IMPAIRED SURFACTANT PROTEIN B SYNTHESIS IN INFANTS WITH
CONGENITAL DIAPHRAGMATIC HERNIA

P.E. Cogo¹, M. Simonato², O. Danhaive³⁴, G. Verlato², G. Cobellis⁵, F. Savignoni⁴, D. Peca⁴, A. Baritussio⁶, V.P. Carnielli⁵

¹ Department of Medical Cardiac & Cardiac Surgical Pediatrics, Bambino Gesù Children's Hospital, Rome, Italy
² Department of Pediatrics, Medical Center & University of Padua, Padua, Italy
³ Department of Pediatrics, University of California San Francisco, San Francisco, CA
⁴ Department of Medical and Surgical Neonatology, Bambino Gesù Children's Hospital, Rome, Italy.
⁵ Neonatal Division & Pediatric Surgery Division, Institute of Maternal-Infantile Sciences, Polytechnic University of Marche and University of Ancona, Ancona, Italy
⁶ Department of Medical and Surgical Sciences, University of Padua, Padua Hospital, Padua, Italy.

Corresponding author
Paola E Cogo, Terapia Intensiva Cardiochirurgica, Ospedale pediatrico Bambino Gesù, Roma
e-mail paola.cogo@opbg.net Voice: ++39 (049) 821 1477 Fax: ++39 (049) 821 3503

The authors have no conflict of interest to report

Running head: SP-B and DSPC turnover in congenital diaphragmatic hernia

Word count for the body of the manuscript: 2517

Copyright 2012 by the European Respiratory Society.
Abstract

Pulmonary hypoplasia and hypertension account for significant morbidity and mortality in neonates with congenital diaphragmatic hernia (CDH). Whether CDH is associated with surfactant dysfunction remains controversial. Therefore, we measure disaturated phosphatidylcholine and surfactant protein B concentration in tracheal aspirates and their synthesis rate in infants with CDH compared to infants without lung disease.

$^2$H$_2$O as precursor of disaturated-phosphatidylcholine and $^{13}$C-leucine as precursor of surfactant protein B were administered to 13 infants with CDH and 8 GA-matched controls. Disaturated-phosphatidylcholine and surfactant protein B were isolated from tracheal aspirates, and their fractional synthesis rate was derived from $^2$H and $^{13}$C enrichment curves obtained by mass spectrometry. Disaturated-phosphatidylcholine and surfactant protein B amounts in tracheal aspirates were also measured.

In infants with CDH, surfactant protein B fractional synthesis rate and amount were 62±27 % and 57±22 % lower than the value found in infants without lung disease (p<0.01 and p<0.05). There were no significant group differences in disaturated phosphatidylcholine fractional synthesis rate and amount.

Infants with CDH have a lower rate of synthesis of SP-B and less SP-B in tracheal aspirates. In these infants, partial SP-B deficiency could contribute to the severity of respiratory failure and its correction might represent a therapeutic goal.

Key words: isotope labeling, metabolism, pulmonary surfactants.
Introduction

Congenital diaphragmatic hernia (CDH) is a developmental abnormality that affects one in 2,500–5,000 live births, with mortality reaching 30-60% [1-2]. Low lung compliance and global lung immaturity are considered important causes of mortality [3]. Surfactant deficiency could also play a role in this disease, but the issue is undecided. Lack of surfactant phospholipids and proteins has been found in animal models of CDH [4-7]. In humans, on the other side, some found no change in amniotic fluid or lung lavage fluid phospholipids [8], while others found decreased levels of Surfactant Protein (SP) A [9]. Recently an autopsy study comparing human fetuses with CDH with age-matched fetuses with no pulmonary diseases found no difference in lung disaturated phosphatidylcholine (DSPC) content and SP expression [10], suggesting that CDH does not impair surfactant storage and that the surfactant yield might be appropriate for lung size. However DSPC content was measured from the whole lung, while the airway content of surfactant components was not studied. Moreover the study gave a still image of the surfactant life cycle and the small number of fetuses in the study did not allow correlating the degree of pulmonary hypoplasia with surfactant status. At present no data are available on the turnover of SP-B in humans with CDH.

Clarifying this question could help to decide whether infants with CDH benefit from surfactant supplementation since, when surfactant was administered to a large series of infants with CDH, an increased need of extracorporeal membrane oxygenation was found in addition to increased chronic lung disease and increased mortality [11], although the majority of such infants were treated with a surfactant preparation containing a relatively low amount of SP-B. This notwithstanding, exogenous surfactant is frequently administered to CDH infants in several nurseries [12].
The aim of the present study was to assess surfactant DSPC and SP-B concentrations in the airway fluid recovered from tracheal aspirates in newborns with severe CDH versus matched controls and to measure the synthesis rate of these critical surfactant components using a dual stable isotope approach.
Material and Methods

Patient population. Thirteen CDH newborns and eight weight- and gestational age-matched controls were studied from 2006 to 2009 with institutional ethical committees approval. All CDH infants had respiratory failure requiring endotracheal intubation: 5 were admitted to Bambino Gesù Hospital NICU, 5 to the University of Padova NICU and 3 to the Polytechnic University of Marche NICU. Nine newborns were enrolled as controls at the neonatal or pediatric intensive care unit of the University of Padova, Italy.

Controls were full term newborns with 1) no history of lung disease, 2) normal chest radiograph, 3) FiO$_2$ less than 30% before and at any time during the study period. They were on mechanical ventilation for abdominal or airways malformations or neurological impairment. Inclusion criteria for both groups were: 1) presence of arterial (right radial in CDH) and venous lines for clinical monitoring, 2) no evidence of infection, 3) no exogenous surfactant administered, 4) written informed parental consent. Exclusion criteria were: presence of liver failure, defined as transaminases (GOT, GPT) > 3 times normal values, and renal failure (creatinine > 2 times normal value).

Protocol: all CDH infants were treated according to shared guidelines among the three centers. All infants were sedated and ventilated by high frequency oscillatory ventilation (HFOV) since birth and subsequently weaned to conventional ventilation if mean airway pressure (MAP) was less than 10 cmH$_2$O and FiO$_2$ less than 40%. Time of surgery was postponed until hemodynamic stability was achieved. Controls were on conventional ventilation. Ventilator parameters and gas exchange were recorded every 6 hours and PaO$_2$/FiO$_2$-ratio, Oxygenation Index (OI), and Alveolar-arterial O$_2$-gradient (AaDO$_2$) were calculated according to standard formula.
Methods and study design:

All patients received a constant intravenous infusion of 2 mg/kg/h of 1-^{13}C Leucine (Cambridge Isotope Laboratories, Andover, MA) dissolved in saline for 24 h. ^2H_2O (Cambridge Isotope Laboratories, Andover, MA) was administered as 1 ml/Kg bolus at study onset, then every 12 h for a 48 h duration, as intermittent boluses corresponding to 0.0625% of total fluid intake, in order to maintain a steady state of deuterium enrichment in body water [13].

Blood (0.6 ml), urine (1 ml) and tracheal aspirates [14] were collected before the start of the isotope infusion, every 6 h until 72 h and then every 12 h until extubation. Tracheal aspirates and blood samples were centrifuged at 400g and 1300g respectively and supernatants were stored at -80°C.

Analysis of tracheal aspirates

Phospholipids phosphorous was measured in tracheal aspirate samples for 48 h [15] from the start of the study, DSPC was extracted and separated from tracheal aspirates as reported [16], and DSPC fatty acids were derivatized as methyl-esters and measured by gas chromatography [14].

SP-B was measured by ELISA on unfractionated tracheal aspirates obtained at the beginning of the study or within 24 h [17] using a rabbit antiserum directed against mature SP-B [18]. DSPC and SP-B concentrations were expressed as percentage of total phospholipids and as concentrations in epithelial lining fluid (ELF) [22].

Isolation of SP-B from tracheal aspirates

SP-B was isolated from tracheal aspirates and hydrolyzed as previously published [19], individual amino acids were derivatized into their N-acetyl-n-propyl derivatives [20] and the ^{13}C enrichment of leucine was measured by mass spectrometry (GC-MS Agilent, Milan, Italy).
Plasma amino acids were derivatized according to Husek [21] and leucine enrichment was measured by gas chromatography mass spectrometry. Results were expressed as Mole Percent Excess.

The $^2$H enrichment of palmitate-DSPC was analyzed by gas chromatography-Isotope Ratio-Mass Spectrometer (IRMS) and expressed in delta ‰ after correction for isotopic contribution of the derivative group [19]. Urine deuterium enrichment was analyzed by a High Temperature Conversion Elemental Analyzer coupled with an IRMS (TC-EA-IRMS, Thermo Scientific, Bremen, Germany) [19].

**Calculations**

Fractional synthesis rate measurement was performed assuming a steady state. The assumption was based on the following considerations: 1) in all patients plasma $^{13}$C leucine and urine $^2$H$_2$O enrichments reached steady state within 6 h from the start of the isotope infusion; 2) the slope of the enrichment curve over time did not deviate significantly from zero between time 6 h and time 24 h for plasma leucine and between time 6 h and time 48 h for urine $^2$H$_2$O; 3) the concentrations of DSPC and SP-B in tracheal aspirates did not change significantly during the first 48 hours of the study.

Fractional synthesis rate was calculated from the linear portion of the enrichment curve rise over time relative to the plateau enrichment of the respective precursors [19, 22].

Variables were expressed as mean±SD or median (interquartile range) as appropriate. Statistical analysis was performed by a non-parametric test (Mann-Whitney test) or by t Student’s test. The kinetic and quantitative data were correlated with respiratory parameters using Pearson’s correlation. Significance was defined as p<0.05. Data were analyzed using the statistical package SPSS 15 (SPSS Inc, Chicago, IL).
Results

We recruited 22 mechanically ventilated newborn infants, 13 with CDH and 9 with no lung disease (controls). One control infant was subsequently excluded for major congenital heart disease diagnosed after study onset, hence 21 patients completed the study. The two groups’ clinical features are reported in Table 1. Demographics were similar between the two groups except postnatal age, whereas ventilator parameters were significantly different, as expected by study design (Table 1). In 12 infants, CDH was diagnosed before 26 weeks gestational age; in one infant the diagnosis was made at birth. Four of the 13 CDH were right-sided and 9 left-sided. They were all sedated and ventilated on HFOV at birth but only 6 were still on HFOV at the start of the study. One infant with CDH died from lung hypoplasia before hospital discharge but after study completion. None of the survivors was oxygen-dependent at discharge.

All 8 controls required intermittent positive pressure ventilation: 2 for respiratory failure (due to a congenital diaphragmatic paralysis and cystic lymphangioma), 3 for urinary and abdominal surgery (2 bladder malformations and 1 hepatic hemangioma) and 3 for neurological failure (2 brain malformations, 1 hydrocephalus). All control infants survived and were discharged from the hospital. Neither CDH nor control infants had evidence of infection during the study period.

In all study infants the isotopic enrichments of urine $^2$H$_2$O and plasma $^{13}$C-leucine were on steady state (linear regression slopes of the enrichment values versus time not significantly different from zero) during the isotope administration (data not shown). Median urine deuterium enrichment was 7603 (7382-7962) delta ‰ vs. 7712 (7620-7970) delta ‰ and $^{13}$C-leucine enrichment was 9.5 (7.6-10.1) MPE vs. 8.1 (5.9-9.3) MPE in CDH and controls respectively (not significant).
DSPC amounts in tracheal aspirates were also stable during the study period. Isotope incorporation in SP-B was measured in all study infants whereas in DSPC was measured in 20 of the 21 infants (one infant mistakenly received one-tenth of the required $^{2}$H$_2$O dose, hence DSPC enrichments were not detectable).

Surfactant composition
Basal DSPC and SP-B values in tracheal aspirates, expressed as percentage of total phospholipids, are shown in Figure 1. DSPC content was not different between the 2 groups [43.2 (36.4-50.4) vs. 44.6 (30.3-53.9) in CDH and controls respectively] while SP-B was significantly lower in CDH than in controls [0.19 (0.14-0.28) vs. 0.41 (0.18-0.49), p<0.05].

Surfactant DSPC and SP-B kinetics
Pooled enrichment curves of palmitate-DSPC and leucine-SP-B from CDH and control subjects are shown in Figure 2.
As previously reported [22], SP-B turned over at a much faster rate than DSPC both in CDH infants and in controls (2.9 to 12.4 times faster in CDH and 14.2 to 16.2 times faster in controls). SP-B FSR was 33.4 (24.4-43.5) %/day in CDH vs. 52.0 (33.0-87.9)%/day in controls (p=0.03), while DSPC FSR were 5.0 (3.5-8.7)%/day and 6.1 (5.2-8.5) respectively in CDH and in controls (p=0.31) (Table 2).
There were a significant direct correlation between SP-B FSR and SP-B percentage of total phospholipids (P=0.544, p=0.029).
In the CDH group DSPC FSR directly correlated with oxygenation index (P=0.632, p=0.021), alveolar arterial O$_2$ gradient (P=0.632, p=0.021) at the start of the study and oxygenation index expressed as mean value during the study (P=0.597, p=0.031).
Discussion

Data regarding surfactant components in CDH are limited and controversial. Previous studies on sheep fetuses with surgically-created diaphragmatic hernia and rat fetuses with nitrofen-induced CDH reported decreased phospholipids and surfactant proteins levels in lung tissue, decreased surfactant protein transcripts and changes in lung compliance [6-7, 23-25]. However, other authors working on similar CDH models did not report changes in alveolar surfactant composition [26] or even increased SP expression [27].

Data from clinical studies suggest that phospholipids production is normal, since in human CDH fetuses examined between 33 and 38 weeks, amniotic fluid lecithin to sphingomyelin (L/S) ratios and phosphatidylglycerol (PG) levels were not different from those of historical control data [28]. Our group showed that PC synthesis is not altered in CDH infants [9] [29]. Finally, an autopsy study found normal total lung DSPC and SP content in CDH compared to normal fetuses, suggesting that surfactant phospholipids endowment in the lungs of CDH infants may be normal [10]; however, this study did not measure airway levels of surfactant components.

Since surfactant content in the whole lung may not reflect its distribution to the airspaces, we reappraised the subject by comparing mature SP-B and DSPC tracheal aspirate concentrations and synthesis rate in newborns with CDH compared to newborns with normal lung function. We found that SP-B airway recovery and synthesis rate were significantly decreased in CDH infants, while DSPC recovery and synthesis did not differ from controls at variance from a previous study where DSPC amount from tracheal aspirates was significantly decreased in CDH infants compared with matched controls [9]. Recovery from tracheal aspirates can be affected by sampling dilution techniques, and thus could be less consistent than kinetic studies that are not affected by dilution.
Accordingly DSPC FSR directly correlated with the degree of respiratory failure, suggesting an efficient compensatory feed-back mechanism, while SP-B FSR did not increase with the severity of respiratory failure. Thus, infants with CDH appear to have relative SP-B deficiency presumably due to decreased synthesis, although changes in degradation, in the context of immaturity and ventilation-induced lung inflammation, cannot be excluded based on our data [30-32].

Of note, our data refer to mature SP-B, unlike other studies that do not discriminate between mature SP-B and precursor forms of uncertain origin and significance [33]. We speculate that partial SP-B deficiency, together with lung immaturity and inflammation, significantly contributes to respiratory insufficiency in infants with CDH.

The fractional synthesis rate of SP-B in our CDH infants spanned from 5 to 50% suggesting a spectrum of gravity in this deficiency. The low number of study infants did not allow us to demonstrate a correlation of SP-B FSR with any clinical outcome parameters. Interestingly, looking at all study infants, we demonstrated a direct correlation between SP-B FSR and SP-B content in tracheal aspirates (P=0.544, p=0.029). It will be interesting to establish in a larger cohort of CDH infants whether a correlation exists between the degree of respiratory insufficiency and the amount of SP-B in the airways. If this relationship will be confirmed, SP-B measurement in tracheal aspirates could assist clinicians in deciding whether to administer exogenous surfactant to infants with CDH. This is not a trivial issue, as surfactant treatment can transiently alter gas exchange and lung compliance, with a major risk for complications such as pneumothorax and/or pneumomediastinum in these hypoplastic lungs.

Our study has two major limitations. First, CDH and controls were not well matched for age at the study and therefore the differences in SP-B concentrations and kinetics could have been attributable to age difference. Unfortunately there is little information on tracheal aspirate SP-B profile in the early postnatal period. One study found that in preterm infants beyond the
first week of life tracheal aspirate SP-B levels were comparable to those reported in term infants [34] and these levels remained constant during the next 9 weeks after birth. We speculate that, if the differences observed were age-related, they should have affected both DSPC and SP-B.

Second all CDH infants were placed on HFOV at birth, but only 6 of them were still ventilated on HFOV at the time of the study, while the other 7 infants were on conventional ventilation as the controls. However, among CDH infants we found no difference in SP-B FSR between those on HFOV and those on SIMV (data not shown). Experiments in sheep showed that SP-B kinetics is not modified by HFOV [35]. Hence, it is unlikely that ventilation mode differences were the only cause of our observations.

In conclusion we analyzed DSPC and SP-B airway recovery and synthesis rate in infants with CDH compared to controls without lung disease. We found that SP-B airway recovery and synthesis rate were significantly decreased in CDH infants compared to controls, while DSPC recovery and synthesis did not differ. Partial alveolar SP-B deficiency could be a major contributor to respiratory insufficiency in CDH, and its correction might represent a therapeutic goal if new-generation synthetic surfactant containing recombinant SP-B will be available.

Acknowledgements:

The authors thank all intensive care nurses who contributed to the sample collection.


Figure 1.

SP-B and DSPC in tracheal aspirates from CDH and control infants.

SP-B and DSPC values (median and interquartile range) are reported as % of total phospholipids (panel A) and as µg/ml ELF and mg/ml ELF respectively (panel B).

* Different from controls (p<0.05 by Mann-Whitney test).
Figure 2.

Palmitate-DSPC and leucine SP-B enrichment curves in CDH and control infants.

Data are mean ± SD. MPE: mole percent excess; Delta: $^{2}H/^{1}H$ vs. smow.
Table 1. Clinical characteristics and ventilator parameters of study patients.

<table>
<thead>
<tr>
<th></th>
<th>CDH (n = 13)</th>
<th>CONTROLS (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (Kg)</td>
<td>2978±447</td>
<td>3160±350</td>
<td>0.34</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>38±2</td>
<td>38±2</td>
<td>0.53</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/5</td>
<td>5/3</td>
<td>0.98</td>
</tr>
<tr>
<td>Age at the study (d)</td>
<td>2±1</td>
<td>11±5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Intubation time (d)</td>
<td>11±8</td>
<td>6±4</td>
<td>0.19</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>92.3%</td>
<td>100%</td>
<td>0.34</td>
</tr>
<tr>
<td>Mean airway pressure (cm H2O)</td>
<td>10±5</td>
<td>6±2</td>
<td>0.02</td>
</tr>
<tr>
<td>AaDO2 gradient (mm Hg) at study start</td>
<td>173±188</td>
<td>46±19</td>
<td>0.03</td>
</tr>
<tr>
<td>PaO2/FiO2 ratio at study start</td>
<td>217±118</td>
<td>374±137</td>
<td>0.03</td>
</tr>
<tr>
<td>Oxygenation index at study start</td>
<td>8±9</td>
<td>2±1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

CDH=Congenital Diaphragmatic Hernia. Values are mean ± SD. AaDO2 = Alveolar arterial oxygen gradient. PaO2/FiO2 = mean oxygen tension in arterial blood/inspiratory oxygen fraction. Comparisons by Student’s “t” test.
Table 2. SP-B and DSPC kinetic parameters in study infants with CDH and age matched control infants

<table>
<thead>
<tr>
<th></th>
<th>CDH (n = 13)</th>
<th>CONTROLS (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-B FSR (%/d)</td>
<td>33.4 (24.4-43.5)</td>
<td>52.0 (33.0-87.9)</td>
<td>0.03</td>
</tr>
<tr>
<td>DSPC FSR (%/d)</td>
<td>5.0 (3.5-8.7)</td>
<td>6.1 (5.2-8.5)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Data are median (interquartile range). Comparisons by Mann-Whitney test.