INDUCED CONGENITAL DIAPHRAGMATIC HERNIA: A MODEL IN RATS

R TENBRINCK

Een bijdrage in de drukkosten werd ontvangen van:

Physio B.V. te Haarlem.

Roche Nederland B.V. te Mijdrecht

Siemens Nederland N.V. te Den Haag

INDUCED CONGENITAL DIAPHRAGMATIC HERNIA: A MODEL IN RATS

GEINDUCEERDE CONGENITALE HERNIA DIAFRAGMATICA: EEN MODEL BIJ RATTEN

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. PWC Akkermans M.A.
en volgens besluit van het college voor Promoties

De openbare verdediging zal plaatsvinden op
31 mei 1995 om 11.45 uur

door

Robert Tenbrinck

geboren te Vlaardingen

PROMOTIECOMMISSIE:

Promotores:	Prof. Dr. D. Tibboel
	Prof. Dr. B. Lachmann
Overige leden:	Prof. Dr. J.C. Molenaar
	Prof. Dr. W. Erdmann
	Dr. J.L.J. Gaillard

Printed by CopyPrint 2000, Enschede

"If people bring so much courage to this world the world has to kill them to break them, so of course it kills them. The world breaks every one and afterward many are strong at the broken places. But those that will not break it kills. It kills the very good and the very gentle and the very brave impartially. If you are none of these you can be sure it will kill you too but there will be no special hurry."

(E. Hemmingway; A farewell to arms [1929])

Aan Annette

CONTENTS

		Page
Chapter 1	Introduction	9
Chapter 2	Literature review	11
2.1	History	
2.2	Diagnosis and clinical situation	
2.3	The lung: Normal development in humans	
2.4	Pulmonary vascular development	
2.5	Development of the diaphragm	
2.6	The closure of the pleuroperitoneal canals	
2.7	Abnormal lung development	
2.8	Pulmonary vascular abnormalities	
2.9	Possible mechanisms in CDH pathogenesis	
2.10	Lung development in different species	
2.11	Experimental models of CDH	
2.12	CDH in mice and rats	
2.13	Pharmacology of nitrofen	
2.14	Possible mechanisms in nitrofen teratogenicity	
2.15	Specific aims	
	References	
Chapter 3	Experimentally induced congenital diaphragmatic hernia in rats (*J. Pediatr. Surg. 1990; 25: 426-429*)	39
Chapter 4	Nitrofen induced diaphragmatic hernia in rats: an animal model (*J. Pediatr. Surg. 1990; 25: 850-854*)	47
Chapter 5	Pulmonary vascular abnormalities in experimentally induced congenital diaphragmatic hernia in rats (*J. Pediatr. Surg. 1992; 27: 862-865*)	55
Chapter 6	The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo (*J. Pediatr. Surg. 1993; 28: 456-463*)	63
Chapter 7	Nitrofen induced diaphragmatic hernia: Pressure volume registration and artificial ventilation in newborn rats (*Accepted for publication*)	76

Chapter 8	Evaluation of lung function changes before and after surfactant application during artificial ventilation in newborn rats with congenital diaphragmatic hernia (*J. Pediatr. Surg. 1994; 29: 820-824*)	88
Chapter 9	Effect of artificial ventilation on pulmonary antioxidant enzyme activities in a congenital diaphragmatic hernia rat model (*Adv. Exp. Med. Biol. 1992; 317: 363-370*)	96
Chapter 10	Epilogue	104
10.1	General	
10.2	Surgically induced CDH	
10.3	Nitrofen induced CDH	
10.4	Hormonal influence on prenatal lung development in rats	
10.5	Differences between "lamb" and "rat" CDH	
10.6	Consequences of animal models for the human situation	
Chapter 11	Summary	112
	Samenvatting	118
	Dankwoord	122
	Curriculum vitae	
	List of publications	

INTRODUCTION

In patients with congenital diaphragmatic hernia (CDH) a defect in the diaphragm exists together with a variable amount of pulmonary hypoplasia. After birth the defect in the diaphragm is corrected by removing the herniated viscera from the chest and closure of the diaphragm. The estimated incidence is 1 in 2000 to 3000 newborns [1,2,3].
There are several types of herniation, named according to the position of the defect: posterolateral hernia with or without a sac (Bochdalek type), parasternal hernia through the foramen of Morgagni, central hernia and eventration of the diaphragm. The Bochdalek type accounts for 85% of all congenital diaphragmatic hernias [1]. Left-sided posterolateral defects occur eight times more frequently than right-sided defects [3].
Serious clinical symptoms, most of them due to pulmonary hypoplasia, occur shortly after birth and, the current, mortality rate range from 40-70% [4,5]. This mortality rate has not dropped dramatically, in disappointing contrast to the enormous efforts (prenatal diagnosis, a variety of vasoactive drugs, improved ventilatory methods) that have been made to surmount the pulmonary problems [4,5].
CDH is universally seen with pulmonary hypoplasia and/or pulmonary hypertension; this results in the clinical picture of respiratory distress and right-to-left shunting that disconcert so many intensivists fighting for the lives of these newborns.
The management of a newborn with (prenatally) diagnosed CDH depends highly on the understanding of the natural history, and pathophysiology, together with a study of (manipulative) prognostic factors that could affect the outcome.
Therefore, the use of animal models is an indispensable necessity in research on CDH. Up to now, there is little detailed knowledge about the etiology and pathogenesis involved in the closing process of the diaphragm and its relationship with lung development. The influence of drugs or hormones on this mechanism also remains obscure. An animal model provides opportunities to study the progress of postnatal complications and their underlying basis.
In this thesis the use of an animal model for the study of congenital diaphragmatic hernia is described [6]. This model is based on the early induction of CDH in fetal rats by using 2,4-dichlorophenyl-p-nitrophenyl ether (nitrofen); this compound was originally used as a herbicide [7,8].

The objectives of this thesis are to:

1. Compare the induced CDH in rats with the human situation and other available animal models.
2. Describe the morphological characteristics of the developing rat lung and diaphragm in congenital diaphragmatic hernia compared with controls.
3. Evaluate the suitability of this model in testing different ventilatory modes and the subsequent reaction of the CDH lung from a biochemical and histological point of view.

These goals are addressed in chapters 3 to 9; the epilogue (chapter 10) discusses the benefits of this model compared to the other models used; future aspects in CDH research and the possible role of the rat model are also discussed.

REFERENCES

1. Butler N, Claireaux AE. Congenital diaphragmatic hernia as a cause of perinatal mortality. Lancet 1962; 133: 659-63.
2. Harrison MR, DeLorimer AA. Congenital diaphragmatic hernia. Surg Clin North Am 1981; 61: 1023-35.
3. Benjamin JR, Juul S, Siebert DR. Congenital posterolateral diaphragmatic hernia: associated malformations. J Pediatr Surg 1988; 23:899-903.
4. Philip N, Gambarelli D, Guys JM. Epidemiological study of congenital diaphragmatic defects with special reference to aetiology. Eur J Pediatriatr 1991; 150: 726-9.
5. Tracy T, Bailey P, Sadiq F, et al. Predictive capabilities of preoperative and postoperative pulmonary function tests in delayed repair of congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:265-70.
6. Tenbrinck R, Tibboel D, Gaillard J, et al. Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1990; 25:426-29.
7. Adler I, Jones B. Nitrofen. From: Analytical methods for pesticides and plant growth regulates. Vol X, page 403-14 (1978); G. Zweig and J. Slierwa (eds). Acad Press.
8. Kimbrough R, Gaines T, Linder R, Chamblee G. 2,4-Dichlorophenyl-p-nitrophenyl ether (TOK). Effects on the lung maturation of rat fetus. Arch Environ Health 1974; 28:316-20.

LITERATURE REVIEW

2.1 History of congenital diaphragmatic hernia

Bonetus described congenital diaphragmatic hernia in an anatomical tome in 1679 [1]. In 1848 Bochdalek gave an accurate description of herniation of the bowel through the posterior part of the diaphragm, the posterolateral defect has from that time borne his name [2]. Heidenhain performed the first diaphragmatic hernia operation in a 9-year-old boy in 1902 [3]. The patient even survived the first World War as a frontline soldier and showed good health on physical examination in 1920 [4].
Greenwald and Steiner's report on a group of 82 children with a diaphragmatic defect (1929) showed that, out of 11 patients who were operated, only 6 recovered. However, these survivors were over 7 months of age at operation and the hernias were described as oesophageal [5]. They considered presentation of the hernia immediately after birth as lethal, and that: "little or nothing can be done from a surgical standpoint".
Ladd and Gross (1940) recommended emergency surgery after their first successful operation of a Bochdalek hernia in children [6] and newborns [7]; in 1953 they reported a mortality of 8 patients in a series of 63 infants and children. All but six of these patients were older than 24 hours when operated [8].

The availability of endotracheal intubation and ventilatory assistance did not provide the success that was expected. Research was aimed at the pulmonary complications encountered; Campanale and Rowland (1955) described the presence of pulmonary hypoplasia and a reduction in the number and differentiation of alveolar ducts and alveoli [9].
Despite the obtained experience in CDH treatment and research since this time, the overall mortality of CDH "stabilized" between 30-60% [10,11]; these figures are disappointing in comparison with the results obtained in the surgical treatment of other major congenital anomalies.

2.2 Diagnosis and clinical situation

CDH can be detected in utero by (routine) antenatal ultrasonography. Herniated abdominal viscera and or stomach can be seen in the chest; a mediastinal shift can also be detected in some patients [12].
Solitary CDH can not be detected at a chromosomal level; the use of diagnostic methods in this field has not led to an early detection of CDH. CDH can be part of major congenital syndromes like Apert [13], Cornelia de Lange, Fryns [14], and Pierre Robin [15] or a chromosomal defect as trisomy 18 or trisomy 21 [16,17]; the prognosis in cases with associated major congenital anomalies is poor [17].

Familial occurrence of congenital diaphragmatic hernia was first described in 1916 by Makela [18].Since then, scattered reports indicating the occurrence in siblings, twins, and in two generations of the same family appeared [19-21]. None of these reports provided a definite explanation of the pattern of inheritance, but multifactorial inheritance was the most probable way [22,23].

The diagnosis CDH has to be differentiated from cystic adenomatoid malformation of the lung and other cystic masses in the mediastinum. The diagnosis of other simultaneous occurring anatomical abnormalities (cardiac, urogenital or cranial malformations) by a specialised radiologist can determine the optimal postnatal policy [24,25]. It allows maternal transport to a specialized centre, planned delivery and immediate resuscitation. However, in the majority of cases, the afflicted infant is born without suspicion of a congenital diaphragmatic hernia and develops respiratory distress within a variable time after the first breath. The abdomen fails to develop the normal protuberance as air is swallowed and remains scaphoid; the anteroposterior diameter of the chest may increase as the bowel distends with air. Breath sounds are absent on the ipsilateral side, and occasionally bowel sounds may be heard in the chest. The apical heartbeat is displaced to the side opposite the diaphragmatic defect. The infant has increasing tachypnea, cyanosis, and retraction and may rapidly die from respiratory failure. Air-filled intestinal loops on chest roentgenogram establish the diagnosis in most patients; usually, only a small portion of lung is visible in the upper part of the chest with displacement of the heart to the opposite side.

While most patients develop symptoms in the first hours of life, the diagnosis may be delayed for days, weeks and sometimes years [4,26]. In general, the later the onset of symptoms, the better the prognosis. Postoperative survival approaches 100% for infants who were not brought to operation until after the first 24-48 hours [27].

With the improvement of intensive care, also the recognition of the mechanisms involved in CDH enlarged. Much of the early published literature about persistent fetal circulation (PFC) or persistent pulmonary hypertension of the newborn (PPHN), is not written about infants with congenital diaphragmatic hernia, but its applicability to this condition is well documented [28,29]. The neonatal hypoxemia due to pulmonary hypertension, and the subsequent right-to-left shunting of unsaturated blood through the foramen ovale and ductus arteriosus was described by Naeye and Letts in newborns [30] and CDH [31]. Kitagawa et al. [32] examined in detail the lungs of a child with CDH and found a reduced number of alveoli, together with pathological, excessive muscularization of pre-acinar arteries. Using similar techniques, Hislop and Reid [33] found that the degree of hypoplasia in the ipsilateral and contralateral lungs was different at birth and this difference was maintained until death in a 2-month-old baby following correction of CDH.

Until 1986 it was normal pediatric surgical practice to treat patients with CDH as an

emergency condition [27]: evacuation of the viscera out of the thoracic cavity would reasonably improve the ventilatory situation. Many newborns in poor clinical condition, due to a combination of hypothermia, asphyxia and low systemic tension were operated. Postoperatively three groups were recognised; in the first group patients improved and survived, in the second group patients improved only temporarily (the so-called honeymoon period) but died later, and the third group consisted of patients who showed no improvement at all [34]. In the 1980s, several centres observed the beneficial effect of preoperative stabilization and subsequent delayed surgery in the overall survival [10,35].

The improvement in preoperative stabilization was possible because of the use of new ventilatory techniques as High Frequency Oscillation (HFO), and Extra Corporeal Membrane Oxygenation (ECMO) [36-38]. Also surfactant replacement [39,40] therapy is reported to improve the oxygenation and reduce the peak pressures needed. The most enigmatic part in CDH treatment consists of the pulmonary hypertension strategy; nitroglycerine (NTG) and nitroprusside, effective in cardiac-related pulmonary hypertension, failed in many patients. The same is true for other drugs, as the serotonin antagonist Ketenserin, prostacyclin [41], $MgCl_2$ [42], and prostaglandin E2 [43]. The reason for this failure is the substantial lack of knowledge on the mechanisms involved in the development of pulmonary hypertension in congenital diaphragmatic hernia.

Research is now aimed at this problem, and the establishment of the effectiveness of nitric oxide [44] and the endothelium derived relaxing factor (EDRF) [45] on regulation of the pulmonary vascular tone is promising.

2.3 The lung: Normal development in humans

The development of the human lung is described by several authors; two of them offered an important, generally accepted standard in the research of lung development. The first, Reid [46,47], described lung development by three laws that offer a framework to interpret and diagnose the nature of disturbed growth and its timing.

Law I

The bronchial tree is developed by the 16th week

Law II

Alveoli develop after birth, increasing in number until the age of 8 years and in size until growth of the chest wall finishes with adulthood.

Law III

The pre-acinar vessels (arteries and veins) follow the development of the airways, the intra-acinar that of the alveoli. Muscularization of the intra-acinar arteries does not keep pace with the appearance of new arteries.

The second author, Burri [48] followed a more histological approach in his description of the five stages in lung development.

Embryonic stage
The lung begins to develop during stage 12 of development (about 26 days) and is first indicated by a median laryngotracheal groove in the caudal end of the ventral wall of the pharynx. The endodermal lining of the laryngotracheal groove gives rise to the epithelium and glands of the larynx, trachea, and bronchi and to the pulmonary lining epithelium. The connective tissue, cartilage, and smooth muscle of these structures develop from the splanchnic mesenchyme located ventral to the foregut.

Pseudoglandular period (5 - 17 weeks)
In this stage the developing lung somewhat resembles a gland. The tubules in the lung tissue are lined by epithelium composed of columnar or approximately cuboidal cells surrounded by mesenchyme. This period is marked by a continuous growth and branching process in which epithelio-mesenchymal interactions play a determining role in regulating this process. Finally, all major pre-acinar elements of the lung have formed, the vessels included, in a pattern corresponding to that of adult age.

Canalicular period (17 - 26 weeks)
This period is characterized by the occurrence of tubules lined by cuboidal epithelium and situated close to the capillary system. By 24 weeks, each terminal bronchiole has given rise to two or more respiratory bronchioles. The mesenchymal tissue thins out, and capillary invasion into these peripheral units is also seen.

Saccular or terminal sac period (24 weeks to birth)
This final developmental stage before birth is characterized by a further thinning of the interstitium and a flattening of most of the epithelium to give a close link between capillaries and epithelial cells. The saccules have a relatively large lumen and rather smooth walls and form clusters.

Alveolar or postnatal period
This final stage, formation of true alveoli, occurs postnatally. It is marked by the occurrence of distinct pouches in the smooth walled saccules. The lining of these pouches is composed of great and small alveolar cells.

Connecting Reid's laws and the stages mentioned by Burri makes it possible to determine in which stage of lung development a possible derailment took place.
In general, with adaptation of the time axis, the same sequence of developmental stages are found in other mammals. Pringle [49] expressed the various phases of lung

development as percentage of the elapsed pregnancy enabling him to compare lung development in different species.

2.4 Pulmonary vascular development

Angiogenesis is first detected in the coats of the developing trachea, esophagus and lung buds at stage 14 (about 32 days after fertilisation). A vascular plexus forms that receives its blood supply both from branches of the aortic sac as well as from numerous branches of the dorsal aorta, the intersegmental arteries [50]. By the end of the 5th week of gestation the primitive pulmonary arteries become incorporated into the sixth aortic arch and the intersegmental arteries involute. Connections with systemic arteries may persist abnormally.

Reid's third law states that the pre-acinar vessels (both arteries and veins) develop at the same time as the airways, so that by the 16th week all pre-acinar artery branches are present [51].

The structure of the pulmonary artery varies with the size of the vessel and also the developmental stage of the lung. The muscular coat of the arteries begins to develop during the canalicular stage [51]. The axial arteries from hilum to the 7th generation are elastic; beyond this point they are muscular, partial muscular or, at the level of the intra-acinar artery, predominantly non-muscular. By definition, an elastic artery has more than five elastic laminae in its media, a muscular between two and five. A partially muscular artery has muscle in only part of its circumference; at this level the completely muscular coat has been replaced by a spiral. A non-muscular artery is similar in structure to an alveolar capillary, but is of a larger diameter.

The smallest muscular and probably also the partially muscular artery represent the resistance arteries. This muscularisation decreases towards the periphery. In the newborn there is one artery for every 20 alveoli; this ratio is reduced to 8:1 in the adult due to formation of new alveoli postnatally [46,47,52,53].

Two types of pulmonary arteries are distinguished: the conventional type that accompany airways branching from the axial airway, and additional or supernumerary arteries which are lateral branches of the conventional arteries that directly supply the peribronchial parenchyma [46,47,51]. The second type are considerably more numerous than the conventional branches, although their diameter is at any level smaller than that of the conventional arteries. They contribute in a significant way to the cross-section of the totally recruited vascular bed. Branching becomes more frequent towards the periphery. At pre-acinar level the supernumerary arteries contribute about 25% of the cross-sectional area of the side branches, whereas at the intra-acinar level they make up 33% [54]. In the normal lung, according to Hislop and Reid [47,51,53] there are usually 23 generations of conventional arteries along the posterior basal artery and 64

supernumerary branches. It has been suggested that, in the normal lung, the supernumerary arteries facilitate blood oxygenation by allowing passage of venous blood to the more remote alveoli adjacent to large arteries, veins, and airways [55].

2.5 Development of the diaphragm

The development of the diaphragm, a musculotendinous separation between the thoracic and abdominal cavity, is described in two parts: first the development of the diaphragmatic primordium and second the closure of the pleuro-peritoneal canals [56].

At the end of the third week of gestation a mass of mesoderm cranial to the pericardial cavity is identifiable as the beginning of the septum transversum. The ingrowth of liver cells into the septum transversum causes it to expand in a ventro-lateral direction. The septum transversum is continuous with the dorsal structures of the embryo via the pulmonary ridges laterally and the gastrohepatic ligament dorsally. The formed pleuro-peritoneal canals are surrounded by the septum transversum (ventral), the pleuroperitoneal membrane (lateral) and the tissues of the mediastinum (medio-dorsal). Due to enlargement and subsequent burrowing of the pleural cavities in the lateral body walls (9th to 12th week) the body wall (costal) part is incorporated into the diaphragm external to the portions derived from the pleuro-peritoneal membranes [57-60].
Embryology in humans is difficult to comprehend by means of an accidentally obtained postmortem piece, because embryology is a continuous changing ongoing process. The use of animals to study embryology is an obvious choice, provided that the conclusions drawn from such animal study is also applicable to the human situation.
Iritani [61] recognized in mice a posthepatic mesenchymal plate (PHMP) as origin for the most muscular part of the diaphragm. Other authors considered the PHMP as a part of the septum transversum.

2.6 Closure of the pleuroperitoneal canals

The mechanisms responsible for the closure of the pleuroperitoneal canals is thought to play a major role in the development of congenital diaphragmatic hernia. However there is ongoing discussion between the several investigators about the mechanisms that lead to closure of the developing diaphragm.
1) The growth of the pleuro-peritoneal membrane leads to the closure of the canal [60,62].
2) The dorsocranial development of the liver is the decisive factor in the closing process which takes place at membranous level [57].
3) The suprarenal glands are especially important for the closure of the canals [58].

4) The continuous growth and pressure of all the organs in this region (liver,suprarenal gland) forced the canal to close [59].
5) The caudal and lateral growth of the PHMP leads to closure of the diaphragm [61].
6) Kluth et al. [56] used scanning electron microscopy in rat fetuses, and remarked that the closure of the pleuroperitoneal canals took place in two layers. First, the underlying organs approach each other closely and second, the canal is closed at a membranous level by folds derived from the pleural serosae of the liver, the suprarenal gland and the pleuroperitoneal membrane. The closing process is more rapid on the right side than on the left.

2.7 Abnormal lung development

Developmental defects of the lung include: 1) agenesis or hypoplasia of both lungs, one lung, or single lobes; 2) tracheal and bronchial anomalies; 3) vascular anomalies; and 4) congenital cysts.
Pulmonary hypoplasia in newborns is a known accompaniment of a number of malformation syndromes. It is diagnosed in 7.8 to 10.9% of neonatal necropsies and in about 50% of the necropsied neonates with congenital anomalies [63-65]. Factors that influence human lung growth unfavourably include amount of intrathoracic space (CDH, cysts) [32,66] and amniotic space (oligohydramnios due to rupture of the membranes or Potter syndrome) [66-68], and decreased pulmonary arterial flow in cardiovascular malformations like tetralogy of Fallot or hypoplastic right heart [69,70].
A working definition of pulmonary hypoplasia was established by retrospective assessment of lung growth both in recognized and hypothetical pulmonary hypoplasia-associated conditions; pulmonary weight with reference to total body weight was a parameter by which cases of pulmonary hypoplasia were selected [67;71]. Several investigators [66,72,73] also performed a radial alveolar count of the distal airways to characterize these affected lungs further. Radial alveolar count was described in detail by Emery and Mithal [74]: From the centre of a respiratory bronchiolus a perpendicular line is dropped on to the nearest and definite connective tissue septum. The number of alveoli cut by this line was then counted. Pulmonary hypoplasia can be found in congenital diaphragmatic hernia [9,32,65,66], anencephalus [66,75], anuric renal anomalies with oligohydramnios [76,77], and skeletal anomalies restricting thoracic volume [78]. There is, however, a spectrum of severity of pulmonary hypoplasia, often proportional to the severity of the underlying condition. The normal lung weights or LW/BW ratios in different series are reported between 0.18 and 0.22 [66,76]; Emery and Mithal excluded the lung with edema and exudate and found a mean LW/BW ratio of 0.13 [74]. Wet lung weight may be a poor measure of tissue mass for the perinatal lung as it is affected by the quantity of lung liquid retained within the airways and interstitial

tissue. The radial alveolar count provides a clearer definition between pulmonary hypoplasia and normal subjects than the LW/BW ratio alone [73].
Other criteria to describe lung growth and maturation are quantitative biochemical assays as used by Wigglesworth. He found that one of the major problems in studying pulmonary hypoplasia is the difficulty of establishing criteria for the diagnosis of hypoplastic lungs at necropsy in babies who may vary widely both in gestational age and in weight for gestation.
DNA measurement as an index of cell population is widely accepted [79]. Wigglesworth and Desai [80] found that total lung DNA near term in many cases of pulmonary hypoplasia was similar to that in normal fetuses at about 20 weeks gestation. He concluded the lung growth must have been impaired at some time before 20 weeks and divided patients with pulmonary hypoplasia into two groups.
The first group consists of fetus with oligohydramnios due to either renal agenesis, urethral obstruction or amniotic fluid leakage in early pregnancy without other malformations. The lungs in this group have the same characteristic histological pattern with narrow airways and impaired maturation of respiratory epithelium, associated with lack of interstitial tissue and failure of normal elastic tissue development around the airways and terminal sacs [79].
The second group (including CDH) has a normal or increased volume of amniotic fluid. In this group the lungs, although small, are of appropriate maturity for gestational age, with normal epithelial maturation, normal phospholipid content, and normal elastin development. However, Wigglesworth and Desai also suspected a relation between growth and maturation, which was indicated by the hypoplastic left lung associated with left-sided CDH in which there is retarded biochemical maturation compared with greater maturity of the right lung [79,80,81]. Lung growth and maturation during the early fetal period is critically dependent on influences outside the lung [82], such as fetal breathing movements and lung liquid secretion. These influences were already described in 1941 by Potter and Bohlender [83] in their review article; they also emphasized the need for animal research.

2.8 Pulmonary vascular abnormalities

Another major clinical problem in the newborn is persistent fetal circulation or persistent pulmonary hypertension of the neonate (PPHN). It is characterized by right-to-left shunting through the ductus arteriosus and foramen ovale. This may be associated with a multitude of other conditions such as persistent ductus arteriosus, pulmonary hypoplasia and meconium aspiration [28,84,85].
To compare the possible differences in pulmonary artery structure, a standardised way of evaluating these vessels is necessary. Hislop and Davies [51,52] described a way to

process lung tissue into histological slides resulting in barium-gelatin filled arteries, dark stained elastin layers easy to distinguish from the surrounding, but empty lung veins. In these slides, external diameter, wall thickness, wall structure (muscular, partially muscular, or non-muscular) is registered for each artery, as is the type of the accompanying airway. The percentage wall thickness (2x wall thickness/external diameter) is calculated [51]. In addition, the use of a radiopacque injection medium allows rapid assessment of the pulmonary circulation through arteriograms [46].

In the normal newborn the alveolar region is virtually free of muscular arteries; in case of pulmonary hypertension there is extension of the muscularised arteries to this alveolar region [51,54,55]. Reduction in the number of arteries will reduce cross-section as does narrowing of the arterial lumen, so the number of arteries in relation to parenchymal structures is also estimated.
Morphometric studies have established differences in artery structure between congenital diaphragmatic hernia [32], rhesus isoimmunisation [86] and renal agenesis [87].
Geggel and Reid [88] distinguished between maladaption, maldevelopment, and under development. Maladaption represents a structurally normal lung at birth save that increase in compliance of small resistance arteries had not occurred. The pulmonary vascular bed is highly reactive and in a vicious circle of acidosis, hypoxia, hypercarbia and pulmonary vasoconstriction pulmonary hypertension may develop.
Maldevelopment indicates the new and precocious muscularisation seen in idiopathic persistent pulmonary hypertension. Causes of this excessive muscularization are hypoplastic left heart syndrome, chronic intra-uterine hypoxia and also meconium aspiration [47].
Underdevelopment represents the reduced size of arteries seen in congenital anomalies associated with pulmonary hypoplasia, such as CDH, renal agenesis or dysplasia, oligohydramnios.

Table 1. Pattern of airway and vessel structural changes in various types of perinatal pulmonary hypertension [88].

Cause	Airway		Intraacinar artery			
	Number of bronchial generations	Number of alveoli per acinus	Muscle extension by position	External diameter	Medial wall Thickness	Number
Excessive muscularization						
PPHN - idiopathic	N	N	↑	N	↑	N
Meconium aspiration-fatal	N	N	↑	N	↑	N
TAPVC-SD	N	N	↑	↑	↑	N
TAPVC-ID	N	N	↑	N	↑	N
Coarctation, VSD, PDA	N	N	↑	↑	↑[1],↓[2]	N
Underdevelopment						
CDH	↓	N	N,↑	↓[3]	↑	↓
Renal agenesis/dysplasia	↓	↓	↑,N,↓	↓[4]	↑,N,↓	↓
Rhesus isoimmunization	↓	N,↓	N	↓[3]	N[5]	↓
Idiopathic (primary)	↓	↓	NA	NA	NA	NA
Maladaption						
VSD	N	N	↑	↓	↑	↑, N

Abbreviations: CDH = congenital diaphragmatic hernia, ID = infradiaphragmatic, PDA = patent ductus arteriosis, PPHN = persistent pulmonary hypertension of the newborn, SD = supradiaphragmatic, TAPVC = total anomalous pulmonary venous connection, VSD = ventricular septal defect, N = normal, NA = not available, ↑ = increase, ↓ = decrease.
Notes: 1, dependent on the severity of coarctation; 2, preacinar arteries; 3, small for age but appropriate for lung volume; 4, small for age but large for lung volume; 5, preacinar medial hypertrophy.

Intra-acinar arteries in the healthy newborns are virtually all non-muscular, however in persistent pulmonary hypertension of the newborn or persistent fetal circulation, most of them are completely muscularized. Geggel et al. [34] gave a detailed morphometric analysis of the lungs in a series of 7 infants with CDH. He divided these patients in two groups; 4 infants who were never able to be adequately ventilated (the no-honeymoon group), and 3 who did well initially following repair of their diaphragmatic hernia, but then developed an increased pulmonary vascular resistance and died (honeymoon group). No-honeymoon patients have smaller lungs, increased muscularization of intra-acinar arteries, and decreased luminal area of pre-acinar and intra-acinar diameter. They concluded that the clinical deterioration in the honeymoon group depends on a vasoconstrictive response in the hypoplastic vascular bed. Persistent hypoxemia in the no-honeymoon group is determined by the severity of pulmonary hypoplasia and by the structural remodelling of the pulmonary arteries.

Studying lungs of CDH patients, Kitagawa et al. [32] first pointed out that there were abnormalities in both the numbers of arterial branches and their muscularisation. They reported that the number of conventional branches was reduced to 14 in the right lung and to 12 in the left lung. A reduction of supernumerary branches to 17 in the right lung, but to only 36 in the left lung was observed. They also described thicker muscular walls in smaller diameter arteries (shift to the left); see Fig 1.

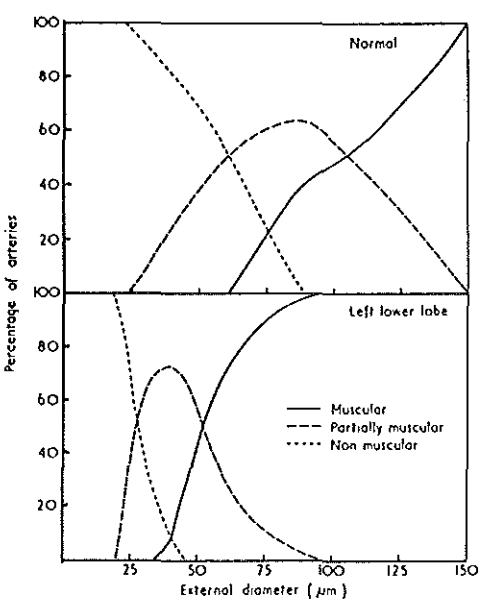


Figure 1. Distribution, in diameter groups, of the muscular, partially muscular, and non-muscular arteries in the normal newborn lung and in the left lower lobe of a case of congenital diaphragmatic hernia. In the latter each structural type is found in smaller arteries than is normal.(Published with permission of the author [32])

Naeye et al., Levin, and Nakamura et al. reported uniform thickening of the pulmonary artery muscle mass in their study of congenital diaphragmatic hernia lungs [31,89,90].

Resistance within the pulmonary circulation drops either because of vasodilatation or by recruitment of arteries [91,92]. The pulmonary artery bed is a low pressure system, considerable increase in flow, as in exercise, is possible without increase in pressure because a large proportion of the arteries is unused at rest and available for recruitment. Vasoconstriction produces an acute rise in pressure or resistance by functional narrowing; in chronic pulmonary hypertension there has to be other, structural changes. Stenmark et al. [92] attempted to integrate the sequence of events starting with the physical stress and ending with the restructured vessel; they distinguished an early and late (chronic) state suggesting an analogous mechanism in pulmonary remodelling as found in systemic hypertension induced vascular diseases.
The exact mechanisms that control the normal resting tone in the pulmonary circulation and their reactivity are not known [91,92]. The presence of smooth muscle cells and connective tissue elements in the wall must be taken into account, but these are influenced by a lot of mediators (e.g. bradykinin, angiotensin II, epinephrine, thromboxane B2, and metabolites of arachidonic acid) in a very complex, until yet unrevealed, cascade [92].

2.9 Possible mechanisms in the pathogenesis of CDH

Since the mechanism of normal diaphragm development is unknown, there will also be much speculation about the pathogenesis of CDH. In the past, several theories had been proposed to explain the occurrence of postero-lateral diaphragmatic defects which include [93]:
1) defects caused by improper development of the pleuro-peritoneal membrane [60,62].
2) failure of muscularisation of the lumbocostal trigone and pleuro-peritoneal canal [62,94].
3) permeation of bowel through the postero-lateral part of the diaphragm (foramen of Bochdalek) [58].
4) Early return of the intestines into the abdominal cavity with a still open canal [62,94].
5) Abnormal persistence of the lung in the pleuroperitoneal canal might prevent closure

of the canal [95].
6) Abnormal development of the embryonic lung and posthepatic mesenchyme causes non-closure of pleuro-peritoneal canals [61].

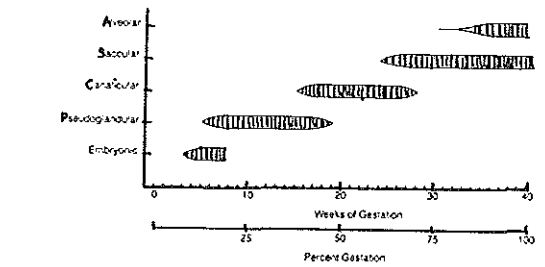
The most common cited theory is the one describing failure of the pleuro-peritoneal membrane to meet the septum transversum. However, Wells [59] thought the disappearance of the pleuroperitoneal canals is caused by the development of the underlying organs. He presumed that non-closure of the canal leads to herniation of the gut; he was not able to render a mechanism for his idea.
The theory of a weak spot in the diaphragm through which the bowel loops are pushed in the thoracic cavity causing pulmonary hypoplasia (point 1-4) has to be considered as well [94].
Little is known about the intra-abdominal pressure in the developing embryo; it is unlikely that the gut returns in the abdominal cavity against a positive abdominal pressure. This return occurs in a relative late stage of lung and diaphragm development [94].

2.10 Lung development in different species

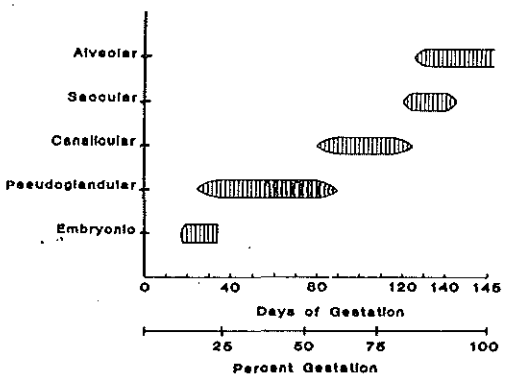
Developmental stages of the normal human lung can also be recognized in animals. However, there are important differences between species regarding the various stages of lung development, especially when they are considered in terms of percentage of the total gestational period.
Figures 2a-d show the outline of fetal lung development in humans, monkey, sheep, rabbits, and rats as they were published by Pringle [49].

According to Pringle [49],three important facts in the study of lung development have to be considered.
First within any species, at any stage of development, there is considerable variation in lung development from fetus to fetus. This makes it impossible to draw sharp boundaries between phases of lung development.
Second, in different areas within one lung, there is often considerable variation of lung development. One part of the lung may develop ahead of, or behind, other parts of the lung.
Third, there is considerable variation between species in the proportion of gestation occupied by the various phases in lung development. This species variation is most marked in the pseudoglandular and alveolar phases of development. This fact makes direct comparison of lung development between species invalid when it is only based on percentage of gestation. Such comparisons can only be made after determining the exact phase of development and a note of the time of gestation in days or weeks and

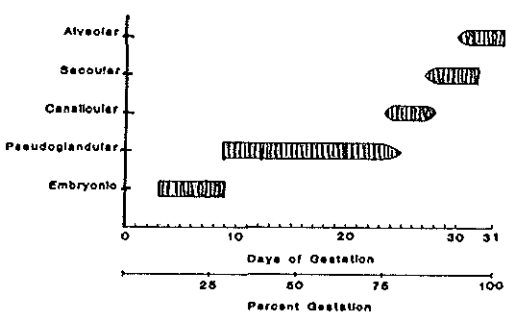
Figure 2 A-D



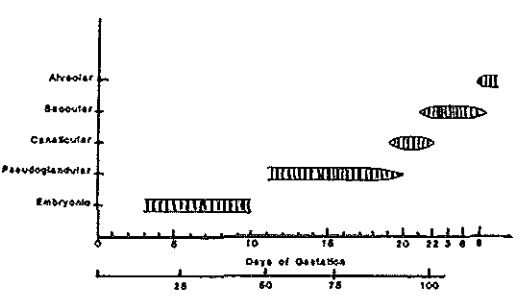
A



B



C



D

Figure 2 (reprint with permission[49]). Schematic outline of lung development in various species. Fig 2a. Human lung. Note the overlap between the phases. The pseudoglandular phase extends to about 20 weeks (50% gestation). The alveolar phase is clearly established by 36 weeks [90% gestation), although many areas of the lung are still in the saccular phase beyond term. Fig 2b. Sheep lung. The pseudoglandular phase extends to 85-90 days (about 60% gestation). The canalicular and saccular phases are foreshortened, but the alveolar phase is clearly established by 125 days (86% gestation) a stage in gestation very similar to the first appearance of alveoli in the human lung. Fig 2c. Rabbit lung. The pseudoglandular phase extends to 23-24 days (75% gestation). Alveoli appear just before birth. Fig 2d. Rat lung. The pseudoglandular phase occupies 86% of gestation. The saccular phase does not begin until the day before birth (95% gestation) and true alveoli do not begin to develop until 4 days after birth.

percentage of gestation.
Ten Have investigated fetal lung development in mice by using immunocytochemical and ultrastructural techniques; this led to a new concept of lung development [96].
Starting at gestational day 9.5, the lung buds give rise to a branching tubular system called the primordial system; this is lined by undifferentiated columnar (primordial) epithelium.
Differentiation of the primordial system into bronchial (columnar) and respiratory (cuboid) system starts around day 14.2 [97]. Immunocytochemistry and electron microscopy detect a sharp demarcation between respiratory (alveolar) system and bronchial epithelium [97,98]. This sharp demarcation supports the view [98] that the bronchial and respiratory systems originate from a separate part of the primordial system.
The cuboid epithelium is composed of type II (precursor) cells. Type II cell is an indicator for all the morphological expressions of this cell; it plays a key role in the pulmonary acinus formation being the stem cell of the complete alveolar epithelium (type I and II cells). It has also, as the only dividing cell, a primary morphogenetic function in pulmonary acinus formation [96,97].
Comparative studies have shown that this concept on mouse lung development is also true for the rat [99] and humans [100].

2.11 Experimental models of CDH

The wide variety in clinical manifestation and the persisting disappointing results in treating CDH resulted in a demand for a model in which the encountered problems could be studied.
Harrison et al. defined in 1980 several characteristics which are necessary to develop

such an animal model [101]:

1. The model must simulate CDH in human infants.
2. The model must allow simulated correction of the diaphragmatic hernia by removal of the space occupying lesion during gestation.
3. The model must permit quantitative assessment of the effect of the simulated lesion and its correction on survival at birth and on pulmonary parenchymal and pulmonary vascular development.

Experimental production of CDH in pregnant animals was reported by DeLorimer et al. [102] in 1967 and Kent et al. in 1972 [103]. They used pregnant ewes and created diaphragmatic hernias in the fetal lambs. Fetal surgery was performed, finally leading to an opening of the left hemithorax at the eight interspace. The lung was retracted and a hole, 1 cm in diameter, was made in the diaphragm through which a loop of small bowel was pulled in the chest. Kent used four fetuses of 69 - 72 days gestation and studied ventilation, hemodynamics and resulting pathologic processes at term to relate them to the effects of CDH in neonates. He concluded that the earlier in fetal life the lesion was produced the more severe the hypoplasia. He measured higher mean pulmonary pressure after delivery in CDH animals; no significant differences were found in cardiac output.

In 1977 Ohi et al. [104] reported creation of CDH in fetal rabbits; these animals were subjected to partial excision of the left diaphragm in utero at a gestational age of 23-26 days. He concluded that the so-called hypoplastic lung in diaphragmatic hernia is small in size but potentially functioning when relieved of compression. Matsuda et al. advanced the operating age to 21-25 days in order to create the defect in the pseudoglandular phase of lung development [105]. They performed histological measurements of the pulmonary vasculature and concluded that developmental pulmonary vascular disorders caused by diaphragmatic hernia result in the absolute reduction of the pulmonary vascular capacity probably causing pulmonary hypertension and PFC.

In 1980 Harrison et al. [101] introduced a conical, silicone-rubber balloon in the left hemithorax of fetal lambs, which was progressively inflated over the last trimester to simulate the compression by growing viscera. They considered that if the pulmonary hypoplasia is a developmental consequence of compression by the herniated viscera, removal of this space-occupying lesion in utero may allow pulmonary development proceed normally. Thus correcting CDH in utero may allow the lung to develop sufficiently to support life at birth.

Harrison's group "corrected" congenital diaphragmatic hernia intra-uterine by deflation of the balloon [106]; this allowed sufficient lung growth and development to alleviate respiratory insufficiency and to assure survival of the lambs delivered by cesarian section. The correction resulted in a significant increase in lung weight, air capacity, compliance, and area of the pulmonary vascular bed [106].

The balloon model could not be used to study the feasibility of correction nor to develop the surgical techniques necessary for actual successful surgical repair. Therefore,

Harrison et al. [107] created CDH by making a hole in the left diaphragm and demonstrated that herniated viscera produced pulmonary hypoplasia comparable to that produced by the balloon. Intra-uterine correction of CDH was performed [107]. Adzick et al. advanced the creation of CDH to 60-63 days gestation [108]; a phase in fetal lamb lung development [109] that corresponds with appearance of CDH in the human situation (8-10 weeks gestation).

The fetus with CDH shows many similarities to the premature, surfactant deficient newborn with respiratory distress syndrome (RDS), considering histological, morphological, and quantitative biochemical criteria [65,80]. Pringle et al. [110] studied pulmonary maturity and morphology in CDH lambs at various stages of gestation with electronmicroscopy. They found an increase in number and size of the type II pneumocyte; these changes affected the ipsilateral greater than the contralateral lung, and these changes were partially reversed by in utero repair. However, in this study no type II pneumocyte function studies or surfactant analyses were performed.
Glick et al. [111] hypothesized that a surfactant deficiency may partly contribute to the pathophysiology of CDH. In term CDH lambs, they found surfactant deficiency with associated decreased compliance. The phospholipid concentrations, as well as the phospholipid composition (decreased phosphatidylcholine (PC)), were significantly changed.

Pulmonary hypoplasia is also encountered in congenital malformations other than CDH such as renal dysplasia [76,77]. This pulmonary hypoplasia can be produced experimentally in fetal rats [112] by amniocentesis, or in fetal lambs by bladder outlet obstruction [113]. These experiments suggest a relationship between pulmonary fluid dynamics and pulmonary growth [113-115]. In 1984 Adzick et al. demonstrated that tracheal ligation in fetal lambs could prevent pulmonary hypoplasia associated with oligohydramnios [116]. This study was recently repeated and extended. Wilson et al. [117] divided 95 gestational day sheep in nephrectomy, nephrectomy + tracheal ligation (TL), tracheal ligation alone and sham operated controls. DiFiore et al. [118] performed diaphragmatic hernia and TL in 90 days gestation sheep.
They concluded: TL in the fetus accelerated lung growth beyond normal limits, even in the absence of fetal kidneys. Lung growth was achieved, at least in part, by cell proliferation rather than hypertrophy. They also found histologically relatively normal lung parenchyma, suggesting that developmental pathways are not markedly disordered by tracheal ligation. Fetal TL produces increased intratracheal pressure [118], which may be responsible for the pulmonary growth observed. Their suggestion that it could also prevent pulmonary hypoplasia in CDH provided the lungs were ligated in an early phase of development was confirmed by Hedrick et al. [119]. They created CDH in lambs at 75 days gestation and ligated the trachea at 120 days. After delivery at 135-140 days

tracheal ligation or plugging the upper airway in CDH lambs reduces the abdominal viscera from the chest, accelerates lung growth and improves oxygenation and ventilation at birth. This plugging may provide a less invasive way of intra-uterine CDH treatment.

2.12 CDH in mice and rats

Iritani postulated in 1984 that an abnormality in the development of the lung is responsible for the diaphragmatic defect [61]. He used pregnant mice; 2,500 ppm 2,4-dichloro-4'-nitrodiphenyl ether (nitrofen) was given in the food from the 5th gestational day until the day of sacrifice (days 9-18). Hypoplasia of the lung buds may affect the later growth of the lung itself and cause abnormality in the vascular system of both lungs, resulting in persistent fetal circulation [61].
The fetal rat model of diaphragmatic hernia [120] which is described in this thesis is derived from the results of Iritani and data of other toxicological studies with Nitrofen in rats.

2.13 Pharmacology of nitrofen (2,4-dichloro-4'-nitrodiphenyl ether)

Originally, nitrofen is a selective pre and early post-emergence herbicide for the control of annual grasses and weeds on a variety of food crops. Nitrofen (or commercially offered under the name of Tok®) is the common name adopted by the Weed Society of America. The Rohm and Haas Company obtained the first registration in 1966 [121].
Almost 20 years of commercial nitrofen use has not produced indications of toxicity in man, with the exception of occasional reports of skin irritation. In adult rats and mice, oral LD_{50} values range from 2.4 to 3.6 g/kg [122]; death in these animals from acute exposure generally occurs within 2-8 days preceded by progressive depression of all vital functions.
The teratogenic properties of nitrofen have been investigated in some 25 studies conducted in several laboratories utilizing different strains and 4 species of laboratory animals [122]. In these studies, the identified LOEL (lowest observable effect level) was 0.15 mg/kg/day for production of malformations with oral treatment on days 5-17 of gestation in the rat. Comparison of this value to oral LD_{50} values in rats indicates that the compound is a potent and selective teratogen in rodents. This raises concern about the tertogenicity of other diphenyl ethers, which constitute a major class of pre- and post-emergent herbicides that also includes bifenox, acifluoren, oxyfluoren and CNP. Diphenyl ethers are also being used industrially as components of heat transfer media, solvents and plasticizers.
In a series of experiments with rats Costlow and Manson [123-125] showed day 11 of gestation to be the most sensitive day for induction of neonatal mortality; oral administration of 116 mg/kg nitrofen to the dam on this day was the LD_{50} for the

neonate. The malformations in the neonates observed were dose related: 70 mg/kg produced hydronephrosis and a slightly delay in ossification. Similarly, doses of 115 mg/kg and higher produced diaphragmatic hernia in term fetuses, and doses over 150 mg/kg decreased body weight gain and produced heart malformations (ventricular septal defect, double outlet right ventricle and transposition of the great vessels).

No increases in chromosomal abberations were found in bone marrow taken from animals killed at approximately 6, 24 and 48 h after a single dose of nitrofen or 6 h after the last of the multiple exposures [126].
Pharmacokinetic studies in non-pregnant rats revealed that within 48 h following the last dose of 125 mg/kg [^{14}C]nitrofen 72 - 100% of the radioactivity was excreted, with 14-21% appearing in the urine and 54-80% appearing in the faeces.
In a study by Costlow and Manson in 1983 [125] a single oral dose of 120 mg/kg [^{14}C]nitrofen was given to pregnant rats on day 11 of gestation. Maternal blood [^{14}C]-concentration peaked 7-9 h after dosing at approximately 10μg/ml ^{14}C (calculated as nitrofen). The half-life in the maternal blood was 8 days. In the embryonic compartment, ^{14}C was first detected 2-3 h after dosing, peaked at 4-6 h after dosing at 50 ppm and declined to half of that initially seen by 24 h (the maximal studied period for the embryo).
A follow-up study by Brown and Manson [127] on day 10 of pregnancy showed accumulation in maternal fat for over 72 h. Peak levels were reached in other maternal organs after 3-12 h; the plasma half-life was estimated to be 42 h. Radioactivity was first detected in the embryonic compartment at 3 h and continued to increase through the 72 h time point. Despite this accumulation of activity in the embryo over time, the actual levels found in the embryonic compartment were low (about 1%). After 48 h there was redistribution of the parent compound from the maternal fat to other maternal organs and the embryo.

2.14 Possible mechanisms in nitrofen teratogenicity

It is suggested [128] that nitrofen exerts its teratogenic effect through specific mechanism(s) of action; the pattern of visceral malformations that occur in the absence of overt maternal toxicity or embryolethality/cytotoxicity suggest that the compound perturbs processes unique or highly selective for embryonic differentiation.

Approximately 20 different metabolic products, including intermediates and conjugates, have been identified in adult rats after nitrofen exposure; only the parent compound (2,4-dichloro-4'-nitrodiphenyl ether) could be detected in the embryo.
Experiments [128] implied that it is the number and position of chlorine groups on the nitrofen molecule and not the nitro group that determines the teratogenic activity. The

absence of the stable endpoint of the nitroreduction pathway, the 4'-amino metabolite, in embryonic tissue suggests that this pathway may not be relevant for its teratogenic effect.

Nitrofen, as a diphenyl ether compound, has a stereochemical configuration similar to thyroid hormone.

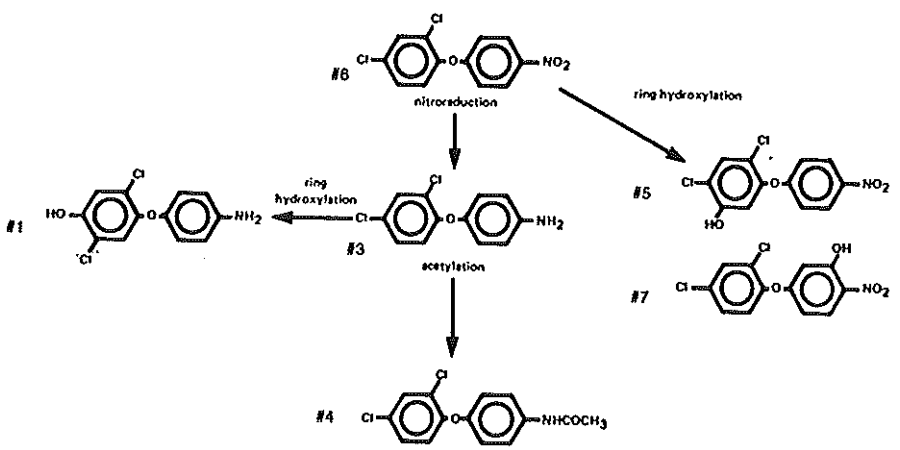


Figure 3. The postulated scheme of nitrofen metabolism in the rat [permission of 125].

Both possess diphenyl ether ring structures in which aromatic rings are inclined at an angle of 120° and the planes of the aromatic rings are perpendicular to each other.

Manson et al. [128,129] examined the influence of nitrofen exposure on the hypothalamic-pituitary-thyroid function in nonpregnant, pregnant and fetal rats and attempted to relate alterations in thyroid hormone status to teratogenicity. They showed that co-administration of T4 with nitrofen to thyroidectomized pregnant rats during days 6 - 15 of gestation, resulted in a 70% reduction of the frequency of malformed fetuses compared with nitrofen exposure alone [128].

The interpretation of this research is that nitrofen exposure results in a thyreomimetic challenge to the conceptus. In the case of the fetal lung, this was manifested as a

transient stimulation of lung differentiation at the expense of lung growth. This concept was already described in 1968 by Hamburgh for nervous tissue [130]; thyroid hormone alters the course of development in neural tissues by inhibiting proliferation and stimulating differentiation.
The real mechanism of nitrofen teratogenesis remains unclear. The proximate teratogen could be the parent compound itself which directly binds to the embryonic nuclear receptors for T3, leading to altered differentiation of target organs. Alternatively, increased availability and placental transport of maternal thyroid hormones could be the proximate source of the thyreomimetic challenge to the embryo.
Zeman et al. [131] described reductions in DNA, RNA and protein contents in the lungs and also other organs except the brain; suggesting that the encountered organ weight deficits are the result of a reduced cell population.
Lau et al. [132] gave nitrofen (20 or 40 mg/kg/day) to pregnant rats during days 10-13 of gestation and investigated the effects on cardiac structure and function in newborns. They found marked depression of heart rate and abnormal electrocardiographicy (ECG) profiles, together with respiratory distress. The postnatal mortality was significant higher than controls. The percentage CDH was 14% and 50% in the mentioned dosage groups; there were no survivors after 24 h in the CDH groups. They observed an overall (CDH and non-CDH) improvement in heart rate after extra oxygen supply; they concluded that other more subtle morphological and physiological factors which contribute to improper systemic delivery and cellular utilization of oxygen may be involved. The high mortality can not be accounted for by the anatomical changes to the rat heart and diaphragm.

In 1988 Lau et al. [133] continued research in this experimental setting and described that the lungs in the nitrofen animals were smaller, did not exhibit any specific pathologic lesions, but had a simplified and immature appearance. The static compliance of the total system, derived from pressure/volume curves of the hysteresis loops was also significantly reduced. They found a CDH percentage of 50% in Sprague-Dawley rats and concluded diaphragmatic hernia does not seem to account for all the deficient lung compliance.

In all these studies there was no intention to use nitrofen as a specific inductor of CDH. It was given to pregnant animals for a prolonged period and never as a pulse dosage at a critical phase in lung development. The prolonged gavage allowed the induction of multiple malformations, greatly influencing the final conclusions.

2.15 Specific aims

The specific aims of this thesis are:

1. To establish a reliable and reproducible animal model of CDH using the herbicide

nitrofen in pregnant rats, and to compare this model to the human situation.
2. To evaluate differences in the embryological/fetal development of the lung in CDH rats.
3. To evaluate biochemical, histological and functional differences following artificial ventilation of healthy and CDH newborns.

REFERENCES.

1. Bonetus T. De Sufficatione. Observatio XLI. Sufficatio excitata a tenium intestorum vulnus diaphragmatis in thoracem ingrestu. Selpulchretum sive anatomia procteia et cadaveribus morbo denatus. Geneve 1679.
2. Bochdalek V. Enige betrachtugen uber die entstehung des angeborenen zwergfellbruches, als beitrag zur pathologischen anatomie der hernien. Vierteljahrschrift Prakt Heilkund 1848; 3:89.
3. Heidenhain L. Geschichte eines falles von chronischer inkarzeration des magens in einer angeborenen zwergfellhernie. Deutsche Zeitschr Chir 1905; 76:4-6.
4. Aue O. Uber angeborene zwergfellhernie. Deutsche Zeitschr Chir 1920; 160:14-35.
5. Greenwald H, Steiner M. Diaphragmatic hernia in infancy and childhood. Am J Dis Child 1929; 38:361-92.
6. Ladd W, Gross R. Congenital diaphragmatic hernia. N Engl J Med 1940; 223:917-24.
7. Gross R. Congenital diaphragmatic hernia. Am J Dis Child 1946; 71:579-85.
8. Gross R. Congenital hernia of the diaphragm. In: Surgery of infancy and childhood. Philadelphia, W. B. Saunders 1953;428-44.
9. Campanale RP, Rowland RH. Hypoplasia of the lung associated with congenital diaphragmatic hernia. Ann Surg 1955;142:176-89.
10. Hazebroek FWJ, Tibboel D, Bos AP, Pattenier AW, Madern GC, Bergmeijer JH, Molenaar JC. Congenital diaphragmatic hernia: the impact of preoperative stabilization. A prospective study in 13 patients. J Pediatr Surg 1988;23:1139-46.
11. Nio M, Haase G, Kennaugh J, et al. A prospective trial of delayed versus immediate repair of congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:618-21.
12. Harrison MR, DeLorimer AA. Congenital diaphragmatic hernia. Surg Clin North Am 1981; 61:1023-35.
13. Cunnif C, Lyons K, Jones MC. Patterns of malformation in children with congenital diaphragmatic defects. J Pediatriatr 1990;116:258-61.
14 Fryns JP. Fryns syndrome: A variable MCA syndrome with diaphragmatic defects, coarse face, and distal limb hypoplasia. J Med Genet 1987; 24:271-4.
15. David TJ, Illingworth CA. Diaphragmatic hernia in the south west of England. J Med Genet 1976; 13:253-62.
16. Benjamin DR, Juul S, Siebert JR. Congenital posterolateral diaphragmatic hernia: associated malformations. J Pediatr Surg 1988; 24:899-903.
17. Adzick NS, Harrison MR, Glick PL, et al. Diaphragmatic hernia in the fetus: Prenatal diagnosis and outcome in 94 cases. J Pediatr Surg 1985; 20:357-61.
18. Makela V. Hernia diaphragmatica congenita spuria. Finska Lak Sallsk Handl 1916; 58:1107-10.
19. Crane JP. Familial congenital diaphragmatic hernia; prenatal diagnostic approach and analysis of twelve families. Clin Genet 1979; 16:244-52.

20. Pollack LD, Hall JG. Posterolateral (Bochdalek) diaphragmatic hernia in sisters. Am J Dis Child 1979; 133:1186-93.
21. Frey P, Glanzmann PF, Nars P. Familial congenital diaphragmatic defect: transmission from father to daughter. J Pediatr Surg 1991; 26:1396-8.
22. Daentl D, Passarge E. Familial agenesis of the diaphragm. Birth Defects 1972; 8:24-6.
23. Wolff G. Familial congenital diaphragmatic defect: a review and conclusions. Hum Genet 1980; 54:1-5.
24. Nakayama DK, Harrison MR, Chinn DH. Prenatal diagnosis and natural history of the fetus with a congenital diaphragmatic hernia: initial clinical experience. J Pediatr Surg 1985; 20:118-24.
25. Manni M, Heydanus R, Den Hollander NS, Stewart PA. Prenatal diagnosis of congenital diaphragmatic hernia: a retrospective analysis of 28 cases. Prenat Diag 1994; 14:187-90.
26. Heij HA, Bos AP, Hazebroek FWJ. Acquired congenital diaphragmatic hernia. Eur J Pediatriatr 1987; 146:440-1.
27. Reynolds M, Luck SR, Lappen R. The critical neonate with congenital diaphragmatic hernia: a 21 year perspective. J Pediatr Surg 1984; 19:364-7.
28. German JC, Bartlett RH, Gazzinga AB. Pulmonary artery pressure monitoring in persistent fetal circulation (PFC). J Pediatr Surg 1977; 12:913-7.
29. Cloutier R, Fournier L, Levasseur L. Reversion to fetal circulation in congenital diaphragmatic hernia: A preventable postoperative complication. J Pediatr Surg 1983; 18:551-5.
30. Naeye RL, Letts HW. The effects of prolonged neonatal hypoxemia on the pulmonary vascular bed and heart. Pediatrics 1962; 30:752-4.
31. Naeye RL, Sochat SJ, Whitman V. Unsuspected pulmonary vascular abnormalities associated with diaphragmatic hernia. Pediatrics 1976; 58:902-4.
32. Kitagawa M, Hislop A, Boyden EA, Reid L. Lung hypoplasia in congenital diaphragmatic hernia; a quantitative study of airway, artery, and alveolar development. Brit J Surg 1971;58: 342-6.
33. Hislop A, Reid L. Persistent hypoplasia of the lung after repair of congenital diaphragmatic hernia. Thorax 1976; 31:450-6.
34. Geggel RL, Murphy JD, Reid L. Congenital diaphragmatic hernia: Arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 1985; 107:457-64.
35. Cartlidge PH, Mann NP, Kapila L. Preoperative stabilisation in congenital diaphragmatic hernia. Arch Dis Child 1986; 61:1226-8.
36. Boros SJ, Mammel MC, Coleman JM. Neonatal high frequency ventilation: 4 years experience. Pediatrics 1985; 75:675-9.
37. Cornish JD, Gerstmann D, Clark RH. Extracorporeal membrane oxygenation and high frequency oscillatory ventilation: potential therapeutic relationship. Crit Care Med 1987; 15:831-4.
38. O'Rourke PP, Lillehei C, Crone RK, Vacanti JP. The effect of extracorporeal membrane oxygenation on the survival of neonates with high-risk congenital diaphragmatic hernia: 45 cases from a single institution. J Pediatr Surg 1991; 26:147-52.
39. Bos AP, Tibboel D, Lachmann B. Surfactant therapy in high-risk congenital diaphragmatic hernia. Lancet 1991; 338:1279.
40. Glick PL, Leach CL, Holm B. Pathophysiology of congenital diaphragmatic hernia III: Exogenous surfactant therapy for the high-risk neonate with CDH. J Pediatr Surg 1992; 27:866-9.
41. Stevenson D, Schreiner R, Bull M. An analysis of tolazoline therapy in the critically ill neonate. J Pediatr Surg 1980; 15:964-7.
42. Abu-Osba YK, Treatment of persistent pulmonary hypertension of the newborn: update. Arch Dis Child 1991;88:74-7.
43. Hammerman C, Lass N, Strates E. Prostanoids in neonates with persistent pulmonary hypertension.

J Pediatr 1987; 110:407-12.
44. Kinsella JP, Neish S, Ivy D, Abman S. Clinical responses to prolonged treatment of persistent pulmonary hypertension of the newborn with low doses of inhaled nitric oxide. J Pediatr 1993;123:103-8.
45. Johns R. Endothelium derived relaxing factor: basic review and clinical implications. Cardio Thor Vasc Anesth 1991; 5:69-79.
46. Reid L. The pulmonary circulation: remodelling in growth and disease Am Rev Respir Dis 1979; 119:531-53.
47. Reid L. Lung growth in health and disease. Br J Dis Chest 1984; 78:113-34.
48. Burri PH. Fetal and postnatal development of the lung. Ann Rev Physiol 1984; 46:617-28.
49. Pringle KC. Human lung development and related animal models. Clin Obstet Gynec 1986; 29:502-13.
50. Rabinovitch M. Morphology of the developing pulmonary bed: pharmacologic implications. Pediatr Pharm 1985; 5:31-48.
51. Hislop A, Reid LM Intrapulmonary arterial development during fetal life: branching pattern and structure. J Anat 1972; 113:35-48.
52. Davies G, Reid LM. Growth of the alveoli and pulmonary arteries in childhood. Thorax 1970; 25:669-81.
53. Hislop A, Reid LM Pulmonary arterial development during childhood: branching pattern and structure. Thorax 1973; 28:129-35.
54. Meyrick B, Reid LM. Pulmonary hypertension: Anatomic and physiologic correlates. Clin Chest Med 1983; 4:199-217.
55. Meyrick BO. Structure of the normal pulmonary vasculature and changes with disease. In: The lung in health and disease, ed M Dekker. Pulmonary hypertension ed... 1991; 215-55.
56. Kluth D, Petersen C, Zimmermann HJ. The developmental anatomy of congenital diaphragmatic hernia. Pediatr Surg Int 1987; 2:322-6.
57. Broman I. Uber die entwicklung des Zwergfells beim Menschen. Verh Anat Gesellschaft 1902; 16:9-17.
58. Bremer JL. The diaphragm and diaphragmatic hernia. Arch Pathol 1943; 36:539-49.
59. Wells LJ. Development of the human diaphragm and pleural sacs. Contr Embryol Carneg Inst 1954; 35:107-37.
60. Grosser O, Ortmann R. Grundriss der Entwicklungsgeschichte des Menschen; 7th ed. 1970; Springer, Berlin.
61. Iritani I. Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 1984; 169:133-9.
62. Gray SW, Skandalis JE. Embryology for surgeons. Saunders, Philadelphia 1972; 359-85
63. Driscoll SG, Smith CA. Neonatal pulmonary disorders. Ped Clin North Am 1962; 9:325-52.
64. Pryse-Davies J. Pathology of the perinatal lung. Proc Roy Soc Med 1972; 65:823-4.
65. Wigglesworth JS, Desai R, Guerrini P. Fetal lung hypoplasia: biochemical and structural variations and their possible significance. Arch Dis Child 1981; 56:606-15.
66. Reale FR, Esterly JR. Pulmonary hypoplasia: A morphometric study of the lungs of infants with diaphragmatic hernia, anencephaly, and renal malformations. Pediatr 1973; 51:91-6.
67. Potter EL. Bilateral renal agenesis. J Pediatr 1946; 29:68-72.
68. Fantel AG, Shephard TH. Potter syndrome: nonrenal features induced by oligoamnios. Am J Dis Child 1975; 129:1346-7.
69. Hislop A, Sanderson M, Reid LM. Unilateral congenital dysplasia of lung associated with vascular anomalies. Thorax 1973; 28:435-41.

70. Haworth SG, Reid LM. Quantitative structural study of pulmonary circulation in newborn with pulmonary atresia. Thorax 1977; 32:129-33.
71. Gruenwald P, Minh HN. Evaluation of body and organ weights in perinatal pathology. Am Clin Pathol 1960; 34:247-53.
72. Areechon W, Reid LM. Hypoplasia of lung with congenital diaphragmatic hernia. Br Med J 1963; 1:230-3.
73. Askenazi SS, Perlman M. Pulmonary hypoplasia: lung weight and radial alveolar count as criteria of diagnosis. Arch Dis Child 1979; 54:614-8.
74. Emery JL, Mithal A. The number of alveoli in the terminal respiratory unit of man. Arch Dis Child 1960; 544-7.
75. Naeye RL, Blanc W. Organ and body growth in anencephaly. Arch Path 1971; 91:140-7.
76. Potter EL. Bilateral absence of ureters and kidneys: a report of 50 cases. Obstet Gynecol 1965; 25:3-12.
77. Perlman M, Levin MR. Fetal pulmonary hypoplasia, anuria, and oligohydramnios: clinicopathologic observations and review of the literature. Am J Obstet Gynecol 1974; 118:1119-23.
78. Finegold MJ, Katzew H, Genieser N, Becker B. Lung structure in thoracic dystrophy. Am J Dis Child 1971; 122:153-9.
79. Enesco M, Leblond CP. Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. J Embryol Exp Morphol 1962; 10:530-62.
80. Wigglesworth JS, Desai R. Use of DNA estimation for growth assessment in normal and hypoplastic fetal lungs. Arch Dis Child 1981; 56:601-5.
81. Blackburn WR, Logsdon P, Alexander JA. Congenital diaphragmatic hernia: studies of composition and structure. Am Rev Resp Dis 1977; 115:suppl 275.
82. Wigglesworth JS, Desai R. Is fetal respiratory function a major determinant of perinatal survival? Lancet 1982; 264-7.
83. Potter EL, Bohlender GP. Intrauterine respiration in relation to development of the lung. Am J Obst Gynecol 1941; 42:14-22.
84. Drummond WH. Persistent pulmonary hypertension of the neonate (persistent fetal circulation syndrome) Clin In Perinat. 1984 Year Book Med Publishers 61-91.
85. Walther FJ, Benders M, Leighton JO. Early changes in the neonatal circulatory transition. J Pediatrics 1993; 123:625-32.
86. Chamberlain D, Hislop A, Hey E, Reid LM. Pulmonary hypoplasia in babies with severe rhesus isoimmunisation: a quantitative study. J Pathol 1977; 122:43-52.
87. Hislop A, Hey E, Reid L. The lungs in congenital bilateral agenesis and dysplasia. Arch Dis Child 1979; 54:32-8.
88. Geggel RL, Reid LM. The structural basis of PPHN. Clin Perinatol 1984; 3:525-49.
89. Levin DL. Morphologic analysis of the pulmonary vascular bed in congenital left-sided diaphragmatic hernia. J Pediatr 1978; 92:805-9.
90. Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T. Pulmonary acinar development in diaphragmatic hernia. Arch Pathol Lab Med 1991; 115:372-6.
91. Voelkel NF. Mechanisms of hypoxic pulmonary vasoconstriction. Am Rev Respir Dis 1986; 133:1186-95.
92. Stenmark KR, Badesch D, Durmowicz A, Voelkel N. Control of pulmonary vascular tone and cell proliferation. In: Developmental physiology of the lung. 1993; pp 169-87.
93. Kluth D, Petersen C, Zimmermann HJ, Mulhaus K. The embryology of congenital diaphragmatic hernia. Mod Probl Paediatr, Puri P (ed), 1989; 24:7-21.
94. Holder RM, Ashcraft KW. Congenital diaphragmatic hernia. In: Ravitch MM, et al. Pediatric

Surgery 3rd edn(1979). Year Book Medical Publishers. Chicago, 432-45.

95. Gattone VH, Morse DE. A scanning electron microscopic study on the pathogenesis of posteriolateral diaphragmatic hernia. J Submicrosc Cytol 1982; 14:483-90.
96. Ten Have-Opbroek AAW. Lung development in the mouse embryo. Exp Lung Res 1991; 17:111-30.
97. Ten Have-Opbroek AAW. The development of the lung in mammals: An analysis of concepts and findings. Am J Anat 1981; 162:201-19.
98. Ten Have-Opbroek AAW. Immunological study of lung development in the mouse embryo: II the first appearance of the great alveolar cell, as shown by immunofluorescence microscopy. Dev Biol 1979; 69:408-23.
99. Otto-Verberne CJM, Ten Have-Opbroek AAW. Development of the pulmonary acinus in the fetal rat lung: A study based on an antiserum recognizing surfactant associated proteins. Anat Embryol 1987; 175:365-73.
100. Otto-Verberne CJM, Ten Have-Opbroek AAW, Balkema JJ, Franken C. Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant associated proteins. Anat Embryol 1988; 178:29-39.
101. Harrison MR, Jester JA, Ross NA. Correction of congenital diaphragmatic hernia in utero.I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. Surgery 1980; 88:174-82.
102. DeLorimier AA, Tierney DF, Parker HR. Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. Surgery 1967; 62:12-7.
103. Kent GMK, Olley PM, Creighton RE, Dobbinson T. Hemodynamic and pulmonary changes following surgical creation of a diaphragmatic hernia in fetal lambs. Surgery 1972; 72:427-33.
104. Ohi R, Suzuki H, Kato T, Kasai M. Development of the lung in fetal rabbits with experimental diaphragmatic hernia. J Pediatr Surg 1976; 11:955-9.
105. Matsuda K, Kato K, Hebigichi K, Koyama K. Histometrical investigation of pulmonary vascular system in experimental diaphragmatic hernia. Keio J Med 1988; 37:24-36.
106. Harrison MR, Bressack MA, Churg AM, DeLorimier AA. Correction of congenital diaphragmatic hernia in utero. II. Simulated correction permits fetal lung growth with survival at birth. Surgery 1980; 88:260-8.
107. Harrison MR, Ross N, DeLorimer AA. Correction of congenital diaphragmatic hernia in utero. III. Development of successful surgical technique using abdominoplasty to avoid compromise of umbilical blood flow. J Pediatr Surg 1981; 16:934-42.
108. Adzick NS, Outwater KM, Harrison MR, Davies P. Correction of congenital diaphragmatic hernia in utero IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 1985; 20:673-80.
109. Alcorn DG, Adamson TM, Maloney J, Robinson PM. A morphometric analysis of fetal lung development in sheep. Anat Rec 1981; 201:655-67.
110. Pringle KC, Turner JW, Schofield JC, Soper RT. Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 1984; 19:131-40.
111. Glick PL, Stannard VA, Leach CL, Rossman J. Pathophysiology of congenital diaphragmatic hernia II: the fetal lamb CDH model is surfactant deficient. J Pediatr Surg 1992; 27:382-8.
112. Moessinger AC, Bassi GA, Blanc WA. Experimental production of pulmonary hypoplasia following amniocentesis and oligohydramnios. Early Hum Dev 1983; 8:343-50.
113. Docimo SG, Luetic T, Crone RK, Davies P. Pulmonary development in the fetal lamb with severe bladder outlet obstruction and oligohydramnios: a morphometric study. J Urology 1989; 142:657-60.
114. Moessinger AC, Harding R, Adamson TM, Singh M. Role of lung fluid volume in growth and maturation of the fetal sheep lung. J Clin Invest 1990; 86:1270-7.
115. Peters CA, Reid LM, Docimo S, Luetic T. The role of the kidney in lung growth and maturation

in the setting of obstructive uropathy and oligohydramnios. J Urology 1991; 146:597-600.

116. Adzick SN, Harrison MR, Glick PL, Villa RL. Experimental pulmonary hypoplasia and oligohydramnios: relative contributions of lung fluid and fetal breathing movements. J Pediatr Surg 1984; 19:658-63.

117. Wilson JM, DiFiore JW, Peters CA. Experimental fetal tracheal ligation prevents the pulmonary hypoplasia associated with fetal nephrectomy: possible application for congenital diaphragmatic hernia. J Pediatr Surg 1993; 28:1433-40.

118. DiFiore JW, Fauza DO, Slavin R, Peters CA. Experimental fetal tracheal ligation reserves the structural and physiological effects of pulmonary hypoplasia in congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:248-57.

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

120. Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Lachmann B, Molenaar JC; Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1990;25: 426-9.

121. Adler I, Jones B. Nitrofen. From: Analytical methods for pesticides and plant growth regulates. Vol X, page 403-14 (1978); G. Zweig and J. Slierwa (eds). Acad Press.

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

123. Costlow R, Manson JM. Effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (nitrofen) on fetal lung development in rats. Toxicol 1981; 20:195-207.

124. Costlow R, Manson JM. The heart and the diaphragm: target organs in the neonatal death induced by nitrofen. Toxicol 1981; 20:209-27.

125. Costlow R, Manson JM. Distribution and metabolism of the teratogen nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) in pregnant rats. Toxicol 1983; 26:11-23.

126. Kiryishin VA. Mutagenic action of various pesticides in case of their successive entry into the body of albino rats. Gig Sanit 1974; 9:43-52.

127. Brown TJ, Manson JM. Further characterization of the distribution and metabolism of nitrofen in the pregnant rat. Teratology 1986; 34:129-39.

128. Manson JM. Mechanism of nitrofen teratogenesis. Environ Health Perspec 1986; 70:137-47.

129. Manson JM, Brown T, Baldwin DM. Teratogenicity of nitrofen (2,4-dichloro-4'-nitrodiphenyl ether) and its effects on thyroid function in the rat. Toxicol Appl Pharmacol 1984; 73:323-35.

130. Hamburgh M. An analysis of the action of thyroid hormone on development based on in vivo and in vitro studies. Gen Comp Endocrin 1968; 10:198-213.

131. Zeman FJ, Heng H, Kavlock RJ. Cell number in selected organs of fetuses of rats malnourished and exposed to nitrofen. Terat Carc and Mutag 1986; 6:339-47.

132. Lau C, Cameron AM, Irsula O. Effects of prenatal nitrofen exposure on cardiac structure and function in the rat. Toxicol Appl Pharmacol 1986; 86:22-32.

133. Lau C, Cameron AM, Irsula O. Teratogenic effects of nitrofen on cellular and functional maturation of the rat lung. Toxicol Appl Pharmacol 1988; 95:412-22.

EXPERIMENTALLY INDUCED CONGENITAL DIAPHRAGMATIC HERNIA IN RATS

(J Pediatr Surg 1990;25:426-9)

Abstract

We report our experiments to induce congenital diaphragmatic hernia (CDH) in rats by means of administering a single dose of 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen) on the tenth day of gestation.The rat model, including a control group, was used to evaluate lung development and the presence of lung hypoplasia by morphometrical analysis. We found that the single dose of Nitrofen given five days before the normal closure of the diaphragm in the rat leads to a high incidence of diaphragmatic hernia -mainly on th right side- and highly abnormal lung development (hypoplasia) comparable with the human situation.Both lung weight/bodyweight index as well as radial alveolar counts were significantly lower in animals with CDH ($p < 0.05$). This animal model offers a good opportunity to study abnormal lung development in relation to ventilatory capacity and pulmonary vascular reactivity.

INTRODUCTION

The high mortality rate due to congenital diaphragmatic hernia (CDH) remains a major challenge for pediatric surgeons and neonatologists. Many questions concerning pulmonary vascular abnormalities, the extent of lung hypoplasia and its reaction pattern on artificial ventilation remain (1,2,3). These factors are hard to evaluate in the clinical situation. For this reason several workers (4-9) have used sheep for experimental studies. In a relatively late stage of fetal development diaphragmatic hernia was induced. Only Adzick (10) interfered with pulmonary development early in gestation. All these experimental series suffer from relatively small numbers, high costs and the need for surgical intervention to obtain a CDH model.

In 1984 Iritani (11) published successful procurement of neonatal CDH after oral administration of 2,4-dichlorophenyl-P-nitrophenyl (Nitrofen) for prolonged periods during gestation in mice. However, no details about pulmonary development or morphometrical analysis of the lungs were presented. Nitrofen has been reported earlier as a teratogen (12) leading to a high incidence of CDH in certain rat strains, especially Sprague-Dawley. In our experiments to induce CDH, Nitrofen was used to study lung development and to evaluate the presence of lung hypoplasia by morphometrical analysis.

MATERIAL AND METHODS

Female Sprague-Dawley rats, weighing between 200-269 g bodyweight (Centraal Proefdierenbedrijf Harlan, Zeist, The Netherlands) were mated overnight (during 12 hours) with proven Sprague-Dawley males. The day of mating was considered as day 0 of pregnancy.The animals were housed separately in an airconditioned room; water and food (Rat Diet, Hope Farms, Woerden, The Netherlands) were supplied ad libitum. After mating, females were divided into two group: Control and Nitrofen. At day 10 of pregnancy 115 mg/kg bodyweight of 2,4-dichlorophenyl-P-nitrophenyl (Nitrofen) dissolved in olive oil was given as a single dose through a gastric tube.The control group were given the same volume of olive oil without Nitrofen. Pregnancy was continued without specific measurements.

Table 1. Congenital Diaphragmatic Hernia in Rats

	No.	Body Weight (g) [SE]	Lung Right (mg) [SE]	Lung Left (mg) [SE]	Lung Total (mg) [SE]	L/B Ratio (mg/g) [SE]	R/L Ratio (mg/mg) [SE]
Before Birth							
Control	24	5.7 [0.3]	99.7 [10.0]	52 [8.7]	151.7 [17.3]	26.76 [8.30]	1.91 [0.03]
Nitrofen	23	5.0 [0.3]*	86.0 [8.0]*	46.5 [4.3]*	132.5 [11.2]*	26.34 [2.84]*	1.85 [0.12]
CDH	11	4.8 [9.3]*†	58.5 [15.3]*†	33.5 [9.3]*†	91.9 [24.1]*†	19.29 [4.35]*†	1.78 [0.22]
After Birth							
Control	32	5.9 [0.3]	73.4 [14.3]	38.1 [8.6]	111.6 [22.6]	18.9 [4.0]	1.94 [0.12]
Nitrofen	34	5.7 [0.4]*	57.9 [8.0]*	31.5 [4.0]*	89.4 [11.7]*	16.8 [1.70]*	1.84 [0.14]
CDH	33	5.51 [0.4]*	48.2 [7.6]*†	26.5 [4.6]*†	74.8 [11.0]*†	13.5 [1.50]*†	1.83 [0.26]

Abbreviations: SE, standard error; L/B, lung weight/body weight; R/L, right lung/left lung.

*Significant from control, $P < .05$.

†Significant from Nitrofen, $P < .05$.

In both groups a part of the mothers were allowed to deliver spontaneously on day 22.5 while on the remaining part a ceasarean section was performed on day 21 of gestation. The number of live births and the number of living newborns with respiratory insufficiency was evaluated. Following determination of bodyweight all animals were killed by intraperitoneal injection of an overdose of pentobarbital (Nembutal, Sanofi, The Netherlands) followed by autopsy (Stuckhardt 1984).

The number of animals with a diaphragmatic defect, the position (right or left sided) and the size of the defect as well as the nature of the intrathoracic contents (liver, bowel, pancreas, stomach) were evaluated. The lungs were dissected and left and right wet lung weights were determined using a balance (Mettler, Haarlem, The Netherlands) enabling calculation of lung weight/bodyweight ratio.

In 26 newborns with CDH, left and right lung weight were measured and related to the size of the diaphragmatic defect and the intrathoracic contents.

For our microscopic studies the pulmonary arterial trunk was cannulated and perfusion was performed with Davidson solution (40 vol% ethanol 100%; 5 vol% acetic acid 96%; 10 vol% formaldehyde 37%; 45 vol% saline, pH 7.3) under standard conditions until the pulmonary veins no longer contained blood.To prevent lung collapse after tracheal cannulation, the lungs were inflated with room air under a constant pressure of 20 cm H_2O while arterial perfusion with Davidson solution was performed. Following perfusion the trachea was ligated and the whole animal was placed in fixative for at least 24 hours (13). After fixation the lungs were dissected out of the thoracic cavity and processed for routine histology after embedding in paraffin. Six micron slides were made and stained with haematoxylin eosin (HE), Elastica Van Giesson (EVG), Alcian Blue or Azan.

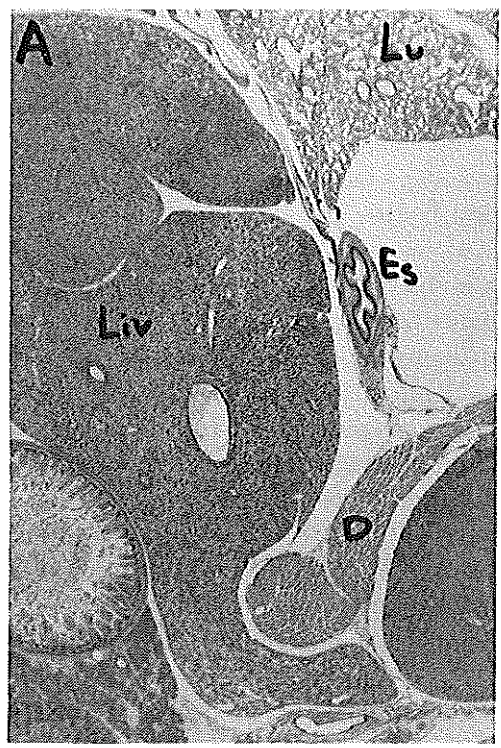
Morphometric analysis to determine radial alveolar count (RAC) (14,15) was performed from at least 3 different slides using magnification 250x. At least 10 RAC's per slide were evaluated.

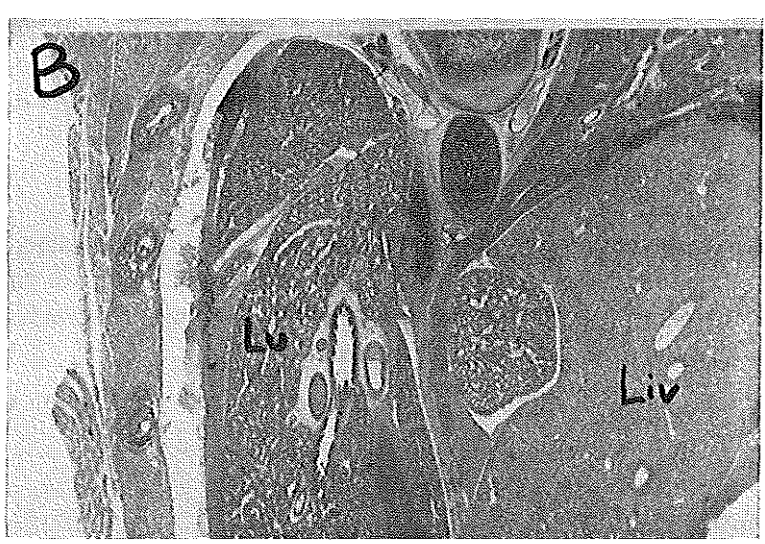
In the group of newborn animals with CDH radial alveolar counts were plotted versus lung weight/bodyweight index according to Askenazi (16). The same procedure was performed in a number of animals (N=10 for each group) irrespective of the presence of a CDH in the Nitrofen group as well as in the untreated controls.

Statistical Analysis

All data are reported as mean $\pm$ Standard Deviation, unless otherwise stated.Differences between the means were tested by the Mann-Whitney-Wilcoxon test for unpaired samples. Where it was allowed the Student T-test was also used. P values less than 0.05 were considered to be statistically significant.

Figure 1. [A] Typical example of congenital diaphragmatic hernia in newborn rat. Lu lung; Liv liver; Es esophagus; D remaining part of the diaphragm [B] Posterior section observed through the upper half of the thorax. Hernia is located on the left side. (H&E, 6µm slide original magnification x60)





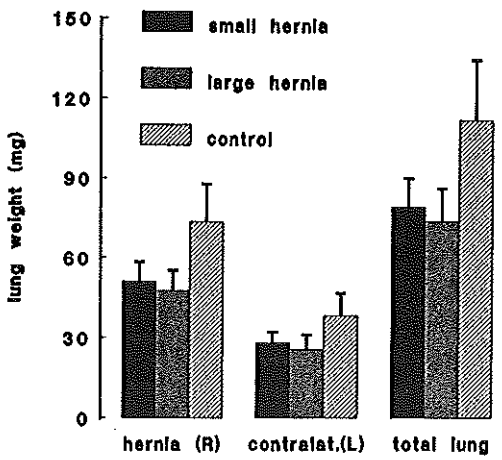


Fig 2. Ipsilateral and contralateral lung weights in CDH compared with controls.

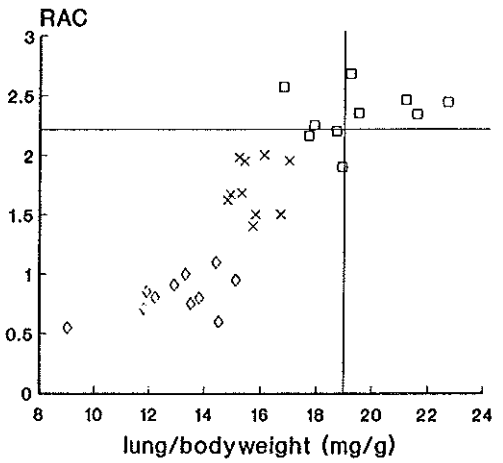


Fig 3. Radial alveolar count versus the lung weight/body weight index in CDH (◊), Nitrofen exposed without CDH (X), and control (□) animals.

RESULTS

Only in the newborn group with CDH was severe respiratory insufficiency observed leading to death in more than 80% of the animals within two hours postpartum. High ventilatory rate, persistent cyanosis with the inability to suck spontaneously were observed. The total percentage of CDH in the treated group ranged between 30-80% with a mean value of 40 %. In animals who did not acquire CDH no obvious respiratory difficulties were notified, like in the control group. Mean body weights, lung weights, lung/body weight ratio (L/B) and right/left lung weight (R/L) index including standard deviations are shown in Table I. The mean bodyweight differed significantly in the Nitrofen treated group compared with controls. The same was observed for total lung weight both at day 21 as well as post partum.

Right sided CDH occurred eight times more than left sided CDH. In most animals the defect was observed in the posterior region of the right diaphragm, while in the minority of cases an almost total absence of the right hemi-diaphragm was observed. A part of the whole right liver lobe extended in the right hemi-diaphragm directly adjacent to the right lung (see figure 1). In a number of cases bowel loops and pancreas were positioned in the right hemi-thorax. Eventration and laxation of the diaphragm was never observed.Figure 2 shows contra- and ipsilateral lung weight in CDH are shown in relation to the size of the diaphragmatic defect. No significant correlation was found between the size of the defect and the ipsilateral lung weight. In all cases a significant

decrease in lung weight was also observed on the contralateral side. This is also illustrated by the values of the R/L index : throughout the six groups there is no significant difference (table I). In all cases of right sided diaphragmatic hernia the right and left lung weight was significantly lower than in control animals. No correlation was found between the presence of liver and/or bowel loops, the extent of the diaphragmatic defect and the corresponding lung weight.

Microscopic evaluation in case of diaphragmatic hernia revealed thick intra-alveolar septa, retarded development in comparison with controls resembling the canalicular stage of development. Only a few number of sacculi which are normally present at birth were notified. Radial alveolar count was significantly lower in CDH and Nitrofen treated animals than in controls (Figure 3). Animals treated with Nitrofen without CDH, however, showed high radial alveolar counts than animals with diaphragmatic defect. The radial alveolar counts plotted against lung-bodyweight index indicate severe hypoplasia of the lung in case of diaphragmatic hernia.

DISCUSSION

CDH has been induced in sheep to mimic the diminished lung growth and the effect of antenatal repair on the developing lung (4-9). Besides the technical problems related to the manipulation of these animals, interference with pulmonary development in these animals has been rather late (second trimester) or resulted in high mortality following early intervention (10).

It is generally accepted that a diaphragmatic hernia in human is the result of defective development of the diaphragm during the first trimester of gestation, leading to abnormal lung development far before the critical period (17) in lung development (between 20th and 24th week of gestation). Although Iritani (11) reported the incidence of CDH in a strain of mice after prolonged exposure to Nitrofen and a high rate of mortality, his study focused mainly on the abnormal development of the diaphragm itself in relation to the phrenic nerve. Detailed information about the morphological development of the lung and consequently of the significance of this animal model for understanding the etiology and pathogenesis of the human situation remains unclear from his report.

Our results show that a single dose of a herbicide given five days before normal closure of the diaphragm in the rat (18) leads to high incidence of diaphragmatic hernia (mainly on the right side) and severely abnormal lung development (hypoplasia) comparable with the human situation. We cannot explain why the diaphragmatic defect occurs in a high incidence on the right side. Data from relevant literature show that timing of the administration of the herbicide is essential to induce a high incidence of CDH suggesting a critical period of development to interfere with normal diaphragmatic development (12,19).It is speculated that a different time of Nitrofen administration could lead to a different ratio of right and left sided CDH. The abnormal lungs in the Nitrofen exposed

rats without CDH suggest that the lungs are the primary target organ for Nitrofen (19). The significance of this observation and the fact that no clear connection was found between the size and place of the the missing part in the diaphragm and the ipsilateral lungweight may lead to new thoughts about the primary problem in CDH.

The idea that pulmonary hypoplasia and diaphragmatic hernia is dependent only on the compression of the early developing lung as well as the position of the bowel loops in relation to the diaphragmatic defect during early stages of development needs re-evaluation.

It is concluded that CDH can be successfully induced in rats at an early stage of development before the normal time of closure of the diaphragm. This animal model offers a new opportunity to study abnormal lung development related to both the ventilatory capacity as well as to pulmonary vascular reactivity.

REFERENCES

1. Vacanti JP, Crone RK, Murphy JD: The pulmonary hemodynamic response to perioperative anesthesia in the treatment of high risk infant with congenital diaphragmatic hernia. J Pediatr Surg 1984; 19:672-9.
2. Geggel RL, Murphy JD, Langleben D: Congenital diaphragmatic hernia: arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 1985; 107:457-64.
3. Shochat SJ: Pulmonary vascular pathology in congenital diaphragmatic hernia. Pediatr Surg Int 1987; 2:331-5.
4. Haller JA, Signer RD, Golladay ES, et al: Pulmonary and ductal hemodynamics in studies of simulated diaphragmatic hernia of fetal and newborn lambs. J Pediatr Surg 1976; 11:675-80.
5. Harrison MR, Jester JA, Ross NA: Correction of congenital diaphragmatic hernia in utero. I. The model. Intrathoracic balloon produces fetal pulmonary hypoplasia. Surgery 1980; 88:174-82.
6. Harrison MR, Bressack MA, Churg AM, et al: Correction of congenital diaphramatic hernia in utero. II. Simulated correction permits fetal lung growth with survival at birth. Surgery 1980; 88:260-8.
7. Harrison MR, Ross NA, de Lorimer AA: Correction of congenital diaphragmatic hernia in utero. III. Development of a successful surgical technique using abdominoplasty to avoid compromise of umbilical blood flow. J Pediatr Surg 1981; 16:934-42.
8. Pringle KC, Turner JW, Schofield JC, Soper RT; Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 1984; 18: 131-140.
9. DeLuca U, Cloutier R, Laberge JM, Fournier L and Guttman FM,1987, Pulmonary barotrauma in congenital diaphragmatic hernia: experimental study in lambs, J Pediatr Surg, 22: 311.
10. Adzick NS, Outwater KM, Harrison MR, et al: Correction of congenital diaphragmatic hernia in utero. IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 20: 673-680, 1985
11. Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169: 133-139, 1984
12. Costlow RD, Manson JM. The heart and diaphragm: target organs of nitrofen. Toxicology 1981;20:209-27.
13. Burri P. Postnatal growth of the rat lung I. Morphometry. Anat Rec 1974; 178:711-30.

14. Emery Jl. The number of alveoli in terminal respiratory unit of man during late intrauterine life and childhood.Arch Dis Child 1960; 35:544-7.
15. Cooney TP, Thurlbeck WM. The radial alveolar count method: A reappraisal I. Postnatal lung growth. Thorax 1982; 37:572-9.
16. Askenazi SS, Perlman M: Pulmonary hypoplasia: lung weight and radial alveolar count as criteria of diagnosis. Arch Dis Child 54: 614-618, 1979
17. Inselmann LS, Mellins RB. Growth and development of the lung J Pediatr 1981; 98:1-15.
18. Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W; Nitrofen-induced diaphragmatic hernia in rats: an animal model. J Ped Surg 1990;25:850-4.
19. Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Perspect 70: 137-147, 1986

Nitrofen-Induced Diaphragmatic Hernias in Rats: An Animal Model

(J Pediatr Surg 1990; 25:850-854)

Abstract

In embryological terms, pathogenesis of congenital diaphragmatic hernia (ICDH) associated with pulmonary hypoplasia is still unclear. However, it is known since 1971 that Nitrofen (2,4 dichloro-phenyl-p-nitrophenyl ether) can induce anatomical malformations in rats including diaphragmatic hernias. On order to establish an animal model of the embryogenesis of CDH, the effect of Nitrofen on the developing diaphragm was studied. Thirty-three pregnant female rats were exposed to Nitrofen. Five unexposed pregnant rats served as controls. In the first set of experiments, single doses of Nitrofen were given between the 9th and 13th day of pregnancy. In the second set of experiments, dosages of 50, 100, and 150 mg per animal were given on day 11 of pregnancy only. Postnatally the litters (469 newborn rats) were dissected to record the incidence of diaphragmatic malformations. The results were: (1) most hernias occurred after administration of 100 mg Nitrofen on day 9 (42%) and 11 (59%); (2) left-sided hernias were observed only after exposure to Nitrofen on day 9; (3) after exposure on day 10 or later all hernias were on the right side; and (4) Fifty-nine percent of the newborn rats exposed on day 11 had CDH. These results show that this model is suitable for further embryological investigations on the development of CDH.

The pathogenesis of congenital diaphragmatic hernia (CDH) associated with pulmonary hypoplasia is poorly understood[1]. Most authors speculate that the intestine enters the thoracic cavity through a dorsolateral diaphragmatic defect with subsequent secondary hypoplasia of the lungs. However, this sequence of embryological events has not been studied previously. Progress has been hampered by lack of an appropriate animal model allowing definition of morphological changes during embryogenesis specific for this malformation.

Since 1971, the embryotoxicity of the herbicide Nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether) has been well known[2]. After exposure to Nitrofen several malformations were observed in rats, including diaphragmatic hernias[3-6].

In order to use this substance for the establishment of an animal model for diaphragmatic hernias, the following questions need to be answered: (1) what percentage of newborn rats will present with diaphragmatic hernias after exposure to Nitrofen during embryogenesis? (2) what dosages of Nitrofen are necessary to induce these malformations? and (3) in what gestational age is the embryo most sensitive to Nitrofen?

MATERIALS AND METHODS

Adult female Sprague-Dawley rats weighing between 210 and 315 g (mean, 271 g), were bred under standard laboratory conditions. After controlled overnight mating, pregnancy was verified by means of the vaginal smear method. The day of positive smear was rated as day zero of pregnancy.

The pregnant rats were exposed to orally administered Nitrofen (Wako Chemicals, Neuss, West Germany). The substance was mixed with 20 g of moistened commercial rat chow (Altromin, Lage, West Germany) and offered to the rats after a 24-hour period of fasting. Rats not consuming this mixture within 12 hours were excluded. Using this method, 33 pregnant rats were successfully exposed to Nitrofen. Thereafter the rats were again supplied with food and water ad libitum and spontaneous delivery was allowed.

Five animals, after undergoing a 24-hour fast, served as controls. Two experimental designs were used. In a first set of experiments, a single dose of 100 mg Nitrofen was given to rats on day 9, 10, 11, 12, or 13 of pregnancy. There were five rats in each group. In a second set of experiments, dosages of 50, 100, or 150 mg of Nitrofen were given to rats on day 11 of pregnancy only. There were four rats in each groups receiving 50 or 150 mg Nitrofen. The animals comprising the 100-mg group were taken from the first experimental set (five animals).

After spontaneous delivery all newborn rats were killed, promptly dissected microscopically, and than fixed in Bouin's solution. The number of CDHs per litter, the size, and the location were recorded. Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance test in experiment 1 and regression analysis (linear model) in experiment 2.

RESULTS

A total of 469 newborn rats were dissected microscopically (Table 1). Among 388 rats born to mothers exposed to Nitrofen during pregnancy, 94 had diaphragmatic hernias (24%). In all cases of CDH the liver was in the thoracic cavity. Additionally, in 15 of 94 newborns with CDH (16%) the stomach or the intestine was also present in the thoracic cavity.

Table 1. Overall Results

Group	No. of Female Rats	No. of Newborns
Nitrofen	33	388
Control	5	81
Total	38	469
Observed hernias (total)		94 (24%)

NOTE. Hernias were observed in the Nitrofen group only.

Malformations of other organ systems were not observed on routine dissections in this sample. Eighty-one newborns (control group) were not exposed to Nitrofen in utero. None of these newborns had CDH.

The results of the first set of experiments are shown in Tables 2, 3, and 4. In Table 2, the relative frequency of diaphragmatic hernias, the maternal body weight, and the number of siblings per litter are recorded. The relative frequency of diaphragmatic hernias was 42% for the day 9 group and 59% for the day 11 group. This was statistically significant compared with the data from groups 10, 12, and 13 ($P < .002$). Maternal body weight and number of siblings were equally distributed. Statistical analysis failed to detect any impact of maternal body weight or number of siblings on the relative frequency of diaphragmatic hernias.

Table 2. Results of the First Set of Experiments

Day of Exposure	Sibs/Litter (avg)	Weight (mat) in g (avg)	Hernias (%) (avg)
9	11.4	260.8	41.7*
10	10.8	290.4	18.8
11	10.4	275.0	59.0*
12	11.2	284.6	7.2
13	14.0	270.0	6.0

NOTE. Neither the number of siblings per litter nor maternal weight influenced the relative number of observed congenital diaphragmatic hernias. The day of exposure was the only variable that significantly (*) influenced the number of hernias.

Abbreviations: sibs, siblings; mat, maternal.

*$P < .001$.

Table 3. Size of Observed Hernias (First Experiment)

Size	No.
Small	21
Medium	16
Large	36

In Table 3 the size of CDH is recorded. The size of the hernia was considered as small when it was totally covered by the lung, and as medium when liver and bowel loops represented less than 50% of the thoracic contents. The size of the hernia was considered large when over 50% of the thoracic contents were liver and/or bowel loops. According to this score, 21 small hernias (Fig 1), 16 medium-size hernias (Fig 2), and 36 large hernias (Fig 3) were observed. Table 4 shows the location of the observed hernias

Table 4. Location of Hernias

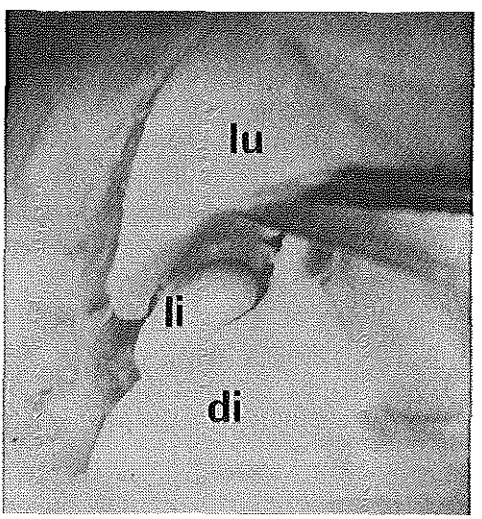
Day of Exposure	Left	Right	Bilateral
9	1	14	3
10		8	
11		31	
12		4	
13		4	

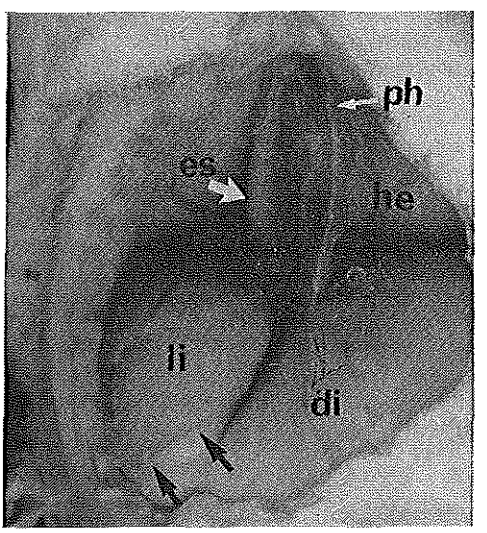
on the right or left side depending on the day of Nitrofen exposure. The vast majority were seen on the right side. The left side was involved only in animals exposed on day 9. These rats also showed bilateral hernias.

The results of the second set of experiments are shown in Table 5. The highest number of diaphragmatic hernias was observed in the group receiving 100 mg of Nitrofen per animal. Using regression analysis, there was no linear correlation between the number of hernias observed and the dosage of Nitrofen exposure in the range between 172 and 641 mg/kg body weight (correlation coefficient .4447, r^2 = 19.8%).

No left-sided hernias were observed in this experiment. The size of the hernias was always small in the 50-mg group, although in the 100- and 1 50-mg groups medium and large hernias were regularly observed.

Fig 1. Typical view of a small right-sided CDH in a newborn rat after Nitrofen exposure. The small knob-like structure is liver (li) and represents the dorsally located CDH: lu, lung (lifted with forceps); di. diaphragm.
Fig 2. Typical view of a medium-sized right-sided CDH in a newborn rat after Nitrofen exposure. Lung is dissected out. The liver (li) herniates into the thoracic cavity through a dorsal diaphragmatic defect (black arrows). di, Ventral diaphragm; he, heart; es, esophagus; ph, phrenic nerve with vein cava inferior.





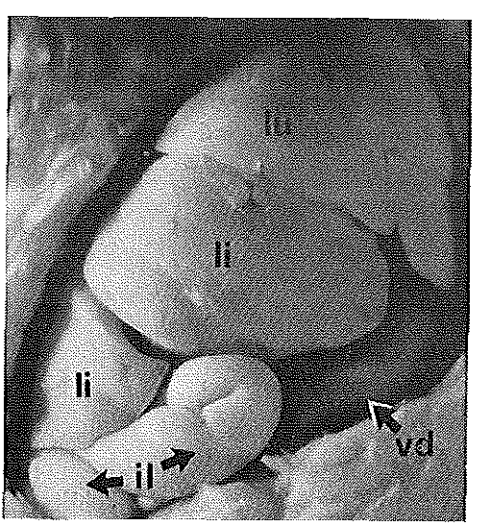


Fig 3. Large right-sided CDH in a newborn rat after Nitrofen exposure. Two lobes of liver (li) and intestinal loops herniate through 8 dorsal diaphragmatic defect. The lung (lu) is small; vd, ventral part of diaphragm.

DISCUSSION

Although surgical treatment of CDH is simple, the mortality associated with this malformation is still high. This is due to the fact that in many of these children the lungs are too hypoplastic to allow normal extrauterine life even in full-term babies [7,8]. Many investigators state that lung hypoplasia results from intrathoracally displaced viscera preventing normal development of the lungs[9-13]. This view has been supported by data of DeLorimier et al[14], and Harrison et al[15], obtained in a sheep model of CDH. As a consequence, fetal surgery has been advocated, including in utero correction of CDH, aimed at preventing severe intrauterine lung damage[16]. However, the beneficial effects of intrauterine intervention, effective in the sheep model, proved of limited value in humans[17]. Pathogenesis of CDH and the associated hypoplasia of the lungs remained elusive. To gain precise informations about the natural history of CDH, its development must be studied in a suitable animal model using embryos in different stages.
This model should meet the following criteria. The number of hernias per litter should be high. Phases of high and low sensitivity to the substance used to induce the malformation should be clearly discernible. Using 100 mg Nitrofen on day 11 of pregnancy, diaphragmatic hernias could be observed in almost 60% of the newborns.

Table 5. Results of Second Set of Experiments

Total Dosage of Nitrofen (mg)	Siblings/Litter (avg)	Maternal Weight (g) (avg)	Hernias (%) (avg)	Dosage mg Nitrogen/kg (avg)	Small Hernia (%)	Large Hernia (%)
0	15.2	284.6	0	0	0	0
50	11.8	249.0	11.1	202.6	100	0
100	10.4	275.0	59.0*	369.0	51.6	48.5
150	11.3	250.5	21.8	602.0	57.1	42.9

NOTE. The number of hernias depends only on the dosage of Nitrofen. A peak results after the 100 mg dosage; a higher dosage does not result in more hernias.
*$P < .002$.

This number is adequate for further embryological studies. These data are in accordance with the study of Iritani[6], who demonstrated CDH in up to 80% of newborn mice exposed to Nitrofen continuously between day 5 and 15 of pregnancy. These data were also confirmed by a previous study from our laboratories[18]. Pulse-feeding of Nitrofen for a maximum period of 12 hours demonstrates the presence of two sensitivity peaks, one on day 9 and a second on day 11 of pregnancy. This is precisely the time when lung anlage and diaphragmatic primordium start to develop in rat embryos[19]. Moreover, in this period the early anlage of the lung and primordium of the diaphragm are in close spatial relationship[20]. After day 12, Nitrofen sensitivity sharply declines, confirming observations made earlier by Iritani[6]. This means that sensitivity to Nitrofen is minimal at the time when the pleuroperitoneal canals disappear. This has been shown to take place on day 17 of pregnancy in normal rat embryos[20]. Consequently, Nitrofen seems to interfere with the formation of the diaphragmatic anlage rather than with the closure of its so-called pleuroperitoneal canals. Interestingly, left-sided diaphragmatic hernias

were only observed after exposure to Nitrofen on day 9. If Nitrofen was given on day 10 or later only rightsided hernias were observed. Thus, Nitrofen sensitivity peaks earlier on the left side than on the right side. This means that in early embryological stages the left diaphragm anlage is more advanced in development than the right anlage, contrary to what had been assumed heretofore in human embryos[9-13].

These results demonstrate that the described model is an valuable tool for further embryological investigations of CDH and the associated pulmonary hypoplasia. This model allows us to address the following questions: (1) which parts of the diaphragm are affected during early stages of the malformation? (2) at what time signs of pulmonary hypoplasia become visible? and (3) does pulmonary hypoplasia occur as a primary event or as a result of displaced, intrathoracic viscera?

REFERENCES

1. Kluth D, Petersen C, Zimmerman HJ: The developmental anatomy of congenital diaphragmatic hernia. Pediatr Surg Int 2:322-326, 1987
2. Ambrose AM, Larson PS, Borcelleca JF, et al: Toxicological studies on 2,4-dichlorophenyl-p-nitrophenyl ether. Toxicol Appl Pharmacol 19:263-275, 1971
3. Kimbrough RD, Gaines TB, Linder RE: 2,4-Dichlorophenyl-pnitrophenyl ether (TOK), effects on the lung maturation of rat fetus. Arch Environ Health 28:316-320, 1974
4. Nakao Y, Iritani I, Kishimoto H: Experimental animal model of congenital diaphragmatic hernia induced chemically. Teratology 24:11A, 1981 (abstr)
5. Costlow RD, Manson JM: The heart and diaphragm: Target organs in the neonatal death induced by nitrofen (2,4-dichloro-phenylp-nitrophenyl ether). Toxicology 20:209-227, 1981
6. Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984
7. Harrison MR: Fetal diaphragmatic hernia, in Puri P (ed): Congenital Diaphragmatic Hernia: Modern Problems in Paediatrics, vol 24. Basel, Switzerland, Karger, 1989, pp 130- 142
8. Bohn D, Tamura M, Perrin D, et al: Ventilatory predictors of pulmonary hypoplasia in congenital diaphragmatic hernia, confirmed by morphologic assessment. J Pediatr 111:423-431, 1987
9. Broman I: Uber die Entwicklung des Zwerchfells beim Menschen. Verh Anat Ges 16:9-17, 1902
10. Bremer JL: The diaphragm and diaphragmatic hernia. Arch Pathol 36:539-549, 1943
11. Wells LJ: Development of the human diaphragm and pleural sacs. Contr Embryol Carnegie Inst 35:107-137, 1954
12. Gray SW, Skandalakis JE: Embryology for Surgeons. Philadelphia, PA, Saunders, 1972, pp 359-385
13. Starck D: Embryologie. New York, NY, Thieme, 1975, pp 488-500
14. deLorimier AA, Tierney DF, Parker HR: Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. Surgery 62:12-17, 1976
15. Harrison MR, Jester JA, Ross NA: Correction of congenital diaphragmatic hernia in utero. I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. Surgery 88:174- 182, 1980
16. Harrison MR, Bressack MA, Churg AM: Correction of congenital diaphragmatic hernia in utero. II. Simulated correction permits fetal lung growth with survival at birth. Surgery 88:260-268, 1980
17. Harrison MR, Langer JC, Adzick NS, et al: Fetal diaphragmatic hernia: Prognostic factors and preliminary experience with repair before birth. Presented at the 20th Annual Meeting of the American Pediatric Surgical Association, Baltimore, MD, May 28-31, 1989
18. Tenbrinck R, Tibboel D, Gaillard JLJ, et al: Experimentally induced congenital diaphragmatic hernia

in rats. J Pediatr Surg 25:426-429, 1990

19. Witschi E: Development of the rat, in Altman P, Dittmer DS (eds): Growth Including Reproduction and Morphological Development. Washington, DC, Fed Soc Experiment Biol, 1962, pp 304-414

20. Kluth D, Petersen C, Zimmermann HJ, et al: The embryology of congenital diaphragmatic hernia, in Puri P (ed): Congenital Diaphragmatic Hernia: Modern Problems in Paediatrics, vol 24. Basel, Switzerland, Karger, 1989, pp 7-21

PULMONARY VASCULAR ABNORMALITIES IN EXPERIMENTALLY INDUCED CONGENITAL DIAPHRAGMATIC HERNIA IN RATS.

(J Pediatr Surg 1992; 27:862-5)

ABSTRACT

In infants with congenital diaphragmatic hernia (CDH), abnormalities of the pulmonary arteries are present consisting of increased medial wall thickness and decreased external diameter. This forms the morphological substrate for persistent pulmonary hypertension, one of the leading causes of the high mortality in these patients. To elucidate the significance of these abnormalities, experimental models are required that mimic as close as possible the human situation.

In our rat model we are able to study the hypoplastic CDH lungs extensively. In this study we performed a histological evaluation of the pulmonary arterial bed in the control group and the nitrofen treated group in which the latter was subdivided in CDH and normal aspect diaphragm. We examined the newborn rats after perfusion of the pulmonary arteries with barium gelatine with subsequent fixation.

At the level of the respiratory bronchioles significant differences in the vessels were found consisting of decreased external diameter, increased wall thickness as percentage of the external thickness in CDH lungs compared to controls. Abnormal muscularization of the peripheral branches of the CDH pulmonary arteries was also found.

We concluded that the rat model strongly resembles the human situation concerning the arterial bed in the lungs.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a serious anomaly of the diaphragm and lungs in newborns; the mortality rate of approximately 30 to 60 percent has not changed significantly in the last 30 years [1]. The incidence of this disease is about 1:3000 newborns. The high mortality and morbidity of patients with CDH is due to the presence of pulmonary hypoplasia, uni- or bilateral [2] in combination with microscopically well defined abnormalities of the pulmonary vessels [3-5].

The course of CDH is often unpredictable and progress in ways of treatment has been hampered by lack of an appropriate animal model to investigate the morphological, biochemical and physiological changes during embryogenesis and immediately after birth without the effects of intensive care treatment. Clinical research is based on comparison of different ventilation techniques [6] (e.g. conventional, high frequency oscillation) or alternative methods of treatment (e.g. extracorporeal membrane oxygenation) [7].

Besides pulmonary hypoplasia, persistent fetal circulation (PFC) determines the final outcome in CDH patients. A strong and often unpredictable vasoconstrictive response of a hypertrophied hypermuscularised pulmonary arterial bed leading to increased pulmonary vascular resistance appears to be one of the most important mechanisms leading to the often fatal hypoxemia in these newborns. A broad spectrum of vasoactive drugs [8] have been used in an attempt to treat pulmonary hypertension, however, without success in many patients. To date research in animals has been in sheep with induced CDH at different stages of pulmonary development [6,9-10]. Only Adzick interfered with pulmonary development early in gestation in sheep (60-63 days). However, detailed studies on the presence of pulmonary vascular abnormalities are scarcely available in these animal studies[10].

Since 1971 the embryotoxicity of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) has been known in rats [11] and mice [12]. The heart and lungs are suggested as primary target organs [13].

In former studies on induction of CDH in rats we reported differences in lung weight and morphological changes that established pulmonary hypoplasia [14].The dose and time related effects of nitrofen application in relation to the incidence and side of diaphragmatic defect were also described [15]. This study aims to investigate the presence of pulmonary arterial abnormalities in a CDH model. Data are compared to those of a control group of newborn rats (less than 4 hours after birth) and to data in humans with CDH as described in the literature [3-5,16].

MATERIAL AND METHODS

Three groups of animals were used: 1: Control (C) receiving only olive oil, 2: nitrofen (N) despite of receiving nitrofen, they developed an intact diaphragm and 3: CDH group (H) animals receiving nitrofen and developing a one-sided diaphragmatic hernia. Each group consisted of 6 animals; the group H comprised of 4 left-sided and 2 right-sided

hernias.

Animals were obtained as described earlier [14], for this study only spontaneously delivered animals were used. These newborns were killed by an intraperitoneal injection of sodium pentothal (200 mg/kg of body weight).The chest wall was removed and the pulmonary arterial bed was injected and perfused through a cannula into the right ventricle with barium-gelatin mixture at 60°C and constant pressure [17].The perfusion was stopped when this white solution reached the visceral pleura in all segments leading to so-called 'snow flocks'. Subsequently tracheal cannulation and lung fixation with Davidson solution (40 vol % ethanol 100%; 5 vol % acetic acid 96%; 10 vol % formaldehyde 37%; 45 vol % saline; pH 7.3) was performed; fixation was maintained under constant pressure of 20 cm water[17]. After fixation (for 2 days), the lungs were dissected out of the thoracic cavity. The number of animals with a diaphragmatic defect, the position (right or left sided) and the size of the defect as well as the contents of the thorax (liver, bowel, stomach) were observed. An arteriogram was taken of each pair of lungs.

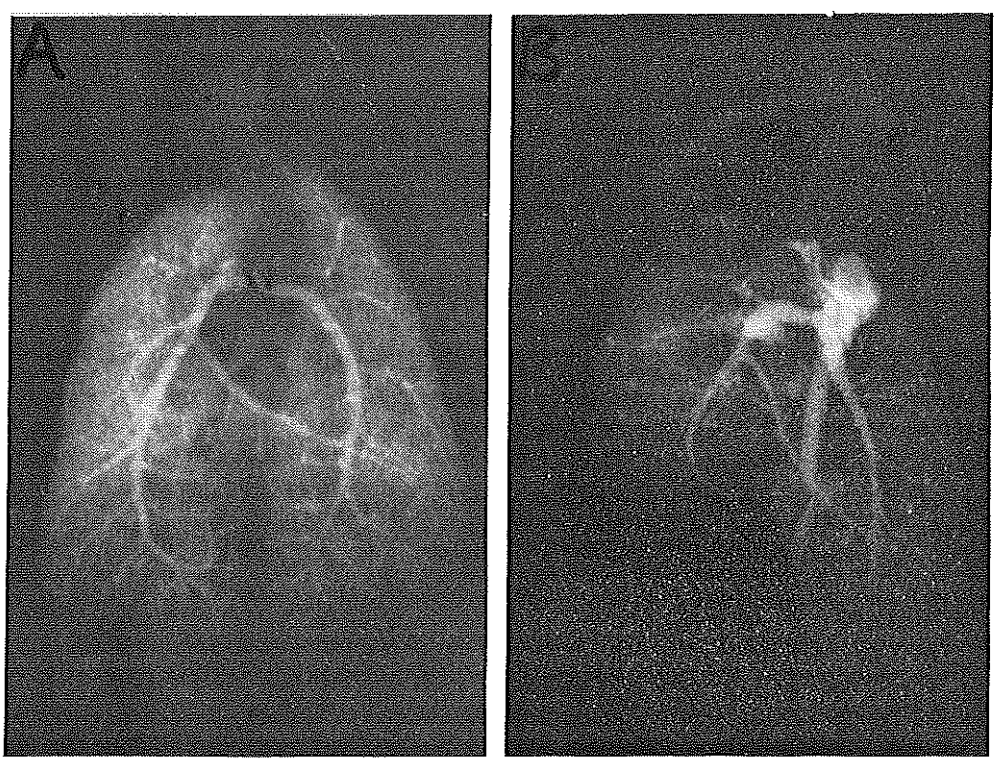


Fig 1. Arteriogram showing a control lung (a) and a right-sided congenital diaphragmatic hernia lung(b). There is no apparent reduction in the number of arteries after injecting the pulmonary trunk with barium-gelatin mixture.

Histologic preparation and light microscopic techniques.

The dissected lungs were processed for routine histology resulting in paraffin embedding of total lungs. Six μm frontal sections showing both lungs were made and stained with haematoxylin-eosin and Lawson combined with elastica van Gieson staining. Two different sections per pair of lungs were observed (12 sections per group). Analysis of each section was carried out in a blind fashion by means of an ocular micrometer (1 unit is 1.48 μm, Zeiss Optical Industry, Germany) and standard magnification (10 x 63) A total of at least 35 arteries of each animal (15 from each section) were examined. For each artery external diameter, wall thickness, wall structure (muscular, partially muscular, or nonmuscular) was noted [5,18]. Also the airway that they accompanied was noted (conducting airway, (CA), or respiratory bronchiole, (RB)). Wall thickness was defined as the distance between the luminal surface and the adventitia. External diameter was defined as the distance between the outer edges of the adventitia. The percentage wall thickness (2x wall thickness/external diameter) x 100% was calculated [17]. The mean value for each group was calculated with respect to the accompanying structure. No correction was made for processing and shrinkage factors [5]. A frequency tabulation of the artery structure was made depending on the accompanying airway structure [19].

Statistics

All data are reported as mean $\pm$ standard deviation. Differences between the means were tested by analysis of variance. Further tests used were the Student T-test and the Mann-Whitney-Wilcoxon test. P-values less than 0.05 were considered to be statistically significant; a specification of the p-values is not indicated.

Table 1. Number, Size, and Structure of Arteries in Three Groups of Newborn Rats

Accompanying Airway Landmark	No. of Arteries Examined per Animal	External Diameter (μm)	Wall Thickness (% ext diameter)	Wall Structure		
				Muscular (%)	Partial Muscular (%)	Nonmuscular (%)
Conducting airways						
Control	20	115 (59)	8.1 (2.1)	76	14	10
Nitrofen	21	98 (45)*	9.4 (3.6)	75	18	7
CDH	19	65 (29)*†	18.9 (7.3)*†	90	6	4
Respiratory bronchioli						
Control	22	46 (14)	11.7 (6.4)	13	23	64
Nitrofen	24	46 (19)	11.1 (3.7)	20	47	33
CDH	20	39 (14)*†	18.3 (7.6*†	45	24	31

NOTE. All values are mean (SD). Wall structure is expressed in relative frequency.

*$P < .05$ v control.

†$P < .05$ v nitrofen.

RESULTS

The body weights of the animals were similar to our former study [14] C: 6.0 $\pm$ 0.3g; N: 5.9 $\pm$ 0.3g; H: 5.6 $\pm$ 0.4g. Lung weights were not determined in this study because of the use of barium gelatin instillation.

The arteriograms showed remarkable structural conformity of the arterial tree in the

three groups with respect to the branching pattern and number of arteries. The dense background haze, produced by vessels too small to show up as individual lines was difficult to assess in these small lungs (Fig 1).

The external diameter was reduced in the CDH group (Table 1). The wall thickness expressed as percentage of the external diameter was increased in the CDH group at both levels. This is due to decreased external diameter and to an increased wall thickness as well.

A frequency distribution of the muscular, partial muscular, and nonmuscular arteries in the C, N, CDH groups, which is plotted against the external diameter (as described by Kitagawa [5]), revealed that in the CDH group each structural type is found in smaller arteries than in the C and N groups. Our distribution data are similar to those found by Kitagawa.

Also the frequency distribution of the wall structure (M: P: N), independent of external diameter, but plotted against the accompanying airway landmark (Table 1.), shows that the percentage of muscular arteries in CDH lungs differs from that of the control and nitrofen group at both surveyed levels; it shows a more peripheral extension of arterial muscularization in the CDH-lungs. Fig 2 shows the difference at the alveolar level between the C and H group arteries.

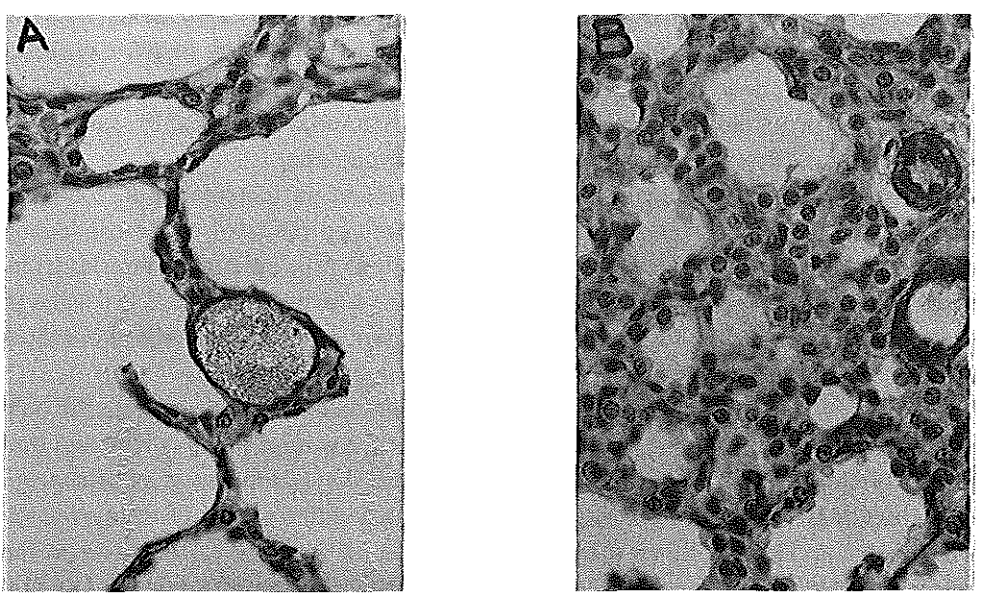


Fig 2. The difference between control lung (a) and left sided congenital diaphragmatic hernia lung (b) at the same magnifications (original 400x; Lawson/elastica van Giesson staining). The difference in structure between controls and CDH is evident; the latter showing thick walled sacculi and a denser structure despite the same pressure at fixation. The difference in artery structure on the alveolar level is evident. Thick muscularized walls in the CDH versus thinner walls in controls; also the external diameter of the control larger. This leads to the increased wall thickness as percentage from external diameter.

DISCUSSION.

Lung hypoplasia in human newborns with congenital diaphragmatic hernia has been described by several authors [2-6].

Kitagawa [5] described the pulmonary vascular bed in a CDH patient; he reported the distribution of the muscular, partial muscular and nonmuscular arteries showing a shift to the left, indicating an extension of muscle into smaller vessels as far as preacinar arteries than is normal. Analysis of the wall structure revealed hyperplasia of the muscular coating. Little difference between left and right lung was found. Levin [4], Bohn [6] have analyzed the pulmonary arterial bed in the lung of infants who died of CDH; they found an increase in the muscle mass (medial width/external diameter ratio) and a reduction in the total number of vessels. Geggel [3] revealed structural differences in the lungs between infants with CDH who enter a honeymoon period after surgical correction and those who do not. In all the infants the cross-sectional area of the pulmonary arterial bed is decreased, but this is more severe in the no-honeymoon group. In the latter the arteries are smaller and their media thicker causing a greater reduction in luminal diameter.

Adzick [10] created a diaphragmatic defect during the pseudoglandular phase of lung development in sheep; this resulted in gross lung hypoplasia, a marked decrease in the size of the pulmonary vascular bed, and abnormal extension of muscle in the intra-acinar vessels. Wallen [20] found that ligation of the left pulmonary artery in 105 day sheep caused significant decreases in left lung weight, volume displacement, future airspace, parenchyma tissue and capillary contents. Pulmonary arterial flow must be considered in evaluation of factors associated with pulmonary hypoplasia.

In our model of experimental induced CDH, we found an increased wall thickness expressed as the percentage of external diameter both on the level of the conducting airways and respiratory bronchiole. The muscularization of the pulmonary arteries starts at arteries with a smaller external diameter, suggesting a further peripheral extension of the muscular arteries in CDH lungs. The arteriograms showed no large differences between the three groups in branching pattern and number of arteries.

There are few data in the literature with which to compare our control group; the only one found was a study from Geggel [19]. Our data of the control group correspond well to that of Geggel. This author found a lower value for the wall thickness as percentage of the external diameter, this could be due to a slightly different way of measuring the wall thickness. He also found a slightly lower value for the external diameter in the respiratory bronchiole area with almost the same subdivision in wall structure. Lau [13] studied the lungs in nitrofen fed rats but he did not make a difference between the N and H groups. Also the pulmonary vessels structure was not considered in his study. Care has to be taken in comparing the models since we know that the time of administering nitrofen is important [15].

Comparing our model to the human situation we found a similar survival pattern as

described by many authors [3,4,6]. We were unable to differentiate between the ipsi and contralateral lungs in the group H; because of the very dense structure on the herniated side we could not distinguish the accompanying airway landmarks very well in all the CDH lungs.

CONCLUSION.

Congenital diaphragmatic hernia can be induced in rats successfully in an early stage of fetal development. According to our study we can conclude that the pulmonary vascular changes in this new animal model strongly resembles the human pathology in the case of CDH, so our model provides many opportunities to study the problems of CDH and its pre and postnatal development.

REFERENCES

1. Hazebroek FWJ, Tibboel D, Bos AP, Pattenier AW, Madern GC, Bergmeijer JH, Molenaar JC Congenital diaphragmatic hernia: the impact of preoperative stabilization. A prospective study in 13 patients. J Ped Surg 1988;23:1139-46.
2. Askenazi SS, Perlman M: Pulmonary hypoplasia: lung weight and radial alveolar count as criteria of diagnosis. Arch Dis Child 54: 614-618, 1979
3. Geggel RL, Murphy JD, Langleben D: Congenital diaphragmatic hernia: arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 107: 457-464, 1985
4. Levin D; Morphologic analysis of the pulmonary vascular bed in congenital left-sided diaphragmatic hernia. J Pediat 1978;92:805-9.
5. Kitagawa M, Hislop A, Boyden EA, Reid L; Lung hypoplasia in congenital diaphragmatic hernia; a quantitative study of airway, artery, and alveolar development. Brit J Surg 1971;58: 342-6.
6. Bohn D, Tamura M, Perrin D, Barker G, Rabinovitch M. Ventilatory predictors of pulmonary hypoplasia in congenital diaphragmatic hernia, confirmed by morphologic assessment. J Pediatr 1987; 111:423-31.
7. Weber TR, Connors RH, Pennington G et all. Neonatal diaphragmatic hernia. An improving outlook with extracorporeal membrane oxygenation. Arch Surg 122:615-8 (1987).
8. Abu-Osba YK, Treatment of persistent pulmonary hypertension of the newborn: update. Arch Dis Child 1991;88:74-7.
9. Pringle KC, Turner JW, Schofield JC, Soper RT; Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 1984; 18: 131-140.
10. Adzick NS, Outwater KM, Harrison MR, et al: Correction of congenital diaphragmatic hernia in utero. IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 20: 673-680, 1985
11. Manson JM: Mechanism of Nitrofen teratogenesis. Environ Health Perspect 70: 137-147, 1986
12. Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169: 133-139, 1984
13. Lau C, Cameron AM, Irsula O, Antolick LL, Langston C, Kavlock RJ; Teratogenic effects on cellular and functional matuartion of the rat lung. Toxicol Appl Pharmacol 1988; 95: 412-22.
14. Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Lachmann B, Molenaar JC; Experimentally induced congenital diaphragmatic hernia in rats. J Ped Surg 1990;25: 426-9.
15. Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W; Nitrofen-induced

diaphragmatic hernia in rats: an animal model. J Ped Surg 1990;25:850-4.

16. Davies G, Reid L Growth of the alveoli and pulmonary artries in childhood. Thorax 1970; 25:669-81.
17. Hislop A, Reid L; Intrapulmonary arterial development during fetal life: branching pattern and structure J Anat 1972; 113: 35-48.
18. Meyrick B, Reid L, Pulmonary and alveolar development in normal postnatal rat lung. Am Rev Respir Dis 1982; 125:468-73.
19. Geggel RL, Aronovitz MJ, Reid LM. Effects of chronic in utero hypoxemia on rat neonatal pulmonary arterial structure. J Pediatr 1986;108:756-9.
20. Wallen LD, Perry SF, Alston JT et all. Morphometric study of the role of pulmonary arterial flow in fetal lung growth in sheep Ped Res 27;122-127 (1990).

THE NATURAL HISTORY OF CONGENITAL DIAPHRAGMATIC HERNIA AND PULMONARY HYPOPLASIA IN THE EMBRYO

(J Pediatr Surg 1993; 28:456-63)

Abstract

Up to now, descriptions of the natural history of congenital diaphragmatic hernia (CDH) associated with pulmonary hypoplasia (PH) are based exclusively on observations made in the fetal period. However, nothing is known about the events that take place in an embryo with CDH. Recently, an animal model of CDH and PH has been established in rat embryos to study the embryology and natural history of this lesion. We exposed 36 pregnant Sprague-Dawley rats to a single dose of 100 mg nitrofen on day 11 of pregnancy. A total of 356 staged embryos and fetuses from day 13 to day 21 were studied by light and scanning electronmicroscopy. The litters of 9 untreated rats (124 normal age-matched embryos and fetuses) served as controls. The abnormal development of the diaphragmatic anlage was first seen in embryos aged 13 to 14 days. A defect appeared in the dorsal part of the diaphragm, normally on the right side. The liver grew through this defect early on. Gut was found in an intrathoracic position only in the very late stages (day 21/22) and newborns. Compared to controls, lungs of nitrofen-embryos with CDH were smaller, depending on the size of liver found in the thoracic cavity. Histologically, compression of lung was absent at these stages. Most authors speculate that CDH results because the pleuroperitoneal canals fail to close at the end of the embryonic period (ie, week 8 to 10 in human development) leading to a defect in the dorsolateral region of the diaphragm. However, contradictory to this assumption, our findings indicate that diaphragmatic defects develop in early embryonic life. They are easy to identify even in rat embryos as early as 14 days. The early ingrowth of liver into the thoracic cavity through these defects is the crucial step in the pathogenesis of PH in CDH, because it is the liver and not the gut that reduces the thoracic cavity in the embryo. Normal lung growth will be hampered in early embryonic stages according to the size of the liver mass inside the thoracic cavity. Recent reports suggest that the presence of liver in the fetal thoracic cavity affects the outcome of a fetus with CDH. This observation is in accordance with our findings, suggesting that the major events of pathogenesis take place in the embryo and not in the fetus, as often assumed.

Presented at the 23rd Annual Meeting of the American Surgical Association, Colorado Springs, Colorado, May 13-16, 1992. Supported in part by "Werner Otto Stiftung"

The outcome of a newborn with congenital diaphragmatic hernia (CDH) depends on the severity of the associated pulmonary hypoplasia (PH). Most authors consider lung hypoplasia as a secondary abnormality: delayed or incomplete closure of the so-called pleuroperitoneal canals may result in the herniation of intestine into the thoracic cavity leading to abnormal compression of the developing lungs[1,2]. Studies on the natural history of CDH in humans and data derived from experimental animal models[3-6] seem to support this assumption. These studies, however, are based exclusively on observations made in the fetal period while data that may elucidate the natural history of CDH in the embryonic period are not at hand. An animal model of CDH and PH has been recently established in rat embryos using nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether) as teratogen[7,8]. We used this model to define the specific morphological changes of CDH and PH during the embryogenesis of this lesion.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats weighing between 196 and 319 g (mean, 261 g), were bred under normal laboratory conditions. After controlled overnight mating, pregnancy was verified by means of the vaginal smear method. The day of positive smear was rated as day zero of pregnancy. The pregnant rats were exposed to a single dose (100 mg/rat) of orally administered nitrofen (Wako Chemicals, Neuss, Germany) on day 11 of pregnancy. The substance was mixed with 20 g of moistened commercial rat chow (Altromin, Lage, Germany) and offered to the rats after a 24-hour period of fasting. Rats not consuming the mixture within 12 hours were excluded. Using this method, 36 pregnant rats could be exposed successfully to nitrofen. There after the rats were again supplied with food and water ad libitum. Nine other rats, also undergoing a 24-hour fast, served as controls. Four rats from the nitrofen group plus one control rat were killed on each day between day 13 and 21 of pregnancy. A total of 480 embryos and fetuses (356 from the nitrofen group, 124 controls) were removed by laparotomy and were then staged after Witschi[9]. One litter from each treated group and three embryos from each control rat (92 nitrofen embryos plus 27 control embryos) were fixed in Bouin, embedded in paraffin, cut in serial sections (7μm), and stained with hematoxilin-eosin for light microscopy (LM).

The remaining embryos and fetuses (264 nitrofen embryos and 97 control embryos) were fixed in glutaraldehyde for 24 hours and then microdissected in 80% ethanol. After dehydratation, the embryos were dried by critical point method, gold-sputtered, and then examined and photographed with a DMS 940 scanning electronmicroscope (SEM) (C. Zeiss, Oberkochen, Germany).

RESULTS

SEM Findings

In the first group (13 days) 30 nitrofen embryos and 5 embryos of the control group were studied. No deviation in the morphology of the diaphragm was found, when we compared the nitrofen group with the control group. Furthermore, we found no differences between the nitrofen group and the control animals when we studied the development of the lungs at this stage (Fig 1).

In the second group (14 days) 41 nitrofen embryos and 4 controls were analyzed. In this age group there was clear evidence of disturbed diaphragmatic development in the embryos of the nitrofen group while evidence of disturbed lung development was absent. In the majority of these embryos, a defect was found in the dorsal part of the diaphragm (Fig 2A). All defects appeared exclusively on the right side. They resulted in an abnormal diaphragm, leaving parts of the liver uncovered. Compared to controls, the left dorsal diaphragm was found to be normal in all studied embryos.

In the next two age groups (15 days and 16 days) 34 and 32 nitrofen embryos and 7 and 6 age-matched controls were studied. In most litters, the majority of embryos had a right diaphragmatic defect. In all these embryos, the liver was clearly scen inside the thoracic

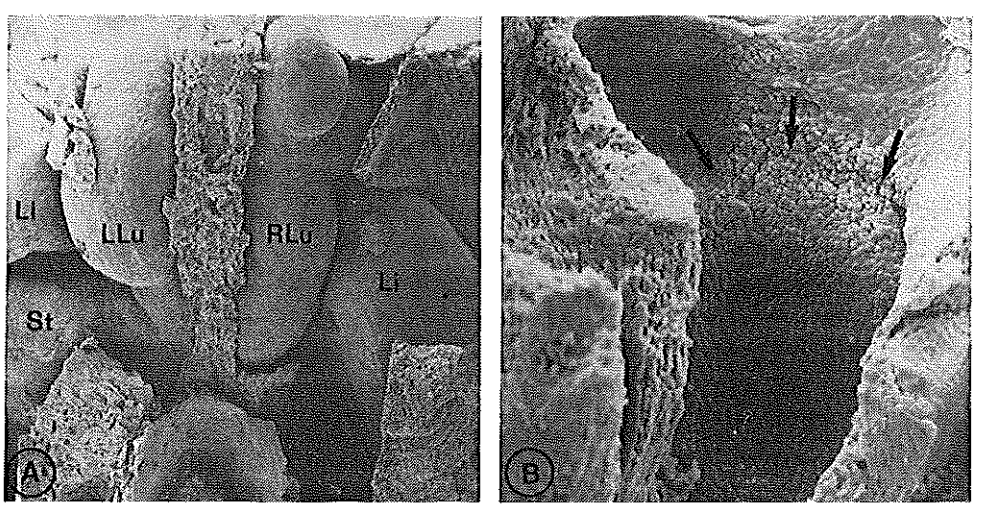


Fig 1. SEM picture of a 13-day-old rat embryo; this stage corresponds to a human embryo 5 weeks of age.(li, liver; St, stomach; LLu, left lung; RLu right lung).A) Dorsal view. The lungs are still in situ. B) Dorsal view into the thoracic cavity; the right lung is removed. Arrows indicate the lower border of the diapragm.

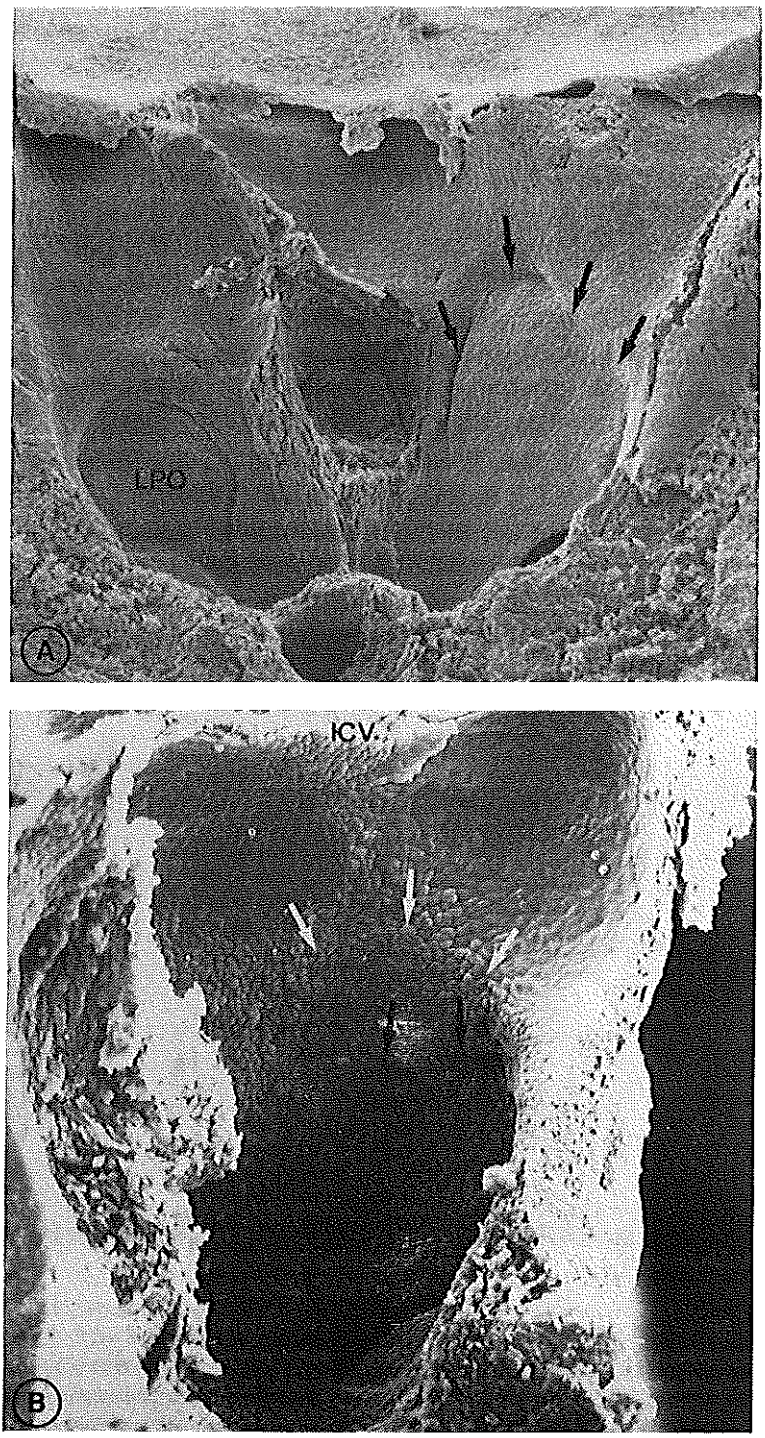
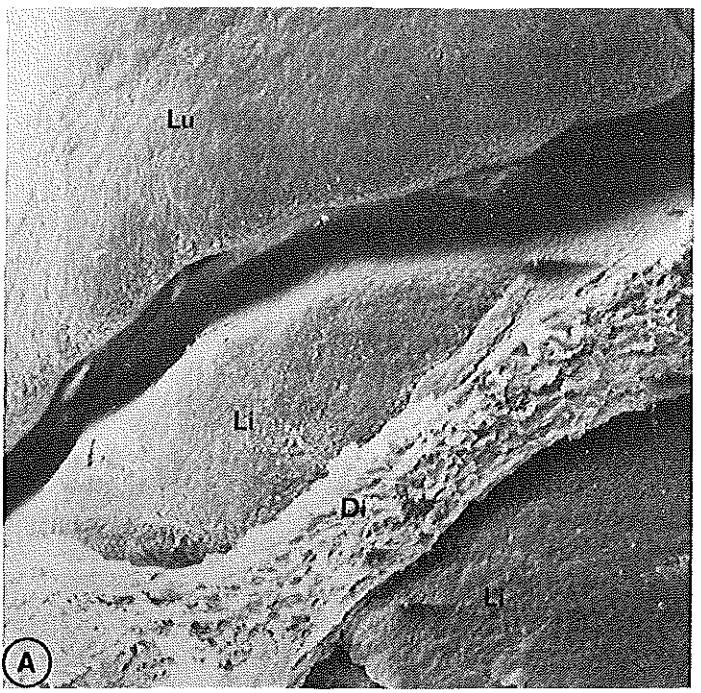


Fig 2 (A) SEM picture of a 14 day-old rat embryo (nitrofen group). Dorsal view. The lungs are removed. The left pleuroperitonealopening (lpo) is normal. A large defect is seen in the right diaphragm (black arrows). The diaphragm ends abnormally high, leaving parts of the liver uncovered (B) SEM picture of a 13.5-day-old rat embryo (control group). Black arrows indicate the border of the normally developed diaphragm White arrows indicate the site of this border in a 13-day-old embryo (white arrows). The downgrowth of the diaphragm is notable. icv, inferor vena cava. (This stage corresponds to a human embryo 5 to 6 weeks of age)



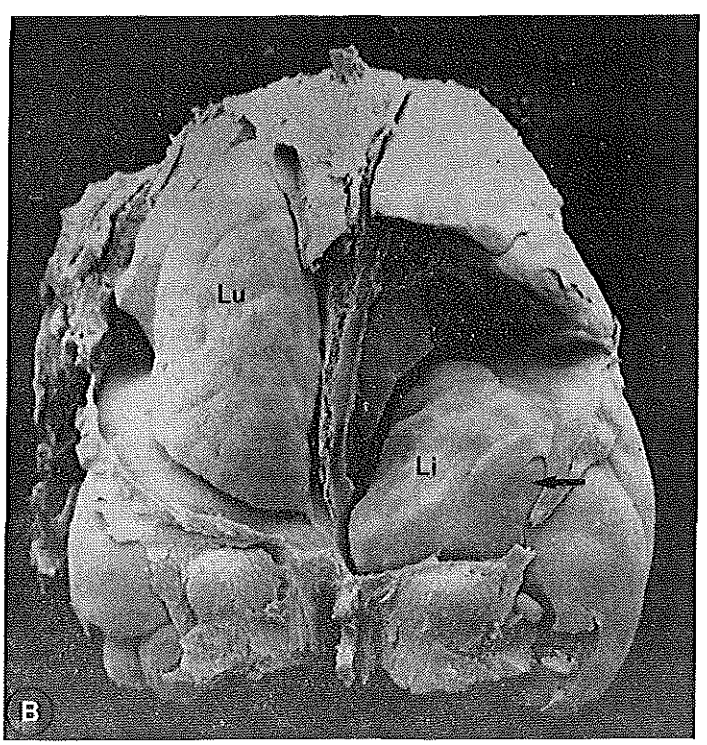


Fig 3 IA) SEM picture of a 16-day-old rat embryo (nitrofen group). Lateral view. A small part of the liver (li) protrudes diaphragmatic defect (di) and is now inside the thoracic cavity. Note the close contact between liver (li) and lung (lu). (B) SEM picture of a 16-day-old rat embryo (nitrofen group). Dorsal view. The right lung is partially removed. The large defect with the intrathoracally displaced portion of the liver (li) is seen. Due to the ingrowth of liver, the opening of the right pleuroperitoneal "canal" fails to close (arrow). Note that this opening represents only a small part of the whole defect. (This stage corresponds to a human embryo 7 weeks of age).

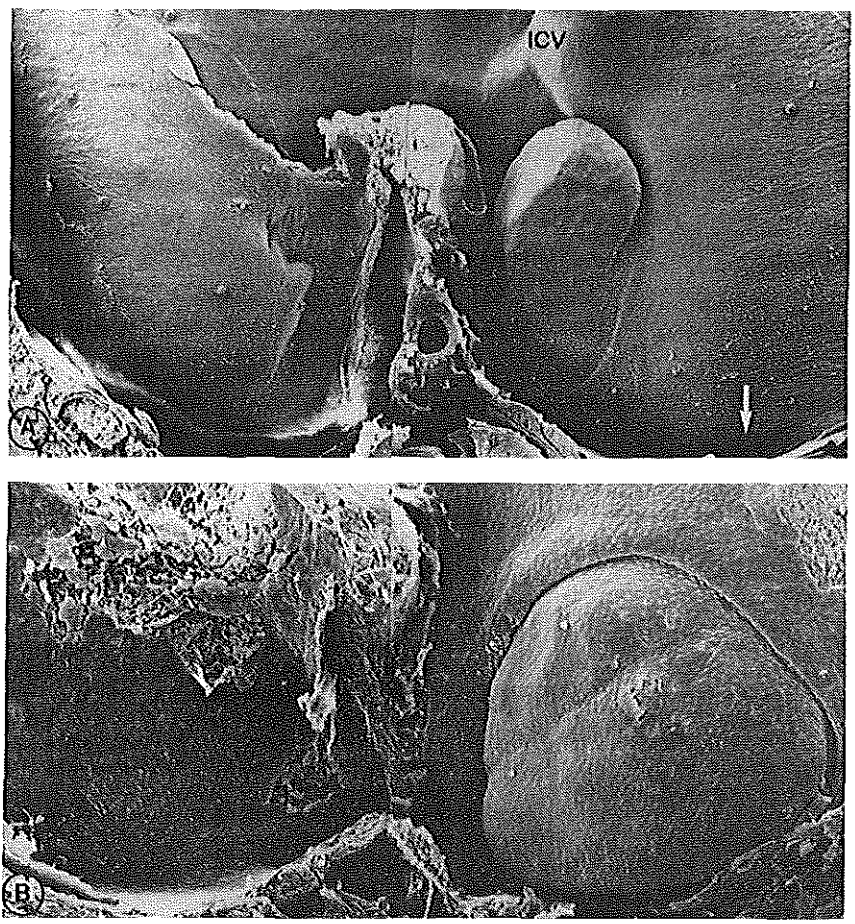


Fig 4. (A) SEM picture of a small diaphragmatic defect. Typically, these are in close contact with the inferior vena cava (icv) and are thus more centrally located. The white arrow indicates the region, where the opening of the right pleuroperitoneal "canal" has closed. (B) SEM picture of a large diaphragmatic defect. This defect incorporates the dorsolateral region ("Bochdalek-type"). (This stage corresponds to a human embryo 8 weeks of age).

cavity (Fig 3A). Two distinct forms of the malformation could be discerned. Typically, a minor form of the defect was found centrally, close to the inferior vena cava (Fig 4A). In these cases the so-called pleuroperitoneal canals closed properly. In the major form (Fig 4B), the defect extended from the inferior vena cava to the dorsolateral region of the diaphragm, thus incorporating the openings of the pleuroperitoneal "canals". In these age groups, the gross aspect of the lungs appeared to be normal. The lungs often covered

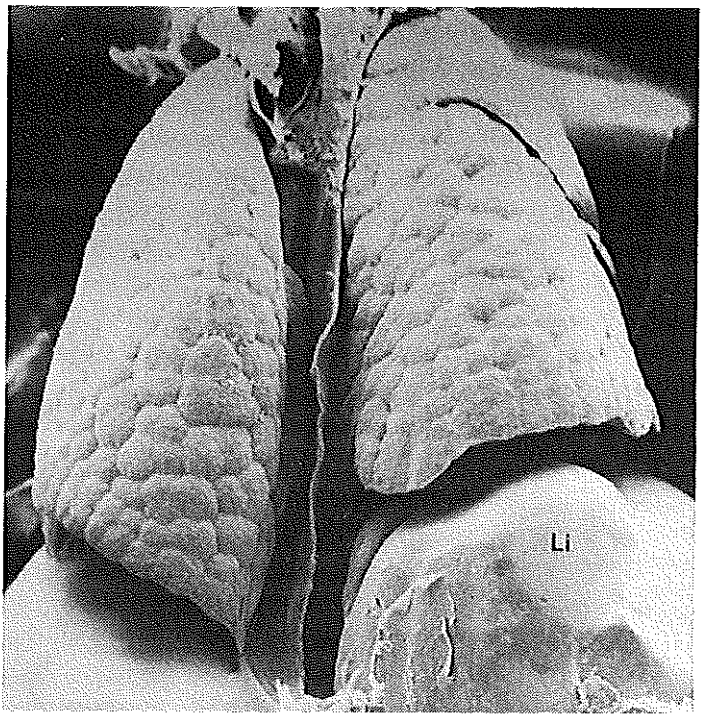


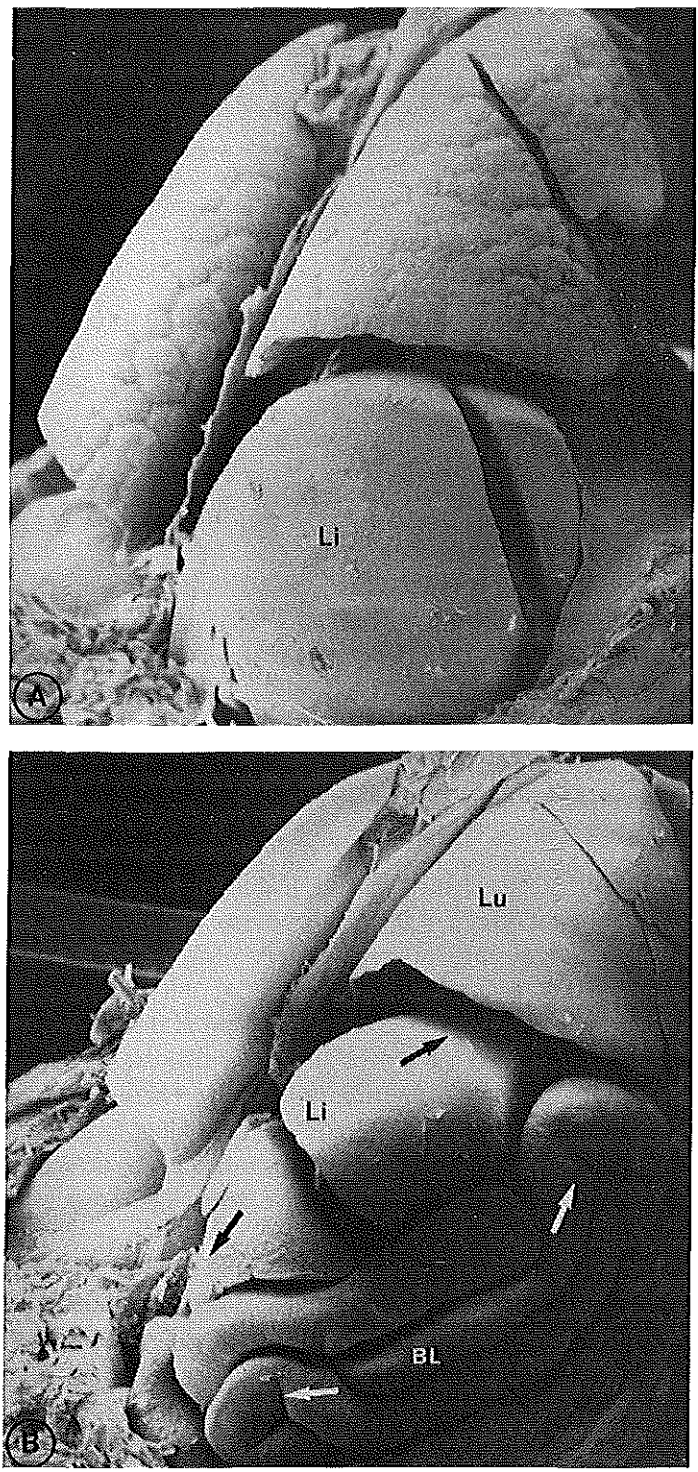
Fig 5. SEM picture of a 18-day-old rat embryo (nitrofen group). View from dorsal. The liver (li) occupies nearly one third of the thoracic cavity. The right lung is reduced in size. Bowel loops are absent (This stage corresponds to a human embryo 10 weeks of age).

the diaphragmatic defect, making it difficult to detect. However, after removing the lungs, the total extend of the defect became apparent (Fig 3B). In all normal 16-day-old embryos, the openings of the pleuroperitoneal "canals" were found to be closed on the right and left side of the diaphragm. At this stage, bowels loops were still found exclusively inside the extraembryonic celom of the umbilicus.

In the following two age groups (17 days and 18 days) 28 and 25 nitrofen embryos were compared with 7 and 8 controls, respectively. There was a remarkable difference in the growth pattern of the more "central" defects (minor forms) and the "dorsolateral" defects (major forms). In some of the embryos with a major defect, the liver occupied one third of the thoracic cavitv (Fig 5). In these cases, right lungs were markely reduced in size compared to normal controls.

In the minor defects, only a small portion of the liver reached the thorax. In these cases, the lungs were near normal except for a small indention in the area of the protruding liver. In all 18-day embryos, the intestine was found inside the abdominal cavity while in the embryos of the 17-day age group, intestinal loops remained still in the extraembryonic celomic cavity of the umbilicus.

Fig 6. (A) SEM picture of 20-day-old rat fetus. Half of the thoracic cavity is occupied by liver (li). The lung is markedly reduced. Bowel loops are still absent (original magnification x 20). (B) SEM picture of 21-day-old rat fetus (original magnification x 8). Bowel loops (bl) finally have entered the thoracic cavity. The liver (li) occupies two thirds of the thoracic cavity. The lung (lu) is small. (These stages correspond to human fetuses older than 18 weeks).



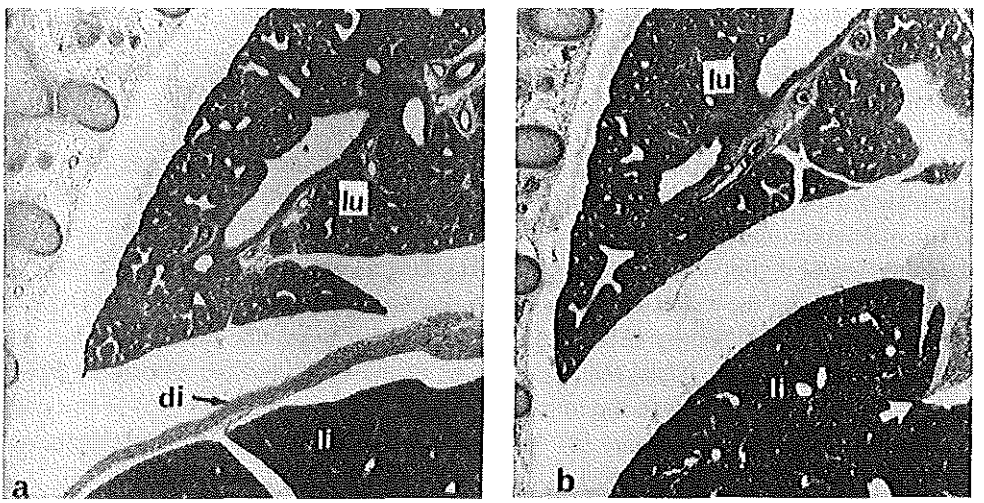


Fig 7. LM pictures of serial sections of 18-day-old rat embryos. (A) Control embryo. (B) Nitrofen-embryo (original magnification x 100). Clear signs of lung compression by the ingrowing liver can not be discerned. di, diaphragm; li, liver; lu, lung. White arrow indicates the site of the diaphragmatic defect. (This stage corresponds to a human embryo 10 weeks of age).

The next age groups (19 days, 20 days, and 21 days) represent the fetal stage of development. Here, the whole spectrum of the malformation could be observed. In some of the 19- and 20-day-old rat fetuses, over half of the chest was occupied by liver masses (Fig 6A), while in others the ingrowth of the liver was less obvious. In those with massive liver protrusion, the lungs were small and hypoplastic while they appeared near normal when only small parts of liver were found inside the chest. Intrathoracally displaced bowel loops were first observed in 21-day-old fetuses (Fig 6B). In these, the lungs were found to be reduced in size due to amount of liver occupying up to two thirds of the available thoracic space.

LM Findings

Our anatomical SEM findings were confirmed by serial histological sections. The defect was first identified in 15-day-old embryos. It was always situated in the dorsal diaphragm and was found in close contact with the inferior vena cava. Starting on day 16, the liver gradually expanded into the thoracic cavity. However, clear signs of lung compression

were not found in our embryos (Fig 7). Comparison of the lungs and the thoracic cavities in age groups 16 and 18 demonstrated that in the embryo, these structures were expanding despite the ingrowth of liver (Fig 8).

DISCUSSION

Since Broman's investigations[10] on the diaphragmatic development, most authors have speculated that CDH results because the pleuroperitoneal canals fail to close at the end of the embryonic period (ie, week 8 to 10 in human development) leading to a defect in the dorsolateral region of the diaphragm[1,2,11]. Bowel loops may then herniate through this defect into the chest with subsequent hypoplasia of the developing fetal lungs[1,2,11]. However, the natural history of this sequence of embryological and fetal events has not been previously studied. Thus, the pathogenesis of the CDH and the associated PH is still elusive and the answers to the following questions remain unknown: (1) When does the diaphragmatic defect appear? (2) Are the pleuroperitoneal canals the precursors of the diaphragmatic defect? (3) Why is the lung hypoplastic in cases of CDH?
Since 1971, the embryotoxicity of the herbicide nitrofen (2,4 - dichloro - phenyl - p - nitrophenyl ether) is well known[12]. In 1981, Nakao et al[13] noticed that this teratogen can induce CDH in rats and mice. Since then, this substance
has been widely used to study its effect on the lung and diaphragm[14-16]. However, only the work of Iritani[16] adresses the natural history of CDH in the embryo. In our study, we used the pulse feeding technique. Pregnant rats were exposed to a single 100-mg dose of nitrofen on day 11 of pregnancy. This day has been previously identified by us to be highly sensitive to the teratogenetic effect of nitrofen[7,8].

The Onset of CDH

Most authors speculate that in the human fetus with CDH, the pleuroperitoneal canals fails to close late in the embryonic period between the 8th and 10th week of gestation[1,2,11]. At the same time, the intestine returns into the abdominal cavity[1,2,11,17]. In rats, this developmental stage is equivalent to that of 17 or 18 days of gestation. At this time, the openings of the so-called pleuroperitoneal canals are normally closed[18]
In contrast to this assumption, it is evident from our study that the diaphragm is already malformed in the 14-day-old embryo (Fig 2A). This age group is equivalent to that of a 5- to 6-week-old human embryo. In a 13-day-old rat embryo, signs of a disturbed diaphragmatic development are still missing (Fig 1B). However, the onset of the malformation takes place at this stage. The diaphragmatic anlage of a 13.5-day-old embryo (Fig 2B) is too advanced to serve as the starting point for the maldevelopment. These findings are in accordance with observations made by Iritani[16]. He found signs of disturbed diaphragmatic development in 11-day-old mouse embryos exposed to nitrofen continuously from day 5 to 11 after conception. Thus, we conclude that in rat

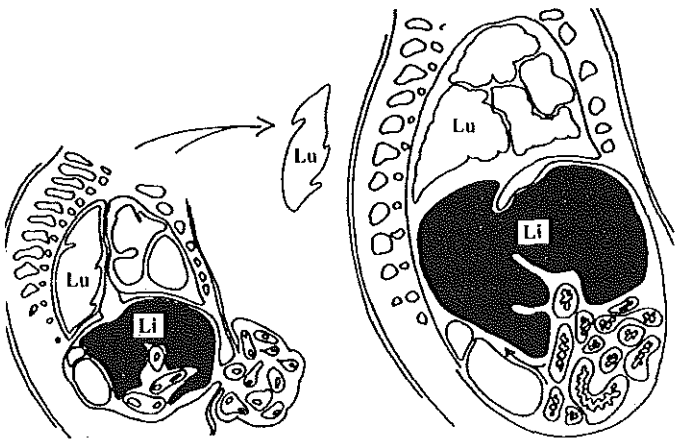


Figure 8 When two embryos of different age groups are compared (16 days versus 18 days), the expansion of the thoracic cavity becomes obvious. Thus, the lung still continues to grow, while part of the chest is occupied by liver. In a growing embryonic body, compression is unlikely. (Sketch after serial sections in same magnification).

embryos the onset of the diaphragmatic defect clearly lies in the early embryonic period. This phase is comparable to that of a human embryo in the 5th gestational week.

The Site of the Defect

Iritani[16] was the first to notice that nitrofen induced diaphragmatic hernias are not caused by an improper closure of the pleuroperitoneal openings but rather the result of a defective development of the so-called posthepatic mesenchymal plate (PHMP). The observed defects were also localized in the PHMP in our SEM study (Fig 2A). We could identify two distinct types of defects: (1) large "dorsolateral" defects and (2) small "central" defects. Large defects extended into the region of the pleuroperitoneal openings. In these cases, the closure of the pleuroperitoneal openings was usually impaired by the massive ingrowth of liver (Fig 3B). If the defects were small, they were consistantly isolated from the pleuroperitoneal openings closing normally at the 16th or 17th day of gestation. Thus, in our embryos with CDH, the region of the diaphragmatic defect was a distinct entity and was seperated from that part of the diaphragm where the pleuroperitoneal "canals" are localized. We conclude therefore that the pleuroperitoneal openings are not the precursors of the diaphragmatic defect.

The Lung in CDH

Primary pulmonary hypoplasia.

Nakao et al[14], Ueki et al[15], and Iritani[16] postulated that the lung is primarily hypoplastic. In his study, Iritani even drew the conclusion that the hypoplastic lung may

induce a defective PHMP which may again result in CDH[16].
However, in our young embryos (14 days gestational age) with a diaphragmatic defect, disorders of the early lung anlage could not be observed. This finding is in sharp contrast to previously published results by Ueki et al[15]. They observed reduced lung growth in young mouse embryos continuously exposed to nitrofen from postconceptual days 5 to 11. However, this reduced lung growth may be the result of nitrofen interference with the very early lung anlage. It is well known that developing embryonic organs have specific periods of greatest sensitivity to teratogens. Continuous administration of nitrofen for a longer period must thus result in an combination of malformations. In our study, we avoided this combination by pulse feeding of nitrofen on day 11. The diaphragm has proved to be highly sensitive to the teratogenetic action of nitrofen on this day[7,8].

Secondary pulmonary hypoplasia.
It is generally assumed in the literature, that lung hypoplasia in cases of CDH is of secondary origin[1,2,4,5,11] and that this hypoplasia is caused by herniated bowel loops compressing the developing fetal lung[11]. In contrast to this assumption, our study indicates that pulmonary hypoplasia develops in the embryonic period. Soon after the onset of the defect in the 14-day-old embryo, liver grows through the diaphragmatic defect into the thoracic cavity. This indicates that from this time on the available thoracic space is reduced and further lung growth hampered. In 15- or 16-day-old embryos, the amount of liver inside the chest is only small (Fig 3A). Therefore, the lungs are nearly normal in these age groups. However, in embryos aged 17 or 18 days, more than one third of available thoracic space may be occupied by liver masses. In these cases, the impaired lung growth is obvious. At first sight, in our embryos PH seems to be the result of compression caused by the ingrowing liver. However, serial section analysis shows that clear signs of lung compression are absent (Fig 7). Furthermore, it is obvious (Fig 8) that in the embryo, the thoracic cavity expands despite the ingrowth of liver. Lung compression is therefore unlikely in a fast growing embryo.
Herniated gut was found in our embryos and fetuses only in late stages of development (21 days and newborns). In all of these, the lungs were already hypoplastic, when the bowel entered the thoracic cavity (Fig 6B). Based on our observations, we conclude that the early ingrowth of the liver through the diaphragmatic defect is the crucial step in the pathogenesis of PH in CDH. The presence of liver inside the fetal thoracic cavity was recently considered to be of major prognostic importance in the outcome of a fetus with CDH[19]. Our pathogenetic concept clearly explains this observation. We found that impaired lung development is proportional to the size of the liver mass inside the thoracic cavity. This indicates that growth impairment is not the result of lung compression in the fetus but rather the result of growth competition in the embryo: the liver that grows faster than the lung reduces the aviable thoracic space. If the remaining space is too small, PH will result.

REFERENCES

1. Wells LJ: Development of the human diaphragm and pleural sacs. Contr Embryol Carneg Inst 35: 107-137, 1954
2. Gray SW, Skandalakis JE: Embryology for surgeons. Philadelphia, PA, Saunders, 1972, pp 359-385
3. deLorimier AA, Tierney DF, Parker HR: Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. Surgery 62:12-17, 1976
4. Harrison MR, Jester JA, Ross NA: Correction of congenital diaphragmatic hernia in utero. I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. Surgery 88:174- 182, 1980
5. Harrison MR, Bressack MA, Churg AM: Correction of congenital diaphragmatic hernia in utero. II. Simulated correction permits fetal lung growth with survival at birth. Surgery 88:260-268, 1980
6. Adzick NS, Harrison MR, Glick PL, et al: Diaphragmatic hernia in the fetus: Prenatal diagnosis and outcome in 94 cases. J Pediatr Surg 20:357-361, 1985
7. Kluth D, Kangah R, Reich P, et al: Nitrofen-induced diaphragmatic hernia in rats: An animal model. J Pediatr Surg 25:850-854, 1990
8. Tenbrinck R, Tibboel D, Gaillard JLI, et al: Experimentally induced congenital diaphragmatic hernia in rats J Pediatr Surg 25:426-429, 1990
9. Witschi E: Development of the rat, in Altman P, Dittmer DS (eds): Growth Including Reproduction and Morphological Development. Washington, DC, Federation of Societies for Experimental Biology, 1962, pp 304-414
10. Broman I: Uber die Entwicklung des Zwerchfells beim Menschen. Verh Anat Ges 16:9-17, 1902
11. Harrison MR: The fetus with a diaphragmatic hernia: Pathophysiology, natural history, and surgical management, in Harrison MR, Golbus MS, Filly RA (eds): The Unborn Patient. Fetal Diagnosis and Treatment (ed 2). Philadelphia, PA, Saunders, 1990, pp 295-319
12. Ambrose AM, Larson PS, Borcelleca JF, et al: Toxicological studies on 2,4-dichlorophenyl-p-nitrophenyl ether. Toxicol Appl Pharmacol 19:263-275, 1971
13. Nakao Y, Iritani 1, Kishimoto H: Experimental animal model of congenital diaphragmatic hernia induced chemically. Teratology 24:11A, 1981 (abstr)
14. Nakao Y, Ueki R: Congenital diaphragmatic hernia induced by nitrofen in mice and rats: Characteristics as animal model and pathogenetic relationship between diaphragmatic hernia and lung hypoplasia. Congen Anom 27:397-417, 1987
15. Ueki R, Nakao Y, Nishida T, et al: Lung hypoplasia in developing mice and rats induced by maternal exposure to nitrofen. Congen Anom 30:133-143, 1990
16. Iritani I: Experimental study on embryogenesis of congenital diaphragmatic hernia. Anat Embryol 169:133-139, 1984
17. Starck D: Embryologie Stuttgart, Germany, Thieme, 1975, pp 488-500
18. Kluth D, Petersen C, Zimmermann HJ, et al: The embryology of congenital diaphragmatic hernia, in Puri P (ed): Congenital Diaphragmatic Hernia. Mod Probl Paediatr vol 24. Basel, Switzerland, Karger, 1989, pp 7-21
19. Harrison MR, Langer JC, Adzick NS, et al: Correction of congenital diaphragmatic hernia in utero, V. Initial clinical experience. J Pediatr Surg 25:47-57, 1990

NITROFEN INDUCED DIAPHRAGMATIC HERNIA: PRESSURE-VOLUME REGISTRATION AND ARTIFICIAL VENTILATION IN NEWBORN RATS

(accepted for publication)

Abstract

Congenital diaphragmatic hernia (CDH) can be introduced in rat fetuses by administering the herbicide nitrofen to pregnant rats.

Artificial ventilation of newborn rats with intermittent registration of pressure-volume (P-V) diagrams is described. The first experiment shows that delivery of the fetus by hysterotomy is followed by inadequate ventilation during the first hour after delivery ($p < 5\%$; hysterotomy vs spontaneous delivery). This delayed lung adaptation is characterized by impaired compliance values.

In the second experiment lung mechanics and histology were compared immediately after spontaneous birth (0 h) and after 1 and 6 hours postpartum in controls (C), and nitrofen exposed animals with (NH) or without (NN) CDH. The differences between the C and NH groups considering the volumes during all points of measurement (0, 1, 6 hours) are significant. For example: at $P = 10$ cm H_2O, the corresponding volumes (0, 1, 6 hours) for C are 137 $\pm$ 92; 189 $\pm$ 33; 301 $\pm$ 93 µl and for NH are 17 $\pm$ 28; 75 $\pm$ 73; 147 $\pm$ 78 µl. The mortality in the NH group with postmortem proven severe CDH was high; 80% did not survive the 6 hour period. Because the mortality rate in the less affected NH group was lower (35%), the NH group showed a paradoxical improvement in time due to a relative increase during time of the better performing, less affected CDH.

Conclusion: newborn rats can be ventilated for several hours. Since improvement in lung function induced by, e.g. drugs, will result in altered P-V relations, this model is suitable for testing new modes of treatment during the period immediately after birth.

Congenital diaphragmatic hernia (CDH) remains a major problem in neonatology and pediatric surgery. The reported incidence of this anomaly ranges from 1 in 2200 to 1 in 5000 births [1-3].
Even today, mortality of patients with CDH remains over 40% as a result of pulmonary hypoplasia with or without pulmonary hypertension. Infants with severe respiratory distress need maximal ventilatory support and are often subjected to high inflation pressures and high percentages of oxygen. The side effects of prolonged respiratory treatment are well known [4] and the clinical picture of bronchopulmonary dysplasia is increasingly encountered in neonatal intensive care units [5].
The enormous variety in presentation of CDH, from minimal respiratory symptoms in the days to years following birth to severe acidosis and hypercarbia accompanied by acute pulmonary hypertension immediately after birth, makes it difficult to evaluate the efficacy of new therapeutic techniques. The evaluation of these techniques, such as delayed surgery, extracorporeal membrane oxygenation (ECMO), high frequency oscillation ventilation (HFOV) and surfactant replacement therapy [6-9] is further impaired by the effects of the necessary aggressive and often combined procedures to overcome acute respiratory insufficiency. Improvement of therapies for CDH patients with respiratory distress requires not only insight into normal and abnormal pulmonary development such as morphology and biochemistry, but also the effect of ventilation on these parameters.

In a fetal rat model, CDH and subsequent pulmonary hypoplasia is induced by application of 2,4 dichlorophenyl-P-nitrophenyl ether(nitrofen) at day 10 of gestation [10,11].
Until now there are few reports in the literature on artificial ventilation of newborn [12], or premature born [13] rats. The chosen method for ventilating the rats in the present study was first described by Lachmann et al. [14] for the ventilation of neonatal rabbits. The purpose of this communication is to provide basic information on ventilation of small rats, and to demonstrate the effects on lung mechanics in the neonatal rat with or without CDH during artificial ventilation.

MATERIALS AND METHODS

Animals

All experiments were approved by the Animal Ethical Committee of the Erasmus University Rotterdam. Female Sprague Dawley rats (Harlan, Zeist) weighing 240-280 grams were mated during 1 hour; this time was considered as day 0 of gestation. On day 10.5 of gestation 115 mg nitrofen dissolved in 1 ml oil, was administered by gastric tube to the nitrofen (N) group; controls received 1 ml oil as described before [10]. Normal gestation in these rats lasts about 22 days. We either performed a hysterotomy at gestational day 22 or awaited spontaneous birth (most animals were delivered at mean 22.2 days); animals with unknown time of spontaneous birth were excluded from the

experiment.
Three groups of newborns were recognized: Controls (C); nitrofen exposure without resulting diaphragmatic defect (NN) and with diaphragmatic hernia (NH). It was not possible to sort the NN and NH animals immediately after delivery, since the dams treated with nitrofen delivered both.

Preparation
For the hysterotomy the dam was killed by an intraperitoneal injection of sodium pentobarbital (250 mg/kg) and the fetuses were delivered.
Immediately after birth or hysterotomy, the newborns were weighed and received pentobarbital (20 mg/kg every 3 hours) and pancuronium bromide (0.08 mg/kg every hour) intraperitoneally. In a pilot study we found a clear time saving in favour of intubation versus tracheostomy (2 min vs 5 min) while no influence on ventilation parameters was observed.
The animals were intubated with a metal canula. The canulas used throughout the experiment were made from syringe needles (internal diameter 0.5 mm; external diameter 0.7 mm); after intubation the canula was fixed to the animal's head by a strip of plaster. The intubated animals were immediately transferred to a multichambered, pressure-constant body plethysmograph heated to 38°C. The connection with the bodybox was performed by a flexible tube to provide an adequate connection between the trachea and the bodybox. The maximum number of ventilated animals per litter was 9. The NN/NH determination was made after autopsy.
Lung weights were only determined after birth before ventilation, and were compared with the values found in former studies [10].

Experimental set-up
In the first experiment, lung mechanics were compared between spontaneous born rats and those delivered by hysterotomy. Measurements were made immediately after birth or delivery and after 6 hours ventilation.
The second experiment included only spontaneous born animals. These animals were ventilated for maximal 6 hours. Pressure-volume relations were determined at 0, 1 and 6 hours. Zero-hour animals were intubated immediately after delivery before any sign of spontaneous breathing. If any sign of spontaneous breathing was visible, the animal was excluded from this group. Animals without visible heart action, pneumothorax or other complications related to insufficient ventilation were also excluded from the study. After the experiment the animals were killed by an overdose of pentobarbital before preparation of the lungs.
Due to the use of some animals for histology and the death of several others (mainly from the large-sized [10] CDH group) during measurement, the composition of the groups varied in time.

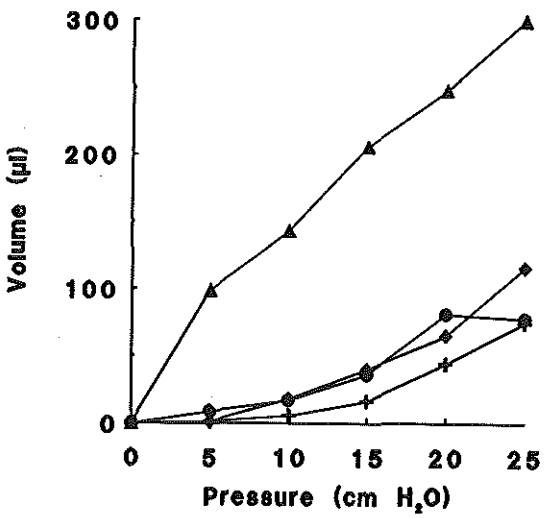


Figure 1. Effect of hysterotomy and spontaneous birth on pressure-volume relations immediately after birth (T=0 h). Pressures (cm H_2O) on the horizontal axis, the corresponding volumes (in µl) on the left axis. There is a significant difference between the spontaneous born controls (triangles) and controls born after hysterotomy (cross). Both other lines represent CDH lungs; no significant difference was found between CDH and hysterotomy control (P< 1%).

Ventilation

The body plethysmograph was connected to a ventilator (Servo 900B, Siemens Elema Sweden) as described by Lachmann [14]. This equipment provides pressure-generated ventilation with decelerating flow using excess flow through the ventilator system.

The ventilator settings throughout the experiment were FiO_2 1.0; frequency 40/min; I/E ratio 1:2; peak pressure 17 cm H_2O; PEEP = 0 cm H_2O.

At 0, 1, and 6 hours, the pressures were varied from 6 cm H_2O to maximal 28 cm H_2O and back to 6 cm H_2O by steps of approximately 5 cm H_2O; the other ventilator settings remained unchanged. Volumes were determined with a specially designed Fleisch-tube [14] connected to the body plethysmograph, a differential pressure transducer (Siemens Elema EMT 34), amplifier (EMT 31), and integrator unit (EMT41). Airway pressure was measured by means of a pressure transducer; P-V data were recorded simultaneously (Siemens-Elema Mingograph 81).

Heart action was controlled visually, because it was not possible to have reliable ECG tracings in the bodybox.

Histology

All lungs used for histology were fixed in situ; a part was prepared as reported previously

by inflation of the lungs during fixation with Davidson solution [15], or by fixing them for several days by submerging the unopened thorax in Davidson solution. Routine histologic preparation (6 μm slides; HE staining) was followed by microscopic examination with particular reference to expansion pattern, hemorrhage, interstitial emphysema, inflammation, and other lesions that may have influenced lung mechanics.

Calculations and statistical analysis

By means of the measured P-V points, an inflating and deflating limb (PV-curve) was plotted for each animal at the specified times.

For the deflation limb of individual P-V curves the volumes (in μl) at the standardized pressures 0, 6, 10, 15, 20 and 25 cm H_2O were determined.

From the expiratory limbs of the P-V curves two other parameters were calculated:

1. Compliance at 15 cm H_2O (ml/cm H_2O)
2. Compliance normalized for body weight at 15 cm H_2O (ml/cm H_2O/kg bw)

In each group (C, NN, NH), at the mentioned time (0, 1, 6 hours), a mean volume and derived parameter was calculated. This enabled to compare the measured volumes and their derivatives in all groups at a certain pressure.

Comparison of the differences between the groups and their statistical significance were determined by means of T-test or analysis of variance (data were normally distributed). The P-values are indicated.

RESULTS

The body weights (4-6 g) and lung weights in this experiment correspond well with those reported in an earlier study [10].

First experiment

We considered the first experiment as a pilot study to establish differences between spontaneous born and hysterotomy delivered rats (controls and CDH rats). Several animals were lost because they were born at times when it was impossible to start measurements immediately.

Spontaneous born rats (Spon); CSpon (n=9) and NHSpon (n=8), and rats delivered by hysterotomy (Hyst); CHyst (n=9) and NHHyst (n=7) were compared at T=0 h and T=6 h. At T=0 h there was a significant difference in P-V relations between CSpon and the three other groups. At P=15 cm H_2O, the volumes of CSpon, CHyst, NHSpon and NHHyst were 210, 30, 40 and 40 μl, respectively. (Fig. 1; $p<1\%$). After 6 hours ventilation no significant difference was found between the control animals born spontaneously or obtained after hysterotomy. Because of this observed distinction in the control group, we decided to use only spontaneous born animals with known time of birth for the second experiment.

Second experiment

All animals were delivered spontaneously, intubated immediately and P-V measurements were made (Fig. 2). The total number of animals in each group is given in Table 1. In the NH groups left and right-sided CDH are present; their numbers are not specified. The size of the hernias were comparable at 0 h and 1 h: 70% was defined as "large dorsal", the remaining 30% as "medium dorsal", although marked differences in mechanical behaviour between the same type of hernia were observed.

Table 1.

Hours	C	NN	NH
0	23	22	15
1	28	20	31
6	30	31	14

Numbers of newborn rats used during the second experiment. C = Control; NN = Nitrofen without CDH; NH = Nitrofen with CDH. Numbers indicate animals used for obtaining P-V values.

The mortality in the NH group with postmortem proven severe CDH (coinciding with very low volumes) was higher: almost 80% did not survive the 6 hour period. In the less severe affected CDH this was about 30-40%, against 5% and 7% in the C and NN group, respectively. These mortality rates included the deaths after T=0 due to technical problems such as plugged canula or missed (re)-intubation.

After intubation and starting ventilation, the NH group showed almost no lung expansion, there was only a slowly change in colour of the fetus from blue to pink. In the C group this colour change was performed within two breaths, while in the NN group 6 animals had a similar colour change pattern as in the NH group. This small group of 6 animals is outnumbered by the other 16 animals from which several performed better than the controls.

Volumes

(See also Fig 3). At T=0 the NH group (n=15) has significantly lower volumes at matched pressures than the NN (n=22) and C (n=23) ($p<1\%$).

At T=1 and T=6 the difference between the NN and NH groups has almost disappeared, but both still show a significant difference compared to C ($p<1\%$).

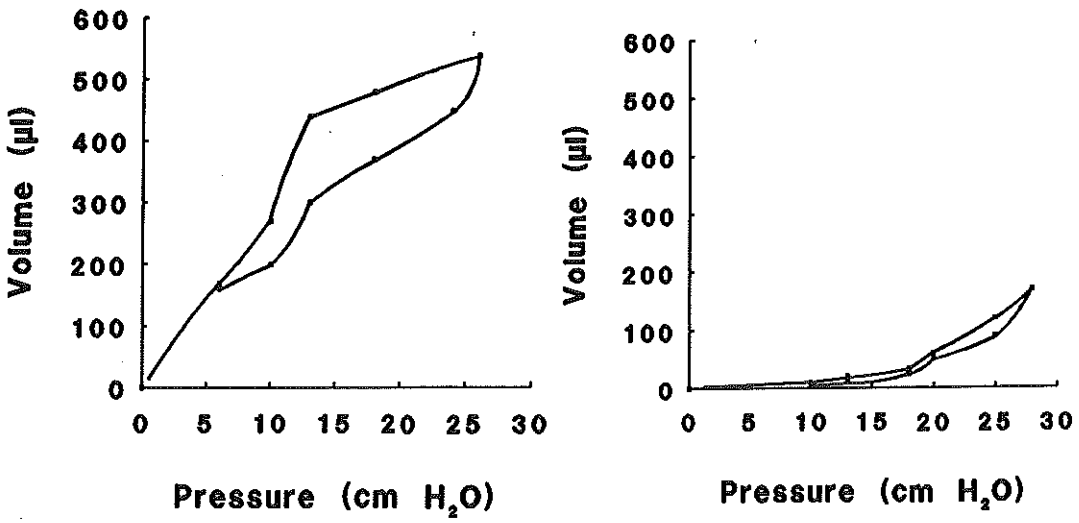


Figure 2. Two pressure-volume registrations from individual animals (T = 1 h): E70/5 (control) left and E68/2 (left-sided CDH) right. Pressures (cm H_2O) on the horizontal axis, the corresponding volumes (in µl) on the left axis. The CDH lung shows no stabilisation of the airways, resulting in high inflation pressures for small changes in volume; and a drop almost to zero at rather high expiratory pressures.

Compliance

Compliance and compliance normalized for body weight are significantly ($p < 1\%$) lower in the CDH rats. The body weight does not influence the compliance significantly.

Histology

Lungs obtained at T= 0, 1 and 6 hours ventilation, were examined. The lungs of CDH rats, irrespective at which hour they are examined, show very poor saccular air expansion, i.e. the appearance of most terminal air spaces corresponds to the fetal, fluid-filled state. There is almost no stabilization of the smaller airways. Instead, the thick-walled, densely compacted cells dominate the view (Fig. 4b). Even in the full-term born control and NN animals after 6 hours ventilation, aeration of the lungs was generally incomplete and irregular (Fig. 4a). In all groups, intrasaccular hemorrhage of varying degree was observed in most lungs, but there were no widespread hemorrhagic lesions. There were also clear signs of interstitial emphysema and, in a few cases, also interstitial edema.

DISCUSSION

In the present study, changes in lung mechanics due to induced CDH are evaluated. The results of the first experiment showed the effect of the passage through the birth canal on the first breaths and after 6 hours of ventilation. After 6 hours no significant differences were seen between the animals obtained by hysterotomy and those born spontaneously in the same group. The difference found between CSpon and NHSpon at T=0 h was not observed between CHyst and NHHyst ($p<1\%$). To provide adequate aeration of the liquid filled lung at birth, the insufflation pressure must be high enough to overcome capillarity in the finer conducting airways [16]. This opening pressure must be applied for a period long enough to overcome the viscosity of the fluid in the larger airways because the air-liquid interface is not founded in these larger airways and a considerable extra volume has to be absorbed by the saccules. Another important characteristic is to establish the functional residual capacity (FRC); this is organized quickly after birth and is slowed in hysterotomized animals [17]. Because of the result of the first experiment; a negatively influenced establishing of FRC due to hysterotomy, we decided to use spontaneous born animals for the second experiment.

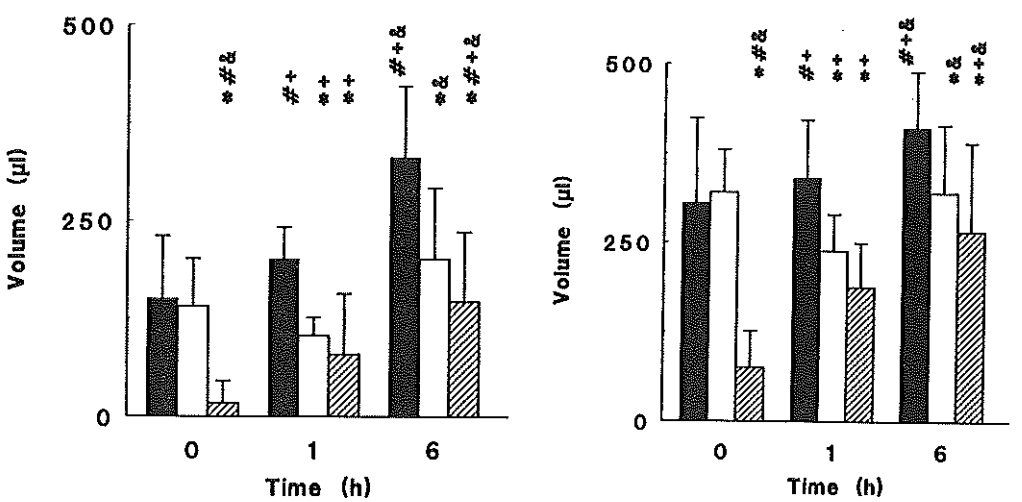


Figure 3. Mean volumes calculated from the individual expiratory loops at pressure 10 cm H_2O (3a) and 25 cm H_2O (3b) during the six-hour study. The horizontal axis represents the time (h), the corresponding volume (in µl) and standard deviation is shown on the vertical axis. Three different groups C (left bar, dark area), NN (middle bar, dense striped area), and NH (right bar, light striped area) are shown.

* = *significant from control at the same time*

\# = *significant from nitrofen at the same time*

\+ = *significant from same group at T=0 h*

& = *significant from same group at T=1 h*

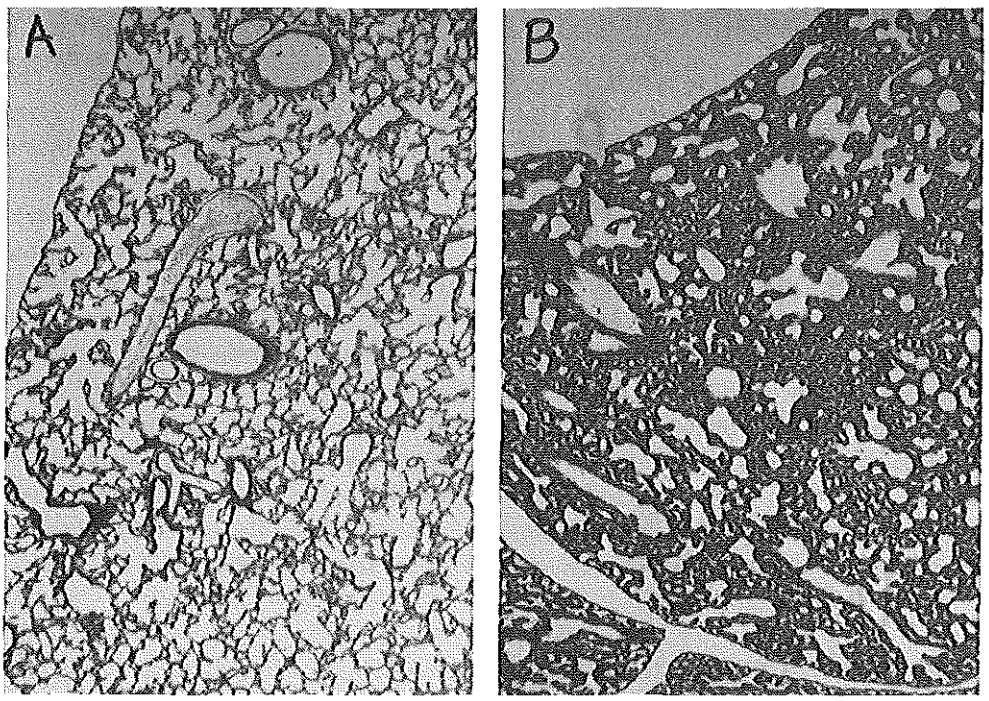


Figure 4. Control lung (left) and NH lung (right) both ventilated for 6 hours. Same original magnification (2.5 x), HE staining after Davidson fixation [15].

The results of the second experiment show the CDH rat model is suitable to evaluate different ventilatory regimens. Rats weighing 4-6 g can be intubated and ventilated for several hours. The mortality rate of about 80% in the very severe CDH group corresponds with the human situation [1,10]. Death is caused by pneumothorax, severe ventilatory insufficiency or pulmonary hypertension. This significantly influenced the results of our experiments after 6 hours. Careful examination of the data obtained in the second experiment suggests that an unforeseen natural selection took place in time; the NH survivors at $t=6$ h already showed at $t=0$ h, and 1 h better PV-relations than the mean value of the group ($p<5\%$). This could explain the gradual improvement in the NH group during the experiment. It may also be concluded that (as in the human situation) the very severe CDH animals die within 6 hours after birth, irrespective of their treatment. This important result has to be considered in future experiments.

It is well established that artificial ventilation influences normal cardiovascular and respiratory function [18]. Moreover, an important factor is that ventilation itself can lead to decreased lung compliance and dysfunction of gas exchange; the underlying causes are formation of atelectasis, pulmonary edema and interstitial emphysema [19]. To date, no

adequate explanation of the pathophysiological basis of all these changes due to artificial ventilation has been documented. However, there is evidence that in at least a part of these alterations, the surfactant system is involved [20]. The recording of pressure-volume (P-V) diagrams is the only direct in vivo test for characterization and indication of damage to the surfactant system and lung function [14,20].
Reports on the surfactant system and its function in CDH lungs in humans are scarce; they only show the lecithin/sphingomyelin ratio, which is an inaccurate method of testing surfactant function. This ratio was reported to be lowered [21] or unchanged [22].

In the present set of experiments, the ventilatory settings were kept constant because we were only interested in the effect of ventilation on CDH mechanics in our rat model; the influence of different ventilatory settings in our model are subject of later studies. It is demonstrated that the presence of CDH is associated with decreased total lung capacity (TLC) and compliance, as was also found in the lamb model [23-25]. It can be expected that the total lung volume will be proportional decreased, but there has to be other contributing factors, so the enormous reduction in TLC can only partly be explained by relating it to pulmonary hypoplasia. Pringle et al [26] suggested three possible factors based on lamb studies: first, a failure of development of the respiratory surfaces; second, the increased thickness of the barrier to gaseous exchange; and third pulmonary hypertension. The presumed surfactant deficiency has an important role [25], it is suggested to have an increased inhibitory effect of the alveolar proteins on surfactant function; on the other hand a decreased production of surfactant.
In the rat model a retarded differentiation of cuboid type II into squamous type I cells has been reprted, as well as morphometric proven smaller lung volumes and lung tissue volumes, smaller airspace/tissue ratios, and smaller epithelial surface areas [27,28]. Suen et al [29] found a lower amount of total disaturated phosphatidylcholine (DSPC) per mg lung and per μg DNA in the rat model .
Another indication for involvement of the surfactant system is found in the low compliance of CDH lungs in the present study. The airways are not stabilized during deflation so they tend to collapse, resulting in high inflation pressures throughout the experiment.
The NN group shows the same intermediate position as in the RSC study [10], suggesting a sliding scale from healthy lung to CDH. This implies new evidence to the theory that the lung is primarily affected and that the defect diaphragm is perhaps secondary to this anomaly.
In conclusion, the results of this study demonstrated the rat model to be an effective tool in CDH research for functional test of drugs or ventilatory effects.

REFERENCES.

1. Fauza DO, and Wilson JM. Congenital diaphragmatic hernia and associated anomalies: their

incidence, identification, and impact on prognosis. J Pediatr Surg 1994;29:1113-7.

2. Philip JR, Gambarelli D, and Guys JM. Epidemiological study of congenital diaphragmatic hernia: associated malformations. J Pediatr Surg 1991; 23:899-903.
3. Manni M, Heydanus R, Den Hollander NS, and Stewart PA. Prenatal diagnosis of congenital diaphragmatic hernia: a retrospective analysis of 28 cases. Prenat Diag 1994;14:187-90.
4. Northway WH, Rosan RC, and Porter DY. Pulmonary disease following respiratory therapy of hyaline membrane disease. N Engl J Med 1967; 267:357-362.
5. Bancalari E, Gerhardt T. Bronchopulmonary dyplasia. Pediatr Clin North Am 1986; 33:1-23.
6. Nio M, Haase G, Kennaugh J, et al. A prospective trial of delayed versus immediate repair of congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:618-21.
7. O'Rourke PP, Lillehei C, Crone RK, and Vacanti JP. The effect of extracorporeal membrane oxygenation on the survival of neonates with high risk congenital diaphragmatic hernia: 45 cases from a single institution. J Pediatr Surg 1991; 26:147-152.
8. Bos AP, Tibboel D, Hazebroek FWJ, Molenaar JC, Lachmann B, and Gommers D. Surfactant replacemant therapy in high risk congenital diaphragmatic hernia. Lancet 1991; 338:1279.
9. Glick PL, Leach CL, and Holm B. Pathophysiology of congenital diaphragmatic hernia III: Exogenous surfactant therapy for the highrisk neonate with CDH. J Pediatr Surg 1992; 27:866-869.
10. Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Lachmann B, and Molenaar JC. Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1990;25: 426-9.
11. Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, and Lambrecht W. Nitrofen-induced diaphragmatic hernia in rats: an animal model. J Pediatr Surg 1990;25:850-4.
12. Bos AP, Tenbrinck R, Tibboel D, Molenaar JC and Lachmann B. Artificial ventilation in newborn rats with experimentally induced congenital diaphragmatic hernia. Pediatr Res 1990;27 Suppl:A1761.
13. Scheffers EC, IJsselstijn H, Tenbrinck R, Lachmann B, De Jongste JC, Molenaar JC et al. Evaluation of lung function changes before and after surfactant application during artificial ventilation in newborn rats with congenital diaphragmatic hernia. J Pediatr Surg 1994;29:820-4.
14. Lachmann B, Grossmann G, Freyse J, and Robertson B. Lung-thorax compliance in the artificially ventilated premature rabbit neonate in relation to variations in inspiration:expiration ratio. Pediatr Res 1981; 15:833-838.
15. Tenbrinck R, Gaillard JLJ, Tibboel D, Kluth D, Lachmann B, and Molenaar JC. Pulmonary vascular abnormalities in experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1992; 27:862-865.
16. Agostini E, Taglietti A, Agostini A, and Setnikar I. Mechanical aspects of the first breath. J Appl Physiol 1958;13:344.
17. Klaus M, Tooley WH, Weaver KH, and Clements JA. Lung volume in the newborn infant. Pediatrics 1962;30:111.
18. Price HL, Connnor EH, and Dripps RD: Some respiratory and circulatory effects of mechanical respirators. J Appl Physiol 1954; 6:517.
19. Nilsson R, Grossmann G, and Robertson B: Lung surfactant and the pathogenesis of neonatal bronchiolar lesions induced by artificial ventilation. Pediatr Res 1978; 12: 249-55.
20. Bos JAH, Lachmann B. Surfactant function: is it influenced by artificial ventilation? In: The surfactant system in the lung. Cosmi EV, Di Renzo GC, Ancesschi MM (eds.), Houndmills and London, Macmillan Academic and Professional Ltd, 1991; 96-107.
21. Hisanaga S, and Shimokawa H. Unexpectedly low lecithin/sphingomyelin ratio associated with fetal diaphragmatic hernia. Am J Obstet Gynecol 1984;149:905.
22. Sullivan KM, Hawgood S, Flake AW, Harrison MR, and Adzick NS. Amniotic fluid phospholipid analysis in the fetus with congenital diaphragmatic hernia. J Pediatr Surg 1994;29:1020-3.
23. Adzick NS, Outwater KM, Harrison MR, Davies P, Glick PL, de Lorimier AA et al: Correction of

congenital diaphragmatic hernia in utero. IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 1985;20:673-80.

24. Pringle KC, Turner JW, Schofield JC, and Soper RT. Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 1984;18:131-40.
25. Glick PL, Stannard VA, Leach CL, et al: Pathophysiology of congenital diaphragmatic hernia II: The fetal lamb CDH model surfactant deficient. J Pediatr Surg 1992;27:382-8.
26. Pringle K. Lung development in congenital diaphragmatic hernia. In: Puri P, editor. Congenital diaphragmatic hernia. Mod Probl Paediatr. Basel: Karger, 1989;24:28-53.
27. Brandsma AE, Ten Have-Opbroek AAW, Vulto IM, Molenaar JC and Tibboel D. Alveolar epithelial composition and architecture of the late fetal pulmonary acinus: An immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Exp Lung Res 1994;20:491-515.
28. Brandsma AE, Tibboel D, Vulto IM, Egberts AAW, Ten Have-Opbroek. Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus: A comparison between normal and hypoplastic lungs using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Microsc Res Techn 1993;26:389-99.
29. Suen H, Catlin E, Ryan DP, Wain JC, and Donahoe PK. Biochemical immaturity of lung in congenital diaphragmatic hernia. J Pediatr Surg 1993;28:471-7.

EVALUATION OF LUNG FUNCTION CHANGES BEFORE AND AFTER SURFACTANT APPLICATION DURING ARTIFICIAL VENTILATION IN NEWBORN RATS WITH CONGENITAL DIAPHRAGMATIC HERNIA

(J Pediatr Surg 1994; 29:820-824)

Abstract

Patients with congenital diaphragmatic hernia (CDH) have unilateral or bilateral hypoplasia of the lungs including delayed maturation of the terminal air sacs. Because these lungs are highly susceptible to barotrauma and oxygen toxicity, even in full-term newborns, continued research into optimal ventilatory regimen is essential to improve survival rate and to prevent ongoing lung damage. Against this background, the effect of exogenous surfactant application is evaluated. In newborn rats, CDH was induced after a single dose of 2,4 dichloro-4'-nitrophenyl (Nitrofen) (400 mg/kg) on day 10 of gestation. The newborn rats were intubated immediately after hysterotomy, transferred to a heated multichambered body plethysmograph, and artificially ventilated. Inspiratory peak pressures were initially set at 17 cm H_2O, with positive end-expiratory pressure at 0 cm H_20 and FiO_2 at 1.0. The pressure was raised in steps of 5 cm H_20, from 5 to 30 cm H_20, to obtain pressure-volume diagrams at 0, 1, and 6 hours of artifical ventilation. These measurements were obtained in controls and in CDH rats with and without endotracheal installation of bovine surfactant (n = 4 to 10 in each group). Significant differences in lung volume between CDH and control rats were observed at all time-points. Surfactant application had a positive effect on lung volume, especially in control rats at t = 1 hour. No significant differences were observed between the CDH groups at t = 1 or t = 6 hours. In this animal model, the effect of artificial ventilation as well as the beneficial short-term effect of exogenous surfactant application have been evaluated. A continued positive effect on lung volume in CDH lungs could not be determined. Routine administration of exogenous surfactant in human CDH patients is not supported by these experimental results.

Supported by the Sophia Foundation for Scientific Research and the Dutch Astma Foundation.

In patients who have congenital diaphragmatic hernia (CDH), abnormal morphological development of both lungs and the intrapulmonary blood vessels is present[1-5]. However, reports on the biochemical maturation of the lung in CDH are scarce.
Hisanaga et al[6] noted low lecithin:sphingomyelin ratios (0.56 and 0.57, respectively) in two infants with CDH. They argued that lung hypoplasia actually means a reduced number of type II pneumocytes and reduced production of surfactant. The lecithin:sphingomyelin ratio reflects to some extent the maturation of the fetal lung. However, the question remains: are the ratios low because of the total reduction in lung tissue or because the type II cell in CDH functions at a lower level[7]?
Hashimoto et al[8] investigated the morphological characteristics of the type II cell in a fetal lamb model of CDH. Surprisingly, they found that type II cells were 5 to 10 times more abundant in the lungs of animals with a diaphragmatic defect. No ultrastructural changes of immaturity were observed in type II cells.
Glick et al[9] studied lung surfactant production in the fetal lamb after experimental induction of CDH and showed (1) a marked decrease in pulmonary compliance, (2) a reduction of the total amount of phospholipid in bronchoalveolar lavage fluid, and (3) a reduction in the synthesis rate of phosphatidylcholine by type II cells. The authors concluded that CDH in the fetal lamb leads to profound lung hypoplasia and apparent immaturity of the surfactant system.
In neonatal rats, CDH can be induced by feeding of 2,4 dichloro-4'-nitrophenyl ether (Nitrofen, Rohm & Haas Co, Philadelphia, PA) to the mothers during gestation, as documented previously[10-12]. In this model, the lungs of CDH animals showed hypoplasia and lower content of disaturated phosphatidylcholine per microgram DNA and total disaturated phosphatidylcholine, as shown by Suen[13].
The conflicting results obtained in different animal models has guided us to investigate the effect of exogenous surfactant application in relation to changes in lung volume after artificial ventilation in newborn rats with CDH.

MATERIALS AND METHODS

Female Sprague Dawley rats (Harlan, Zeist, The Netherlands) weighing 240 to 280 g were mated during 1 hour (day 0 of gestation). On day 10 of gestation, 100 mg of 2,4 dichloro-4'-nitrophenyl ether (Nitrofen), dissolved in 1 ml of olive oil, was administered by gastric tube to the Nitrofen group; controls received 1 ml of olive oil. Food and water were supplied ad libitum during the entire period. Hysterotomy was performed on gestational day 22.
The animals born spontaneously were excluded from the experiment because the period of spontaneous breathing could not be determined in detail and would influence lung function parameters. There were three groups of newborns: controls, animals with a diaphragmatic hernia, and animals without a diaphragmatic defect after Nitrofen administration (10%-20% of the animals receiving Nitrofen).

Before the hysterotomy, the dam was anaesthetized with N_2O and enflurane-inhalation, and the fetuses were delivered. Immediately after hysterotomy, the newborns were weighed and received pancuronium bromide (0.08 mg/kg each second hour) and pentobarbital (40 mg/kg each third hour) intraperitoneally, followed by intubation with a metal canula. The cannulas used throughout the experiment were made from syringe needles (internal diameter, 0.5 mm; external diameter, 0.7 mm).
The intubated animals were immediately transferred to a multichambered, pressure-constant body plethysmograph heated to 38°C. This procedure lasted 1 to 2 minutes for each animal. A flexible tube provided an adequate connection between the trachea and the body-box. The maximum number of ventilated animals per litter was nine.

Artificial Ventilation

The body plethysmograph was connected to a modified ventilator (Servo 900B; Siemens-Elema, Solna, Sweden), as routinely used in the I.C.U., and as described by Lachmann et al[14]. This equipment provides pressure-generated ventilation with decelerating flow, using excess flow through the ventilator system.
The ventilator settings throughout the experiment were as follows: FiO_2, 1.0; frequency, 40/min; I:E ratio, 1:2; inspiratory peak pressure, 17 cm H_2O; positive end-expiratory pressure (PEEP), 0 cm. These settings were changed only to obtain pressure-volume relations, ie, the pressures were raised from 7 cm H_2O, in steps of approximately 5 cm H_2O, to a maximum of 30 cm H_2O. The other settings remained the same throughout the experiments. The pressure-volume relations were determined for each fetus with a specially designed Fleisch-tube[14] connected to the body plethysmograph, a differential pressure transducer (EMT 34; (Siemens Elema) and amplifier (EMT 31), an integrator unit (EMT 41), and a recorder Mingograf 81; Siemens-Elema).
The animals were ventilated for a maximum of 6 hours. Pressure-volume relations were determined at 0, 1, and 6 hours. Zero hours applied to the animals that were intubated immediately after hysterotomy because they showed no signs of spontaneous breathing. The animals without visible heart action, pneumothorax, or other complications related to insufficient ventilation or technical problems were excluded from the study. The same holds true for animals without CDH after Nitrofen administration and those with huge diaphragmatic defects that died less than 6 hours after birth. After the experiments, the animals were killed by an overdose of pentobarbital.

Surfactant Application

After intubation and the start of artificial ventilation (with peak inspiratory pressures of 17 cm H_20, PEEP of 0 cm H_2O, and FiO_2 of 1.0), both control and Nitrofen animals received either a bolus of bovine surfactant (0.05 ml of a 25-mg/ml solution) or nothing. Pressure-volume diagrams were obtained as described before, at $t = 1$ and $t = 6$ hours.

Calculations

By means of the measured pressure-volume points, a pressure-volume curve was plotted for each animal at each time-point. The volume at the standardized pressures of 7, 10, 15, 20, 25, and 30 cm H_2O was determined from these curves and recorded. For each group, the means of volume were calculated at standardized pressures. These means were compared, and statistical significance was determined by means of the Mann-Whitney or Student's t test.

RESULTS

For each group, the values of lung volume at the peak pressures of 15 and 25 cm H_20 (with standard deviation) as well as the number of animals at each time-point are shown in Table 1.

Table 1. Pressure/Volume Relations

Time (hours)		Control			
		No Surfactant		Surfactant	
		15 cm H_2O	25 cm H_2O	15 cm H_2O	25 cm H_2O
0	Mean	24.8	81.4	—	—
	SD	17.6	32.1		
	n	15			
	Significance				
1	Mean	109.8	160.4	108.6	216.9
	SD	34.9	41.1	59.2	61.9
	n	9		12	
	Significance	@	@	@	@*
6	Mean	141.3	165.1	146.5	190.4
	SD	50.8	63.3	45.9	61.5
	n	8		10	
	Significance	@	@	@	@

Time (hours)		Hernia			
		No Surfactant		Surfactant	
		15 cm H_2O	25 cm H_2O	15 cm H_2O	25 cm H_2O
0	Mean	5.9	22.4	—	—
	SD	5.5	23.6		
	n	9			
	Significance	#	#		
1	Mean	34.1	95.3	26.9	83.5
	SD	13.9	38.2	17.1	20.6
	n	6		6	
	Significance	@#	@#	@#	@#
6	Mean	41.6	87.8	44.9	85.6
	SD	27.3	25.3	30.4	38.6
	n	6		4	
	Significance	@#	@#	@#	@#

Note. For all values, $p<5\%$.

Abbreviations: @, significant from T=0 in the same group;
**, significant from the same group without surfactant;*
#, significant from control at the same time.

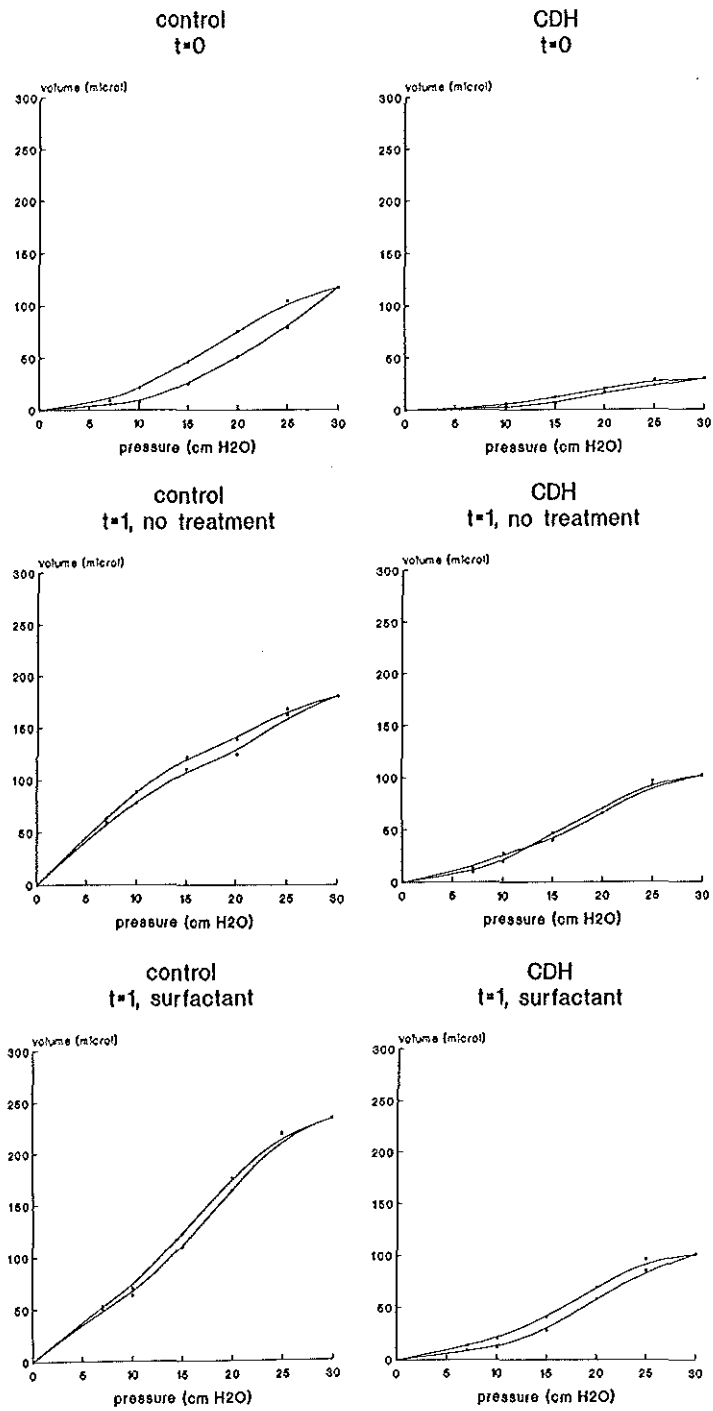


Fig 1. Representative pressure-volume curves at t=0 and t=1 in control rats, CDH rats without treatment, and CDH rats with surfactant.

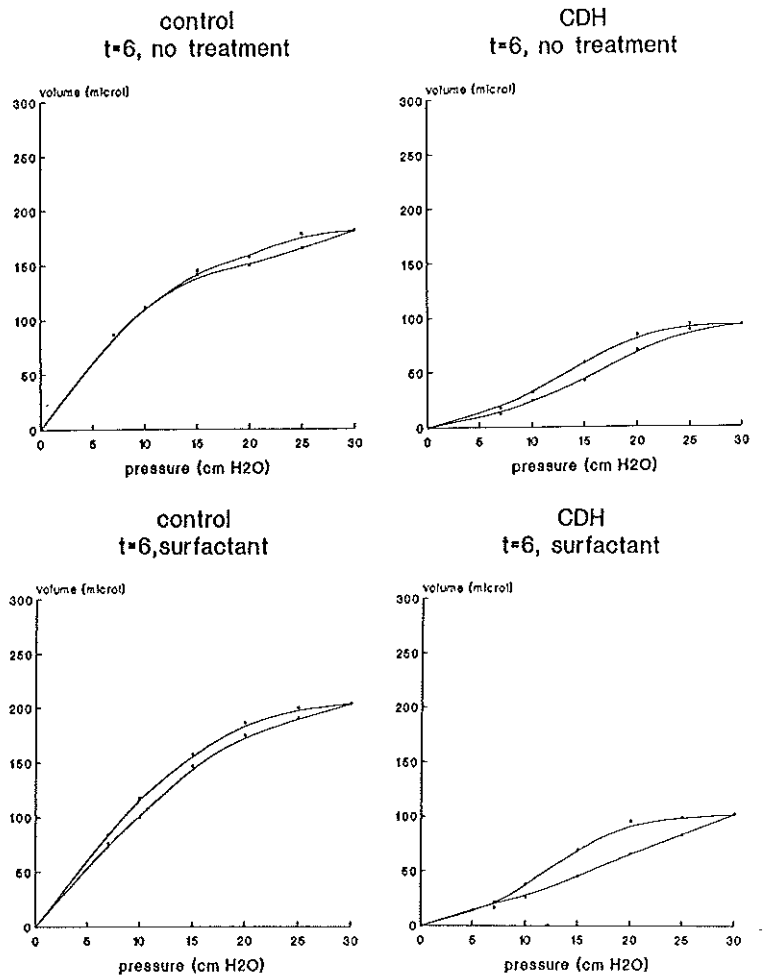


Fig 2. Representative pressure-volume curves at t=6 in control rats, CDH rats without treatment, and CDH rats with surfactant.

Representative pressure-volume diagrams are shown in Figs 1 and 2. There are significant differences for each time-point between control and CDH rats, with CDH rats having lower lung volumes. The effect of surfactant application is greater in controls than in CDH rats. A positive effect of surfactant application in control rats was found at t = 1 hour. There were no significant differences at t = 1 or t = 6 hours between the CDH groups.

DISCUSSION

Animal Data

Our study shows not only that artificial ventilation of neonatal rats is possible but also that the effect of exogenous surfactant application can be studied in detail. Following the classic approach of inducing CDH in sheep (by Harrison et al[15]), Hashimoto et al [18] investigated the morphological characteristics of the type II cell in a fetal lamb model of CDH. Surprisingly, they found that type II cells were 5 to 10 times more abundant in the lungs of animals having a diaphragmatic defect. No ultrastructural changes of immaturity were observed in type II cells.

In contrast to the above-mentioned study, various other research groups have documented or suggested that in the hypoplastic lungs of CDH rats, immaturity of the lungs exists[12,13,16]. Suen et al[13], using whole-lung homogenates, noted significantly lower desaturated phosphatidylcholine (DSPC) per microgram DNA and total DSPC in CDH rats, and Brandsma et al[16] using bronchoalveolar lavages of control and CDH rats, came to the same conclusion. Recently, Suen et al[17] documented the positive effect of antenatal glucocorticoid treatment on the DSPC content of whole lung homogenates in rats with Nitrofen-induced CDH.

The reason we were not able to demonstrate a continuing positive effect on lung volume in CDH rats might be related to the method of delivery, dosage, timing, or volume of surfactant application; the effect of introducing PEEP should be determined as well.

Human Data

Increasing evidence shows that the hypoplastic lung in CDH is developmentally retarded[1-3]. A lower lecithin-sphingo myelin (L/S) ratio in the amniotic fluid[6] as well as morphological findings showing hyaline membranes in full-term infants with CDH emphasize the significance of a further characterization of the developing terminal lung unit in these patients. For a patient with CDH, accurate determination of the presence of a surfactant deficiency, either primary or secondary, will be of great significance in the selection of appropriate treatment modalities[18]. Moreover, the high incidence of bronchopulmonary dysplasia in surviving patients[19] might be prevented by early administration of surfactant, either prophylactically or therapeutically, as has been shown for premature infants with respiratory distress syndrome[20].

Treatment of CDH can be individualized by using standard procedures such as bronchoalveolar lavages, as documented by Stenmark et al[21] newborns with persistent pulmonary hypertension, to detect surfactant deficiency. Prospective randomized trials in CDH patients, using exogenous surfactant as either prophylaxis or rescue therapy, should be undertaken in the near future to test the value of this approach.

REFERENCES

1. Wigglesworth JS, Desai R, Guerrini P: Fetal lung hypoplasia: Biochemical and structural variations and their possible significance. Arch Dis Child 56:606-615, 1981
2. George DK, Cooney TP, Chiu BK, et al: Hypoplasia and immaturity of the terminal lung unit (acinus) in congenital diaphragmatic hernia. Am Rev Respir Dis 136:947-950, 1987
3. Nakamura Y, Yamamoto I, Fukuda S, et al: Pulmonary acinar development in diaphragmatic hernia. Arch Pathol Lab Med 115:372-376, 1991
4. Levin DL: Morphologic analysis of the pulmonary vascular bed in congenital left-sided diaphragmatic hernia. J Pediatr 92:805-809, 1978
5. Geggel RL, Murphy JD, Langleben D, et al: Congenital diaphragmatic hernia: Arterial structural changes and persistent pulmonary hypertension after surgical repair. J Pediatr 107:457-464, 1985
6. Hisanaga S, Shimokawa H, Kashiwabara Y, et al: Unexpectedly low lecithin/sphingomyelin ratios associated with fetal diaphragmatic hernia. Am J Obstet Gynecol 89:905-906, 1984
7. Kikkawa Y, Motoyama EK, Cook CD: The ultrastructure of the lungs in lambs: The relation of osmiophilic inclusions and alveolar lining layer to fetal maturation and experimentally produced respiratory distress. Am J Pathol 47:877-879, 1965
8. Hashimoto EG, Pringle KC, Soper RT, et al: The creation and repair of diaphragmatic hernia in fetal lambs: Morphology of the type II alveolar cell. J Pediatr Surg 20:354-356, 1985
9. Glick PL, Stannard VA, Leach CL, et al: Pathophysiology of congenital diaphragmatic hernia II: The fetal lamb CDH model is surfactant deficient. J Pediatr Surg 27:382-388, 1992
10. Tenbrinck R, Tibboel D, Gaillard JLJ, et al: Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 25:426-429, 1990
11. Kluth D, Kangah R, Reich R, et al: Nitrofen-induced diaphragmatic hernia in rats: An animal model. J Pediatr Surg 25:850-854. 1990
12. Alfonso LF, Vilanova J, Aldazabal P, et al: Lung growth and maturation in the rat model of experimentally induced congenital diaphragmatic hernia. Eur J Pediatr Surg 3:6-11, 1993
13. Suen HC, Catlin EA, Ryan DP, et al: Biochemical immaturity of lungs in congenital diaphragmatic hernia. J Pediatr Surg 28:471-477, 1993
14. Lachmann B, Grossman G, Freyse J, et al: Lung-thorax compliance in the artificially ventilated premature rabbit neonate in relation to variations in inspiration:expiration ratio. Pediatr Res 19:833-838, 1981
15. Harrison MR, Jester JA, Ross NA: Correction of congenital diaphragmatic hernia in utero: I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. Surgery 88:174-182, 1980
16. Brandsma A, Tibboel D, Vulto IM: Ultrastructural features of alveolar epithelial cells in the late fetal pulmonary acinus; A comparison between nommal and hypoplastic lungs, using a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. Micros Res Techn 26:389-399, 1993
17. Suen HC, Bloch D, Donahoe PK: Antenatal glucocorticoid treatment corrects the pulmonary immaturity of congenital diaphragramatic hernia. J Pediatr Surg (in press)
18. Bos AP, Tibboel D, Hazebroek FWJ, et al: Surfactant replacement therapy in high-risk congenital diaphragmatic hernia. Lancet 338:1279, 1991 (letter)
19. Bos AP, Hussain SM, Hazebroek FWJ, et al: Radiographic evidence of bronchopulmonary dysplasia in high-risk congenital diaphragmatic hemia survivors. Pediatr Pulmonol 15:231-234, 1993
20. Jobe AH: Pulmonary surfactant therapy. N Engl J Med 328:861-868, 1993
21. Stenmark KR, James SL, Voelkel NF, et al: Leukotriene C_4 and D_4 in neonates with hypoxemia and pulmonary hypertension. N Engl J Med 309:77-80, 1983

EFFECT OF ARTIFICIAL VENTILATION ON PULMONARY ANTIOXIDANT ENZYME ACTIVITIES IN A CONGENITAL DIAPHRAGMATIC HERNIA RAT MODEL

(Adv Exp Med Biol 1992; 317:363-70)

INTRODUCTION

Treatment of infants with congenital diaphragmatic hernia (CDH) developing respiratory insufficiency within a few hours after birth remains unsatisfactory. The incidence of CDH is about 1:3000 newborns (Hazebroek et al, 1988), mortality for these high-risk infants ranges from 30%-60%. These infants require aggressive respiratory support, including high pressures and oxygen concentrations. Frequently the clinical course is complicated by pulmonary hypertension. Compared to premature infants, CDH survivors have a high incidence (40%) of bronchopulmonary dysplasia (BPD) (Molenaar et al, 1991; Redmond et al, 1987; O'Rourke et al, 1991). Because this disease occurs almost exclusively in premature infants who receive mechanical ventilation with increased inspiratory oxygen concentration, it was postulated (Northway et al, 1967; Crapo, 1986) that oxygen alone is toxic to the lung parenchyma.Other factors that may play a role in BPD include gestational age, barotrauma, infection, the presence of a persistent ductus arteriosus (PDA), pulmonary hypertension and reperfusion damage. It is difficult to separate the effect of oxygen from those of other factors that may influence the development of BPD. Therefore the need for a reliable animal model (preferably with CDH) to study the pathogenesis of BPD and investigate protective measurements has augmented. DeLuca described barotrauma in ventilated CDH lambs; there was no specific mention of oxygen toxicity or its defense mechanisms (DeLuca et al, 1987).

The defense mechanism against oxygen damage has been extensively described (Tanswell and Freeman, 1984; Gerdin et al, 1985; Frank and Sosenko, 1987a,b), but none of them deals with ventilated newborn animals. From the results of the cited O_2 toxicity studies has evolved the concept that baseline antioxidative enzyme activity (AOA) levels are of much less importance in determining resistance or susceptibility to O_2-induced lung damage than are the responses of the AOA to hyperoxic challenge.

We are able to ventilate newborn rats with induced congenital diaphragmatic hernia (Tenbrinck et al, 1990) and investigated the AOA levels in these CDH newborns in comparison with the data from similar treated control animals.

This study consists of two experiments: the first to detect whether the development of

the baseline AOA levels in CDH rats differs from that of controls. We describe the developmental pattern of superoxide dismutase (CuZnSOD) (EC.1.15.1.1), catalase (EC.1.11.1.6) and glutathione peroxidase (GPX) (EC.1.11.1.9) in the CDH lung during late gestation from day 19 up to birth.
In the second experiment, animals were ventilated during 5 h with air or oxygen to establish different responses in AOA to hyperoxidative stress.

MATERIALS AND METHODS

CDH was induced in pregnant Sprague Dawley rats by means of the herbicide nitrofen (Tenbrinck et al, 1990). Two groups of animals were studied: C = controls and N = nitrofen. In the N group animals were obtained with CDH = NH and without CDH = NN. The distinction between NN and NH groups could only be made after autopsy.
In the first experiment, imaginable differences in late gestational development of AOA profiles were studied; in a second experiment the changes in AOA during 5 h ventilation with either air or 100% oxygen were observed.

Animals

In the first experiment, the fetuses were obtained by hysterotomy (days 19, 20, 21 of gestation) or after spontaneous birth at day 22 of gestation. After determination of bodyweight the fetuses were killed by an intraperitoneal injection of pentobarbital (2 g/kg) (Tenbrinck et al, 1990).
The thoracic cavity was opened, the presence of a possible diaphragmatic defect and its size noted; after this the lungs were perfused with phosphate buffered saline (PBS, 0.07 mol/l, 4°C) via the pulmonary artery until they turned pale white. The perfused lungs were taken out, weighed, frozen in liquid nitrogen and stored at -70°C for biochemical assay.
For the second experiment adult animals from groups C and N were allowed to deliver the pups after a gestation period of approximately 22 days. The pups were anesthetized (pentobarbital 35 mg/kg every 4 h), relaxed (pancuroniumbromide 0.1 mg/kg/h) and intubated. The tubes were connected with a body box (Lachmann, 1981) and the pups were ventilated with either FiO_2 = 0.21 or FiO_2 = 1.0 for 5 h to establish a possible difference in AOA during ventilation between C, NN and NH groups. The ventilator settings of the Servo 900B (Siemens Elema, Sweden) were the same throughout the experiment in C, NN and NH groups: pressure controlled 17/2 cm H_2O; respiration rate 40/min; I:E ratio 1:2. The animals were treated as in the first experiment after they had completed their 5 h ventilation period.

Biochemical analysis

After weighing, each obtained lung was coded and homogenized separately so no pools were made. In the homogenate we determined the protein (Lowry et al, 1951) and DNA (Labarca et al, 1980) content expressed per mg wet lung weight. For AOA estimation the

suspensions were centrifuged at 20,000 g for 30 min. The activity of the most prevailing SOD isoenzyme in the lung, the copper-zinc SOD (CuZnSOD) was assayed by the inhibition of xanthine xanthineoxidase catalyzed reduction of ferricytochrome-c at pH 10.2 in the presence of EDTA to chelate free copper; the unit of SOD activity was defined according to Fridovich. (Hayatdavoudi et al, 1981; Biemond et al, 1984). Catalase was measured as described earlier by Bergmeyer (Bergmeyer, 1955). Glutathione peroxidase (GPX) activity was assayed according to Paglia (Paglia et al, 1967). The activities of SOD, catalase and GPX were expressed as units per mg lung DNA to eliminate lung weight differences.

Statistical analysis

The data are presented as mean with one standard deviation (SD). After rank transformation treatment effects were evaluated by analysis of variance (Conover, 1981).If a significant F-value was found, Bonferroni's correction method for multiple comparisons was used to identify differences among the groups (Glantz, 1987). A difference was considered statistically significant when the p-value was < 5%. No further indication of p-values are made; however lower values were found.

RESULTS

Wet lung weights increased in C, NN and NH groups during gestation (Table 1), but the mean lung weight of NN and NH was significantly lower compared to C. The differences between NN and NH were also significant. No significant differences between body weights were found. The L/B ratio was also significantly lower in the NH group before birth, suggesting that lung hypoplasia develops before birth. Lung protein and DNA content expressed per mg wet lung remained virtually unchanged in all groups; this is shown by the protein/DNA ratio which did not alter over time and amounted to a mean value of 7 for all groups.

The CuZnSOD activity did not change significantly within the three groups between day 19 to birth (Fig 1A). Catalase activity showed a significant increase in activity between day 21 and birth (Fig 1B); however there was no significant difference between the groups. GPX activity (Fig 1C) increased in each group during gestation (time dependency in the three groups $P < 0.001$). The activity measured at birth for C, NN and NH was respectively 173%, 161% and 185% of the initial GPX activity at day 19 of gestation.

The effect of ventilation on AOA

In the NH group only 2 animals survived the 5 h ventilation period with $FiO_2 = 0.21$, the others died within a few hours after start of ventilation. Therefore it was decided to use data of animals immediately after birth as reference values rather than those who were not ventilated for 5 h, because none of the NH animals survived the 5 h without ventilation. There were no significant differences in lung weight, body weight, L/B ratio,

Table 1. Late gestational changes (day 19 until birth) in groups C, NN, NH in several lung parameters.

		LW mg	BW g	L/B ratio mg/g	DNA μg/mg LW	protein μg/mg LW	P/D ratio
C	19	43 (7)	1.51 (0.15)	28.2 (2.4)	10.4 (0.7)	71.5 (4.3)	6.9 (0.1)
	20	90 (5)	2.73 (0.3)	33.8 (1.7)	8.2 (1.4)	71.1 (4.7)	9.0 (1.8)
	21	122 (8)	3.90 (0.01)	33.5 (0.9)	9.6 (0.7)	62.8 (3.9)	6.6 (0.7)
	birth	136 (21)	5.43 (0.32)	28.1 (2.6)	10.3 (1.1)	64.1 (7.8)	6.5 (0.8)
NN	19	41 (4)	1.57 (0.14)	25.8 (1.6)	11.0 (0.5)	72.7 (4.3)	6.6 (0.1)
	20	66 (5) #	2.55 (0.45)	27.1 (1.8)	8.9 (1.4)	61.7 (6.2)	7.2 (0.8)
	21	137 (12)	4.49 (0.42)	30.8 (1.1)	7.5 (1.2)	59.8 (6.3)	8.3 (2.0)
	birth	109 (6) #	5.08 (0.07)	22.6 (1.2)	8.8 (0.5)	65.0 (6.8)	7.4 (0.8)
NH	19	35 (5) #	1.45 (0.14)	24.1 (1.8) #	11.2 (1.0)	76.9 (3.7)	6.7 (0.1)
	20	47 (18) #*	1.98 (0.73)	26.6 (2.7) #	9.7 (1.1)	65.2 (6.7)	6.7 (0.1)
	21	99 (17) #*	4.21 (0.72)	24.8 (5.1) #*	9.2 (0.8)	63.4 (3.5)	7.0 (0.9)
	birth	63 (10) #*	4.96 (0.44)	12.2 (2.1) #*	12.1 (1.6)	83.4 (11.3)	7.0 (0.1)

Abbreviations: values are expressed as mean ± (standard deviation); each parameter is assessed in 6 animals. LW lungweight; BW body weight; L/B ratio lung weight/ body weight; P/D ratio protein content/ DNA content. # significant ($p<0.05$) from C; * significant ($p<0.05$) from NN.

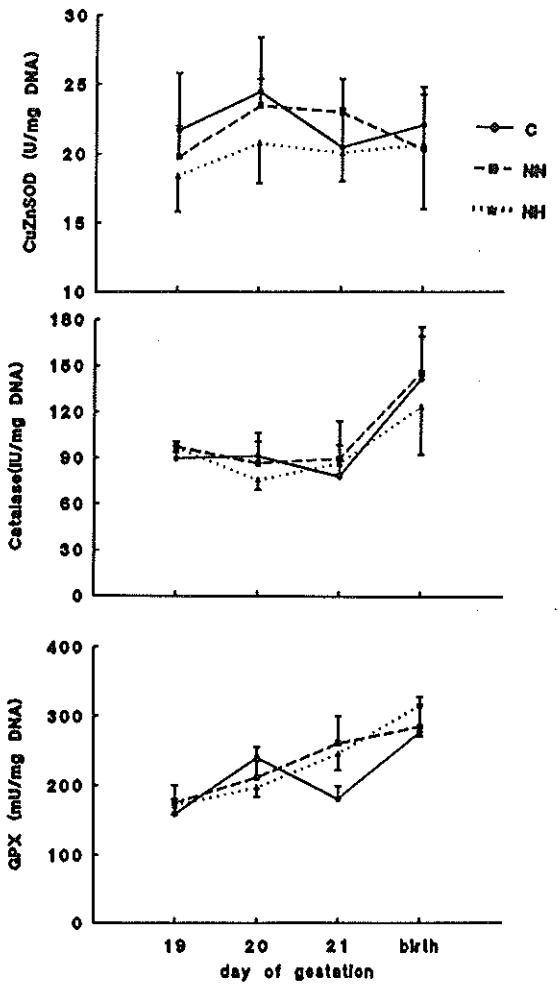


Figure 1. The development of AOA during gestation from day 19 until birth. The continuous lines represent the C; the striped line the NN and the dotted line the NH group. The CuZnSOD (a); catalase (b) and GPX (c) are shown. The values are expressed in activity per mg DNA and given as mean ± SD.

DNA, protein and P/D ratio between the ventilated animals and their initial values immediately after birth. The existing differences between the groups remained the same. Table 2 shows that in the C and NN groups the values for CuZnSOD, Catalase and GPX did not change significantly under the influence of ventilation, neither with air nor with 100% oxygen.

In the NH group CuZnSOD showed a tendency to decrease under both ventilatory conditions to about 85% of initial activity (P=0.18 for NH 100% oxygen). This value was significant from the same value in the C and NN groups. Catalase activity in the NH rats remained at the initial level and was similar to values in the C and NN groups.

Values for GPX activity decreased significantly to 78% and even 68% of initial values after ventilation with room air or 100% oxygen, respectively. Also compared to the C 100% value this means a significant decrease of 21%.

Table 2. The effect of ventilation with either air or 100% oxygen on SOD, catalase and GPX activities in the three groups (C, NN, NH).

		n	SOD (U/mg DNA)	Catalase (IU/mg DNA)	GPX (mU/mg DNA)
	birth	10	22.1 (2.2)	142 (27)	277 (40)
C	air	7	20.9 (3.5)	136 (23)	276 (19)
	100%	8	22.8 (4.5)	126 (14)	271 (41)
	birth	7	19.4 (4.4)	146 (30)	283 (44)
NN	air	6	24.5 (4.6)	117 (12)	264 (12)
	100%	9	24.6 (5.1)	144 (31)	255 (41)
	birth	7	20.7 (4.7)	124 (30)	315 (45)#
NH	air	2	17.5 (0.2)	119 (10)	246 (12)
	100%	6	17.9 (1.2)#	134 (40)	216 (24)*#

Values are expressed as mean ± (SD), * significant (p<5%) from birth value in the same group, # significant (p<5%) from controls.

DISCUSSION

CDH lungs are hypoplastic in humans (Areechon, 1963), as well as in the rat model (Tenbrinck, 1990) this is mainly based on morphological differences, however little is known about the biochemical compound of the hypoplastic lung. Frank reported that in the rat the chronology of development of AOA is very similar to that of the fetal surfactant system (Frank, 1987a). A different content of lecithin-sphingomyelin in

amniotic fluid of fetuses with hypoplastic lungs has been found (Hisanaga, 1984). CDH lungs of humans (Redmond, 1987) and fetal lambs (DeLuca et al, 1987) are very vulnerable to high inflation pressures. Nothing is known about their reaction to high inspiratory oxygen concentrations during ventilation. In healthy lungs the AOA levels are strongly correlated with the degree of protection that may be anticipated from O_2 radical induced lung injury. Increased enzyme activity has been consistently found in association with tolerance to hyperoxia, and reduced AOA usually leads to greater than normal susceptibility of the lung to high O_2 concentrations. This phenomenon was recently demonstrated in healthy premature rabbits compared with term rabbits (Frank and Sosenko, 1991). Literature search revealed no study in which the effect of ventilation on AOA in newborn CDH vs healthy (rat) lungs was described.

In the first experiment of this study we concluded that baseline AOA levels are almost the same throughout the three groups. We also found a similar developmental pattern in late gestation. These results are in accordance with those found by others (Frank and Sosenko, 1987a; Tanswell, 1984; Gerdin et al, 1985; Hayashibe et al, 1990). The lung weights in the NH group were lower than that in C and NN so the total amount of AOA is reduced. This indicates that the CDH lungs does not differ qualitatively from the C and NN lungs, but only quantitatively. This is also supported by the unchanged protein/DNA ratio in the groups.

In the second experiment we concluded that in the NH group the SOD and GPX activities tended to decrease during ventilation both with room air and oxygen. This supports our hypothesis that the CDH lung behaves like a premature lung and unlike C and NN lungs, would fail to mount a protective increase in AOA during ventilation with high FiO_2. This failure could be an explanation for the increased susceptibility to O_2-induced damage in CDH lungs.

Because the NH FiO_2 = 0.21 group consisted of only 2 surviving animals, care has to be taken with the interpretation of these results. Also in the larger NH FiO_2 = 1.0 group care has to be taken with simplification that the decreased SOD and GPX levels alone are the cause of the high incidence of BPD in human CDH survivors. On the basis of these experiments we could only speculate about the consequences of our results for the clinical practice. Of more importance is that this is the first applicable in vivo study that deals with the problem of oxygen toxicity in hypoplastic CDH lungs during ventilation.

REFERENCES

Areechon W, Reid L, 1963, Hypoplasia of lung with congenital diaphragmatic hernia, Br Med J, 1:230.

Bergmeyer HU, 1955, Zur messung von Katalase-aktivitaten, Biochem Z, 327: 255.

Biemond P, Swaak AJG, Koster JF,1984, Protective factors against oxygen free radicals and hydogen peroxide in rheumatoid arthritis synovial fluid, Arthritis Rheum 27:760.

Conover WJ, Iman RL, 1981, Rank transformations as a bridge between parametric and nonparametric statistics, Amer Statist, 35: 124.

Crapo JD, 1986, Morphologic changes in pulmonary oxygen toxicity, Ann Rev Physiol,48:721.
DeLuca U, Cloutier R, Laberge JM, Fournier L and Guttman FM,1987, Pulmonary barotrauma in congenital diaphragmatic hernia: experimental study in lambs, J Pediatr Surg, 22: 311.
Frank L, Sosenko IRS, 1987a, Development of lung antioxidant enzyme system in late gestation: possible implications for the prematurely born infant, J Pediatr, 110:9.
Frank L, Sosenko IRS, 1987b, Prenatal development of lung antioxidant enzymes in four species,J Pediatr, 110:106.
Frank L, Sosenko IRS, 1991, Failure of premature rabbit to increase antioxidant enzymes during hyperoxic exposure: increased susceptibility to pulmonary oxygen toxicity compared with term rabbits, Pediatr Res, 29:292.
Gerdin E, Tyden O, Eriksson UJ, 1985, The development of antioxidant enzymatic defense in the perinatal rat lung: activities of SOD, GPX, and catalase, Pediatr Res, 19:687.
Glantz SA, 1987, Primer of biostatistics, 2nd ed. McGraw-Hill, New York.
Hayashibe H, Asayama K, Dobashi K, Kato K, 1990, Prenatal development of AOE in rat lung, kidney and heart: marked increase in immunoreactive superoxide dismutases, glutathione peroxidase and catalase in the kidney, Pediatr Res, 27: 472.
Hayatdavoudi G, O'Neill JJ, Barry BE, Freeman BA, Crapo JD, 1981, Pulmonary injury in rats following continious exposure to 60% O_2 for 7 days, J Appl Physiol, 51:1220.
Hazebroek FJW, Tibboel D, and Molenaar J, 1988, Congenital diaphragmatic hernia: the impact of preoperative stabilization. A prospective pilot study in 13 patients, J Pediatr Surg 23: 1139
Hisanaga S, Shimokawa H, 1984, Unexpectedly low lecithin/sphingomyelin ratio associated with fetal diaphragmatic hernia, Am J Obstet Gynecol 149:905.
Labarca C, Paigen K, 1980, A simple, rapid and sensitive DNA assay procedure, Anal Biochem, 102:344.
Lachmann B, Grossmann G, Freyse J, Robertson B, 1981, Lung thorax compliance in the artificially ventilated premature rabbit neonate in relation to variations in I:E ratio, Pediatr Res, 15:833.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951, Protein measurements with the folin phenol reagent, J Biol Chem, 193:265.
Molenaar JC, Bos AP and Tibboel D, 1991, Congenital diaphragmatic hernia, what defect?, J Pediatr Surg, 26: 248.
Northway WH, Rosan RC, Porter DY, 1967, Pulmonary disease following respiratory therapy of hyaline membrane disease, N Eng J Med, 267,357.
O'Rourke PP, Lillehei CW, Crone RK, Vacanti JP, 1991, The effect of ECMO on the survival of neonates with high-risk congenital diaphragmatic hernia: 45 cases from one institution, J Pediatr Surg,26:147.
Paglia DE, Valentine WN, 1967, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J Lab Clin Med, 70: 158.
Redmond C, Heaton J, Calix J, Graves E, Farr G and Arensman R, 1987, A correlation of pulmonary hypoplasia, MAP and survival in congenital diaphragmatic hernia treated with ECMO, J Pediatr Surg, 22,1143.
Tanswell AK, Freeman BA, 1984, Pulmonary antioxidant enzyme maturation in the fetal and neonatal rat.I. Developmental profiles, Pediatr Res,18:584.
Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, Lachmann B and Molenaar JC, 1990, Experimentally induced congenital diaphragmatic hernia in rats, J Pediatr Surg, 25: 426.

EPILOGUE

10.1 General

Neonates with congenital diaphragmatic hernia (CDH) experience a high mortality despite intensive medical and surgical treatment. This mortality is caused by a combination of pulmonary hypoplasia and pulmonary hypertension [1-4]. Clinical and postmortem reports show that CDH lungs resemble the immature lungs of premature newborns; they lack adequate surfactant, have poor compliance and demonstrate immature morphology; hyaline membrane formation is also observed [4-8].
Efforts were made to apply scoring systems [9] for the prediction of CDH severity. The implementation of new techniques such as high frequency ventilation, extracorporeal membranous oxygenation, and a variety of vasoactive drugs did not affect the mortality as was expected [3,4,10]. Over the years it became clear that the basic problem in CDH treatment is a substantial lack of knowledge of the developmental process leading to CDH. The translation of the encountered problems into an animal model can yield new ideas, or even enable to find more specific modes of treatment.
Until now two types of animal models concerning CDH exist: Surgically induced CDH in e.g. sheep [11], and a nitrofen-induced CDH model in rats [12].

10.2 Surgically induced CDH

Various animals are used for surgical induction of CDH; the lamb is the most widespread used [11,13,14], but also monkeys [15] and rabbits [16] have been tested.

The timing of CDH induction in lambs is, as in rats, important; the earlier in fetal life the lesion is produced the more severe the hypoplasia [14]. Because it is based on penetration of a balloon [17] or bowels [11,18] through an already closed diaphragm there is a limit in the advancing of the operation time; Adzick et al. created CDH in lambs at gestational day 60-63 [19]. The CDH lamb is surfactant deficient [20]. In the lamb it is possible to evaluate hemodynamics and the influence of ventilation on blood-gas values and morphology [14,18-20]. Recently Wilcox et al. [21] reported on the effect of exogenous surfactant replacement therapy on gas exchange; both lung mechanics and gas exchange were markedly improved.

Harrison's group used the lamb model to study pulmonary hypoplasia that accompanies CDH and the possibility of reversing these changes by correcting the diaphragmatic defect in utero. Fetal therapy is the logical culmination of progress in fetal diagnosis. In other words, the fetus is now a patient [22,23]. This influenced the ideas of Harrison's group on the embryological aspects of CDH: pulmonary hypoplasia was caused by migrated bowels during fetal development and could be corrected by retracting these loops out of the thoracic cavity in an as early as possible stage of development. The lungs will show a compensatory growth which will beneficially influence survival. The results

of the animal experiments conducted by Harrison et al. proved, at least partially, their ideas.
Pulmonary hypoplasia in humans can also be associated with other anomalies such as renal dysplasia [24] and oligohydramnios [24,25]. Animal experiments in sheep revealed a relation between pulmonary fluid dynamics and pulmonary growth [26,27,28]. Tracheal ligation in the fetus accelerates lung growth beyond normal limits, even in the absence of kidneys [29,30,31]. One of the members of Harrison's group, Hedrick [32] ligated the trachea of CDH lambs during fetal development, which resulted in an improved survival of the lamb with CDH after birth. This plugging is considered to provide a less invasive way of intra-uterine CDH treatment. Wallen et al. [33] showed that fetal surgery sham operation had an adverse effect on lung growth: there was a significant decrease in DNA, protein and saturated phosphatidylcholine, but no significant change in lung volume was observed.

10.3 Nitrofen induced CDH

The results of research using this model are extensively described in the preceding chapters. The main conclusions are:
CDH can be successfully induced in large numbers of rats by means of a single dose nitrofen in an early stage of development [12]. The CDH lungs are hypoplastic considering lung weight and radial saccular count, and are affected equally comparing the left-right lung weight ratio [12].
Pulmonary vascular changes in CDH rats [34] strongly resemble the human pathology [35]: At the level of the respiratory bronchioles the pulmonary arteries in CDH lungs show decreased external diameter and increased wall thickness as percentage of the external thickness; this is due to hyperplasia of the muscular coating [34].

The early induction of CDH makes this model suitable for further embryological studies [36]. The first discovery was that the day of nitrofen exposure is important: left-sided congenital diaphragmatic hernia was only observed on day 9 exposure. When nitrofen was given on day 10-12 only right-sided CDH was observed [36]. It also appeared that the pleuroperitoneal openings are not the precursors of the diaphragmatic defect [37].

The rat model can be used to evaluate the biochemical and histological differences in CDH; because the rats can be ventilated for a limited time [38] it also enables to investigate the effect of pre or postnatal administered drugs on postnatal lung function [39-41].

10.4 Hormonal influence on prenatal lung development in rats

Antenatal maternal glucocorticoid therapy is known to accelerate pulmonary development in premature, otherwise healthy neonates, and has a decreased incidence of respiratory distress syndrome and pulmonary complications of preterm neonates of different species [41,42]. Biochemical evidence is found of increased surfactant production [43], and increased antioxidant enzyme activities in fetal lung [44]. Functional the treated lungs show increased maximal lung volume and compliance [45].
Several investigations indicate the benefit of antenatal hormone administration in CDH rats.
Biochemically the CDH lungs in rats are immature or hypoplastic with regard to DNA [38,39], phospholipid [39,46], and antioxidant enzyme activity [38]. Also morphologic measurements show hypoplasia: lowered lung weight, volumes and RSC [12] or immaturity evidenced by a retarded differentiation of cuboid type II cells into squamous type I cells [47].
Suen et al. [48] administered antenatal dexamethasone to nitrofen treated rats and observed increased disaturated phosphatidylcholine content, reduced lung glycogen, reduced saccular septal thickness and increased mean saccular size in rats with severe CDH.
Thyroid hormone acts synergistically with glucocorticoid in stimulating the synthesis of phosphatidylcholine [49,50]. The thyroid hormone is given as thyrotropin-releasing hormone (TRH) which crosses the placenta and increases thyrotropin (TSH), T3, T4 and prolactin concentrations in preterm fetuses [51].
This combined therapy was used in CDH rats [52]; the expected synergism was found to a significant extent.

10.5 Differences between "rat" and "lamb" CDH

Both models have their own (dis)advantages; it depends on the question posed as to which model is the most suitable. Table 10.1 shows some characteristics of both models. Rats are easily obtainable in large numbers; even with an unforeseen large failure percentage this will lead to a minimal delay in experiments. The low weight of the rats, with the recent advancement in biochemical techniques, is hardly a disadvantage. Hemodynamic measurements and the taking of blood samples from rat neonates is unfortunately impossible, as is probably fetal surgery.

Table 10.1 Characteristics of CDH in rats versus lambs

	Rat model	Lamb model
number control	++	-
number CDH	++	-
embryological study	+	-
fetal study	+	+
neonatal study	+	+
pre-partum drugs administration	+	+
post-partum drugs administration	+	+
biochemical characterization	+	+
fetal surgery	-?	++
artificial ventilation	+	+
hemodynamics / blood gas measurements	-	++
cost-effective	++	-

Concerning the properties of the model + = suitable; - = not suitable.

10.6 Consequences of animal models for the human situation

Because Harrison et al. considered the fetus with congenital malformations as a patient [22] and their experiments performing fetal surgery in lambs and monkeys were promising, the operation of human fetuses was a more or less logical step. The mortality rate in the first CDH population (n=83; operation at 24 weeks gestation) was about 58% [53]. Improved, less invasive videofetoscopic techniques [54] will simplify the surgical approach to CDH and the PLUG (Plug the Lung Until it Grows) method will be the first

method of choice [23].
The use of ECMO in CDH treatment is common (provided it is available); it provides a temporary period of rest for the lung, but problems in the post-ECMO period due to artificial ventilation or supplementary oxygen leading to BPD may result in postponed death in a number of patients [55].
Surfactant replacement improves blood gases and pulmonary dynamics [56,57]; trials have yet to reveal the effects on BPD prevention. The use of antioxidative enzymes would decrease the rate of BPD, but until now there is no adequate way to bring these enzymes intracellular for optimal effect [38,58].
The changes in the pulmonary arterial bed, causing pulmonary hypertension in CDH, are often therapy resistant. Many vasoactive drugs have been used, but none of them appeared to represent a breakthrough [4]. The combination of ECMO and the use of tracheal administered nitric oxide is proving beneficial in the modulation of pulmonary vascular tone. But, often, there is a rebound effect after withdrawal [59].

CDH research in future needs to evaluate the effects of hormonal therapy. The work will involve the investigation for specific receptor-expression and their role during organogenesis and fetal development. If these specific receptors are found, more effective drugs can be developed to more effectively stimulate preterm lung growth.
The translation and implementation of these new drugs in the human situation will take several years; to bridge this time, combined prenatal hormonal therapy (TRH plus glucocorticoids) and surfactant replacement therapy, ECMO and nitric oxide postnatal are the tools of choice; perhaps antioxidative drugs will be added to this list in the coming years.
The role of fetal surgery will probably remain limited, due to the ethical aspects involved.

REFERENCES.

1. Harrison MR, DeLorimer AA. Congenital diaphragmatic hernia. Surg Clin North Am 1981; 61:1023-35.
2. Adzick NS, Harrison MR, Glick PL, et al. Diaphragmatic hernia in the fetus: Prenatal diagnosis and outcome in 94 cases. J Pediatr Surg 1985; 20:357-61.
3. Molenaar JC, Bos AP and Tibboel D. Congenital diaphragmatic hernia, what defect?. J Pediatr Surg 1991; 26:248.
4. Tibboel D, Bos AP, Hazebroek FWJ, et al. Changing concepts in the treatment of congenital diaphragmatic hernia. Klin Pädiatr 1993; 205:67-70.
5. Wigglesworth JS, Desai R, Guerrini P. Fetal lung hypoplasia: biochemical and structural variations and their possible significance. Arch Dis Child 1981; 56:606-15.
6. Blackburn WR, Logsdon P, Alexander JA. Congenital diaphragmatic hernia: studies of composition and structure. Am Rev Resp Dis 1977; 115:suppl 275.
7. Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T. Pulmonary acinar development in

diaphragmatic hernia. Arch Pathol Lab Med 1991; 115:372-6.
8. Hisanaga S, Shimokawa H. Unexpectedly low lecithin/sphingomyelin ratio associated with fetal diaphragmatic hernia. Am J Obstet Gynecol 1984; 149:905-6.
9. Tracy T, Bailey P, Sadiq F et al. Predictive capabilities of preoperative and postoperative pulmonary function tests in delayed repair of congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:265-70.
10. O'Rourke PP, Lillehei C, Crone RK, Vacanti JP. The effect of extracorporeal membrane oxygenation on the survival of neonates with high-risk congenital diaphragmatic hernia: 45 cases from a single institution. J Pediatr Surg 1991; 26:147-52.
11. Harrison MR, Bressack MA, Churg AM, DeLorimier AA. Correction of congenital diaphragmatic hernia in utero. II. Simulated correction permits fetal lung growth with survival at birth. Surgery 1980; 88:260-8.
12. Tenbrinck R, Tibboel D, Gaillard JLJ, Kluth D, et al. Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1990; 25: 426-9.
13. DeLorimier AA, Tierney DF, Parker HR. Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. Surgery 1967; 62:12-7.
14. Kent GMK, Olley PM, Creighton RE, Dobbinson T. Hemodynamic and pulmonary changes following surgical creation of a diaphragmatic hernia in fetal lambs. Surgery 1972; 72:427-33.
15. Harrison MR, Anderson J, Rosen M et al. Fetal surgery in the primate. I Anesthetic, surgical and tocolytic management to maximize fetal-neonatal survival. J Pediatr Surg 1982; 17:115-20.
16. Ohi R, Suzuki H, Kato T, Kasai M. Development of the lung in fetal rabbits with experimental diaphragmatic hernia. J Pediatr Surg 1976; 11:955-9.
17. Harrison MR, Jester JA, Ross NA. Correction of congenital diaphragmatic hernia in utero.I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. Surgery 1980; 88:174-82.
18. Pringle KC, Turner JW, Schofield JC, Soper RT. Creation and repair of diaphragmatic hernia in the fetal lamb: lung development and morphology. J Pediatr Surg 1984; 19:131-40.
19. Adzick NS, Outwater KM, Harrison MR, et al: Correction of congenital diaphragmatic hernia in utero. IV. An early gestational fetal lamb model for pulmonary vascular morphometric analysis. J Pediatr Surg 1985; 20:673-680.
20. Glick PL, Stannard VA, Leach CL, Rossman J. Pathophysiology of congenital diaphragmatic hernia II: the fetal lamb CDH model is surfactant deficient. J Pediatr Surg 1992; 27:382-8.
21. Wilcox DT, Glick PL, Karamanoukian H et al. Pathophysiology of congenital diaphragmatic hernia. V. Effect of exogenous surfactant therapy on gas exchange and lung mechanics in the lamb congenital diaphragmatic hernia model. J Pediatrics 1994; 124:289-93.
22. Harrison MR, Golbus MS, Filly RA eds. The unborn patient: prenatal diagnosis and treatment, 2nd ed Philadelphia: W.B. Saunders, 1990.
23. Adzick NS, Harrison MR. Fetal surgical therapy. Lancet 1994; 343:897-902.
24. Reale FR, Esterly JR. Pulmonary hypoplasia: A morphometric study of the lungs of infants with diaphragmatic hernia, anencephaly, and renal malformations. Pediatrics 1973; 51:91-6.
25. Perlman M, Levin MR. Fetal pulmonary hypoplasia, anuria, and oligohydramnios: clinicopathologic observations and review of the literature. Am J Obstet Gynecol 1974; 118:1119-23.
26. Docimo SG, Luetic T, Crone RK, Davies P. Pulmonary development in the fetal lamb with severe bladder outlet obstruction and oligohydramnios: a morphometric study. J Urology 1989; 142:657-60.
27. Moessinger AC, Harding R, Adamson TM, Singh M. Role of lung fluid volume in growth and maturation of the fetal sheep lung. J Clin Invest 1990; 86:1270-7.
28. Peters CA, Reid LM, Docimo S, Luetic T. The role of the kidney in lung growth and maturation in the setting of obstructive uropathy and oligohydramnios. J Urology 1991; 146:597-600.
29. Adzick NS, Harrison MR, Glick PL, Villa RL. Experimental pulmonary hypoplasia and

oligohydramnios: relative contributions of lung fluid and fetal breathing movements. J Pediatr Surg 1984; 19:658-63.

30. Wilson JM, DiFiore JW, Peters CA. Experimental fetal tracheal ligation prevents the pulmonary hypoplasia associated with fetal nephrectomy: possible application for congenital diaphragmatic hernia. J Pediatr Surg 1993; 28:1433-40.
31. DiFiore JW, Fauza DO, Slavin R, Peters CA. Experimental fetal tracheal ligation reserves the structural and physiological effects of pulmonary hypoplasia in congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:248-57.
32. Hedrick MH, Estes JM, Sullivan KM, Bealer JF. Plug the lung until it grows (PLUG): a new method to treat congenital diaphragmatic hernia in utero. J Pediatr Surg 1994; 29:612-7.
33. Wallen L, Perry SF, Alston J, Maloney JE. Fetal lung growth. Influence of pulmonary arterial flow and surgery in sheep. Am J Respir Crit Care Med 1994; 149:1005-11.
34. Tenbrinck R, Gaillard JLJ, Tibboel D, Kluth D, Lachmann B and Molenaar JC. Pulmonary vascular abnormalities in experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1992; 27: 862-5.
35. Kitagawa M, Hislop A, Boyden EA, Reid L. Lung hypoplasia in congenital diaphragmatic hernia; a quantitative study of airway, artery, and alveolar development. Br J Surg 1971;58: 342-6.
36. Kluth D, Kangah R, Reich P, Tenbrinck R, Tibboel D, Lambrecht W. Nitrofen-induced diaphragmatic hernia in rats: an animal model. J Pediatr Surg 1990; 25:850-4.
37. Kluth D, Tenbrinck R, Von Ekesparre M, et al. The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 1993; 28:456-63.
38. Tenbrinck R, Scheffers EC, IJsselstijn H, et al. Pressure-volume relations during artificial ventilation in newborn rats with induced diaphragmatic hernia. In press.
39. Tenbrinck R, Sluiter W, Silveri F et al. Effect of artificial ventilation on pulmonary antioxidant enzyme activities in a congenital diaphragmatic hernia rat model Adv Exp Med Biol 1992; 317:363-70.
39. Suen HC, Catlin EA, Ryan DP, Wain JC, Donahoe PK. Biochemical immaturity of lungs in congenital diaphragmatic hernia. J Pediatr Surg 1993; 28:471-7.
40. Scheffers EC, IJsselstijn H, Tenbrinck R, Lachmann B, et al. Evaluation of lung function before and after surfactant application during artificial ventilation in newborn rats with congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:820-4.
41. Liggins GC, Howie RN. A controlled trial of antenatal glucocorticoid treatment for prevention of the respiratory distress syndrome in premature children. Pediatrics 1972; 50:515-25.
42. Collaborative group of antenatal steroid therapy. Effect of antenatal dexamethasone administration on the prevention of respiratory distress syndrome. Am J Obstet Gynecol 1981; 141:276-87.
43. Kotas RV, Avery ME. Accelerated appearance of pulmonary surfactant in the fetal rabbit. J Appl Physiol 1971; 30:358-61.
44. Frank L, Lewis PL, Sosenko RS. Dexamethasone stimulation of fetal rat lung antioxidant enzyme activity in parallel with surfactant stimulation Pediatrics 1985; 75:569-74.
45. Mitzner W, Johnson JWC, Scott R. Effect of betamethasone on pressure-volume relationship of fetal rhesus monkey lung. J Appl Physiol 1979; 47:377-9.
46. Zimmermann LJL, IJsselstijn H, Den Ouden J, Sauer PJJ et al. Decreased surfactant synthesis in rats with congenital diaphragmatic hernia (CDH) is due to immaturity of both type II pneumocytes and lung fibroblasts. Pediatr Res 1994; 35:45A.
47. Brandsma AE, Tenbrinck R, IJsselstein H, et al. Congenital diaphragmatic hernia: new models, new ideas. Pediatr Surg Int 1995;10: 10-15.
48. Suen HC, Bloch KD, Donahoe PK. Antenatal glucocorticoid corrects pulmonary immaturity in

experimentally induced congenital diaphragmatic hernia in rats. Pediatr Res 1994; 35:523-9.

49. Schellenberg JC, Liggins GC, Manzai M. Synergistic hormonal effects on lung maturation in fetal sheep. J Appl Physiol 1988; 24:166-70.

50 Devaskar U, Nitta K, Szewczyk K. Transplacental stimulation of functional and morphologic fetal rabbit lung maturation: Effect of thyrotropin releasing hormone. Am J Obstet Gynecol 1987; 157:460-4.

51. Ballard R, Ballard PL, Creasy RK, et al. Respiratory disease in very-low-birthweight infants after prenatal thyrotropin-releasing hormone and glucocorticoid. Lancet 1992;339:510-5.

52. Suen HC, Lotsy P, Donahoe PK, Schnitzer JJ. Combined antenatal thyrotropin-releasing hormone and low dose glucocorticoid therapy improves the pulmonary biochemical immaturity in congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:359-63.

53. Harrison MR, Adzick NS, Estes JM, Howell LJ. A prospective study of the outcome for fetuses with congenital diaphragmatic hernia. JAMA 1994; 271:382-4.

54. Estes JM, MacGillivray TE, Hedrick MH, Adzick NS. Fetoscopic surgery for the congenital anomalies. J Pediatr Surg 1992; 27:950-954.

55. Wilson JM, Lund DP, Lillehei GW. Congenital diaphragmatic hernia: predictors of severity in the ECMO era. J Pediatr Surg 1991; 26:1028-34.

56. Gommers D, Lachmann B. Surfactant therapy: does it have a role in adults? Clin Int Care 1993; 4:284-95.

57. Bos AP, Tibboel D, Lachmann B. Surfactant therapy in high-risk congenital diaphragmatic hernia. Lancet 1991; 338:1279.

58. Tanswell AK, Freeman BA. Liposome entrapped antioxidant enzymes prevent lethal O_2 toxicity in the newborn rat. J Appl Physiol 1987; 63:347-52.

59. Kinsella JP, Neish S, Ivy D, Abman S. Clinical responses to prolonged treatment of persistent pulmonary hypertension of the newborn with low doses of inhaled nitric oxide. J Pediatrics 1993;123:103-8.

SUMMARY

Neonates with congenital diaphragmatic hernia(CDH) experience a high mortality despite intensive medical and surgical treatment. The estimated incidence is 1:3000 newborns. The basic anomaly is a defect of the diaphragm left or right sided; this defect can be treated relative easily by pediatric surgeons.
However it is the associated pulmonary hypoplasia, often accompanied by well documented abnormalities in the pulmonary vessels, that gives tremendous pre and post operative problems. CDH mortality has not dropped in contrast to that of other congenital anomalies; it still remains between 30-60%.In surviving patients there is a high morbidity consisting of bronchopulmonary dysplasia(BPD) and gastro-esophageal reflux.
In order to obtain more basic information on CDH and its pulmonary malformations, it was recognized early that CDH research should be performed in animal models. The lamb model, introduced in 1967 by deLorimer, did not reveal much about the real cause of pulmonary hypoplasia.
Because of the disadvantage of the lamb model, this study was initiated with the objective to set up an CDH model in rats where CDH was introduced by a herbicide (nitrofen) in an early stage of embryological development. Investigation of the processes from this early stage of development to birth may reveal new perspectives in CDH research and subsequent treatment.

Chapter 1 is the introduction to the subject of this thesis and presents the aims of the different experiments.

Chapter 2 gives a review of the literature concerning the various subjects involved in CDH research.

The model is introduced in **chapter 3**. Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) can induce anatomical malformations in rats including congenital diaphragmatic hernia. On day 10 of gestation (total gestation 22 days) 115mg/kg body weight nitrofen dissolved in olive oil, was administered to the pregnant rats by means of a gastric tube. This leads to a high incidence -up to 60%- of right sided CDH and subsequent pulmonary hypoplasia in the rat neonates, comparable to the human situation. Both the lung weight/ body weight index as well as the radial saccular count (both generally accepted indices of pulmonary development) were significantly lower in the animals with CDH.

In **chapter 4** we investigated both the effect of changing the day of gestation (9th to 13th day) to give a single dose of nitrofen as well as the effect of different dosages (50, 100, 150 mg/kg bw). The results were: 1] most hernias occurred after administration of 100 mg nitrofen on day 9 (42%) and 11 (59%); 2]left-sided CDH were observed only after exposure to nitrofen on day 9; 3] after exposure on day 10 or later all hernias were on the right side. The outcome in chapter 3 and 4 shows that the model is suitable for

further study of abnormal lung development in relation to ventilatory capacity and pulmonary vascular reactivity.

Chapter 5 is a histological evaluation of the pulmonary vascular abnormalities encountered in CDH rats. We examined the newborn rats after perfusion of the pulmonary arteries with barium gelatine and subsequent fixation. At the level of the respiratory bronchioles significant differences in the vessels were found, consisting of decreased external diameter and increased wall thickness as percentage of the external thickness in CDH lung compared to controls. Abnormal muscularisation of the peripheral branches of the CDH pulmonary arteries was also found. With respect to the pulmonary vasculature the rat model strongly resembles the human situation

Chapter 6 is a further description of the developmental aspects of the CDH lung. Up to now, descriptions of the natural history of CDH and pulmonary hypoplasia are based exclusively on observations made in the fetal period. However nothing is known about the events that take place in the embryo with CDH.
The abnormal development of the diaphragm was first seen in embryos aged 13 to 14 days. A defect appeared in the dorsal part of the diaphragm, normally on the right side(nitrofen was given on day 11 of gestation). The liver grew through this defect early on. The gut was found intrathoracic in late stages (day 21-22 gestation) and newborns. Most authors speculate that CDH results because the pleuroperitoneal canals fail to close at the end of the embryonic period (ie, week 8 to 10 in human development) leading to a defect in the dorsolateral region of the diaphragm. However our findings indicate that diaphragmatic defects develop in early embryonic life. They are easy to identify in rat embryos as early as 14 days. During development the expansion of the thoracic cavity is obvious, so the lung still continuous to grow, while part of the chest is occupied by liver. In a growing embryonic body, compression is unlikely; the growth impairment is rather the result of growth competition in the embryo: the liver that grows faster than the lung reduces the available thoracic space.

The research in **Chapter 7** adds an important factor to the model: newborn rats (Control, CDH and Nitrofen) can be ventilated for several hours. The spontaneous born rats were anesthetized and intubated, and ventilated in a heated multichambered box. The ventilatory settings were: $F_{iO2} = 1$; Inspiratory peak pressure 17 cm H_2O, with positive end expiratory pressure 0 cm H_2O; frequency 40/min; Inspiratory/expiratory ratio is 1/2. The control and nitrofen treated animals were ventilated for 6 hours without much problems, but in the CDH group about 80% of the severe CDH died within this period. By registration of the changing pressures and their matching volumes, we created individual inflation and deflation curves at a distinct time (0, 1, 6 hours). The individual deflation curves of the three groups were compared and analyzed statistically.
The measured pressure- volume registrations showed low compliance values without improvement during time in the CDH group. This suggests an involvement of the

surfactant system.

Chapter 8 describes the effect of surfactant instillation on the pressure-volume registrations. Surfactant application had a positive effect on lung volume,especially in control rats after 1 hour ventilation. No significant differences were observed between the CDH groups after 1 and 6 hours ventilation. A continued positive effect on lung volume could not be determined.

The first study about the effects of the ventilation on antioxidative enzyme activity (AOA) is showed in **Chapter 9.** Compared to mature infants, CDH survivors have a high incidence (40%) of bronchopulmonary dysplasia (BPD), a similar percentage is also found in prematures. One of the presumed causes of this serious disease is a oxygen radical induced damage of the lungs during longer periods of raised inspiratory oxygen concentrations. The primary defense against radical induced damage are the AOA; during radical challenge the AOA has to go up. In our experiment we found a not significant (in the short 6 hour study period) trend of decreasing AOA. These results are to fragile to speculate about clinical significance, but further research might bring more definite results.

Chapter 10. compares the rat model and the lamb model; both models have their own field of optimal application. Using both research models will perhaps lead to results that can decrease mortality and morbidity of CDH.

SAMENVATTING

Dit proefschrift beschrijft de ontwikkeling van een diermodel met de kenmerken en symptomen van congenitale hernia diafragmatica (CHD). Congenitale hernia diafragmatica komt voor bij 1 : 3-5000 pasgeborenen. Het is een aangeboren afwijking waarbij de buikorganen via een defect in het middenrif in de borstholte herniëren. Het defect kan betrekkelijk gemakkelijk worden geopereerd door kinderchirurgen. De bijbehorende longhypoplasie, vaak vergezeld van afwijkingen in het pulmonale vaatstelsel, geven pre- en postoperatief grote problemen. In tegenstelling tot andere aangeboren afwijkingen is de mortaliteit van CHD nauwelijks gedaald. Al jarenlang ligt dit percentage tussen de 30 en 60%. In overlevende patiënten wordt een hoge incidentie van broncho-pulmonale dysplasie (BPD) gevonden. Deze longaandoening komt anders eigenlijk alleen voor bij te vroeg geborenen.

Om inzicht te krijgen in de pulmonale afwijkingen bij CHD werd duidelijk dat het onderzoek gebruik zou moeten maken van diermodellen. In 1967 introduceerde deLorimer een model in schapen. Dit gaf echter geen duidelijke informatie over het ontstaan van de longhypoplasie. Bij de rat kan CHD worden geïnduceerd door in een vroeg stadium van de embryonale ontwikkeling het herbicide nitrofen aan de zwangere moeder toe te dienen. Onderzoek van de orgaanontwikkeling, in de periode van de vroeg embryonale ontwikkeling tot de geboorte, kan perspectieven bieden in het CHD onderzoek, met mogelijke gevolgen voor behandeling.

In **hoofdstuk 1** wordt het probleem van CHD en longhypoplasie geïntroduceerd. Het bevat tevens de doelstellingen van de verschillende experimenten.

Hoofdstuk 2 geeft een overzicht van de bestaande literatuur, die betrekking heeft op de verschillende onderwerpen in het CHD onderzoek.

Het rattenmodel van CHD wordt geïntroduceerd in **hoofdstuk 3**. Nitrofen (2.4-dichlorophenyl-p-nitrophenyl ether) kan verschillende anatomische afwijkingen induceren bij de rat, waaronder congenitale hernia diafragmatica. Op dag 10 van de zwangerschap (totale zwangerschapduur 22 dagen) wordt 115 mg/kg lichaamsgewicht nitrofen opgelost in olijfolie en oraal aan de zwangere ratten toegediend. Dit leidt uiteindelijk tot een hoge incidentie - tot 60% - van rechtszijdige CHD in de pasgeboren ratten. Zowel de long-/lichaamsgewichtindex als de radial saccular count (beide in de literatuur geaccepteerde indicatoren voor pulmonale ontwikkeling) zijn significant lager in de dieren met CHD. Deze situatie lijkt sterk op die bij de mens.

In **hoofdstuk 4** hebben wij het effect onderzocht van verandering van het tijdstip van de nitrofengift (9 tot 13 dagen), alsmede het effect van verschillende doseringen (50, 100 en 150 mg/kg lichaamsgewicht). Dit resulteerde in de volgende bevindingen: 1] de

meeste hernia's werden gevonden bij een dosering van 100 mg/kg nitrofen op dag 9 en 11; 2] linkszijdige hernia's werden alleen gevonden na toediening van nitrofen op dag 9; 3] na toediening op dag 10 of later werden uitsluitend rechtszijdige hernia's gevonden. De conclusie van hoofdstuk 3 en 4 is dat het model geschikt is voor verdere studie van abnormale longontwikkeling.

Hoofdstuk 5 is een histologische evaluatie van de pulmonale vaatafwijkingen die worden gevonden in de CHD-ratten. Het pulmonale vaatbed van de pasgeboren ratten werd geperfundeerd met een barium gelatine oplossing en daarna in-situ gefixeerd; bij de CHD-ratten werden significante verschillen gevonden in de vaatstructuren op het niveau van de respiratoire bronchiolen. Het betrof hier een kleinere externe diameter met een toegenomen wanddikte als percentage van de externe dikte. Tevens werden abnormale muscularisatie van de perifere pulmonale arteries gevonden; de pulmonale vaten in de rat leken sterk op de humane situatie.

Hoofdstuk 6 beschrijft een aantal ontwikkelingsaspecten van de CHD-long. Tot nu toe zijn de beschrijvingen van de ontwikkelingen van CHD met longhypoplasie eigenlijk uitsluitend gebaseerd op waarnemingen die in de foetale periode gedaan zijn. Er is niets bekend over de ontwikkelingen die in de embryonale fase plaatsvinden. De abnormale ontwikkeling van het middenrif(diafragma) werd het eerst gezien in embryo's van 13 tot 14 dagen oud. Het defect verscheen in het dorsale gedeelte van het middenrif, meestal aan de rechterzijde (nitrofen werd op dag 11 gegeven). De lever groeide door dit defect heen, dit was al in een vroeg stadium zichtbaar. Darm werd pas in een later stadium intrathoracaal gevonden (dag 21 tot 22). Deze studie toonde aan dat het defect in het middenrif in een vroeg stadium van de embryonale ontwikkeling ontstaat. Het defect kan worden gevonden bij ratte-embryo's vanaf 14 dagen. Tijdens de ontwikkeling blijft er een duidelijke groei van de thoracale holte, zodat ook de long blijft groeien. Echter, een deel van de borstholte wordt ingenomen door de lever. Het idee van compressie van de long door lever of darm in een groeiend lichaam is daardoor onwaarschijnlijk; de groeibelemmering is waarschijnlijk meer te wijten aan een competitie in het groeiende embryo: de lever groeit sneller dan de long en reduceert daardoor de beschikbare plaats in de thoracale holte.

Het onderzoek beschreven in **hoofdstuk 7** voegt een belangrijke factor toe aan het model: pasgeboren ratten kunnen worden beademd gedurende een aantal uren. Spontaan geboren ratten krijgen algehele anaesthesie met intubatie en beademing in een verwarmde meerkamer box. De ventilator instelling is als volgt: F_{IO2} = 1; Inspiratoire piekdruk 17 cm H_2O, met een PEEP = 0 cm H_2O; frequentie 40/min; I/E ratio 1/2. De controlegroep en de met nitrofen behandelde dieren worden beademd gedurende 6 uur. In de herniagroep sterft rond de 80% in deze periode. Van de individuele ratten worden de beademingsdrukken en de bijbehorende volumes geregisteerd op de tijdstippen 0, 1 en 6 uur. De aldus verkregen inspiratoire en expiratoire curves worden vergeleken en

statistisch geanalyseerd. De CHD-groep heeft een lage compliantie met weinig verbetering gedurende de beademingsperiode. Dit kan duiden op een betrokkenheid van het surfactant-systeem.

Hoofdstuk 8 beschrijft de effecten van het toedienen van surfactant op de druk-volume curves. Surfactant toediening had een positief effect op het longvolume, vooral in de controle ratten na 1 uur ventilatie. Er werden geen significante verschillen gevonden tussen de CHD-groepen, na 1 en 6 uur beademen. Een blijvend positief effect op het longvolume kon niet worden aangetoond.

De eerste studie over de effecten van ventilatie op de antioxidatieve enzymactiviteit (AOA) is omschreven in **hoofdstuk 9**. In vergelijking met à terme geboren kinderen hebben CHD-overlevenden een hoge incidentie (40%) van bronchopulmonale dysplasie. Dit percentage is ongeveer gelijk aan dat wat in prematuren gevonden word. In dit experiment vonden wij een niet-significante trend van een dalende AOA. Deze resultaten zijn te mager om te speculeren over een eventuele klinische relevantie. Verder onderzoek in deze richting zou misschien betere resultaten kunnen opleveren.

Hoofstuk 10 vergelijkt het rattemodel en het lamsmodel; beide modellen hebben een eigen toepassingsgebied. Gebruik van beide modellen zou uiteindelijk tot resultaten kunnen leiden die effect hebben op de mortaliteit en morbiditeit van de CHD.

DANKWOORD

Na een aantal jaren onderzoek als student op de afdeling experimentele anaesthesiologie, werd mij in 1988 door Dr. D. Tibboel gevraagd om een diermodel in ratten op te zetten met de kenmerken van CHD. Gedurende de ruim twee jaar dat ik als medewerker van de afdeling kinderchirurgie hieraan kon werken, alsmede in de periode hierna, heb ik advies, hulp en steun gehad van vele personen. Ook degenen wier naam ik waarschijnlijk volledig onterecht niet heb vermeld, dank ik voor hun bijdrage.

Prof. Dr. D. Tibboel, beste Dick, ik wil je graag bedanken voor de niet aflatende steun die ik van je heb gehad tijdens de productie van dit boekwerk en, veel belangrijker, voor de vrijheid en vertrouwen tijdens het onderzoek. Kortom, een modelpromotor.

Prof. Dr. Dr. B. Lachmann. Dit jaar is het 10 jaar geleden dat u mij het boeiende onderzoekswoud introk om te ondervinden hoe onderzoek gedaan kan worden. Hard werken gedurende vele uren met daarnaast voldoende gelegenheid voor sociale activiteiten (Happy Palace) met de andere onderzoekers kenmerken de afdeling experimentele anaesthesie. Deze periode, voorafgaand aan het CHD-onderzoek, bleek een goede basis. Dat u als promotor wil optreden verheugt mij dan ook zeer.

Prof. Dr. J.C. Molenaar, als hoofd van de afdeling kinderchirurgie altijd geïnteresseerd naar de vorderingen van het onderzoek, bedankt dat u ook in de promotiecommissie heeft willen plaatsnemen.

Dr. J.L.J. Gaillard. Vele microscopische uren brachten uiteindelijk interessante zaken aan het licht; de gastvrijheid die ik genoot op het Laboratorium voor Cytodiagnostiek waren ongekend. Hiervoor moet ik natuurlijk ook Dr. Noorduyn bedanken.
Op het Laboratorium voor Cytodiagnostiek leerden Ingrid en Carmelo mij hoe, van een stukje met fixatief doordrenkt weefsel, een fatsoenlijk gekleurd 6μm dik op glas geplakt weefsel te maken. Ook bedankt voor het inbedden/snijden/kleuren van coupes als ik er geen tijd voor had. De anderen van het LvC nog bedankt voor de steun.

Het hele onderzoek begon met ratten en hier was ook altijd Thijs van Aken. Zeker toen besloten werd om de "fok in eigen hand te nemen" leverde dit jou zeer veel extra werk op. Elke avond om 19.00 draaide de PEEP-show; een vruchtbaar uurtje voor ratten die willen. Het prepareren van de beesten onder de microscoop, het geven van gif aan "moes", constructies voor de fixatie altijd weer Thijs. Bedankt.

Prof. Dr. K Slob bedankt voor de adviezen om een getimede zwangerschap op te zetten bij de rat. Door deze bijdrage kon het model goed gaan draaien.

Arie Kok. Tussen de andere experimenten, en met koffie, Volkskrant en Van Nelle

onder handbereik, altijd bereid te adviseren/controleren van de body-box met meetgerei.

Laraine Visser. Thenk you very much, my englisch improfed a lot during these years, but I dare not sent a letter away without your magic eye.

Biochemisch gezien veel dank aan Dr. W Sluiter en de gastvrijheid van zijn lab: Regina en Alfredo hebben een duidelijke inbreng gehad bij het bewerken van de longen voor de antioxidatieve enzym activiteit bepalingen. Wim, nu wordt het tijd om de biochemie van het Belgische bier eens te bestuderen, in plaats van de etiketten.

Dr. D. Kluth. Dietrich, die Zusammenarbeit war ziemlich erfolgreich. Es freudt mich sehr dass du auch am 31 Mai dabei sein sollst. Auch Dank für Marianne, wegen der sehr freunlichen Empfang in Norderstedt während meine Besuchen an Hamburg.
Prof. Dr. W. Lambrecht und Dr. P. Reich. Auch Ihnen möchte ich bedanken; die beste Mändelhörnchen kauft man in Eutin während die Festspiele; Bäume in Hamburg sind besser gesätzlich geschützt wie in Holland.

De opvolgers van het onderzoek: Attie (dit onderzoek is een goede voorbereiding op gynecologie want een bevalling om 4 uur 's nachts is het mooiste dat er is: deze ratten bevallen toch altijd tussen 3 en 6 uur?); Elke (waarom worden ze niet zwanger?); Hanneke (ik doe een sectio want dan vallen er minder dieren uit het protocol; bovendien is dat een goede voorbereiding op kindergeneeskunde). Kortom ieder heeft op haar eigen wijze de tolerantie drempel verhoogd.

Annelies Brandsma. De evenwijdige onderzoekskoers bracht heel andere problemen, maar uiteindelijk werd toch de haven bereikt.

De heren portiers van faculteit (ik moet even naar het lab; past deze ladder ook in de lift?) en van het SKZ (de correspondentie met prof. Tibboel).

Ook de medewerkers van het Chirurgisch Laboratorium worden bedankt voor de hulp: Pim en Lidia voor de analyses, Kas voor de beestjes.

Dr. C. Moreley, Colin thank you for your hospitality in Cambridge and the facilities and expertise of your laboratry on phospholipid chemistry.

Annemarie voor het kontakt tussen mij(onbereikbaar) en Dick(altijd weg).
Sharida, Amina en Janine bedankt voor de secretariële werkzaamheden en de briefjes in mijn postvak.

Mensen van de experimentele anaesthesie: Govinda, Diederik, Annemarie en Pneu, Serge, Edwin, Erwin(2x), Stephan, Arthur en uit de oude doos ook nog Siggi, Wolfgang,

Jelle, Erik, Geert Jan en Frits(halothaan) Smit. Altijd bereid problemen op te lossen of de helpende hand toe te steken, bedankt.
Andere "medestrijders" Leon, Freek, Arthur, Johan.

Jelle Bos; uitzicht op zee neem je wel zeer letterlijk, maar ik hoop dat je ook nog eens de diepte in kan gaan. Ik zal de "snij-sessies" met jou en Erik Eijking boven (in?) Sorbonne niet licht vergeten. Erik E., collega/vriend het leven is meer dan een video en Melief.

Geert Jan en Olav, paranymfen, wij kennen elkaar al zo lang dat woorden tekort zullen schieten. Zeilen, roeien, onderzoeker van het eerste uur, directeur.

Mijn opleider Prof. Dr. W. Erdmann dank ik voor het beoordelen van mijn manuscript als secretaris van de promotiecommissie.

De verpleegkundigen, stafleden en assistenten van de afdeling anaesthesiologie van het AZR, dank ik voor de steun cq belangstelling gedurende de jaren.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 21 juli 1960 te Vlaardingen. In 1980 behaalde hij het diploma Atheneum aan de scholengemeenschap "Ring van Putten" te Spijkenisse. De studie geneeskunde aan de Erasmus Universiteit te Rotterdam werd in 1981, een jaar na aanvang, onderbroken voor de militaire dienst.
De studie geneeskunde werd in 1982 voortgezet; het doctoraalexamen werd in februari 1988 behaald. Gedurende zijn studie vervulde hij een student-assistentschap bij Prof. Dr. B. Lachmann op de afdeling Experimentele Anaesthesiologie van de Erasmus Universiteit. Na zijn doctoraal examen trad hij in dienst als wetenschappelijk medewerker bij de afdeling Kinderchirurgie van het Sophia Kinderziekenhuis (hoofd Prof. Dr. J.C. Molenaar). In een samenwerkings verband tussen Kinderchirurgie(Prof. Dr. D. Tibboel), Experimentele Anaesthesiologie (Prof. Dr. B. Lachmann) en Stichting voor Cytodiagnostiek (Dr. J.L.J. Gaillard) werd een model in ratten ontwikkeld met de kenmerken van congenitale hernia diaphragmatica. Spoedig werd ook samengewerkt met de afdeling "Kinderchirurgie van Universitäts Klinik Eppendorf" te Hamburg, Duitsland (Prof. Dr. W. Lambrecht en Dr. D. Kluth).
In 1991 werd de artsenopleiding afgerond. In november 1991 kwam hij in dienst van de afdeling Anesthesiologie (hoofd Prof. Dr. W. Erdmann) van het Academisch Ziekenhuis Dijkzigt.
Vanaf 1988 tot heden werd gewerkt aan de uitbreiding van het diermodel dat de basis vormt voor dit proefschrift.

LIST OF PUBLICATIONS

Concerning CDH:

1. **Tenbrinck R**, Tibboel D, Gaillard JL, Kluth D, Bos AP, Lachmann B, Molenaar JC. Experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1990; 25:426-429.
2. Kluth D, Kangah R, Reich P, **Tenbrinck R**, Tibboel D, Lambrecht W. Nitrofen induced diaphragmatic hernias in rats: an animal model. J Pediatr Surg 1990; 25:850-854.
3. **Tenbrinck R**, Gaillard JL, Tibboel D, Kluth D, Lachmann B, Molenaar JC. Pulmonary vascular abnormalities in experimentally induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1992; 27:862-865.
4. **Tenbrinck R**, Sluiter W, Silveri F, Bos AP, Scheffers EC, Go ATJI, Bos JA, Tibboel D, Lachmann B. Effect of artificial ventilation on pulmonary antioxidant enzyme activities in a congenital diaphragmatic hernia rat model. Adv Exp Med Biol 1992; 317:363-370.
5. Sluiter W, Bos AP, Silveri F, **Tenbrinck R**, Kraak-Slee R, Tibboel D, Koster JF, Molenaar JC. Nitrofen induced diaphragmatic hernia in rats: Pulmonary antioxidant enzyme activities. Ped Res 1992; 32:394-398.
6. Kluth D, **Tenbrinck R**, Von Ekesparre M, Kangah R, Reich P, Brandsma A, Tibboel D, Lambrecht W. The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 1993; 28:456-62;
7. Scheffers EC, IJsselstijn H, **Tenbrinck R**, Lachmann B, De Jongste J, Molenaar JC, Tibboel D. Evaluation of lung function changes before and after surfactant application during artificial ventilation in newborn rats with congenital diaphragmatic hernia. J Pediatr Surg 1994; 29:820-824.
8. Brandsma AE, **Tenbrinck R**, IJsselstijn H, Scheffers EC, Gaillard JLJ, Kluth D, Ten Have-Opbroek AAW, Lachmann B, Tibboel D. Congenital diaphragmatic hernia: new models, new ideas. Pediatr Surg Int 1995; 10:10-15.
9. Bos AP, Sluiter W, **Tenbrinck R**, Kraak-Slee R, Tibboel D. Angiotensin-converting enzyme activity is increased in lungs of rats with pulmonary hypoplasia and congenital diaphragmatic hernia. Exp Lung Res 1995; 21:41-50.
10. **Tenbrinck R**, Scheffers EC, IJsselstein H, Go ATJI, Tibboel D, Lachmann B. Nitrofen induced diaphragmatic hernia: Pressure volume registration and artificial ventilation in newborn rats. Accepted for publication.

Books

1. Tibboel D, **Tenbrinck R**, Bos AP, Gaillard JLJ, Kluth D, Van Aken T, Molenaar JC. An experimental model of congenital diaphragmatic hernia. In: Defauts congeniteaux de la paroi abdominale. Beaufils F,Aigrain Y, Nivoche Y, editors.

Paris: Arnette, 1990; 99-105.

2. **Tenbrinck R**, Brandsma A, Bos AP, Sluiter W, Kluth D, Ten Have-Opbroek AAW, Lachmann B, Molenaar JC, Tibboel D. Pathogenic and functional aspects of congenital diaphragmatic hernia(CDH); an experimental approach. In:Interdisziplinare Probleme in der Perinatalmedizin. Zwergfell-Hernie Reitzhemen - Retinopathia prematurorum - Ethische und Juristische Problematik. Karlsruhe, G. Braun Fachverlage 1993: 9-11.

Other publications

1. Tenbrinck R, Schairer W, van Daal GJ, Kuypers MH, Steeghs GF, Lachmann B. Evaluation of a heparin-coated PO2 electrode for continuous intravasal PO_2 monitoring. Adv Exp Med Biol 1989; 248:157-162
2. Van Woerkens LJ, Lachmann B, van Daal GJ, Schairer W, Tenbrinck R, Verdouw PD, Erdmann W. Influences of different routinely used muscle relaxants on oxygen delivery to and oxygen consumption by the heart during xenon-anesthesia. Adv Exp Med Biol 1989; 248:673-678.
3. Van Daal GJ, Lachmann B, Schairer W, Tenbrinck R, Van Woerkens LJ, Verdouw P, Erdmann W. The influence of different anesthetics on the oxygen delivery to and consumption of the heart. Adv Exp Med Biol 1989; 248:527-532.
4. Trouwborst A, Van den Broek WG, Tenbrinck R, Groenland TH, Bucx M, Faithfull NS. Alterations in oxyhemoglobin dissociation curve during normoxic acute normovolemic hemodilution. Adv Exp Med Biol 1989; 248:419-425.
5. Bos JA, Schairer W, Schaffers JT, Tenbrinck R, Ten Have-Opbroek AA, Bakker WH, Wollmer P, Lachmann B. Effects of high frequency jet ventilation on the pulmonary claerance of 99mTc-DTPA in respiratory failure in rabbits. Br J Anaesth 1989; 63:59S-64S.
6. Gommers D, So KL, Armbruster S, Tenbrinck R, Van Remortel JLM, Van Eyk JE, Lachmann B. Human surfactant derived from vaginal delivered amniotic fluid improves lung mechanics as effectively as natural surfactant derived from bovine lungs. Prog Respir Res 1990; 25:302-304.
7. Trouwborst A, Van Woerkens EC, Van Daele M, Tenbrinck R. Acute hypervolaemic haemodilution to avoid blood transfusion during major surgery. Lancet 1990; 336:1295-1297.
8. Trouwborst A, Tenbrinck R, Van Woerkens EC. Blood gas analysis of mixed venous blood during normoxic acute isovolemic hemodilution in pigs. Anesth Analg 1990; 70:523-529.
9. Trouwborst A, Tenbrinck R, Fennema M, Bucx M, Van der Broek WG, Trouwborst-Weber BK. Cardiovascular responses, hemodynamics and oxygen transport to tissue during moderate isovolemic hemodilution in pigs. Adv Exp Med Biol 1990; 277:873-879.

10. Van Daal GJ, De Jong PT, Tenbrinck R, Mouton JW, Petzoldt K, Bergmann KC, Lachmann B. Oral immunization with bacterial lysate against infection with Streptococcus pneumoniae in mice. Respiration 1990; 57:229-232.
11. Trouwborst A, Tenbrinck R, Van Woerkens EC. S35: a new parameter in blood gas analysis for monitoring the systemic oxygenation. Scand J Clin Lab Invest Suppl 1990; 203:135-142.
12. Van Daal GJ, So KL, Mouton JW, Van 't Veen A, Tenbrinck R, Bergmann KC, Lachmann B. Oral immunization with a polyvalent bacterial lysate can reduce mortality by infection with S. pneumoniae or influenza A in mice. Pneumologie 1990; 44:1180-1182.
13. Eijking EP, Strayer DS, Van Daal GJ, Tenbrinck R, Merritt TA, Hannappel E, Lachmann B. In vivo and in vitro inactivation of bovine surfactant by an anti-surfactant monoclonal antibody. Eur Respir J 1991; 4:1245-1250.
14. Eijking EP, Van Daal GJ, Tenbrinck R, Luijendijk A, Sluiters JF, Hannappel E, Lachmann B. Effect of surfactant replacement on Pneumocystis carinii pneumonia in rats. Intensive Care Med 1991; 17:475-478.
15. Van Woerkens EC, Trouwborst A, Tenbrinck R. Accuracy of a mixed venous saturation catheter during acutely induced changes in hematocrit in humans. Crit Care Med 1991; 19:1025-1029.
16. Eijking EP, Van Daal GJ, Tenbrinck R, Sluiters JF, Hanappel E, Erdmann W, Lachmann B. Improvement of pulmonary gas exchange after surfactant replacement in rats with Pneumocystis carnii pneumonia. Adv Exp Med Biol 1992; 316:293-298.
17. Van Woerkens ECM, Trouwborst A, Snel L, Tenbrinck R. Comparative study of the accuracy of two fiberoptic mixed veous saturation catheters (Spectracath vs Opticath) during acute changes in hematocrit and cardiac output in humans. Adv Exp Med Biol 1992; 317:509-514.
18. Trouwborst A, Van Woerkens ECM, Tenbrinck R. Hemodilution and oxygen transport. Adv Exp Med Biol 1992; 317:431-440.
19. Van Daele MERM, Trouwborst A, Van Woerkens ECSM, Tenbrinck R, Fraser AG, Roelandt JRTC. Transesophageal echocardiographic monitoring of preoperative acute hypervolemic hemodilution. Anesthesiology 1994;81 :602-9.