Serial Lecithin/Sphingomyelin Ratios and Surfactant/Albumin Ratios in Tracheal Aspirates from Term Infants with Respiratory Failure Receiving Extracorporeal Membrane Oxygenation

Andrea Lotze, Crystal Y. Stroud, and Steven J. Soldin

Serial tracheal aspirate samples were obtained for determination of lecithin/sphingomyelin (L/S) ratios from 47 term infants in respiratory failure. Phospholipids were extracted with Folch solution (chloroform:methanol, 2:1 by vol) and analyzed by HPLC with use of a silica column and a mobile phase of acetonitrile:methanol:water (48:31:21, by vol). Surfactant/albumin (S/A) ratios were determined with the TDx Fetal Lung Maturity Assay® (Abbott Labs). L/S ratios increased significantly over time in all patients (F = 19.42, P < 0.0001). The S/A ratio correlated with the L/S ratio (r = 0.554, P < 0.001). This study suggests that postnatal surfactant deficiency in term newborns with respiratory failure is a component of the newborn's lung injury.

Indexing Terms: lung disorders/pediatric chemistry/phospholipids/hyaline membrane disease/meconium/pulmonary hypertension

In 1959, Avery and Mead recognized that hyaline membrane disease in the premature infant is the result of a primary surfactant deficiency (1). In the past 5 years, surfactant replacement has become a standard form of therapy for hyaline membrane disease and has led to the investigation of the possible role of surfactant deficiency in other types of neonatal respiratory failure. Secondary deficiency of surfactant has been demonstrated in meconium aspiration syndrome, with inhibition of pulmonary surfactant activity occurring at meconium concentrations much lower than those required for inhibition by plasma protein or blood cell components (2, 3). Development of pneumonia in infants with sepsis can result in leakage of fluid into the alveoli and interstitium, with destruction of lung surfactant (4). Damage to the alveolar capillary barrier as a result of mechanical ventilation and hyperoxia alone can result in cell necrosis with protein leak, pulmonary edema, or hemorrhage; such protein leak can result in significant alteration of surfactant function (5, 6). Cardiopulmonary bypass has also been shown to alter surfactant activity in children (7).

Significant intra- and interpatient variability is seen

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6 Nonstandard abbreviations: L/S, lecithin/sphingomyelin; S/A, surfactant/albumin; ECMO, extracorporeal membrane oxygenation; and Cl, lung compliance.
tococcal sepsis with pneumonia, documented by either a positive result on a urinary Wellcogen test (Wellcome Diagnostic, Research Triangle Park, NC) or a positive blood culture result; (c) hyaline membrane disease; or (d) idiopathic primary pulmonary hypertension of the newborn, defined as increased pulmonary artery pressure without evidence of another primary disease process. Infants in each diagnostic category were then randomly assigned to either the surfactant or the control group (Table 1). Infants in the surfactant group received a total of four doses of surfactant (beractan intratracheal suspension, a modified bovine surfactant (investigational new drug no. 33 750) supplied by Ross Labs., Columbus, OH). Nurse practitioners administered all drug and placebo doses and were not responsible for the patients' clinical management. Bedside personnel, ECMO physicians responsible for the patients' daily management, and investigators participating in the study were not permitted to assist with drug administration. The infants' beds were screened from bedside personnel during dosing, and tinfoil was wrapped around the end of the endotracheal tubes after drug delivery.

Sample Collection

Tracheal aspirates were collected before surfactant administration (pre) and daily until the infant was removed from bypass. For analysis purposes, we used the samples collected before surfactant administration (pre), on the median day on bypass (mid), and on the day of removal from bypass (last). Tracheobronchial aspiration was performed by instilling a total of 3 mL of 9 g/L NaCl in 0.5-mL aliquots into the endotracheal tube. After each 1-mL aliquot of saline, the baby was hand-ventilated to disperse the saline solution, and the trachea was suctioned via a catheter slightly beyond the tip of the endotracheal tube. Secretions were collected into a pediatric Leuken trap; the suction catheter was then rinsed with 1 mL of saline into the collection trap to remove secretions adhering to the inside of the suction catheter. Samples were immediately placed on ice, vortex-mixed, and then centrifuged at 950g, 4 °C, for 5 min. The cell pellet was discarded and the clear supernate saved for determination of the L/S and S/A ratios.

The role of centrifugation in the measurement of amniotic fluid has been previously described (13). Intact particles representative of fetal lung surfactant precipitate at very low g forces, resulting in removal of as much as 20–40% of the surfactant in amniotic fluid. The extent of their removal by centrifugation can therefore result in alteration of L/S ratio measurements. We measured variation in the mean lecithin peak area, the mean sphingomyelin peak area, and the L/S ratios when samples were centrifuged at 950g vs not centrifuged. Surfactant recovery with centrifugation averaged 96.7% of the amount recovered from uncentrifuged samples. Various characteristics of the media, such as viscosity, composition, and concentration, might have contributed to the higher recovery of surfactant from tracheal aspirate fluid.

Determination of L/S Ratios by HPLC

Phospholipid extraction. From the clear supernate, 0.5 mL was pipetted into siliconized centrifuge tubes. The phospholipids were extracted by adding 10 mL of a Folch solution of chloroform:methanol (2:1, by vol) and treating with an ultrasonic membrane disruptor for 20 min. Protein was then removed by adding 2 mL of 500 mg/L CaCl₂ to the solution and shaking the entire mixture; after separation into two phases, the top layer, consisting of methanol and protein, was discarded. The clear lower phase (chloroform) was evaporated under nitrogen and the dry residue redissolved in 50 μL of methanol. The extract was then transferred to an Eppendorf microtube and centrifuged at 2000g for 5 min; 10 μL was then used for each injection. This technique was compared with the more-often-used extraction procedure for amniotic fluid (involving a mixture of equal volumes of fluid and methanol, followed by the addition of chloroform equal to the aqueous phase), and found to yield no significant difference in recovery.

Chromatography. The normal-phase HPLC equipment consisted of a System Gold chromatographic system from Beckman Instruments (Palo Alto, CA). A stainless steel 5-μm silica column (Alltech (Deerfield, IL) Adsorbosphere HS, 250 × 4.6 mm) was used with a silica column guard. A modification of the isocratic HPLC procedures described by Jungawala et al. (14) and later D'Costa et al. (15) was used to accelerate elution of lecithin and sphingomyelin without compromising resolution. To identify the optimum mobile phase, we chromatographed calibrators in the presence of different mobile phases composed of acetonitrile: methanol:water and varied the flow rate. The proportion of acetonitrile was successively decreased while keeping the proportion of methanol:water at 3:2. Optimum peak separation was achieved with a mobile phase consisting of acetonitrile:methanol:water in a volume ratio of 48:31:21 and a flow rate of 2.3 mL/min. Chromatography was at ambient temperature and detection was by ultraviolet absorption at 205 nm. With this mobile phase, lecithin and sphingomyelin eluted at mean retention times of 3.2 min and 4.0 min, respect-

<table>
<thead>
<tr>
<th>Table 1. Distribution of diagnostic categories between treatment and control groups.</th>
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<tr>
<td>Gestion</td>
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<tr>
<td>Disease</td>
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<tr>
<td>MAS</td>
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<tr>
<td>Sepsis</td>
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<td>HMD</td>
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<tr>
<td>PPHN</td>
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<td>Total</td>
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MAS, meconium aspiration syndrome; HMD, hyaline membrane disease; PPHN, persistent pulmonary hypertension of the newborn.
tively, and L/S ratios were computed from peak areas by means of the Beckman computerized integrator (Fig. 1). Using calibrators for phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine, we have previously demonstrated that these phospholipids elute early, with or near the solvent front. Extraction efficiencies for lecithin and sphingomyelin from a mixed calibrator (4 g/L lecithin:1 g/L sphingomyelin in equivolume methanol:chloroform) were 91.7% and 94.4%, respectively. The precision study of a series of five consecutive runs within a day gave a CV of 8.6% (3.70 ± 0.32 g/L, mean ± SD) for lecithin and 9.3% (0.86 ± 0.08 g/L) for sphingomyelin. The CV for five between-day runs was 10.0% (4.01 ± 0.4 g/L) for lecithin and 13.1% (0.99 ± 0.13 g/L) for sphingomyelin. All chromatographic analyses were complete within 6 min of sample injection.

Determination of S/A Ratio with Abbott TDx/TDxFx

Preliminary analyses of tracheal aspirate fluids for albumin content with the Behring (Somerville, NJ) Nephelometer 100 Analyzer revealed that very low concentrations (0.043–0.968 g/L) of albumin were present. Therefore, for 43 of those samples with undetectable concentrations of albumin, preparation for assay by the TDx included the addition of 20 μL of 25 g/L human albumin to each milliliter of sample to establish a final concentration in tracheal aspirate sample of at least 0.5 g/L albumin. This is the minimum albumin content recommended for the TDx Fetal Lung Maturity Assay for adequate determination of the S/A ratio. Each sample was then placed in a syringe and passed through a 13-mm (pore size) filter into a clean glass tube. From this tube, at least 500 μL was pipetted into the sample well of the Fetal Lung Maturity Assay unit dose cartridge. Likewise, 44 samples were analyzed without the addition of albumin and yielded S/A ratios within the linearity of the instrument. The Fetal Lung Maturity Assay was set up as outlined for unit dose in the Operation section of the Abbott TDx operation manual.

Lung Compliance

C_L was measured with the Star Calc pulmonary function machine (Infrasonics, San Diego, CA). Pressure changes were measured by comparing with a double-sided pressure transducer the transpulmonary pressures from a standard esophageal balloon filled with 0.1 mL of air and positioned in the mid-to lower third of the esophagus, with the pressure at the proximal end of the infant’s endotracheal tube (16). Except for measurements obtained while the infant was still paralyzed or recovering from paralysis, only spontaneous breath data were analyzed.

Statistical Analysis

Demographic variables, including gestational age, race, and primary diagnosis, were assessed between treatment groups by using Pearson χ² analysis. Student’s t-test was used to compare birth weight, gestational age, age at initiation of ECMO, and hours of intubation before ECMO. For L/S and S/A ratios, three serial values were selected for analysis: presurfactant (pre), the patient-specific median day on ECMO (mid), and the day of decannulation (last). Repeated-measures analysis of variance (ANOVA) was used to compare L/S and S/A ratios in surfactant vs control infants and to evaluate changes across time. An additional factor assessed in the S/AANOVA was the effect of supplementary albumin. Simple linear regressions of S/A vs L/S, and L/S or S/A vs C_L, were also performed. For these regressions, S/A measurements were evaluated separately for samples to which human albumin was added. To normalize the distribution and stabilize the variance, we used the natural log transformation of L/S and S/A for all analyses. Significance was assessed at α = 0.05, and only patients with complete data were included in the repeated-measures ANOVA.

Results

Twenty-five patients were enrolled in the surfactant group and 22 in the control group. The two groups were similar with respect to gender, gestational age, birth weight, Apgar scores, age at ECMO, and hours of intubation before ECMO; arterial blood gas values and ventilator settings were also similar before ECMO. Diagnostic categories were evenly distributed between the groups, with the most common diagnosis being meconium aspiration syndrome (Table 1).

Before calculation of L/S ratios, the peak areas of lecithin and sphingomyelin were adjusted individually according to their recovery rate (multiply by 1.09 for lecithin and 1.06 for sphingomyelin). For the 137 specimens analyzed, the L/S ratio varied from 1.7 to 100. For all infants, the natural logarithm of L/S increased significantly in both surfactant (n = 25) and control (n = 22) groups (F = 19.42, P < 0.0001), with no difference between the two treatment groups. Table 2 summarizes the untransformed L/S ratio data of all infants.
with complete sets of data (pre-, mid- and last-day samples). Of these sets, 23 were in the surfactant group and 21 were in the control group. Increases in L/S ratios correlated with increases in C_t (r = 0.345, P <0.001). There were no differences in the L/S ratios between surfactant and control groups (Fig. 2) and no difference among diagnostic groups (Fig. 3).

A total of 87 aspirates was also analyzed by TDx methodology for the determination of S/A ratios, with values ranging from 3.2 to 160 mg/g. Samples obtained from 12 infants did not require the addition of albumin; samples from 13 infants did, because of low albumin content. The natural logarithms of S/A ratios for infants with complete sets of data (pre-, mid-, and last-day samples), evaluated by a two-way repeated-measures ANOVA, were significantly greater in the surfactant group than in the control group (P = 0.0007, Fig. 4). This grouping factor was also significant (F = 7.27, P = 0.0135). Untransformed S/A ratio data for infants with pre-, mid-, and last-day samples are summarized in Table 3. For the 12 sets of samples to which albumin did not need to be added, 5 belonged to the surfactant group and 7 to the control group. For the 13 sets of samples to which albumin was added, 8 belonged to the surfactant group and 5 to the control group.

For those samples to which albumin was not added (n = 44), S/A increased significantly as L/S ratios increased (r = 0.582, P <0.001). With the addition of albumin (n = 43), S/A ratios also increased significantly as L/S increased (r = 0.554, P <0.001, Fig. 5) and as C_t increased (r = 0.552, P <0.001). For these correlational studies, all available samples were included in the analyses.

**Discussion**

In our study, tracheal aspirate L/S ratios obtained from full-term infants in respiratory failure were initially low and then increased significantly over time in both surfactant and control groups, with no difference between the two treatment groups.

Measurement of an amniotic fluid L/S ratio has been extensively used as an obstetrical tool for many years, and continues to be one of the most accurate predictors of prenatal pulmonary maturity (17, 18). Determination of postnatal pulmonary maturity in premature infants by measuring tracheal aspirate L/S ratios by HPLC has also been reported by D’Costa et al., with ratios ranging from 3 to >100 (19). All infants with

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**Table 2. Untransformed L/S ratios in surfactant and control groups (infants with complete sets of data).**

<table>
<thead>
<tr>
<th>Time</th>
<th>Surfactant (n = 23) Mean ± SD (range)</th>
<th>Control (n = 21) Mean ± SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>6.63 ± 3.65 (2.0-18.4)</td>
<td>10.55 ± 15.84 (1.7-76.3)</td>
</tr>
<tr>
<td>Mid</td>
<td>6.62 ± 2.66 (2.3-14.0)</td>
<td>11.60 ± 12.29 (1.8-48.3)</td>
</tr>
<tr>
<td>Last</td>
<td>13.10 ± 7.86 (2.7-35.3)</td>
<td>23.45 ± 24.08 (4.4-100)</td>
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</table>
respiratory distress syndrome had L/S ratios <15. They also compared L/S ratios in tracheal aspirates with those in amniotic fluid obtained within 2 h of delivery and found significant correlation, the tracheal fluid L/S ratios being about three times higher than those in amniotic fluid. To date, HPLC has not been used to determine tracheal aspirate L/S ratios over time in term newborns with respiratory failure.

Several studies have demonstrated the profile of other phospholipids, e.g., phosphatidylglycerol and phosphatidylinositol, as additional indices of fetal lung maturity (20–23). These phospholipid profiles have also been used as predictors of respiratory distress syndrome (24–27). However, it is difficult to achieve complete and simultaneous separation of all phospholipids in a timely manner by HPLC. Analysis of the other phospholipids was not included in our study.

Jungawala et al. introduced an HPLC procedure for the separation and quantification of phospholipids by using a silica gel column, ultraviolet absorption detector, and a gradient of acetonitrile:methanol:water (14). Subsequent advances in HPLC technology have facilitated phospholipid analyses with the use of microbore (23) and diol columns (28, 29), gradient elution programs involving a variety of mobile phase compositions (28, 29), and detection systems such as the differential refractometer (22), the automated phosphorus analyzer (30), and mass detectors (31).

We found a modification of the HPLC procedure established by Jungawala and later clinically validated by D’Costa et al. (14, 15) to be suitable for achieving our goal of determining L/S ratios in a timely manner. The mobile phase modification significantly shortened the retention times of both lecithin and sphingomyelin, thereby providing sharper peaks and enabling quantification of peak areas in small volumes (500 µL) of tracheal aspirate samples. It involved routine HPLC equipment and rendered reproducible data in <6 min from time of injection. A limitation of this procedure, however, is the inability of the HPLC to accurately quantify lecithin. With low-wavelength detection, the carbon bonds in the fatty acid moieties of unsaturated phospholipids produce greater signals than the carbonyl groups of saturated dipalmitoyl lecithin, the main component of lecithin. Though saturated dipalmitoyl lecithin absorbs in the ultraviolet spectrum and gives a measureable peak area, ultraviolet detection monitors the changes in both the unsaturated and saturated lecithins. The inability to predict the ratio of unsaturated to saturated compounds therefore prohibits the method from being accurately standardized. As a result, lecithin could not be quantified in absolute terms (15).

The TDx Fetal Lung Maturity Assay involves the fluorescence polarization of a dye added to a solution of fluid in buffer and comparison with a calibration curve to determine the relative concentrations of surfactant and albumin in the sample (32–35). Albumin and pulmonary surfactant compete for a ligand that, when bound to albumin, demonstrates a high fluorescence polarization. Once the ligand is added to amniotic fluid, the overall polarization quantified by the analyzer reflects the S/A ratio (36). This assay works well for amniotic fluid samples, since amniotic fluid albumin concentrations remain relatively constant throughout the third trimester. With amniotic fluid samples, the S/A value has a sensitivity of 90% and a specificity of 87% for predicting hyaline membrane disease, with values <30 mg/g considered consistent with pulmonary immaturity (32, 34).

The TDx Fetal Lung Maturity Assay has not been used previously for tracheal aspirates from term newborn infants. The manufacturer discourages the use of samples with visible blood contamination. Whereas whole blood containing phospholipids in the membranes of intact erythrocytes increases the S/A ratio, serum and hemolized blood tend to lower the ratio (32). The effect of blood on tracheal aspirate samples was not directly investigated in the present study. However, fresh samples were centrifuged before analysis to remove red cells and cellular debris. Samples with visible blood were excluded from the study.

Because the development of the TDx Fetal Lung Maturity Assay was based on the relatively constant concentration of albumin found in amniotic fluids, measurement of S/A ratios in other fluids with various

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**Table 3. Untransformed S/A ratio data in surfactant and control groups, with and without albumin added (infants with complete sets of data).**

<table>
<thead>
<tr>
<th>Time</th>
<th>Surfactant</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No albumin added</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Pre</td>
<td>77.5 ± 50.4 (28.6-160.0)</td>
<td>59.5 ± 68.9 (10.0-160.0)</td>
</tr>
<tr>
<td>Mid</td>
<td>121.6 ± 44.8 (54.2-160.0)</td>
<td>82.3 ± 63.5 (14.9-160.0)</td>
</tr>
<tr>
<td>Last</td>
<td>148.9 ± 24.5 (105.1-160.0)</td>
<td>105.4 ± 58.3 (21.0-160.0)</td>
</tr>
</tbody>
</table>

| Albumin added |            |         |
| n            | 8          | 5       |
| Pre          | 52.1 ± 31.5 (10.4-98.1) | 39.2 ± 67.6 (3.2-160.0) |
| Mid          | 69.7 ± 42.2 (12.3-135.1) | 29.2 ± 40.5 (6.8-100.5) |
| Last         | 77.6 ± 39.0 (4.2-127.8)  | 60.1 ± 40.3 (10.6-101.6) |

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**Fig. 5. S/A ratios compared with L/S ratios for samples with albumin added (P < 0.001).**
albumin concentrations may yield erroneous results. Detection of S/A ratios by this assay in our sample population was difficult if albumin content was low or variable. For example, albumin concentrations within the samples were variably diluted, depending upon the amount of saline retained within the infant’s lung during the suctioning procedure. For those infants with low tracheal aspirate albumin content, the S/A ratio could not be quantified; therefore, albumin was added to their samples as previously described. Determination of S/A ratios might be further improved by initially quantifying sample albumin content, then adding exogenous albumin to some constant concentration that would shift the S/A ratio into the analytical range of the TDX test, as reported by McCarroll and Van Lente (37), who found that this modified ratio could be mathematically converted to the S/A ratio in the original sample. This methodology may be promising as a rapid “bedside” test for determination of surfactant sufficiency.

C\textsubscript{L} data continue to yield excellent adjunctive information regarding infants’ clinical status (38, 39). We surmise that such information, combined with the use of serial L/S ratios in tracheal aspirate fluids of infants on ECMO, will enhance the clinical assessment of the individual patient’s need for surfactant. The timing and number of surfactant doses may be modified on the basis of determinations of tracheal aspirate surfactant composition during the acute phase of the infant’s pulmonary disease.

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References
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