Study on a New Index of Fetal Respiratory Function:
a Novel Optical Analysis of the Amniotic Fluid
by the Vacuum-dried Infrared Attenuated Total
Reflection (ATR) Method

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For the purpose of detecting fetal pulmonary maturity with real-time convenience but reliable accuracy, infrared spectrophotometry of amniotic fluid lipids was introduced using our newly developed optical device. The peak areas at the wave number of 1740 cm⁻¹ on the infrared spectra (called "PA₁₇₄₀") were measured in 29 specimens sampled from 29 pregnant women, and compared with the Lecithin/Sphingomyelin (L/S) ratio of the same samples. Statistical analysis revealed that the PA₁₇₄₀ was almost as reliable as the L/S ratio, and these indexes illustrated pulmonary immaturity in cases with a PA₁₇₄₀ < 0.9 and an L/S ratio < 2.0. PA₁₇₄₀ is absolutely independent of the effect on the concentration of lecithin in the amniotic fluid because of a combination of the vacuum-drying process and the absorbance correction, and can be completed in less than 20 minutes from sampling of the amniotic fluid by a very simple manipulation. In conclusion, the new index, PA₁₇₄₀, will be a more practical method for prediction of the fetal pulmonary maturity than methods such as the well known L/S ratio.

(Key words: amniotic fluid, fetal respiratory function, infrared spectroscopy, attenuated total reflection (ATR) method)

INTRODUCTION

Since the respiratory distress syndrome (RDS) is the leading cause of death in the pediatric age group (1), the importance of information concerning fetal pulmonary maturity is widely appreciated. The most useful source of such information is the amniotic fluid, and many amniotic fluid parameters have been investigated for prediction of fetal pulmonary maturity.

Because these analytic methods are time-consuming, complicated and not sufficiently accurate, their routine and widespread clinical use has been precluded. Therefore, a device that enables clinicians to obtain useful fetal information from a minute amount of amniotic fluid with real-time convenience but reliable accuracy has been awaited with great interest.

Gluck et al. have already shown in 1971 that fetal pulmonary maturity is closely associated with the amount and composition of the pulmonary surfactant and can be predicted by determination of phospholipids (particularly lecithin itself or its ratio to sphingomyelin) in...
the amniotic fluid (11, 12, 13).

On the other hand, since Freeman et al. reported on infrared spectra of some lipoproteins and related lipids in 1955 (10), infrared spectral analysis in the medical field has mostly been performed in the laboratory to determine the fine structures of proteins (7, 8, 18) and to detect deterioration in the surface of biomedical materials (2, 3, 6).

Recently, we introduced newly developed infrared optical equipment based on the attenuated total reflection (ATR) method and succeeded in quickly and precisely measuring the infrared spectra of a small amount of amniotic fluid (14, 15). The spectral peaks originating from phospholipids were further identified in these spectra (16).

The present study was performed to confirm the usefulness of a new index illustrating fetal pulmonary maturity by investigating the correlations between these peaks originating from phospholipids and the fetal pulmonary outcome.

MATERIALS AND METHODS

As reported elsewhere (16), since a clear peak at the wave number of 1740cm⁻¹ on the infrared spectra of lecithin has been identified with our equipment, the infrared peak at 1740cm⁻¹ seen in amniotic fluid (Fig. 1) was also thought to be derived from lecithin. After confirming the derivation of this infrared peak found in amniotic fluid, we attempted to determine lecithin in the amniotic fluid quantitatively from this peak, and to evaluate the fetal pulmonary maturity.

Amniotic fluid samples

Amniotic fluid samples from 29 pregnant women of accurately established gestational periods were examined. Gestational ages ranged from 15 to 40 weeks. Among 29 amniotic fluid samples examined, 20 were obtained from the vaginal pool at the time of amniotomy, four were obtained by transabdominal amniocentesis, and five were obtained at the time of cesarean section. No sample showed gross blood or meconium contamination. In all cases, the fluids were centrifuged exactly for 5 minutes in a calibrated centrifuge at 2,000 × g (3, 200rpm) within 1 hour of collection to remove cells and sediments. The supernatant fluid was first frozen and kept at −20°C. Before measurement, these fluids were thawed.

Preparation of amniotic fluid samples (extraction of lipids)

Lipids were extracted from the amniotic fluid samples to improve the sensitivity and detectivity with respect to the peaks originating from phospholipids, especially at 1740cm⁻¹, on the infrared spectra of amniotic fluid. According to the method of Folch et al. (9), these lipids were extracted from 1 ml of amniotic fluid by adding an equal volume of absolute methanol and two volumes of chloroform and stirring on a vortex mixer. The resulting emulsion was broken down by centrifugation (2,000 × g; 3,200rpm, 5 min.) and the chloroform layer containing the soluble lipids was used for measurement in the following manner.

Instrumentation

The infrared spectra of the amniotic fluid lipids were measured and recorded with new optical equipment of our own development. This instrument is illustrated in Fig. 2.

The infrared spectrophotometer is of the Fourier transform type based on the theory of Michelson's interferometry (Japan Spectroscopic Co., Type FT/IR-3). To improve the sensitivity and detectivity, a HgCdTe detector was employed and a special chamber including mirrors and a pentagonally shaped prism was interposed between an infrared light source and the detector to combine the attenuated total reflection (ATR) method with a vacuum-drying technique (Fig. 3).

In this chamber, in order to avoid optical degradation of the ATR prism material itself by placing the sample solution directly on its surface, the ATR prism was made of zinc-gerenide (ZnSe) crystal, which is almost insoluble in solvents. The transmitted light beam (about 8mm in diameter) was reflected at the boundary between the ATR prism and the sample on the prism. The ATR prism was installed horizontally because a drop of specimen can be securely held on the prism during the vacuum-drying process. The suction duct for evaporation of the solvent was arranged beside the ATR prism.

With the above instrumentation, we succeeded in precisely obtaining specific infrared spec-
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Fig. 1 Infrared spectra of amniotic fluid

Fig. 2 Schematic diagram of the measurement system
tra for all solutes in solutions, such as lipids in chloroform solution (14, 15, 16).

About 2ml of the above-mentioned chloroform solution was placed on the ATR prism and vacuum-dried. The suction pressure was controlled to prevent scattering of the sample by sudden vaporization. The suction was continued for 5 minutes. When the film of dry solutes (lipids) was completely fixed to the ATR prism surface, the infrared spectra measurement was started. Examples of infrared spectra obtained from normal amniotic fluid lipids are shown in Fig. 4.

A new index of fetal pulmonary maturity

To evaluate the peak area at 1740 cm\(^{-1}\) as a new index of fetal pulmonary maturity (Fig. 5), we measured the area encircled by this peak above the base line on the infrared spectra of the amniotic fluid lipids and compared these measured values with the fetal pulmonary outcome.

Since differences in the film thickness resulted in an apparent difference in overall absorption when measuring the peak area at 1740 cm\(^{-1}\), we normalized the infrared spectra as the mean, and the variances of the infrared spectra from 700 cm\(^{-1}\) to 2000 cm\(^{-1}\) were 0.3 (mean) and 0.15 (variance). We called this index the "Peak Area at 1740 cm\(^{-1}\)" abbreviated as PA_{1740} in the clinical evaluations in this study.

The fetal pulmonary outcome was analyzed by reviewing the pediatric charts and X-rays, in close consultation with the attending neonatologist. The diagnosis of RDS was based on the generally accepted clinical and radiographic criteria (grunting, flaring, retracting, ventilatory support needed for longer than 24 hours, and radiographic signs of RDS such as hypoxpansion, reticulogranular pattern and air bronchograms).

Confirmation of derivation of the peak at 1740 cm\(^{-1}\)

To confirm whether the PA_{1740} was derived from lecithin or not, we dissolved lecithin made from egg (Sigma Chemical Co., St. Louis, U.S.A.) in normal amniotic fluid before extracting lipids (between 4 mg and 32 mg per 100 ml of amniotic fluid) and measured these samples with our equipment. We assessed how PA_{1740} changed with the increase of lecithin in the amniotic fluid, and prepared a working curve.

Lecithin/sphingomyelin (L/S) ratio determination

For the purpose of comparing conventional fetal pulmonary indices with the new index, PA_{1740}, we measured the L/S ratio, the most widely used amniotic fluid test, in all amniotic fluid samples. Determination of the LS ratio was performed with a flame ionization detector using the principle introduced by Gluck et al (13).

RESULTS

The relationship between PA_{1740} and the dissolved amount of lecithin in normal amniotic fluid is shown in Fig. 6. The sample fluid before dissolving lecithin showed a PA_{1740} value of 1.65. The figure indicates a linearly increasing trend of PA_{1740} with the addition of dis-
solved lecithin. Regression analysis of $PA_{1740}$ ($y$) for the dissolved amount of lecithin ($x$) indicated a positive correlation:

$$y = 1.65 + 4.47 \times 10^2 x, \quad r = 0.967.$$ 

Accordingly, it appeared that the peak at $1740\text{cm}^{-1}$ was derived from lecithin in the amniotic fluid and $PA_{1740}$ was proportional to the concentration of lecithin in this fluid.

**Prediction of fetal pulmonary outcome with $PA_{1740}$ versus the L/S ratio**

Fig. 7 shows $PA_{1740}$ versus the L/S ratio with known fetal pulmonary outcomes. $PA_{1740} < 0.9$ and the L/S ratio $< 2.0$ were used as cutoff values for indicating immature fetal lung. Using these criteria, a comparison of $PA_{1740}$ and the L/S ratio in 29 cases is given in Table 1. Among the 29 total samples, 17
Fig. 6 Relationship between PA1740 and the dissolved amount of lecithin in normal amniotic fluid

\[ y = 1.65 + 4.47 \times 10^{-2}x \]
\[ r = 0.967 \]

Fig. 7 PA1740 versus the L/S ratio with known fetal pulmonary outcomes (N = 29)

Table 1 Relationship between PA1740 and the L/S ratio

<table>
<thead>
<tr>
<th>PA1740</th>
<th>L/S ratio</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 0.9 )</td>
<td>( \geq 2 )</td>
<td>17</td>
</tr>
<tr>
<td>( \leq 0.9 )</td>
<td>( &lt; 2 )</td>
<td>1</td>
</tr>
<tr>
<td>( &lt; 0.9 )</td>
<td>( \geq 2 )</td>
<td>2</td>
</tr>
<tr>
<td>( &lt; 0.9 )</td>
<td>( &lt; 2 )</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>29</td>
</tr>
</tbody>
</table>
were predicted to be mature and nine immature by both indices. In the remaining three cases, the fetal pulmonary outcome predicted by PA1740 and by the L/S ratio did not coincide. We also compared both indices with the fetal pulmonary outcome. These results are seen in Tables 2 and 3. Because PA1740 predicted RDS in one out of 17 cases, not suffering from RDS, the false positive rates was 5.9%. However the L/S ratio correctly predicted RDS (the false positive rate was 0%). On the other hand, both indices falsely predicted in two out of 12 cases with RDS (false negative rates of 16.7%). Fisher's exact probability test revealed equally significant associations between both indices and the fetal pulmonary outcome (p<0.01).

In Table 4, the reliability of these two indices is summarized. The sensitivity of both methods was 83.3% (10 out of 12 cases). The specificity and two predicted values of both methods were also basically comparable. Both indices had a low false maturity rate, which is the most important in clinical use since only two of the 18 cases with mature results (11.1%) by PA1740 and two of the 19 cases with mature results (10.5%) by the L/S ratio were associated with the development of RDS. There was no instance of RDS associated with a PA1740 greater than 1.3. Among 10 cases with an L/S ratio greater than 10.0, however, RDS developed in one neonate.

DISCUSSION

Since the L/S ratio was introduced by Gluck et al. in 1971 (11), it has become the standard index for detection of fetal pulmonary matur-

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Table 2  Relationship between PA1740 and fetal pulmonary outcome

<table>
<thead>
<tr>
<th>PA1740</th>
<th>No RDS</th>
<th>RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 0.9</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>&lt; 0.9</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

N = 29; p<0.01

Table 3  Relationship between the L/S ratio and fetal pulmonary outcome

<table>
<thead>
<tr>
<th>L/S ratio</th>
<th>No RDS</th>
<th>RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 2</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>&lt; 2</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

N = 29; p<0.01

Table 4  Reliability of PA1740 and the L/S ratio in predicting fetal pulmonary outcome

<table>
<thead>
<tr>
<th>Immature result</th>
<th>Mature result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>Predictive value of RDS</td>
</tr>
<tr>
<td>PA1740</td>
<td>83.3% (10/12)</td>
</tr>
<tr>
<td>L/S ratio</td>
<td>83.3% (10/12)</td>
</tr>
</tbody>
</table>
ty in most laboratories (4, 5). Therefore the L/S ratio was compared with our own PA1740, as proposed in this study. In 29 uncontaminated amniotic fluid samples, we evaluated the usefulness of both PA1740 and the L/S ratio in predicting the fetal pulmonary outcome.

The critical significance of the indices predicting the fetal pulmonary outcome depended on the false values. With PA1740 < 0.9 and L/S ratio < 2.0 as the indices of fetal pulmonary immaturity, two cases in a total of 12 cases studied showed false mature values in both indices with one common case. There was one case showing a false immature value for PA1740 while no such cases were found for the L/S ratio (Tables 2 and 3). However, the critical consequence of false mature values is less severe than that of false mature values, and the risk involved may cause only unwarranted delay in the delivery of a mature infant (4). Because of the small sample size, the difference in reliability between the PA1740 and the L/S ratio were not statistically significant, but it appeared that PA1740 illustrated almost the same degree of the reliability as the L/S ratio.

From the methodological aspect, we measured the infrared spectra of the total lipid extracted from the amniotic fluid using the method of Folch et al. (9) with our equipment, and estimated PA1740 from the peak at 1740cm⁻¹ after making an absorbance correction. On the other hand, coincidence between this peak and the addition of dissolved lecithin (Fig. 6), i.e., the concentration of lecithin, showed that PA1740 reflects the amount of lecithin in the amniotic fluid, and this finding agreed with the fact that CH₂OCOR found in the structural formula of lecithin has infrared absorption at 1740cm⁻¹ (17). Therefore, the new index, PA1740, indicates the ratio of lecithin in the total lipid of the amniotic fluid because the absorbance correction also corrects variations in the amount of lipids in the amniotic fluid.

Compared with this PA1740, the L/S ratio evaluates the relative amounts of lecithin (dissaturated phosphatidylcholine to be exact) against sphingomyelin. The reason for using the L/S ratio has been that since there are variations in both amniotic fluid volume and in the concentration of lecithin, these variations are assumed to be canceled out to some extent by using the ratio between them. Therefore, a convenient way to detect lecithin concentration by avoiding this effect on condensation or dilution is to compare the variations of lecithin with those of sphingomyelin, the amount of which is comparatively stable throughout the gestational period. In this case, sphingomyelin, a minor fraction of phospholipid, is useful as an internal standard, but it is not absolute.

PA1740 is completely independent of the effect on concentration (condensation or dilution) by vacuum-drying on the ATR prism and the absorbance correction. Vacuum-drying limits the sample processing to the lipid solute. The absorbance correction makes the amount of lipids constant. This is especially important in disorders of the amniotic fluid in view of the possibility of false immature results in cases of polyhydramnios and false mature results in cases of oligohydramnios. From the theoretical point of view, PA1740 is of greater advantage with respect to the effect on the concentration of samples than the L/S ratio. Other attractive points of PA1740 are the rapidity and the convenience of its procedure. In most cases, it can be completed in less than 20 minutes from amniotic fluid sampling with a very simple manipulation. The L/S ratio has been widely used because of its high reliability, but it is not feasible in emergencies because it is time-consuming and technically difficult to perform (5).

With PA1740, we feel confident that we can predict fetal pulmonary maturity even in emergencies. At the present stage, however, the system for determination of PA1740 involves an infrared spectrophotometer for professional use, which is expensive and the availability of such a system is limited to larger institutions. Fortunately, we can simplify the infrared spectrophotometer by limiting it to measurements of PA1740.

In conclusion, although there is still room for further investigation, we believe that the new index, PA1740, is more practical in predicting fetal pulmonary maturity than the conventional LS ratio.

ACKNOWLEDGMENTS

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REFERENCES


