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Effects of Prenatal Glucocorticoids and Postnatal Nitric Oxide Inhalation on the Survival and Lung Maturation of Newborn Rats with Congenital Diaphragmatic Hernia

DISSERTATION

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1 INTRODUCTION

1.1 Topic

Congenital diaphragmatic hernia (CDH) is a simple defect that results in a complicated syndrome with an often lethal outcome. A diaphragmatic defect, usually left-sided, allows abdominal contents to herniate into the thoracic cavity, leading to pulmonary hypoplasia [119], lung immaturity [162], and often left heart hypoplasia [176] causing persistent pulmonary hypertension of the newborn [142]. CDH occurs in approximately 1 in every 2,000 to 5,000 newborns [30, 114, 133, 136, 159, 179]. Despite major advances in neonatal resuscitation and intensive care, newborns with CDH still suffer from a high morbidity and mortality [129]. Although studies over the past two decades have brought insight into the embryology, pathophysiology, and natural history of CDH, as well as new treatment options such as high-frequency oscillatory ventilation, extracorporeal membrane oxygenation, surfactant therapy, inhaled nitric oxide, and prenatal surgery, the mortality rate in newborns with antenatally diagnosed CDH is still 27.5-79% [3, 74, 133, 185]. A hidden mortality rate (deaths before admittance to a treatment center) in CDH ranges between 19.2% and 53% [77, 179].

The high mortality rate in newborns with CDH is directly proportional to the severity of the pulmonary hypoplasia and the associated pulmonary hypertension. It is not necessarily the size of the impaired lung that is detrimental to survival, but rather the associated pulmonary disorders such as surfactant deficiency, decreased ant-oxidant activity, increased vascular reactivity with decreased nitric oxide, and increased endothelin-1 activity [196]. The pulmonary hypertension is pathophysiologically in turn due to a reduced pulmonary vascular bed and an excessive muscularization of the pulmonary arteries [129].

Most of the modern treatment options mentioned above address the postnatal neonate that already has a hypoplastic lung. Although some neonates benefit from these therapeutic strategies, most will not survive. It is, therefore, important to improve antenatal therapies directed at altering lung growth early in utero in order to minimize pulmonary hypoplasia [196]. It is only through a combined antenatal and postnatal therapy that the mortality of newborns with CDH will be decreased.

1.2 Goal of this study

It has been shown in the nitrofen induced CDH rat model that prenatal maternal glucocorticoid administration improves biochemical maturity, increases lung compliance, and enhances lung morphology [125, 190]. There is already a widespread use of antenatal glucocorticoids to induce lung maturation in fetuses at risk of premature delivery suggesting a potential for a therapeutic effect in other fetuses with impaired lung development [56].

A previous research group from this department showed that inhaled nitric oxide (NO), as a sole intervention, significantly improved the survival rate of newborn rats with CDH [108]. Inhaled nitric oxide is a selective pulmonary vasodilator [158] improving oxygenation without producing a decrease in systemic vascular resistance [85]. This occurs because NO is inactivated immediately upon entering the blood by binding to hemoglobin and forming methemoglobin.

The goal of this study is to combine this antenatal and postnatal therapy regiment on the CDH rat model to determine the following:

a. Perform a survival analysis and study the degree of lung maturation in CDH rats given only antenatal dexamethasone, antenatal dexamethasone and postnatal nitric oxide, and just postnatal nitric oxide (without cortisone).

b. To study the following parameters and to quantify or correlate their further effects: degree of hernia, lung morphology, mortality, and therapy regiment.

1.3 Normal development of the diaphragm

The development of the diaphragm is best described in two parts: first the development of the diaphragmatic primordium and second the development of the pleural cavity and the closure of the pleuroperitoneal canals [112].
At the end of the third week of gestation a mass of mesoderm, which is bordered by the epithelium of the pericardial cavity cranially and the epithelium of the peritoneal cavity caudally, represents the beginning of the septum transversum. At this stage, dorsal passages exist between the thorax and the abdominal coelum through the pericardioperitoneal canals. As development continues, liver cells grow into the septum transversum causing it to expand in a ventrolateral direction. This septum is continuous to the dorsal structures of the embryo via the pulmonary ridges laterally and the gastrohepatic ligament medially [112]. Thus, the borders of the pleuroperitoneal canal are laterodorsally the pleuroperitoneal membrane (evolving from the pulmonary ridge), mediodorsally the mesentery of the esophagus, and ventrally the septum transversum.
The pleuroperitoneal membrane continues to grow (4^{th}-8^{th} week) medially and ventrally joining the mesenterium and septum transversum, thereby forming the pleural cavities and dividing the abdominal and thoracic coelum. The pleural cavities enlarge further and come into contact with the mesenchyme of the lateral body wall. This creates a ledge for myoblasts to build the muscular portion of the diaphragm. This is regarded as the final closure of the pleuroperitoneal canals.

1.3.1 Pathogenesis of CDH in the human

The mechanism responsible for the closure of the pleuroperitoneal canals is thought to play a major role in the development of CDH [196]. The present opinion is that the pleuroperitoneal membrane does not properly fuse with the surrounding tissue, thereby not closing the pleuroperitoneal canal. The canal then allows herniation of abdominal content into the thorax (also called Bochdalek hernia).
The process of closure, however, has never been fully studied [112]. There are various theories as to how the diaphragm closes. Some authors believe that the closure depends mainly on the normal development of the pleuroperitoneal membranes [135, 170]. Other theories include: continuous growth and pressure of the surrounding abdominal organs (liver, adrenal glands) forces the canal to close [211]; the suprarenal gland causes pleuroperitoneal canal closure [27]; and growth of a third structure, the posthepatic mesenchymal plate (seen in mice), leads to closure of the diaphragm [94]. Furthermore, most congenital diaphragmatic hernias occur on the left side, approximately 80%, due to earlier or more rapid closure of the right side – again for unknown reasons.

1.3.2 Pathogenesis of CDH in the rat model

The nitrofen induced CDH in the rat model has been extensively studied by Nakao [145, 146], Iritani [94], Kluth [108-112], and Tenbrinck [194]. The diphenyl ether herbicide nitrofen (2,4-dichlorophenyl 4'-nitrophenyl ether) is a potent teratogen, affecting many organ systems while causing little or no maternal mortality [39, 67] (the teratogenic potency, mechanisms of action, and chemical properties of nitrofen will be discussed in section 2.2.1 and 2.2.2).
In the rat model it has been determined that nitrofen given on day 9 of gestation induces a left-sided hernia. If nitrofen is given on day 10 or later only right-sided hernias are observed. The dosage necessary varies upon author; however, our laboratories have had consistent results with a single dose of 100 mg of nitrofen - producing hernias in almost 60% of the newborn rats [109, 194].
Embryologic studies on the morphology of the diaphragmatic anlage in Sprague-Dawley rat embryos after nitrofen ingestion have been carried out using the scanning electron microscope [110-112]. These studies have shown that a disturbed development of the diaphragmatic anlage was evident on day 13 for left sided defects after ingestion of nitrofen on day 9.5, and day 14 for right sided defects after ingestion of nitrofen on day 11.5. The septum transversum did not appear to be involved in the abnormal development; but rather, all developmental defects were found to be restricted to the posthepatic mesenchymal plate (PHMP) and the neighboring liver [112]. The liver anlage seems to show an abnormal growth pattern, and interferes with the normal growth of the PHMP. In 14 and 15 day old embryos (left-sided and right-sided defects, respectively), cells of the liver had already migrated into the thoracic cavity. It was concluded in these studies that in diaphragmatic development, the growth of the liver is in sequential balance with the PMHP, and that in the nitrofen treated rat embryos this balance of growth is disturbed leading to the CDH.

2 MATERIALS AND METHODS

2.1 Animals

The animal specimens used for this project were newborn Sprague-Dawley rats. The parent Sprague-Dawley rats were obtained from Charles River (Sulzfeld, Germany). The weight of the maternal rats ranged between 200 and 250 grams, that of the paternal rats between 250 and 300 grams. The animals were then cared for in the experimental animal laboratory of the University of Hamburg, University Hospital Eppendorf, Germany. The animals were kept in a standardized room environment with a room temperature of 22º C, 50% +/- 5% relative humidity, and a controlled 12-hour light/dark cycle. Nutrition consisted of altromin and water ad libidum.

2.1.1 Mating and conception confirmation

Mating took place between male and female Sprague-Dawley rats breed in the same animal farm (Charles River, Sulzfeld, Germany). Each pair was mated overnight. Conception was determined based on a positive finding of sperm in the vaginal smear taken during the next light-cycle. The day of observation was designated as day 0 of

gestation. The average weight of the animals upon mating ranged between 200g-300g, and the age averaged between 8-10 weeks.

2.1.2 Approval of study by ethics committee

This study was approved by the Ethics Committee of the Hamburg Federal Board of Veterinary Medicine and Animal Care on February 24, 2000 (Az: G8151/591-00.33). The initial application for the study was submitted on December 13, 1999.

2.1.3 Grouping of Sprague-Dawley rats

The data for the control groups (group 1 and 2) were taken from a previous study from this institution [22]. Both groups consist of offspring of nitrofen treated rats (n=12 dams) that were independently allocated to group 1 or 2. Animals in group 1 were exposed to artificial air postnatally. Group 2 animals were exposed to nitric oxide postnatally.
The 20 dams in this study (groups 3 and 4) were also given nitrofen on day 11.5 of gestation in order to induce a right-sided congenital diaphragmatic hernia in the newborn litters (same procedure as for groups 1 and 2).
In order to evaluate the effects of dexamethasone on survival rate and lung maturity all 20 rats were injected with 0.25 mg/kg of dexamethasone intraperitoneally on day 18.5 and 19.5 of pregnancy.
These 20 rats were then independently divided into two groups. The offspring of some were exposed to artificial air postnatally (group 3), and those of the others (group 4) were exposed to nitric oxide at 80 ppm.

Experimental Groups	
Group 1 (n = 88) (Control group) Newborn rats of nitrofen treated dams, with exposition to artificial air postnatally (Nitrofen)	Group 3 (n = 153) Newborn rats of nitrofen treated dams, given dexamethasone prenatally, and exposed to artificial air postnatally (Nitrofen + Dex)
Group 2 (n = 104) Newborn rats of nitrofen treated dams, with exposition to nitric oxide postnatally (Nitrofen + NO)	Group 4 (n = 146) Newborn rats of nitrofen treated dams, given dexamethasone prenatally, and exposed to nitric oxide postnatally (Nitrofen + Dex + NO)

Table 1: Description of Groups

2.2 Induction of CDH with nitrofen

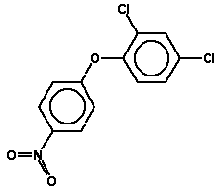
The herbicide Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) was obtained from WAKO Chemicals (Tokyo, Japan). Nitrofen was dissolved in peanut oil and administered in a single oral dose of 100mg to the pregnant rats on day 11.5 of gestation to induce right-sided CDH (as per protocol described in 2.4.1).

2.2.1 Teratogenic potency and mechanisms of action of nitrofen

It has been known since 1971 by studies conducted by Ambrose, et al, that the diphenyl ether herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) is a potent teratogen after maternal ingestion [9]. Nitrofen is the most potent teratogen of its class, causing a multitude of fetal defects in many organ systems, while causing little or no change in prenatal and maternal morbidity and mortality [57]. The extent of the teratogenic potency of a diphenyl ether correlates positively to the number of chlorine substituents and to their steric arrangement.
The mechanism of nitrofen teratogenicity is not known. Nitrofen affects the pituitary-hypothalamic-thyroid axis of the fetus due to its similar structure to thyroxine [39, 67]. However, the exact relationship between thyroid development and nitrofen teratogenicity has not been recognized [131].

2.2.2 Chemical properties of nitrofen

The chemical structure of nitrofen is $C_{12}H_7Cl_2NO_3$. It is a relative hydrophobic substance that has a good solubility in organic solutions. Its molecular weight is 284.1, the melting point is 70ºC, the boiling point is 180-190ºC, and the water solubility is less than 0.1 g/100 ml at 21ºC. It exists as a colorless crystal at room temperature. The apparent color of nitrofen ranges from white to dark brown.

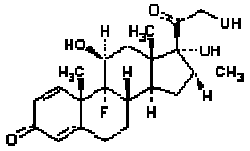


2.3 Dexamethasone treatment

Dexamethasone was given to all 18 dams by intraperitoneal injection on days 18.5 and 19.5 of pregnancy (section 2.4.1). The control groups I and II consisted of offspring from twelve dams that did not receive dexamethasone treatment (table 1) examined in the previous study [22].

2.3.1 Chemical properties of dexamethasone

The chemical structure of dexamethasone is $C_{22}H_{29}FO_5$. Its molecular weight is 392.5, the melting point is 262-264ºC, and the water solubility is less than 0.1 g/100 ml at 25ºC. It exists as a white to off-white crystalline powder at room temperature.



9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione

2.3.2 Antenatal dexamethasone therapy

Animal studies have shown that antenatal steroid therapy in experimentally induced CDH has led to accelerated surfactant synthesis and release [189], increased lung compliance [126, 173], improved morphogenesis [82], and prevention of pulmonary vascular wall thickening [151, 192]. The improved pulmonary vascular resistance at birth following steroid administration seems to be mediated by increased nitric oxide synthase activity, but the timing and duration of steroid administration appear to be critical to the response [70].
There is a widespread use of glucocorticoids to induce lung maturation in fetuses at risk of premature delivery, as well as case-reports of favorable outcomes in fetuses with CDH after antenatal betamethasone [56]. However, numbers are small and clinical trials of maternally administered corticosteroids are also few [51, 126].

2.4 Nitric oxide treatment

Immediately following spontaneous delivery on day 21 or 22 the newborn rats were taken from their mother and, after being tagged for identification, were when allocated to group 4 (and group 2 in the previous study) placed in a plastic chamber serving as a microincubator with a constant flow of 80 ppm nitric oxide. The newborns in group 3 (and group 1 in the previous study) received artificial air consisting of 21 vol% O_2, 79 vol% N_2, and 0.5 vol% miscellaneous.
The chamber had a volume of approximately 2 liters with several circular openings of approximately 1 cm on the upper side to allow an exhaust and prevent toxic concentration and build-up of NO_2. The plastic chamber was placed on a heating plate (Medax Nagel Type 13800) set at 39ºC in order to help the newborn rats keep their physiological body temperature.
The nitric oxide and artificial air were purchased from AGA, Bocholt, Germany.

2.4.1 Properties of nitric oxide

The chemical structure of nitric oxide is NO. Its molecular weight is 30, the melting point is -164ºC, the boiling point is -151.7ºC, and the vapor density is 1.04. It is a colorless gas with a sharp, sweet odor that turns brown at high concentrations in air. NO is extremely lipophilic and can readily permeate biological membranes.

Authentic NO gas is exceedingly unstable and is oxidized (in the presence of O_2) within seconds to nitrogen dioxide (5-fold toxic potency) and higher oxides of nitrogen [90]. The rate of NO_2 formation increases with the F_{IO2}. Dilute solutions of NO exposed to oxygen have a half life of less than 10 seconds due to rapid oxidation to inorganic nitrate and nitrite.
NO is inactivated as it binds to hemoglobin, forming methemoglobin. Excessive concentrations of methemoglobin can alter oxygen-carrying capacity, worsening hypoxia and potentially aggravating hypoxic pulmonary vasoconstriction.

2.4.2 Actions and properties of endothelium derived nitric oxide

The simple molecule nitric oxide (NO), remarkably, was shown in 1987 [92, 154] to be identical to or an intermediate of endothelium derived relaxing factor. Nitric oxide is an endogenous vasodilator produced by the endothelium in response to both chemical and physical stimuli [91, 154].
The vascular endothelium produces NO from the released terminal-nitrogen after conversion of L-arginine to L-citrulline. The lipophilic gas then permeates into the vascular and smooth muscle, interacting with the heme moiety of guanylyl cyclase [138]. Guanylyl cyclase catalyzes the formation of cyclic GMP (cGMP) which acts directly on cell proteins as a second messenger. Regulation of cellular events by cGMP is accomplished by the interaction of the molecule with specific classes of target proteins, such as cyclic nucleotide-regulated protein kinases (for cGMP called G-kinases) [121]. Activation of G-kinases by cGMP may lower intracellular Ca^{2+} by phosphorylating and activating Ca^{2+}-ATPase or components associated with the transporter [121, 172]. Lowered intracellular calcium concentration leads to less contraction, and therefore smooth muscle relaxation and vasodilation [137].
Another mechanism by which nitric oxide mediates vasodilation is the cGMP-dependent activation of Ca^{2+}-activated K^+ channels [72]. Chronic hypoxia has been shown to reduce nitric oxide and cGMP-mediated activation of the Ca^{2+}-dependent K^+ channels by decreasing phosphorylation of the channel [157]. This could explain hypoxic vasoconstriction and refractory pulmonary hypertension in newborns following in utero hypoxia [209].
Other mechanisms of nitric oxide mediated vasodilation include: activation of Ca^{2+}-dependent K^+ channels directly in the absence of cGMP or G-kinases [24]; down-regulation of the angiotensin II type I receptor expression [90]; and necessity for a physiologic balance between endothelin-1 and nitric oxide activity [164].
Nitric oxide is a selective pulmonary vasodilator that does not alter systemic vascular resistance. This occurs because NO is quickly inactivated after diffusion into the bloodstream by binding to hemoglobin. Due to its high affinity for hemoglobin (1500x that of CO), inhaled NO causes a relaxation of the pulmonary microcirculation in the ventilated areas of the lung for a short-lived effect of 3 to 5 seconds. Unwanted systemic side-effects, such as a decrease in systemic blood pressure, are extremely rare. Lastly, nitric oxide inhibits platelet aggregation and inhibits platelet adhesion to endothelial cell surfaces [163]. It, therefore, also has vasoprotective and anti-arteriosclerotic effects.

2.5 Design of study

The study is structured in a three-part experimental phase, a subsequent phase of preparing the extracted lung specimens for the histological evaluation, and the final phase of lung morphology evaluation by means of the light microscope.

2.5.1 Experimental phase

1. Induction of right sided diaphragmatic hernias in utero: Nitrofen was given to all 20 mother rats in this study, and to the 12 mother rats in the control groups of the previous study, on day 11.5 of gestation at the standardized time of 6:00 pm. The average weight of these Sprague Dawley rats at this point of gestation was 344g. Nitrofen (100 mg) was dissolved in peanut oil and administered in a single oral dose. The mother rats had to consume the herbicide within an hour in order to be included in the study. The animals were then further cared for in the standardized room environment as described in section 2.1.

2a. Observation and clinical evaluation of the newborn rats was performed at 2-hour intervals for a period of 12 hours. Vitality of the animals was evaluated every two hours by a modified APGAR score, the "RAT score," based on skin color, spontaneous activity, and reactivity.

2b. The heart rate and percutaneous oxygen saturation were monitored continuously and recorded every 2 hours during which the animals were receiving artificial air or nitric oxide.

The results of the vitality RAT score (2a) and oxygen saturation (2b) will be evaluated in a further dissertation and will, therefore, not be further mentioned in this work.

3. Animals that died during the observation period were promptly inspected, weighed, and dissected for diaphragm inspection and lung recovery. All other animals were sacrificed by decapitation after 12 hours, and likewise dissected to confirm the presence or absence of CDH and record its size.
After the weight and length of each animal was recorded, a thoracotomy and microdissection took place by use of the Olympus model SZ/SZH microscope; care was taken to not injure the diaphragm, the lungs, or any herniated organs in the thoracic cavity.

2.5.2 Classification of hernias

The hernias were classified based on size and the abdominal content of the thorax due to herniation:

Size of Hernia	Morphology of Thorax
0	No Hernia

1	Small Hernia which is totally covered by the lungs and can only be seen after lung elevation. **< 30% of the entire thorax diameter**
2	Hernia encompasses at least 30% of the thorax diameter. **> 30% but < 50% of the thorax diameter**
3	Hernia takes up at least 50% of the thorax diameter and there are no other abdominal structures other than the liver in the thorax. **> 50% of the thorax diameter, no abd. content**
4	Hernia takes up at least 50% of the thorax diameter and abdominal content other than the liver is present in the thorax. **> 50% of the thorax diameter with abd. content**

Table 2: Classification of Hernias

2.5.3 Preparation of lung specimens

2.5.3.1 Paraffin bedding

In order to embed the lung specimens in paraffin the following steps were necessary:

a. Fixation of the specimens in Bouin solution

b. Dehydration of the specimens was carried out by placement in subsequent alcohol solutions of the following concentration and time period while constantly mixing by means of a magnet pellet:

2-Propanol	
1. [70%] for 2 hours	5. [100%] for 4 hours
2. [70%] for 2 hours	6. [100%] for 2 hours
3. [80%] for 2 hours	7. [100%] at 42ºC for 30 min. in incubator
4. [90%] for 2 hours	

c. Placement of specimens in an equal mixture of 100% 2-propanol and paraffin in a closed container for 3 hours in the incubator at 42-44º C
d. Placement of specimens in an open container of paraffin overnight for 12 hours in the incubator at 42-44º C
e. Placement of specimens in an open container of paraffin for 4 hours at 56-58º C
f. Last placement of specimens in an open container of paraffin for 4 hours at 56-58ºC

g. Lung specimens are then placed in a vertical position in a cubed plastic form measuring approx. 3x3cm. Fresh paraffin is hot dipped into the form. The form is then immediately cooled on ice for hardening
h. After hardening a razor is used to cut off the excess paraffin leaving rectangular forms for cutting

2.5.3.2 Cutting of paraffin blocks

Only the right lung specimens were examined in this study. The fixated right lung specimens in the paraffin blocks were then cut from the apical to the basal end of the lung in 6 µm slices with the rotating-microtome model 2035 Biocut and the Microm HM400, and then placed on slides. Each slide fit approximately 2 rows of 8 axial (horizontal) sections.

2.5.3.3 Staining of slides

Slides were stained in a modified Gomori trichrome stain:

a. Paraffin was removed from material by subsequent placement in decreasing alcohol solutions (100-50% Roti-Histol)
b. Staining of nucleus with Hämalaun
c. Trichrom stain
d. Differentiation in 1% acetic acid
e. Placement in increasing alcohol solutions
f. Lid placement with entellan

2.5.4 Morphometric analysis

After completion of staining the histological morphology of the lungs was examined under an Olympus BX60 light microscope. The histological material was evaluated and described by a study-team member who was not aware of the specimen grouping. In order to evaluate the same area of lung in each specimen, only slides at the level of the hilus were analyzed. The lung specimens were given a histological score by the observer based on the following criteria:

Histology-Score	Definition
1	- normal aerated lung (no atelectasis, no bullae) - thin septal walls - good peripheral perfusion - thin vascular walls
2	- good aerated lung - minimal thickening of alveolar septal walls - good peripheral perfusion - minimal thickening of vascular walls

3	- increased interstitial tissue - internal bleeding into tissue often present - normal septal wall formation often producing large emphysematous bullae and/or minimal atelectasis - reduction of peripheral vascularisation and perfusion with larger central vessels - advanced vascular wall thickening
4	- severe atelectatic lung - intermediary stage between 3 and 4
5	- complete atelectatic lung - only central vessels present - severe vascular wall thickening

Table 3: Defining Criteria for Histology-Score

The transfer and conversion of pictures from the microscope was possible by use of the JVC color video camera model TK 1070E and the corresponding JVC AC-adapter.

2.6 Statistical analysis

Descriptive analysis of parametric data is expressed as means. In order to keep the data of each group independent, the mean (survival time or histology score) of each litter was first established and then averaged with the corresponding mean of each litter in that particular group. Whisker box plots were used to study the distribution of data. Statistical significance was estimated using the Kruskal-Wallis-Test and the Mann-Whitney-U-Test. Survival curves were based on Kaplan-Meier. P values of less than .05 were considered significant. Data tabulations and calculations were made using Microsoft Excel version 2002 and SPSS version 12.0.

3 RESULTS

3.1 General

A total of 491 offspring were studied in this project. As expected, due to the timing and dosage of nitrofen ingestion (detailed in section 1.3.2), right-sided hernias were observed in 398 (81.1%) of the newborn rats. As summarized in table 4, most hernias were large falling into size 3 and 4 of our classification (refer to table 2).

Hernia Size	No.	Percent
Ø (No Hernia)	93	18.9%
1	45	9.2%
2	51	10.4%
3	154	31.4%
4	148	30.1%
Total	491	100%

Table 4: Distribution of hernia size in newborn rats

After spontaneous delivery on day 21 or 22, nitrofen treated litters were divided and allocated to groups 1 to 4 as described in table 1. At that point in time the presence or absence of CDH could not be verified because the rats were still alive. The number of offspring in each group, the number of observed hernias in each group and their percentage are summarized in table 5. The 4 groups of newborn rats had a similar proportion of CDH. There was no evident difference in the distribution of the CDH sizes recorded. The distribution of hernia size is shown graphically in chart 1.

Group	Number of Newborn Rats (%)	Ø Hernia (%)	Grade I Hernia (%)	Grade II Hernia (%)	Grade III Hernia (%)	Grade IV Hernia (%)	CDH Total
I	88 (100)	17 (19.3)	4 (4.5)	12(13.6)	29 (33.0)	26 (29.5)	71
II	104 (100)	30 (28.8)	11(10.6)	12(11.5)	28 (26.9)	23 (22.1)	74
III	153 (100)	18 (11.8)	18(11.8)	18(11.8)	61 (39.9)	38 (24.8)	135
IV	146 (100)	28 (19.2)	12(8.2)	9(6.2)	36 (24.7)	61 (41.8)	118
Total	491 (100)	93 (18.9)	45(9.2)	51(10.4)	154(31.4)	148(30.1)	398

Table 5: Group size and respective frequency of hernia in each treatment group

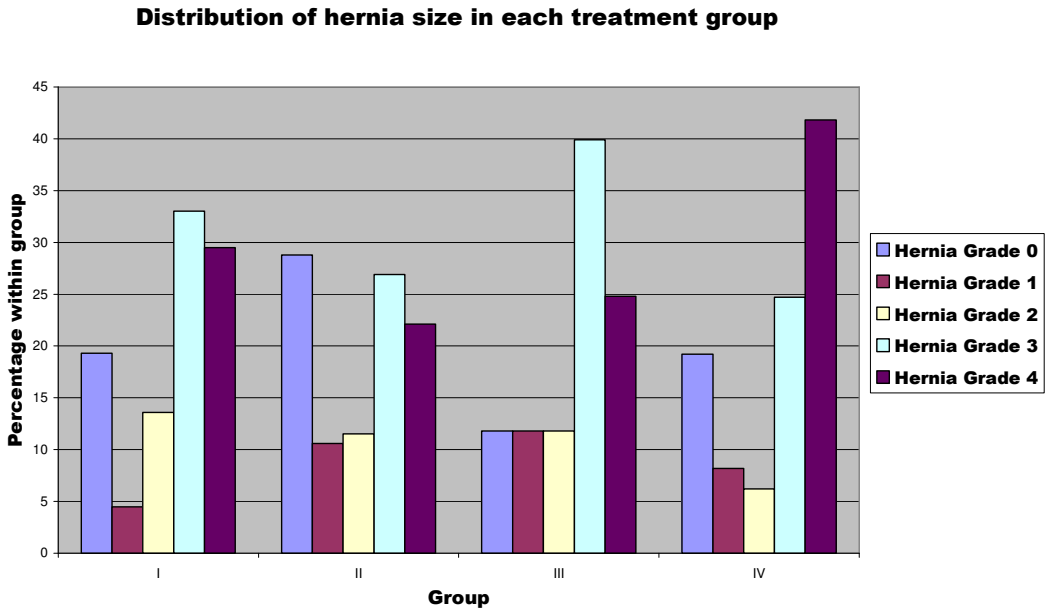


Chart 1: Bar graph of table 5

3.2 Morphometric analysis of right-lung specimens based on histology-score

The *histology-score* is defined in table 3. The score ranges from 1 – for a well developed, good aerated lung specimen, to 5 – for a poorly developed, completely atelectatic lung specimen. At the end of this chapter under section 3.4 are representative pictures of horizontal lung sections for each score.
As can be seen in table 6, we selected 377 right-lung specimens of the 491 newborn rats for histological evaluation. In each respective group the following proportion of specimens were examined: in the control group (group I) 26 of 88 specimens or 29.5%;

in group II 66 of 104 or 63.5%; in group III 145 of 153 or 94.7%; and in group IV 140 of 146 or 95.9%.

Experimental Group		Histology-Score					
		1	2	3	4	5	Total
I (Control)	Count	2	5	7	7	5	26
	% within Group	7.7	19.2	26.9	26.9	19.2	100
	% within All	2.7	8.6	7.2	7.2	18.5	6.9
II (NO)	Count	15	15	12	17	7	66
	% within Group	22.7	22.7	18.2	25.8	10.6	100
	% within All	20.5	25.9	9.8	17.5	25.9	17.5
III (Dex)	Count	13	29	47	46	10	145
	% within Group	9.0	20.0	32.4	31.7	6.9	100
	% within All	17.8	50.0	38.5	47.4	37.0	38.5
IV (NO+Dex)	Count	43	9	56	27	5	140
	% within Group	30.7	6.4	40.0	19.3	3.6	100
	% within All	58.9	15.5	45.9	27.8	18.5	37.1
Total	Count	73	58	122	97	27	377
	% within All	19.4	15.4	32.4	25.7	7.2	100

Table 6: Histology score tabulation

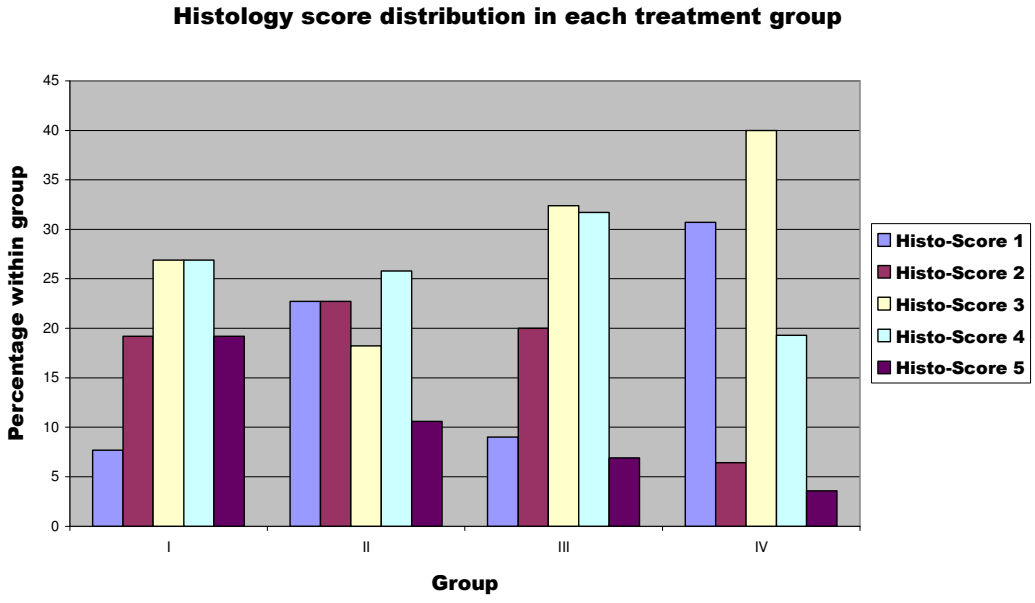


Chart 2: Bar graph of table 6

The entire litter of each mother rat was independently allocated to one of the four groups. In order to determine the histology score mean in each group (I-IV), the mean of each litter was first established and then averaged with the corresponding mean of each litter in that particular group.

The histology-score mean of group I is 3.31, group II is 2.79, group III is 3.08, and group IV is 2.59. The mean histology score was better in all three groups (II-IV) when compared to the control group I, meaning that the alveolar architecture and lung

development improved at least slightly in each treatment group; however, not enough to be statistically significant (p=0.113).
As can be seen in section 3.2.1 on survival times, most newborn rats with hernias of sizes 3 and 4 have high mortality rates (within the 12 hour observation period) irrespective of treatment. Therefore, reevaluation of histology-score means with exclusion of newborns with hernias of sizes 3 and 4 was carried out. This exclusion improves the mean in all four groups. The histology score mean of group I is then 2.90, of group II is 2.16, of group III is 2.15, and of group IV is 1.57. It seems that the anatomically favorable smaller hernias allow a longer period of survival and inhalation of NO; thereby, producing a better aerated lung. There is, of course, also lesser herniation to exert mechanical force on the developing lungs. The effect of prenatal dexamethasone on the alveolar architecture is also more apparent in this subgroup. The combined effect is clearly recognizable with the histology-score mean of 1.57 in group IV. Whisker box plots of the histology-score mean of each litter in each respective group are shown below in chart 3. It can be seen that the variation in data subsequently decreases, with the lowest variation being in group IV data. Furthermore, most data in group IV is within the lower and upper quartiles with just one litter being an outlier with a mean outside of the outer range.

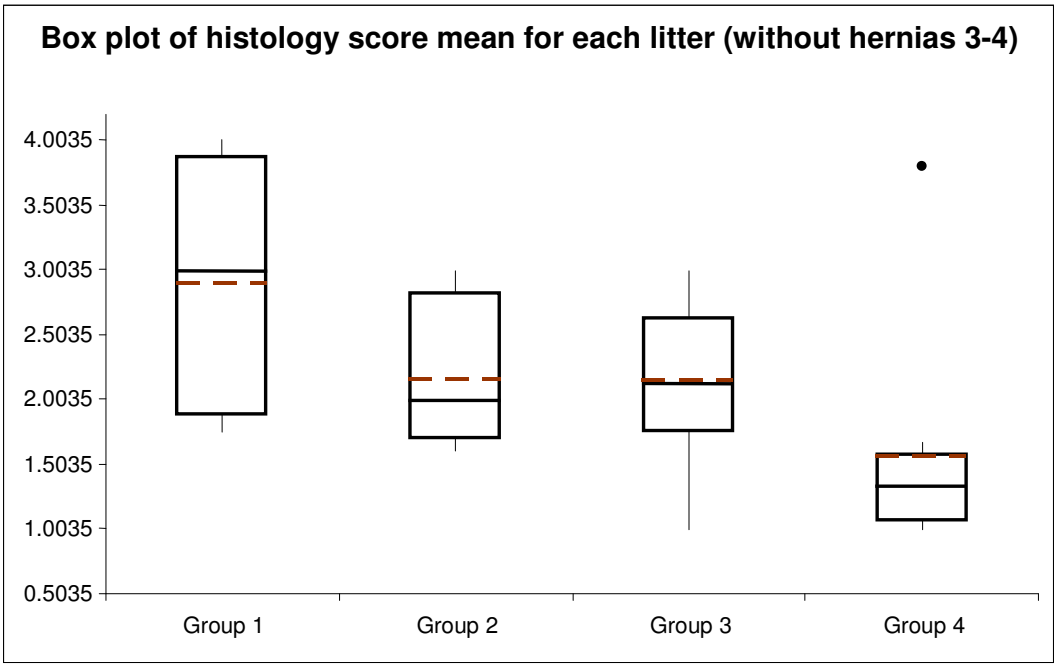


Chart 3: Box plot (red dashed line is the mean)

The Kruskal-Wallis test shows an asymptotic significance of 0.013, with statistical significance in all groups when compared to IV (refer to table 7). This indicates that the combined treatment of dexamethasone prenatally and nitric oxide postnatally (as in group IV) has the most significant effect on lung development. The separate treatment of either dexamethasone prenatally (as in group III) or nitric oxide postnatally (as in group II) was not significant in comparison to the control or to each other. P-values of comparisons are listed in table 7.

Kruskal-Wallis test p-values comparing histology scores for each group (without hernias 3-4)

Group	I	II	III	IV
I	X	0.202	0.206	**0.012**
II		X	0.887	**0.012**
III			X	**0.022**

Table 7: p-values

It has been suggested in a previous study from this department [22] that the nitrofen induced diaphragmatic hernia in our rat model is not the sole factor for the lung hypoplasia, but that nitrofen also causes direct damage to lung development - possibly a decrease in alveolar surface area. Evaluation of histology score means of newborn rats with no hernia (hernia Ø) resulted in a mean of 3.0 for group I, 2.04 for group II, 1.60 for group III, and 1.24 for group IV. One would assume that since the newborns do not have a hernia, then the histology score for all - irrespective of treatment - would be in the range of one. However, the histology scores of the 17 newborns in group I resulted in a mean of 3.0. This definitely suggests a direct degenerative lung change due to the nitrofen ingestion. Statistical comparison of means using Kruskal-Wallis gives a p value of 0.043 in this subgroup (only Ø hernia newborns), as well as significance in the control group compared to group 3 and 4. This indicates that the combined treatment of dexamethasone prenatally and nitric oxide postnatally (as in group IV) as well as the single treatment with prenatal dexamethasone (group III) improved the degenerative changes caused by nitrofen. The improvement in histology score in group II was not significant in comparison to the control group. P-values of comparisons are listed in table 8.

Kruskal-Wallis test p-values comparing histology scores for each group (only null hernias Ø)

Group	I	II	III	IV
I	X	0.106	**0.018**	**0.045**
II		X	0.318	0.152
III			X	0.536

Table 8: p-values

Group	Histology-Score mean		Groups w/o Hernias 3-4	Histology-Score Mean		Groups with only Hernia Ø	Histology-Score Mean
I	3.31		I	2.90		I	3.00
II	2.79		II	2.16		II	2.04
III	3.08		III	2.15		III	1.60
IV	2.59		IV	1.57		IV	1.24

Table 9: Overview of histology score means *(the mean of each litter was first established and then averaged with the corresponding mean of each litter in that particular group)*

3.3 Survival analysis of newborn rats

The observation period of the newborn rats was 12 hours (720 minutes); therefore, the upper limit of survival is 720 minutes, at which point in time the newborns were sacrificed. The survival times were first analyzed based on respective allocation in group, histology score, and size of hernia – as shown in table 10. As done with the histology scores, the survival time mean of each litter was first established and then averaged with the corresponding mean of each litter in that particular group.

Group	Survival Time Mean (min), [w/o Hernia 3-4],		Histology Score	Survival Time Mean (min)		Size of Hernia	Survival Time Mean (min)
I	171 [306]		1	685		Ø	671
II	380 [639]		2	649		1	625
III	285 [585]		3	143		2	421
IV	312 [709]		4	119		3	108
			5	114		4	93

Table 10: Survival time means (survived = 720 min)

There is a gradual increase in survival time as the histology score improves, as well as when the hernia size decreases. As one would expect the rats survive longer when the lung histology is better developed (score of 1) and the hernia size is small or absent (hernia Ø). As can be seen in chart 4 and 5, newborns with a histology score of 1-2 survived considerably longer than those with a histology score of 3-5 irrespective of treatment (groups II-IV).

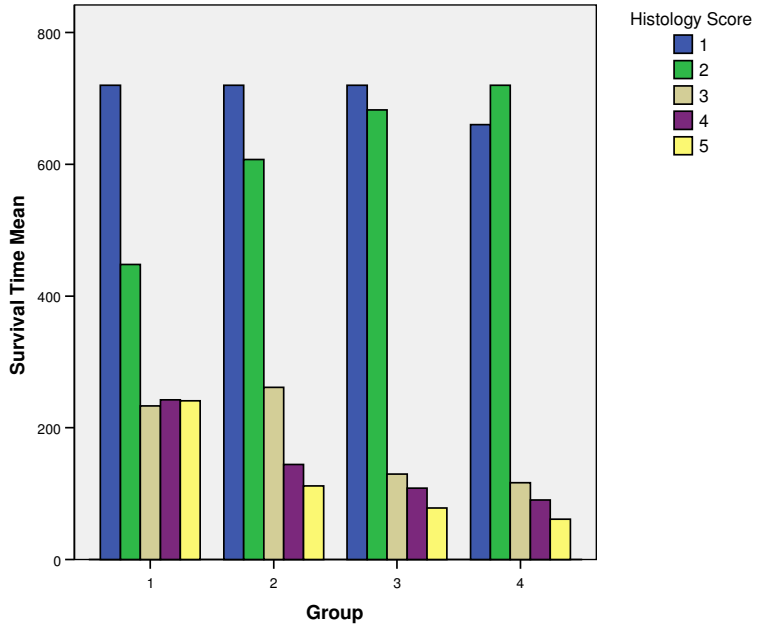


Chart 4: Survival time means based on histology score for each group

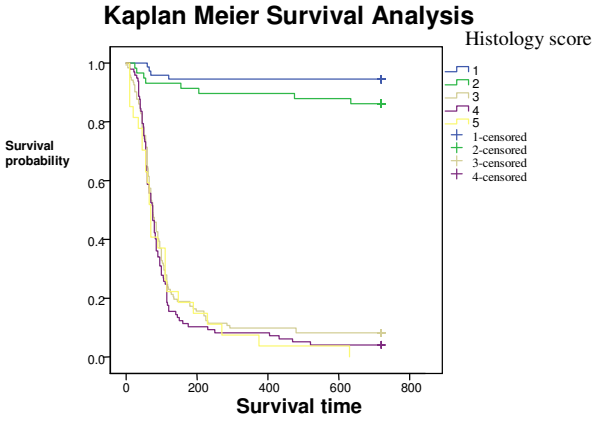


Chart 5: Survival analysis based on histology score *(survivors ≥ 720 minutes censored)*

Statistical comparison of survival time means using Kruskal-Wallis shows no significance in treatment group comparisons (p=0.188). However, it is evident after further breakdown of survival time means based on hernia size (refer to chart 7), that newborns with hernias of size 3 and 4 had considerably worse survival time than those with no or smaller hernias. This is evident in the Kaplan Meier estimator for survival function shown in chart 6.

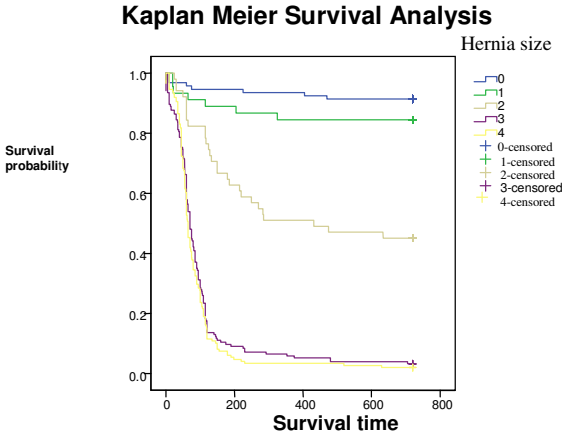


Chart 6: Survival analysis based on hernia size *(survivors ≥ 720 minutes censored)*

Furthermore, irrespective of group allocation therapy regiments did not improve survival in newborns with large hernias. In other words, the experimental evidence shows that the newborns can be divided into a "good" prognosis group (newborns with hernias Ø, 1 and 2) that respond to ante- and postnatal treatment, and a "poor" prognosis group (newborns with hernias 3 and 4) that do not benefit from such care. This belief is further supported by the fact that the mean survival time of all newborns, irrespective of treatment with hernias of size 1-2 is 516 minutes, whereas for newborns with hernias of size 3-4 is considerably lower at 101 minutes.

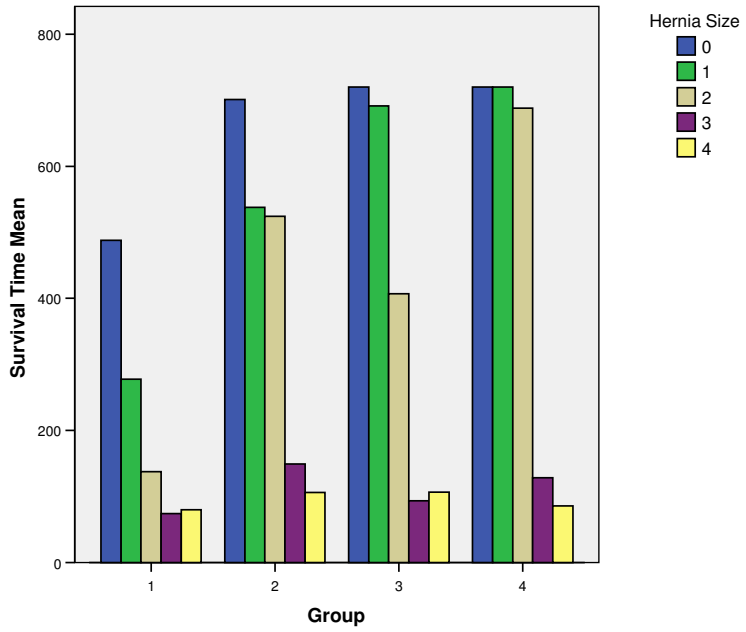


Chart 7: Survival time means based on hernia size for each group

Statistical comparison of survival time means of treatment groups excluding newborns with hernias of size 3 and 4 showed an asymptotic significance of 0.020, with significance in the control group (I) and group II compared to group IV. This indicates that the combined treatment of dexamethasone prenatally and nitric oxide postnatally (as in group IV) improved survival time in comparison to no treatment and the single treatment with nitric oxide. P-values of comparisons are listed in table 11.

Kruskal-Wallis test p-values comparing survival time means for each group (without hernias 3-4)

Group	I	II	III	IV
I	X	0.073	0.075	**0.019**
II		X	0.669	**0.031**
III			X	0.182

Table 11: p-values

Kaplan Meier survival function plots of groups I-IV (excluding hernias 3-4) show the drastic improvement in survival probability when comparing the treatment groups to the control (chart 8). There is an over 60% probability of survival with group IV treatment in comparison to the control, and an over 40% probability of survival with either group II or III treatment in comparison to the control.

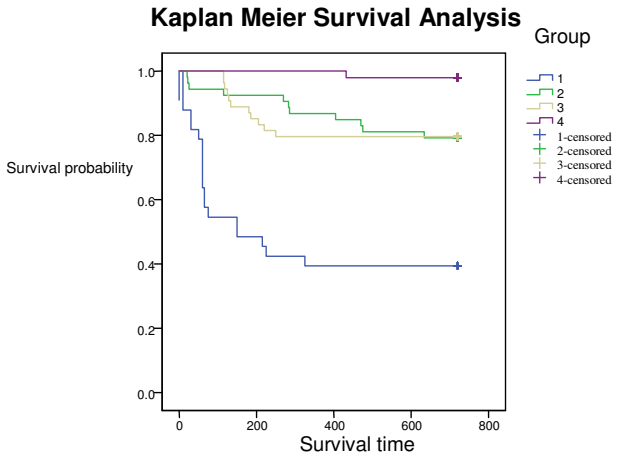


Chart 8: Survival analysis based on group *(without hernias 3-4, survivors ≥ 720 minutes censored)*

In order to further evaluate the effect of nitrofen alone on the outcome, evaluation of survival time means of newborn rats with no hernia (Ø hernia) was also carried out. One would assume that since the newborns do not have a hernia, then the survival time mean - irrespective of treatment - would be in the range of the maximum 720 minutes. However, the survival time mean of the 17 newborns in group I with no hernia resulted in a mean of 488 minutes. This definitely suggests a direct degenerative lung change, due to the nitrofen ingestion, affecting the survival time. However, this change must have not been very severe since the survival time improved drastically in all three treatment groups (group II was 701 min., group III and IV each with 720 minutes). Statistical comparison of means shows an asymptotic significance of 0.026 in this subgroup (only Ø hernia newborns). However, even though all treatment groups had an extreme improvement in the survival time mean, only groups II and IV were statistically significant in comparison to the control group. This indicates that the combined treatment of dexamethasone prenatally and nitric oxide postnatally (as in group IV) as well as the single treatment with postnatal nitric oxide (group II) improved the degenerative changes caused by nitrofen. The improvement in survival time in group III was not significant in comparison to the control group. P-values of comparisons are listed in table 12.

Kruskal-Wallis test p-values comparing survival time means for each group (only null hernias Ø)

Group	I	II	III	IV
I	X	**0.030**	0.093	**0.019**
II		X	0.694	0.694
III			X	0.959

Table 12: p-values

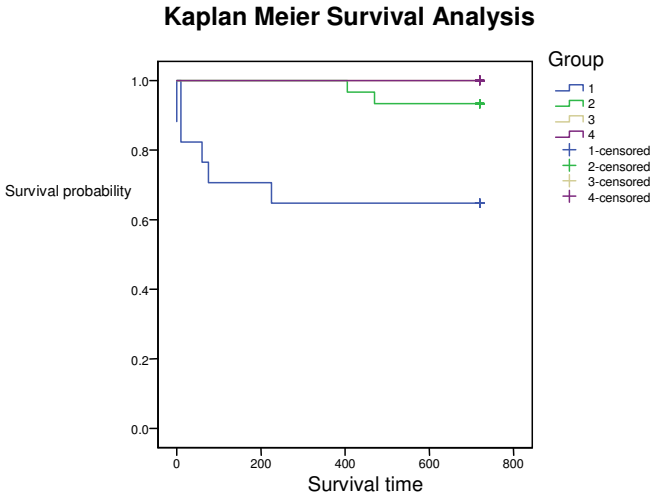


Chart 9: Survival analysis based on group *(only newborns with Ø hernias, survivors ≥ 720 minutes censored)*

3.4 Sample pictures of lung sections

Fixated right lung specimens at the level of the hilus were analysed using a modified Gomori trichrome stain. In figure 1 is a slice of the right lung of a control group newborn (magnification x10) that survived the entire observation period. As can be seen the alveolar architecture is well developed with no atelectasis or bullae. There are thin septal and vascular walls with good peripheral perfusion. This slide corresponds to a histology score of 1.

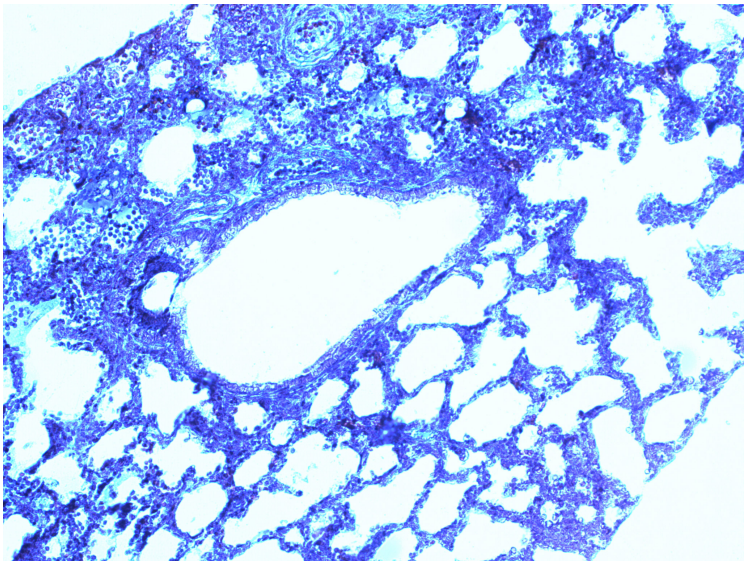


Figure 1: Histology score of 1. Slice of right lung of newborn P1 in group I (magification: x10)

In the next slide (fig. 2) is the right lung specimen of a newborn from group II that also survived the 12-hour observation period. After close examination of the lung structure it is evident that the alveolar walls are somewhat thickened, interstitial tissue is increased, and alveolar air space is slightly diminished. This slide corresponds to a histology score of 2.

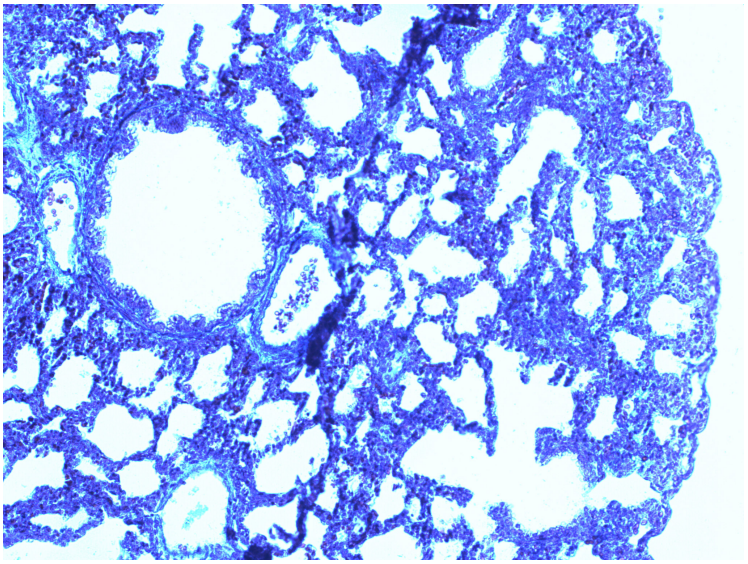


Figure 2: Histology score of 2. Slice of right lung of newborn F1 in group II (magification: x10)

In the next slide (fig. 3) is the right lung specimen of a newborn from group IV that did not survive the observation period. The pulmonary architecture has now further worsened with alveolar walls clearly thickened, interstitial tissue markedly increased, and alveolar air space noticeably diminished. There are also signs of reduction of peripheral vascularisation and perfusion with thickened alveolar-capillary interface. This slide corresponds to a histology score of 3.

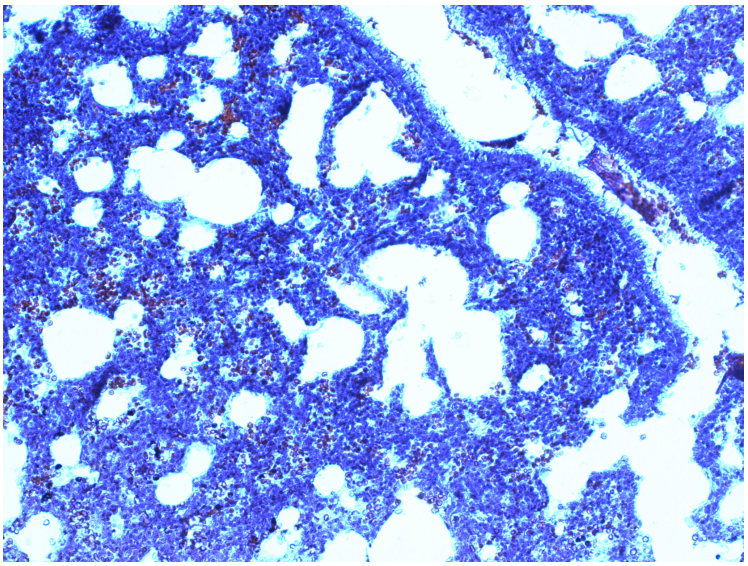


Figure 3: Histology score of 3. Slice of right lung of newborn U2 in group IV (magification: x10)

In the next slide (fig. 4) is the right lung specimen of a newborn from group IV that did not survive the observation period. The lung tissue is dense and hypoplastic. The interstitial and alveolar walls are massively thickened. The alveolar air space is, due to atelectasis and internal bleeding, markedly diminished. This slide corresponds to a histology score of 4.

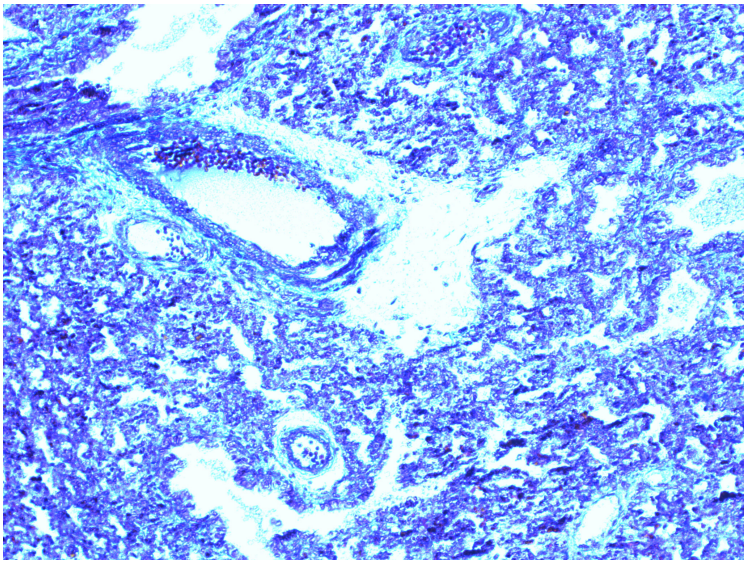


Figure 4: Histology score of 4. Slice of right lung of newborn W1 in group IV (magification: x10)

In the next slide (fig. 5) is the right lung specimen of a newborn from group III that did not survive the observation period. The lung tissue is completely hypoplastic with severe atelectasis. This newborn only survived 70 minutes after birth with postnatal nitric oxide treatment. This slide corresponds to a histology score of 5.

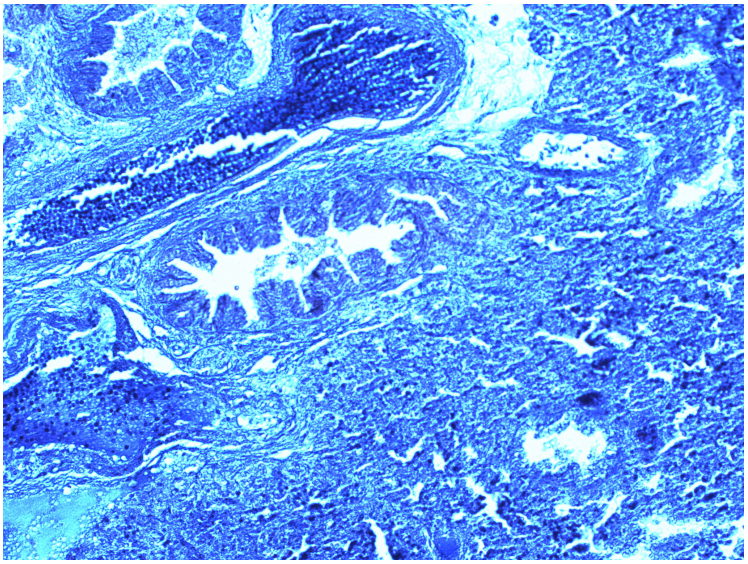


Figure 5: Histology score of 5. Slice of right lung of newborn H1 in group III (magification: x10)

DISCUSSION

4.1 Congenital diaphragmatic hernia: Introduction

Congenital diaphragmatic hernia (CDH) remains a challenge to clinicians with high mortality despite advances in antenatal and postnatal diagnosis, and the advent of new therapeutic modalities. The clinical spectrum of CDH ranges from minimally affected infants who do well with modern neonatal care to severely affected infants who die despite all interventions. The high neonatal mortality is mainly due to pulmonary hypoplasia and pulmonary hypertension. The diaphragmatic defect, usually left-sided, allows abdominal contents to herniate into the thoracic cavity, leading to pulmonary hypoplasia and ultimately postnatal respiratory failure. Although the pulmonary hypoplasia may be partly reversible with antenatal and postnatal care, affected neonates often require months of ongoing treatment [4, 19, 76, 78]. Emphasis now is on prenatal diagnosis, antenatal care, pre-operative stabilization, and delayed repair [149, 191]. The purpose of this study has been to gather further information on therapy regiments for the antenatal care and pre-operative stabilization of newborns with CDH using the nitrofen treated CDH-rat model.

4.1.2 Prenatal diagnosis and prognostic factors

Over the last three decades enormous advances have been made in the understanding, detection of, and approach to fetal abnormalities. Accurate antenatal diagnosis allows possible prenatal care and delivery in an environment with the availability of innovative therapeutic techniques. It further allows for informed counseling of the family with regard to the outcome of the pregnancy and the possible treatment options, such as continuing with or terminating the pregnancy, and in-utero fetal manipulation[81].
Antenatal diagnosis of congenital diaphragmatic hernia by ultrasonography was described toward the end of the 1970s [87, 201]. The improvements in the resolution of ultrasound pictures has made it possible to detect the diaphragmatic hernia early in gestation; however, the timing of the herniation may be as late as the third trimester of pregnancy[28] making it impossible to detect all defects in the second trimester. In a study taken from data of 20 registries of congenital malformations in 12 European countries[62], the overall detection rate was high at 59% but varied significantly between European regions (30%-75%). The mean gestational age at discovery was 24.2 weeks (range 11-38 weeks), indicating that a rather large proportion of cases was diagnosed after the routine second-trimester scan. Although earlier detection of CDH has not yet shown increased survival rate of infants [63, 179, 186, 217] (perhaps due to more severe cases being diagnosed earlier), it does give the parents the option to a termination of pregnancy (TOP), since most European countries have an upper limit for TOP of 22-24 weeks (excluding Germany and France).
The prenatal ultrasound diagnosis of CDH is based on either direct signs, such as bowel loops, stomach and/or liver parts displaced into the thoracic cavity, polyhydramnios, small lung-thorax transverse area ratio, or indirect signs, such as an abnormal position of the heart with mediastinal shift. It has been attempted to use these signs as prognostic factors for poor outcome; however, none of these features have been uniformly predictive of outcome.

Currently, the most reliable prenatal predictor of postnatal outcome for left CDH is liver herniation [6, 134]. Color flow Doppler imaging of the umbilical and portal veins detects liver herniation in utero by following the vessels above the diaphragmatic defect [25]. When combined with amniocentesis to exclude chromosomal abnormalities and prenatal echocardiography to detect cardiac malformations, the presence of an isolated CDH can almost always be confirmed [141]. In difficult cases, the diagnosis can be confirmed with magnetic resonance imaging [89]. In one study the absence of liver herniation predicted 75-80% survival with either fetal or postnatal treatment [73] compared with historical survival of only 20% to 27% for fetuses with prenatally diagnosed CDH [3, 175]. In another study, the absence of liver herniation indicated a good prognosis with a survival of 79%, whereas liver herniation was associated with 41% survival [206].

The right lung area to head circumference ratio (LHR), defined as the two-dimensional right lung area measured at the level of the four-chamber view of the heart divided by the head circumference to normalize for gestational age, is also an important prognostic factor [122, 134]. An LHR less than 1.0 is associated with 100% mortality, whereas an LHR of more than 1.4 is associated with no mortality. For LHRs between 1.0 and 1.4, mortality is approximately 38-60%. The LHR is the first marker which has predicted the severity of lung hypoplasia in left CDH based on ultrasound appearance at 23-26 weeks gestation. It remains uncertain whether LHR is as accurate in predicting outcome in right CDH or in patients with ultrasound data after 26 weeks.

Fetal lung volume in CDH patients can be measured using three-dimensional ultrasound (technique of rotation of the multiplanar imaging) [168, 169]. Results are similar to those obtained by magnetic resonance imaging [53, 128]. Preliminary studies suggest that low fetal lung volume estimated by three-dimensional ultrasound in fetuses with CDH may be associated with pulmonary hypoplasia and neonatal mortality [169].

Lastly, new prognostic potential factors have been reported, such as fetal pulmonary artery diameters [180] or the use of acceleration time/ejection time ratio in pulmonary arteries by doppler blood flow velocimetry [61].

4.1.3 Etiologic and genetic factors

The presence of associated anomalies with co-morbidity affects survival of infants with CDH. Approximately 30-50% of cases with CDH have other malformations, karyotype anomalies, and syndromes [20, 47, 62, 159, 179, 200].

In sporadic cases male infants have more frequent anomalies of the genitalia and kidneys; whereas, female infants have more CNS, heart, gastrointestinal, and liver defects [200]. The distribution in CDH infants shows 56-75% with CNS anomalies, 15-25% with cardiovascular anomalies, and 5-10% with genitourinary anomalies [30, 47, 200]. In familial CDH cardiovascular and genitourinary anomalies occur most frequently in 29% of cases. The frequency of associated malformations is higher in stillborn (92%) than in liveborn infants (23.6%) [156, 160].

The incidence of chromosomal abnormalities varies widely in the literature. Reports show that approximately 11-31% of CDH newborns have a chromosomal abnormality [43, 62, 130, 159, 198]. The association of chromosomal anomalies and CDH significantly worsens the prognosis for these fetuses, and therefore warrants screening when the history or ultrasonography indicates high risk. The reported association of CDH and abnormalities in chromosomal number, chromosomal aberrations (deletions, mutations), and presumed patterns of malformations (syndromes, sequences), are listed

in Table 13. The table acts as a comprehensive list of abnormalities reported up to date with CDH; however, no single abnormality has been shown to be predominant in CDH patients.

Associated Abnormalities in Patients with Congenital Diaphragmatic Hernia		
Based on numbers of chromosomes:	**Recognizable patterns of anomalies:**	**Specific chromosomal abnormalities:**
Trisomy 13	Laterality sequence	Fryns syndrome
Trisomy 18	Pierre-Robin sequence	Denys-Drash syndrome
Trisomy 21	Beckwith-Weidemann syndrome	Fraser syndrome
Partial trisomy 5	Brachmann-de Lange syndrome	Blepharophimosis + 3 p-deletion
Partial trisomy 20	Poland anomaly	Del (1)(q32->q42)
Tetraploidy 21	Pentalogy of Cantrell	Translocation 8q22
Tetrasomy 12p	Goltz-Gorlin syndrome	Translocation 1;21
	Apert syndrome	Translocation 8;14 (q24;q21)
	Klippel-Feil syndrome	Stickler syndrome
	CHARGE association	Tuberous sclerosis
	Rubinstein-Taybi syndrome	46, XY, -9+t(5q;9p)
	Caudal regression sequence	Ring chromosome 4, 7q+, del8
	Collodion baby	Balanced 10:x translocation
	Dicephalus dipus dibrachius	46, XY, -9+t(5q;9p)
	Stickler syndrome	46, XY, 7q-(q32)
		47, XX, + marker
		Turner's syndrome

Multiple Congenital Abnormalities:

A. *Central Nervous system:* Absent corpus callosum, anencephaly, craniorachischis, holoprosencephaly, hydrocephalus, iniencephaly, microcephaly, neural tube defect, spina bifida
B. *Cardian Defects:* Ectopis cordis, double outlet right ventricle, interrupted aortic arch, eleven ribs/right aortic arch, hypoplastic left heart, hypoplastic right heart, pulmonary artery stenosis, transposition of the great vessels, tetralogy of fallot, ventricular septal defect
C. *Pulmonary Anomalies:* Pulmonary sequestration
D. Genitourinary Anomalies: Ambiguous genitalia, bilateral cryptochordism, hypospadies, renal agenesis, potter sequences, uterus didelphys
E. *Skeletal Anomalies:* Congenital hip dislocation, limb reduction defects, polydactyl, vertebral anomalies
F. *Gastrointestinal Anomalie:* Duodenal atresia, imperforante anus, Meckel's diverticulum, Omphalocele: ureteral duplication, volvulus of small or large bowel
G. *Craniofacial anomalies:* Cleft lip, cleft palate
H. *Other:* Cystic hygroma

Table 13 [114, 199]

Most studies show a male predominance in the male/female ratio for both familial and sporadic cases of right and left sided CDH ranging from 1.25 to 3.0 [20, 200]. It appears that sex linked factors do not have greater effect, with respect to the etiology of familial causes, than in nonfamilial cases. Familial cases of CDH have been observed and described regularly. The incidence of familial cases of CDH is estimated to be approximately less than or equal to 2% of all forms of CDH [46, 123, 193, 200]. To date, the mode of inheritance in familial cases of CDH remains unknown. There have been suggestions of autosomal recessive inheritance [156], autosomal dominant inheritance [41], and multifactorial inheritance [220]. The multifactorial mode of

inheritance seems possible due to the heterogeneity of the anatomic nature of the defect, the observation of associated anomalies, and the possible role of exogenous factors.
In 4-6% of all developmental defects in humans, drugs and environmental chemicals are considered to be of etiologic significance [216]. In animal models, exposure to different chemicals has been found to induce CDH. Polybrominated diphenyls, thalomide, nitrofen, quinine, and phenmatrazine have been used to induce CDH in embryos of different species [94]. In human subjects reports exist of CDH after maternal use of phenmetrazine early in gestation [160, 220]. Maternal ingestion of thalomide and quinine has also been mentioned as possibly causing diaphragmatic defects in humans [88, 113]. Geographical distribution in population based studies has shown a slightly higher prevalence rate of CDH in rural areas (2.12 rural vs. 1.45 urban) where elevated exposition to herbicides due to agriculture exists [200]. A maternal vitamin A deficient diet has been shown to cause CDH in various strains of rats [10, 207].

4.1.4 Epidemiology and outcome

Estimations of the incidence of congenital diaphragmatic defects range widely from 0.17 to 0.57 of 1000 births [114, 159, 200]. Geographical differences have been reported, which is suggested to be caused by differences in ascertainment of cases [12, 219].
In a population based study in California of CDH from 1983 through 1987 [200], 237 infants of 718,208 births were born with a CDH. The overall prevalence was 3.13 per 10,000 for live births and 3.30 for live births and stillbirths. In regard to anatomic defect, 95.8% of CDHs were posterolateral, 2.1% were Morgagni, and 2.1% were pars sternalis hernias. Approximately 84% of the posterolateral hernias were left-sided, 13% right sided, and 2% bilateral. The male to female ratio for isolated posterolateral hernias was 1.58.
Reports on congenital diaphragmatic hernia show a variation in mortality rate from 8% to 79% [33, 59, 74, 165, 222]. Mortality rates remain high even though new therapy regiments have been introduced [12, 219]. The mortality rates are influenced by what is known as the “hidden mortality” in CDH neonates. The hidden mortality includes all infants who die, in utero or soon after birth, with CDH that are not recognized. A meta-analysis by Skari and a study by Harrison both report a hidden mortality of 34%[74, 179]. The magnitude of the hidden mortality for CDH cannot be accurately determined retrospectively or from birth defects monitoring programs because many cases go unrecognized unless there is an autopsy. However, due to improvements in prenatal diagnostic imaging over the past two decades, it seems valid to assume that such a hidden mortality has decreased over the past few years and that these cases are not as often missed. This would in turn explain the constant high mortality rate of CDH neonates, since an increasing number can be referred to a fetal treatment center for care. This is in a accordance with a publication reporting a higher proportion of very affected infants in recent years [203].
There seems to be a higher mortality rate in right-sided CDH than in left-sided CDH (range of 20-40% higher mortality) depending on study design [12, 65, 77, 179, 202]. There is no clear association between sidedness and presence of associated major malformations, which suggests that other differences between LCDH and RCDH probably exist contributing to the differences in mortality rates.

4.1.5 Morbidity in survivors of CDH

A paucity of data exists regarding the long-term follow-up of patients that have survived with congenital diaphragmatic hernia. Survivors of CDH remain one of the most complex patients to care for throughout their infancy and childhood. The literature that does exist reflects small patient cohorts with usually short follow-up duration. Associated morbidities include developmental delay, poor growth, gastroesophageal reflux disease, hearing loss, musculoskeletal abnormalities, and pulmonary disease.

A common but not well understood morbidity associated with the CDH survivor is the gastroesophageal reflux (GER) [102, 127, 140, 148, 178]. GER may result in failure to thrive, obstructive airway disease, aspiration pneumonia, esophagitis, and stricture formation [139]. Potential factors that may contribute to its development in CDH survivors include embryonic or surgical factors associated with the diaphragmatic repair. In a study by Muratore et al [140] following 121 survivors of CDH, 21% of the patients required an antireflux operation (fundoplicatio) for GER. Of these patients 68% had had a patch repair. Furthermore, 56% of the survivors were below the 25th percentile of growth within the first year of life. Patients who had prolonged intubation and prosthetic material at the gastroesophageal junction fared worse. To meet caloric needs with fluid limitations, high caloric density formulas are used (32-36 kcal/oz). Fluid or volume restriction together with the use of diuretics is used to manage pulmonary volume overload. Contributing to this growth failure is the concept of oral aversion [140]. It can last through infancy and childhood. It is speculated that the prolonged endotracheal intubation might lead to a delay in the development of the swallowing reflex or the sucking mechanism.

Several follow-up studies have shown functional pulmonary abnormalities in survivors of CDH [54, 80, 177]. Persistent reduction of blood flow in the lung ipsilateral to the defect has been a consistent finding. Ventilation on the hernia side has also been shown to be lower than normal; however, perfusion is worse. In a study by Vanamo et al [204] examining 60 survivors of CDH (having a mean age of 29.6), obstructive or restrictive ventilatory impairment was found in 52% of the patients; 25% had both obstructive and restrictive impairment. As expected, ventilation and perfusion of the lung on the side of the defect were reduced, the latter to a greater extent. It is assumed that the gradual expansion of the hypoplastic lung and consequent alveolar overdistension may result in early closure and obstruction of the small airways. Furthermore, the study showed a high prevalence of bronchial hyperreactivity (in 35% of the patients). The etiology is unknown but may be related to the abnormal bronchial development or to gastroesophageal reflux.

In addition to the nutritional and pulmonary sequelae mentioned above, the next most frequently encountered problems are neurological – manifested by developmental delays, abnormal head CT scans, EEGs, and hearing tests [127]. It is difficult to determine if such neurological sequelae are due to hypoxia at birth, the long recovery period, or perhaps treatment with ECMO. In a follow-up study by Lund et al [127] on 33 survivors, extraaxial fluid collections or enlarged ventricles were present on head CTs of 10 children; four children had clinical seizure activity; seven children required hearing aids; and seven others had abnormal results with brain-stem auditory evoked response testing. A variety of other anomalies and problems have been reported, such as bowel obstruction, cryptorchidism, and recurrent diaphragmatic hernia [54, 80, 102, 127, 140, 148, 177, 178, 204].

It is evident from the above morbidities on CDH survivors that surveillance is necessary at least through early childhood to identify high risk patients and provide adequate care.

4.2 Therapeutic modalities I: Pharmacologic therapy

4.2.1 Inhaled nitric oxide: A pulmonary vasodilator

In the past two decades there have been an incredible amount of reports on the responses of term and near-term newborns with acute hypoxemic respiratory failure to inhaled nitric oxide (iNO). Causes of such respiratory failure have been for example sepsis, asphyxia, dry lung syndrome, acute respiratory distress syndrome, pneumonia, meconium aspiration syndrome, or congenital diaphragmatic hernia. In such newborns pulmonary hypertension may develop and cause right- to-left shunting through the ductus arteriosus and/or the foramen ovale. The resulting hypoxia may further increase pulmonary vasoconstriction and thus trigger a vicious cycle. Intravenous vasodilators, such as tolazoline, are associated with severe side effects due to the associated reduction of systemic arterial pressure causing persistent right-to-left shunting [208]. In addition, they can relieve vasoconstriction in poorly ventilated areas aggravating ventilation-perfusion mismatch.

Nitric oxide (NO) induces smooth muscle relaxation of arterial vessel walls and appears to be identical to or an intermediate of endogenous endothelium derived relaxing factor (EDRF) [91, 92]. NO is an ideal local transcellular messenger because of its small size, lipophilic nature, and short duration of action. NO inhaled as a gas at low levels dilates selectively the pulmonary circulation. Significant systemic vasodilation does not occur because NO is inactivated by rapidly binding to hemoglobin [104, 225]. Inhaled NO maximizes ventilation-perfusion matching by preferentially vasodilating vessels in ventilated lung units only. Besides smooth muscle relaxation, NO has many diverse physiological functions such as neurotransmission, immunoregulation, and inhibition of platelet aggregation and adhesion [7, 98, 225]. It may, however, potentiate bleeding in premature newborns, surgical patients, and patients with hemostatic disorders [188]. Nitric oxide is a free radical, which forms peroxynitrite or nitrogen dioxide in the setting of a high oxygen concentration. Peroxynitrite and nitrogen dioxide are associated with lung injury [34]. Although this toxicity is regarded as low due to the rapid inactivation of NO in contact with blood, concentrations of nitrogen dioxide and methemoglobin should be monitored.

Inhaled NO has been shown to reverse hypoxia-induced pulmonary vasoconstriction both in experimental animals and healthy human volunteers without causing systemic hypotension [60, 166]. Although most studies demonstrate that iNO can be effective in the treatment of persistent pulmonary hypertension (PPHN), no therapeutic range has been established. Since the toxic concentration range for infants is not known, a lower limit of effective concentration of iNO is desired. In most studies, iNO therapy has been initiated with a greater concentration, then decremented to a lower dose for maintenance of vasodilatory effects. For example, Kinsella et al [104] reported a dosing regiment of 20 ppm for 4 hours followed by 6 ppm for 20 hours and demonstrated sustained improvement in oxygenation. On the other hand, Karen et al [100] reported that continuous low doses of iNO (6 ppm) resulted in a comparable improvement in oxygenation without a higher dose of iNO at the initiation of therapy. A review of the literature shows an effective range for infants from 1 ppm to 80 ppm, with most reports stating no further benefit was achieved at higher concentrations above 20 ppm [100, 106]; however, there seem to be early and late responders to iNO and each case should

be handled separately [223]. It is assumed that as the vascular endothelium is repaired and endogenous NO production increases, progressively lower doses of inhaled NO are then needed to produce the desired therapeutic response [143]. Furthermore, it has been suggested that higher concentrations (about 80 ppm) of NO diffuse from ventilated to nonventilated areas, resulting in undesirable vasodilation in poorly ventilated areas and thus increase the intrapulmonary shunt [64, 143].
In comparison to the widely accepted use of inhaled nitric oxide in newborns with persisitent pulmonary hypertension (PPHN) due to various causes [167, 212], the use of inhaled NO in the subset of newborns with PPHN due to CDH remains controversial [1, 36, 118, 174]. The most widely referred to study in the literature is from the NINOS group (Neonatal Inhaled Nitric Oxide Study Group) [1], in which the authors conclude that for term and near-term infants with CDH and hypoxemic respiratory failure unresponsive to conventional therapy, inhaled NO does not reduce the need for ECMO or death based on a randomized, double-masked, controlled multicenter study. This is in comparison to other reports of successful treatment of pulmonary hypertension with iNO in newborn infants with CDH whereby ECMO was prevented [84, 104, 105]. Kinsella et al reported that even in late or protracted pulmonary hypertension 10 ppm of iNO delivered by a nasal cannula reduces the duration of mechanical ventilation and safely treats PH in CDH newborns [105]. Karamanoukian et al reported that iNO requires exogenous surfactant therapy in the lamb model of CDH due to the pathophysiology of CDH involving surfactant deficiency as well as pulmonary hypoplasia [99]. Some authors report the parallel use of surfactant in cases of deficiency, but it is not always decipherable in the literature if surfactant was used. Intratracheal instillation of exogenous surfactant has been shown to improve oxygenation and pulmonary function in infants with RDS [96].

4.2.2 Phosphodiesterase inhibitors

Phosphodiesterase inhibitors (PI) have shown pulmonary vasodilatatory effects similar to that of iNO. The concomitant use of iNO and PIs has shown a synergistic vasodilatatory effect in some studies. Furthermore, the use of PIs to wean patients off inhaled nitric oxide has been successful.
Nitric oxide mediates vasodilation by increasing the activity of soluble guanylyl cyclase. Guanylyl cyclase catalyses the formation of cyclic GMP (cGMP), which acts as a second messenger interacting with G-kinases and activating intracellular pathways. cGMP is hydrolyzed and inactivated by cGMP-specific (type 5) phosphodiesterases (PDE5). Inhibitors of PDE5, such as zaprinast and dipyridamole, have been shown to reduce hypoxic vasoconstriction and pulmonary artery pressure in animal models [38, 210]. Nebulized zaprinast administered directly to the lungs of conscious lambs with pulmonary hypertension caused a sustained increase in the net transpulmonary release of cGMP and selectively dilated the pulmonary circulation without systemic side effects [38, 210]. Further animal studies with combined use of PIs and iNO exhibit synergistic vasodilatory effects.
In contrast to the animal studies, the response of humans to phosphodiesterase inhibitors, as well as in combination with iNO, have been less positive. In pediatric patients with severe resting pulmonary hypertension, combined therapy with inhaled nitric oxide (20 ppm) and dipyridamole decreased the PVR index by over 20% in only 50% of the patients, which was not statistically different from nitric oxide therapy alone [227]. In newborns with PPHN and CDH, two studies reported only transiently

improving oxygenation in infants given dipyridamole and iNO with augmentation of response to iNO [103, 197]. It appears that, since phosphodiesterase inhibitors and exogenous nitric oxide both act by increasing cGMP in pulmonary vascular smooth muscle cells, maximal therapeutic effects might be achieved by optimizing the dosing and conditions of each individual case.
The use of dipyridamole to wean from inhaled nitric oxide has shown consistent positive results. A number of studies have shown that PDE5 inhibition attenuates rebound pulmonary hypertension, which can occur following withdrawal of iNO, in patients with PPHN with and without CDH [5, 11, 31]. Rebound pulmonary hypertension is thought to be related to phosphodiesterase activity and diminished cGMP.

4.2.3 Prostaglandins and prostacyclin

Arachidonic acid is metabolized through the cyclo-oxygenase and lipoxygenase pathways to form prostaglandins and leukotrienes, several of which have been reported to exhibit vasoactive effects in the fetal or neonatal pulmonary ciculations [195]. Prostaglandin I2 (prostacyclin) is an important mediator of pulmonary vasodilation. Patients with severe pulmonary hypertension have been reported to exhibit a deficiency in prostacyclin synthase in lung vessels [210]. A number of studies have also shown that prostacyclin plays a role in vasodilation during the normal transition to extrauterine life [2]. Repeated or continuous administration of prostacyclin to preterm infants has shown to improve oxygenation without causing overt side effects, tachyphylaxis, or clinically relevant alterations in systemic arterial pressure [49].

4.2.4 Endothelin antagonists

The endothelins are a family of endothelial cell derived vasoconstrictor peptides, first identified in 1988 [224]. The isopeptide ET-1 binds primarily to the ET-A receptor, which mediates vasoconstriction. The ET-A receptor is present in smooth muscle cells in fetal lung. The ET-B receptor has been identified on endothelial cells in the fetal and newborn lung [226]. Binding of ET to the ET-B receptor mediates vasodilation by mechanisms involving the cGMP-dependent end pathway of nitric oxide mediated vasodilation [95].
Circulating ET-1 levels are high in the fetal and transitional circulations, and are significantly reduced by the fourth day of life [144]. This suggests that ET-1 may play a physiological role in modulating pulmonary vascular tone during fetal to neonatal transition. ET-A receptor antagonism by exogenous agents has been promoted as a therapy for pulmonary hypertension; however, these efforts have been complicated by the complex interaction between the vasoconstrictor and vasodilator effects of ET-A and ET-B receptors, and by the binding kinetics of ET to these receptors under different physiological conditions. The therapeutic utility of exogenous ET-1 or ET-A blockers in pulmonary hypertension in newborns has yet to be established.

4.2.5 Talazoline

Talazoline is a potent nonspecific vasodilator that has been in use for at least two decades to reduce pulmonary vasoconstriction. Its primary action is as a competitive α-adrenergic antagonist. Talazoline acts directly on smooth muscle, and does not need nitric oxide or endothelium to function [45]. Although intravenous talazoline lowers the mean pulmonary arterial pressure and increases the cardiac index in infants with PPHN, its benefits are often complicated by adverse effects, most notably systemic hypotension.

The most promising new approach is the administration of tolazoline directly into the respiratory system. Animal studies and clinical reports on endotracheal talazoline use on infants with PPHN have shown promising results [44, 155]. However, the specifics of delivery and concentration still need to be determined.

4.2.6 Antenatal Corticosteroid Therapy

Corticosteroids have an effect on both epithelial and mesenchymal cells, affecting both structural development and cell differentiation [15, 17]. Corticosteroids mature fetal lung parenchymal structure in laboratory studies [15], and investigations have demonstrated that a single course of antenatal corticosteroids, given to fetal primates early in gestation at the midcanalicular phase of lung development, accelerates epithelial and interstitial lung maturation when evaluated anatomically 40 days after treatment [29]. In addition, there is an increase in fetal lung compliance and maximum lung volume, as well as an improved ventilatory efficiency index independent of surfactant.

Corticosteroids induce the synthesis of all known components of surfactant [15] and increase the percentage of saturated phosphatidylcholine. This effect is achieved in part by the stimulation of several key enzymes of phosholipid synthesis including fatty acid synthetase, choline phosphate cytidylyltransferase, and lysophosphatidylcholine acyl coenzyme A acyltransferase. The development of type II cells, and both tissue and alveolar concentrations of surfactant are increased. In addition, surfactant-associated proteins A, B, C, and D are increased [16, 52].

Other effects of corticosteroids include increased activity of antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and catalase [58]; and a decrease in fetal lung capillary protein leak with an increase in lung liquid clearance [93]. Endogenous corticosteroids seem to play a significant role in late gestation fetal lung maturation. Circulating unbound cortisol and corticoid conjugates increase during late gestation, in parallel with changes in the lecithin-sphingomyelin ratio, and at a time when the lung acquires differentiated functions [132].

Synthetic corticosteroids, such as betamethasone and dexamethasone, have been studied extensively in human trials because they are not bound by corticoid binding globulin, cross the placenta without being inactivated, and have a greater affinity for steroid receptors than cortisol [15, 132]. Antenatal glucocorticoid therapy causes a transient feedback inhibition to ACTH, with a transient suppression of ACTH, cortisol, dehydroepianderosterone, hydroxyprogesterone, estrogens, and growth hormone. The effects on the maternal adrenal axis rebound within 48 hours of a single course of therapy, whereas the fetal adrenal axis rebounds within 6 days [14, 17]. Those infants exposed to a single course of antenatal corticosteroids remain responsive to stress after birth with an increase in endogenous cortisol and maintain responsiveness to exogenous ACTH [14, 17].

The first reported human trial of antenatal corticosteroids was in 1972 [120]. Both the incidence of respiratory distress syndrome (RDS) and overall mortality were reduced in infants born at less than 32 weeks gestation treated with betamethasone for at least 24 hours before delivery. A meta-analysis of published studies up to 1995 on the use of single course of antenatal corticosteroids demonstrated consistent evidence of beneficial effects on neonatal outcome for those infants born at less than 34 weeks gestation [42]. There was a 50% reduction in the overall incidence of RDS in the treated groups, with an effect persistent for born infants of 24 hours to 7 days after treatment. There was also a reduction in mortality (40%) and intraventricular hemorrhage (62%) among these infants. The antenatal use of corticosteroids has shown to reduce the need for surfactant [101], and multicenter trials of exogenous surfactant therapy have shown an augmentation of the surfactant effect on neonatal respiratory outcome by antenatal steroid treatment [71].
Several follow-up studies of infants with antenatal steroid exposure have been performed [42, 51]. Despite the initial concern that long term neurodevelopmental sequelae may develop due to delays in nerve myelination, there has been no evidence of a negative effect on physical growth and development after antenatal steroid therapy.
There are up to date no extensive studies of antenatal hormone therapy for human fetuses with congenital diaphragmatic hernia. Antenatal hormonal treatment for lung maturity has been studied in animal models of CDH. Antenatal dexamethasone therapy in experimentally induced CDH in the rat model has led to accelerated surfactant synthesis and release [189], increased lung compliance [173], improved morphogenesis, and prevention of pulmonary vascular adventitial and medial wall thickening [151, 192]. There are also associated increases in surface area, airspace volume fraction, and saccule size and volume with a decrease in saccular septal thickness in lung tissue. Antenatal steroid therapy has similarly been shown to suppress pulmonary endothelial angiotensin-converting enzyme activity to levels equivalent to those seen in normal lungs, potentially reducing the predisposition to pulmonary hypertension [152]. A study of ovine fetuses has suggested that the improved pulmonary vascular resistance at birth following steroid administration is mediated by increased nitric oxide synthase activity, but that the timing and duration of steroid administration appear to be critical to this response [70]. In a study of human archival lung tissue from CDH patients an increased mRNA expression of glucocorticoid receptor was found, suggesting that the hypoplastic lung is more sensitive to glucocorticoids than the normal lung [181].

4.3 Therapeutic modalities II: Ventilation strategies

In order to minimize barotrauma or ventilator-inflicted injury and preserve the limited number of alveoli there has been a trend towards less aggressive hyperventilation usually associated with conventional mechanical ventilation (CMV). Studies have shown that this goal can be achieved by e.g. early institution of ECMO, adoption of permissive hypercapnia, institution of high frequency oscillatory ventilation (HFOV) at low pressures, or introduction of new forms of ventilation such as intratracheal pulmonary ventilation (liquid ventilation). There exists no clear cut evidence that any of these modalities offer a significant survival advantage over conventional ventilation alone; however, autopsies performed on nonsurvivors show significant barotrauma on patients after CMV [12, 219].
Recent studies have shown a significant increase in survival of CDH newborns with concomitant decrease in morbidity by the application of gentle ventilation and

permissive hypercapnia [13, 23]. Barotrauma is minimized by largely ignoring the right-to-left shunt. Respiratory support before surgery in such a setting has peak inspiratory pressure (PIP) limited to 30 cm H_20, PEEP to 5 cm H_20, and mean airway pressure limited to 12 cm H_20. Ventilator adjustments are based on maintaining a preductal 0_2 saturation of 90% or greater. Postductal lines are usually not placed and postductal saturations are ignored.
High-frequency oscillatory ventilation used in preoperative stabilisation has also shown to provide correction of hypoxemia, acidosis, and pulmonary hypertension before surgery [182]. It has further been used during surgical correction of CDH, allowing continuity in perioperative management and providing a stable thoracic field [26]. HFOV minimizes barotrauma due to much smaller alveloar pressure and volume variations. Reduction in PCO_2 and alkalosis can be obtained with HFOV in neonates with CDH despite severe pulmonary hypoplasia [50].

4.3.1 Partial liquid ventilation

Partial liquid ventilation (PLV), also known as perfluorocarbon-associated gas exchange, has been in development since the 1960s [35]. It has since then been demonstrated to be effective in experimental models, in mature and immature animals, as well as in term and preterm neonates with various pulmonary pathologies including CDH [161, 214]. However, its role in the management of humans with acute respiratory failure is still not established. Perfluorocarbon is instilled into the lungs to create a liquid functional residual capacity. Ventilation is then carried out using a conventional ventilator that delivers gas tidal volume into the perfluorocarbon-filled lungs. In the total liquid ventilation strategy, the complete substitution of the alveolar gas by perfluorocarbon is performed. Both liquid ventilation strategies, total and partial, are able not only to maintain gas exchange, but also reduce inflammatory changes. While total liquid ventilation remains an experimental technique, partial liquid ventilation could be readily applied, but its implementation still awaits the results from ongoing and future clinical trials [205].

4.4 Therapeutic modalities III: Extracorporeal membrane oxygenation

Extracorporeal membrane oxygenation (ECMO) was first reported by Bartlett in 1976 [18] to increase survival in infants with hypoxemic respiratory failure, since then the use of this therapeutic modality has increased dramatically. ECMO is usually a last line therapy for infants refractive to other forms of medical intervention or directly given to high risk infants in order to improve oxygenation, reduce pulmonary hypertension, and minimize barotrauma that often accompanies conventional ventilation. It involves the use of prolonged extracorporeal circulation to provide pump support for the dysfunctional heart and oxygenation for the failing lungs. The current technique of ECMO includes vascular access via venoarterial means for cardiorespiratory collapse or venovenous means for peripheral collapse, heparin titration based upon whole blood activated clotting time, and lung rest (at low ventilator settings) [183].
ECMO eligibility has changed over the years and varies among institutions; however, main criteria usually include: weight of newborn over 2 kg, gestational age $\geq$ 34 weeks, $PaO_2 < 40$ for 2 hours, pH < 7.25 for 2 hours, oxygen index > 40, no uncorrectable cardiac anomalies, no lethal congenital anomalies. Total annual neonatal ECMO cases

due to respiratory failure have decreased over the past decade from a peak of 1510 cases in 1992 to 786 cases in 1998 [153]. New treatment modalities such as iNO, HFOV, surfactant administration, liquid ventilation, etc., have resulted in fewer patients requiring ECMO [86]. This has led to an increase of sicker infants being put on ECMO, making statistical analysis of survival difficult. Several studies have suggested improved survival rates can be achieved using ECMO in neonates with CDH as an attempt to reverse the cycle of persistent pulmonary hypertension and barotrauma [68, 86, 150]. However, other studies have not shown ECMO to be beneficial in improving survival for neonates with CDH [171, 184]. One explanation for the discrepancies on mortality between studies may be caused by an insufficient number of patients to provide statistical power to prove the null hypothesis. In a statistical analysis of data from the multicenter CDH registry from January 1995 to November 1997, it could be shown that ECMO significantly improves survival rate only for those CDH neonates with a predictive mortality risk $\geq 80\%$ [68].

According to the registered outcomes of 7,647 infants receiving ECMO due to severe respiratory failure up to the end of 1992 reported by the Extracorporal Life Support Organization (started in April 1976), the overall survival rate of infants is 81% [221]. Survival is highly influenced by the underlying diagnosis, with meconium aspiration syndrome faring best with 93% survival, and CDH as worst with 59% survival. Furthermore, 17% of infants experience a cranial infarct or hemorrhage, and of these 57% survive. As of July 2000 the overall survival rate for 15,525 newborns entered into the same registry slightly decreased to 78%. In a recent report on data from this registry (up to June 2001), it was shown that since the late 1980s, survival of infants with CDH requiring ECMO decreased from 63% to 52% [187]. The decreased survival rate was associated with increased rates of prenatal diagnosis, early-term delivery, lower birth weight, longer ECMO runs, and more frequent complications on ECMO. Although most authors assume that improvement in prenatal diagnosis, acute medical support, and transport have allowed sicker infants to reach referral centers and be included in survivor data, thereby offsetting potential improvements from ECMO; it was shown in this report that pre-ECMO respiratory severity had not changed significantly over the different time periods (1976 to 2001) and could not readily explain the decreased survival rates observed.

It should lastly be noted that a number of ECMO treated CDH patients have significant problems seen on late follow-up. These include the presence of chronic lung disease, neurodevelopmental sequelae, and gastroesophageal reflux (GER) [21, 48, 69]. All in all, however, the overall survival rate of approximately 80% for infants with respiratory failure and that of 52% for CDH infants is still quite impressive when one considers how gravely ill these newborns are.

4.5 Therapeutic modalities IV: Surgical procedures

4.5.1 Fetal surgery

The advances in prenatal screening and diagnosis, combined with an understanding of the pathophysiology of congenital anomalies, have brought incredible impetus to the field of fetal surgery. Since the fundamental problem in newborns with CDH is the pulmonary hypoplasia, a number of strategies have been used in an attempt to improve the growth of hypoplastic lungs before they are needed for gas exchange at birth. Initially open fetal (in-utero) repair of CDH proved technically difficult when liver

herniation was present because of the compression of distorted ductus venosus and reduced umbilical venous flow during or after liver reduction from the fetal chest to the abdomen [107]. Although the procedure was feasible in fetuses without liver herniation, a prospective clinical trial comparing fetal CDH repair to postnatal therapy showed no difference in survival: 75% in the fetal surgery group and 86% in the postnatal therapy group survived [75]. The authors concluded that *in utero* repair does not improve survival over standard postnatal treatment in the subgroup of CDH fetuses without liver herniation, primarily because survival in this subgroup is favorable with or without prenatal intervention.
This experience led to the development of a new approach: temporary fetal tracheal occlusion. It has been shown in various animal models that increased fetal lung fluid egress results in pulmonary hypoplasia, whereas decreased fetal lung fluid egress results in large fluid filled lungs [8, 55, 83, 147, 215]. Prenatal tracheal occlusion was shown in the lamb model of CDH to induce lung growth with the reduction of herniated viscera and dramatic improvement in lung compliance and gas exchange. There are presently two approaches to clip the fetal trachea: open fetal surgery and the videofetoscopic technique (Fetendo Clip). Methods have included the use of external metal clips placed on the trachea by means of open hysterotomy, fetoscopic neck dissection, or internal tracheal occlusion with a detachable silicone balloon placed with the use of fetal bronchoscopy through a single 5-mm uterine port [107]. Problems with the Fetendo Clip technique include long operative time, potential laryngeal nerve damage during the dissection, and amniotic fluid leakage. The potential risks of tracheal occlusion include the development of fetal hydrops, and the induction of surfactant deficiency related to decreased type II pneumocytes. In a study by Harrison [79] comparing fetal endoscopic tracheal occlusion to standard postnatal care in high risk fetuses, tracheal occlusion (at 23 to 27 weeks of gestation) did not improve survival or morbidity rates in the cohort of fetuses with CDH. Furthermore, the fetuses that underwent tracheal occlusion were born at an average of 31 weeks of gestational age as a consequence of the intervention in comparison to 37 weeks in infants with standard postnatal care. The optimal timing and duration of occlusion in humans is still not known. Further randomized studies are in process to answer these questions as well as study the use of smaller (2-mm) fetoscopes to prevent preterm labor and delivery. Occlusion earlier in gestation, prior to the pseudoglandular stage of lung development, may lead to more reliable lung growth and there is some evidence that *in utero* reversal of tracheal occlusion gives even better results [215].

4.5.2 Current postnatal surgical management of CDH

Repair of congenital diaphragmatic hernia has changed from an emergent procedure to a delayed procedure in the last decade. The concept of delayed surgery is based on the assumption that pulmonary hypoplasia and not the anatomical defect is primarily responsible for the prognosis of the patient. Some reports suggest that the cardiopulmonary instability of the newborn with CDH is exacerbated by early surgical intervention, and that delayed intervention allows for medical stabilisation before surgery [32, 66, 213]. During the stabilisation period, attempts are made to improve the cardiopulmonary function and maximize oxygen saturation by e.g. HFOV, induced alkalosis, pharmacologic vasodilator therapy, or even ECMO.
However, the benefit of delayed surgery in improving the survival rate of neonates with CDH has been controversial [40, 97, 218]. The premise that delayed repair will

improve pulmonary compliance changes remains unconfirmed. Most studies show a similar mortality rate in early and delayed intervention, although delayed therapy does allow for a stratification of patients for early ECMO or surgical repair, depending on the clinical course [218]. A further advantage of delayed repair may be the avoidance of intraoperative deterioration and iatrogenic pulmonary injury associated with early repair. Although delayed repair of congenital diaphragmatic hernia is still controversial it is at the present time the standard therapy modality in most institutions.
Further controversies in the surgical management of CDH include the use of chest tubes, use of an abdominal versus thoracic approach, and the need for associated procedures. A review of the registry of the CDH Study Group on all live-born infants with CDH during 1995 and 1996 gave a snapshot of current management [37]. Sixty-two centers participated (centers in Europe, North America, and Australia), with 461 patients entered. Overall survival was 63%. The defect was left-sided in 78%, right-sided in 21%, and bilateral in 1%. A subcostal approach was used in 91% of patients; pleural drainage was used in 76%. A patch of some kind was used in just over half (51%) of the patients, with polytetrafluoroethylene being the most commonly used material (81%) in those patients with a patch. Patients with a patch repair had a higher mortality rate than patients repaired primarily (44% vs. 7%).
Synthetic repair of large congenital diaphragmatic defects (>90%) may lead to recurrence, progressive chest wall deformity, and restrictive pulmonary disease. Staged reconstruction with patch closure followed by definitive closure with living, growing tissue such as a reverse latissimus dorsi flap has been shown to be a safe and effective treatment option for patients with large defects [116].

4.6 The rat model of CDH

Due to moral and ethical reasons, as well as the low incidence of CDH requiring large multicenter population pools, human study on experimental strategies for CDH is not feasible. In order to study the pathophysiology and the effectiveness of different therapeutic modalities, various animal models have been developed.
Studies of CDH have been carried out on sheep, rabbits, swine, and mice. The lamb model of CDH has been crucial to our understanding of the pathophysiology of this complex disease. However, most sheep studies have induced a diaphragmatic hernia in a relatively late stage of development. The studies involve rather small numbers, high costs, and the need for surgical intervention to obtain the CDH.
An ideal animal model allows for high reproduction of the desired disease or anomaly, as well as a pathophysiology that corresponds to humans in order to apply the results to the human situation. Furthermore, the animal in the study must be easy to care, easy to breed, with a short gestation period and large litter.
In this experimental study we chose the nitrofen induced CDH rat model due to its proven efficacy as an ideal animal model for this specific malformation. It is generally accepted that a diaphragmatic hernia in humans is the result of a defective development in the diaphragm during the first trimester of gestation leading to abnormal lung development. It has been difficult to study the sequence of embryologic events leading up to the abnormal lung development in an animal model; however, the nitrofen induced CDH rat model has allowed extensive study of morphologic changes during embryogenesis specific for CDH [109-112]. This rat model has been extensively studied and established by Nakao [145, 146], Iritani [94], Kluth [108-112], and Tenbrinck [194].

It has been determined that nitrofen given on day 9 of gestation induces a left-sided hernia. If nitrofen is given on day 10 or later only right-sided hernias are observed. The dosage necessary varies upon author; however, our laboratories have had consistent results with a single dose of 100 mg of nitrofen. In such a manner we produced right-sided hernias in 81% of the offspring in this study with various degrees of severity. Although nitrofen given on day 9 can produce a smaller amount of left-sided hernia similar in location to that of humans, it has been shown the localization of the hernia is of less importance to the interpretation of results in this animal model [109]. Furthermore, nitrofen given earlier in gestation can have a higher teratogenic effect on other organs. We, therefore, gave nitrofen on day 11.5 of gestation and produced right-sided hernias. This high turnover as well as the ease of care and effortlessness in obtaining lung histology has allowed a multitude of data to be gained efficiently in the nitrofen induced CDH rat model.

4.7 Nitrofen as a teratogen

It has been known since 1971 by studies conducted by Ambrose, et al, that the diphenyl ether herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) is a potent teratogen after maternal ingestion [9]. Nitrofen is the most potent teratogen of its class, causing a multitude of fetal defects in many organ systems, while causing little or no change in prenatal and maternal morbidity and mortality [57]. Nitrofen affects the pituitary-hypothalamic-thyroid axis of the fetus due to its similar structure to thyroxine [39, 67]. Thyroid transcription factor 1 has been shown to be decreased in nitrofen exposed pups [124].

Various studies have shown that in the rat model tissues affected include lung, heart, diaphragm, kidney, liver, brain, thyroid, skeleton, and harderian gland; however, only pulmonary and cardiac dysfunction appear to affect short term neonatal survival [115]. In a study examining rats given nitrofen on days 10-13 of gestation (po 20 or 40 mg/kg daily), it was shown that the newborns have lower pulmonary contents of DNA, RNA, and protein [115]. Adrenal catecholemines, which play an important role in surfactant production and fluid resorption in the lung during the transition to air-breathing, were markedly reduced; and red blood cell concentration was significantly diminished. In a further study examining pulmonary growth after nitrofen ingestion, nitrofen was given on the 8^{th} day of gestation, lungs were then removed on the 12^{th} embryonic day and put in culture [117]. In such a manner the authors could evaluate lung growth and development before diaphragm closure and herniation. Nitrofen-exposed lungs had 30% fewer total terminal branches than age-matched controls, and the mRNA expression of proliferative and developmental markers was decreased. This suggests, that nitrofen exposed early embryonic lungs are both hypoplastic and developmentally immature even before the presence of an actual diaphragmatic hernia.

Although many studies report multiorgan pathologies in litter after nitrogen ingestion, it must be noted that nitrofen has usually been given at a much earlier embryonic stage. In order to limit further pathologic changes to other organ systems from nitrofen ingestion, we gave 100 mg of nitrofen on day 11.5 of gestation. Our institution has had consistent results with this dosage and timing with little toxicity to other organs.

Although the exact mechanism of nitrofen teratogenicity is not known, it must be assumed in the rat model that nitrofen causes a certain degree of pulmonary hypoplasia, which is secondarily aggravated by the presence of the diaphragmatic hernia. Whereas in the human disease, the diaphragmatic hernia is seen as the primary cause leading to

the lung hypoplasia. As to what magnitude this discrepancy (or nitrofen toxicity) the correlation of our data to the human disease has - remains to be seen.

4.8 Discussion of study results

A total of 491 offspring from 32 dams were studied in this project. We had a high turn over with 81% of newborn rats having a right-sided hernia. We selected 377 right-lung specimens of the 491 newborn rats (or 77%) for histological evaluation. The nitrofen induced CDH rat model allowed an efficient data collection for the study of lung maturation and survival analysis.

Initial review of the data using bar graphs and survival curves was first carried out to confirm expected tendencies. There is a gradual increase in survival time as the histology score improves, as well as when the hernia size decreases. As one would expect the rats survive longer when the lung histology is better developed (score of 1) and the hernia size is small or absent (hernia Ø). Newborns with a histology score of 1-2 survive considerably longer than those with a histology score of 3-5 irrespective of treatment. Furthermore, newborns with hernias of size 3 and 4 have considerably worse survival times than those with no or smaller hernias (chart 6). Further evaluation showed that this effect of hernia size on survival is so severe that further assessment of results is not possible (p value too high). Only after exclusion of newborns with hernias of size 3-4 can we further evaluate the results. In other words, the experimental evidence shows that the newborns can be divided into a "good" prognosis group (newborns with hernias Ø, 1 and 2) that respond to ante- and postnatal treatment, and a "poor" prognosis group (newborns with hernias 3 and 4) that do not benefit from such care. The hernia size seems to be a good prognostic indicator in our study correlating well to liver herniation and right lung area to head circumference ratio applied to the human newborn (section 4.1.2) indicating poor prognosis when large. The fact that some newborns irrespective of treatment will not survive due to the large hernia or high degree of lung hypoplasia has been the motivating reason for antenatal therapy study. The further discussion of results excludes hernias of size 3 and 4.

The hypotheses going into this study were that antenatal cortisone (as in group III) will help lung maturation producing a better histology score; that inhaled nitric oxide (as in group II) produces better aeration of lung alveoles thereby improving the survival time; and that the combination of these therapies improves both factors. Assessment and comparison of groups II and III shows that their beneficial effect in comparison to the control group (I) is similar. Box plots (chart 3) of the histology score mean for their respective litters show a similar distribution of data, and the mean histology score is almost identical at 2.16 and 2.15, respectively. Although there was an evident improvement in the lung development in both groups compared to the control, it was not statistically significant in either group. Survival analysis also gives similar results with a mean of 639 and 585, respectively; survival probability using Kaplan Meier of roughly 80% for both groups, and an almost equal tendency of significance (table 11) in comparison to the control group ($p=0.073$, $p=0.075$) on survival. Unfortunately, the expected specific improvement in survival for group II newborns, and that for histology score for group III newborns could not be proven. Both therapies showed an equal effect with a moderate improvement in lung maturation and length of survival.

On the other hand, the combined effect of dexamethasone prenatally and nitric oxide postnatally, as in group IV, had a significant effect in comparison to the control group and often to groups II and III in both survival and lung maturation. The histology score

mean of 1.57 is significantly lower in comparison to all groups with little variation of data as shown on the box plot (chart 3). This implies that this combined therapy had the most effect on improving lung morphogenesis and prevention of vascular wall thickening that leads to pulmonary hypertension. This combined effect was significant not only when comparing to the control group, but also to groups II-III, meaning that an amplification of expected result was achieved. It has been mentioned that the improved vascular resistance at birth following steroid administration may be mediated by increased nitric oxide synthetase activity [70]. Based on our results it seems plausible to assume that the antenatal dexamethasone matures fetal lung parenchymal structure and enzyme function to a certain degree, allowing inhaled nitric oxide to enter a larger volume of functional alveolar space in order to selectively dilate the pulmonary circulation. The immature lung parenchyme may also be the reason that NO has been less effective in persistent pulmonary hypertension due to CDH than in other types of PPHN [1, 106].

Survival analysis of group IV shows a significant improvement in survival time not only in comparison to the control but also to group II. This significance ($p=0.031$, table 11) compared to group II perhaps implies that iNO alone has a lesser effect than dexamethasone alone (group III), since the combined therapy was not significantly better ($p=0.182$) than the dexamethasone therapy alone. As mentioned, it seems that the improvement in antenatal lung differentiation is a prerequisite for further postnatal therapy. However, it is again evident from the Kaplan Meier survival curve as well as the survival time mean of 709 minutes for group IV, that the combined therapy significantly improves survival and amplifies the effects of both therapies.

The multiorgan teratogenic effects of nitrofen have been mentioned in section 4.7. Although the nitrofen induced CDH rat model is an established model that has provided consistent results in our department, it does cause direct degenerative changes to lung structure. In order to assess the magnitude of these changes, newborns with no hernia were also evaluated. Since there is no hernia to cause mechanical compression of lung tissue from abdominal content, lung pathology must be directly associated with nitrofen toxicity. One would assume that since the newborns do not have a hernia, then the survival time mean - irrespective of treatment - would be in the range of the maximum 720 minutes. However, the survival time mean of the 17 newborns in group I with no hernia resulted in a mean of 488 minutes. The Kaplan Meier survival curve showed only a 65% probability of survival for the control group, and the histology score mean was 3.00. These results suggest a considerable effect of nitrofen on both lung maturation and survival. However, further evaluation of the therapy groups II-IV shows drastic improvement in both lung histology and survival, as shown in the survival curve (chart 9) depicting an almost 100% survival probability for groups II-IV. In this subgroup (only Ø hernias), group II has a significant effect on survival time (table 11), group III has a significant effect on histology score (table 8), and the combined therapy of group IV improves both parameters. Nitrofen definitely has a detrimental effect on lung maturation thereby affecting survival and of course our study results; however, the changes seem to be minor and very responsive to our therapy regiments.

5 CONCLUSION

Congenital diaphragmatic hernia is one of the most common causes of neonatal morbidity and mortality. Despite intensive clinical and experimental efforts, mortality from CDH remains high. CDH results in various degrees of pulmonary hypoplasia and

severe persistent pulmonary hypertension of the newborn. The clinical spectrum ranges from minimally affected infants who do well with modern neonatal care to severely affected infants who die despite all interventions. Perhaps these newborns would benefit from antenatal treatment directed at altering lung growth early in utero to minimize pulmonary hypoplasia. The use of combination therapies (antenatal and postnatal) will likely prove beneficial in these infants. The goal of antenatal and postnatal therapy is to first stabilize the newborn in order to perform a repair of the hernia, and secondly prevent a postoperative deterioration of cardiopulmonary function.

In this study it was shown that the combined therapy of antenatal dexamethasone and postnatal nitric oxide was successful in improving lung maturation and length of survival for a subgroup (large hernias excluded) of newborn rat litter. This combined therapy in the nitrofen induced CDH rat resulted in improved pulmonary compliance, narrowed septal walls, increased air saccule size, and thinning of the pulmonary interstitium. The greatest benefit of NO may be as adjunctive short term therapy in the preoperative stabilisation and delayed repair of infants with CDH to allow improvement of respiratory mechanics and reduction of operative risk. However, this benefit can only be achieved when a certin degree of lung maturation is present (perhaps histologically equivalent to our histology score of 3). The fact that severely affected pups were refractory to therapy regiments, similar to human clinical data, further underscores the need for antenatal therapy study. The severe lung hypoplasia must be addressed before birth, so that postnatal therapy can be successful. Early and reliable assessment of prognosis for fetuses with CDH at risk of death will become increasingly important in the identification of fetuses most likely to benefit from antenatal therapies and may eventually lead to a decrease in the mortality in this population. The challenge for the future is to continue development of therapeutic approaches in order to improve survival of neonates with CDH.

6 REFERENCES

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