Surface tension of amniotic fluid lipid extracts: Prediction of pulmonary maturity

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The surface tension (ST)–lowering properties of an amniotic fluid lipid extract can provide a rapid and reliable means of predicting pulmonary maturity. One hundred and eleven samples from 91 patients were analyzed. A surface tension of less than 56 dynes per centimeter at 120 μl of extract and less than 46 dynes per centimeter at 220 μl of extract denoted pulmonary maturity. Values greater than these indicated immaturity. Among fluid samples studied within 48 hours of delivery for the presence or absence of respiratory distress syndrome (RDS) in 71 patients, there were no false positive ST values, while 7 of 22 patients with immature values developed RDS. Surface tension correlates well with the lecithin/sphingomyelin (L/S) ratio but provides clearer definition than the L/S ratio when compared to outcome. Blood and meconium contamination make the surface tension of fluid from babies with pulmonary immaturity appear mature. Identical twins with dissimilar ST values and outcome, as well as serial samples from individual patients, are analyzed and discussed. (AM. J. OBSTET. GYNECOL. 128: 591, 1977.)

NEONATAL SURVIVAL is mainly dependent on the presence of adequate quantities of pulmonary "surfactant" for the prevention of respiratory distress syndrome (RDS).[1–8] The ability to predict this antenatally would greatly reduce the incidence of RDS and improve neonatal survival. The current standard in clinical use is the lecithin/sphingomyelin (L/S) ratio, which is dependent on indirect biochemical analysis of surfactant. Tiwary and Goldkrand[9] have established a method whereby this prediction can be made by directly measuring the surface tension–lowering properties of an amniotic fluid lipid extract. The intent of this paper is to present a modified and simplified method of performing the surface tension analysis and to correlate the results directly with neonatal outcome.

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Background

As early as 1929, Von Neergaard[10] noted the effect of a liquid monolayer on lowering surface tension to facilitate the air inflation of pulmonary alveoli. Prattle,[11] in 1955, observed longer air retention by bubbles from cut lung than by plasma and proposed that there was a detergent, derived from the internal surface of the lung, which maintained the surface tension of the bubble and resisted collapse. Clements[12] isolated, from lung extract, a substance which demonstrated a decrease in surface tension on compression and prevented alveolar collapse on expiration. Avery and Mead[1] established the absence of such a surface tension–lowering compound from the lungs of babies dying from hyaline membrane disease. Enhorning[13] found little difference in the surface tension of amniotic fluid at term compared to that of water, but he did not concentrate the surface-active compounds. However, in the study of Enhorning and Kirschbaum[14] of guinea pig tracheal aspirates, there was a decrease in surface tension with increased weight and gestational age. Shelley and associates,[15] utilizing surface tension area diagrams, and Müller-Tyle and associates,[16] using a Wilhelmy balance, noted a progressive increase in surface activity and decrease in surface tension with increasing gestational age.

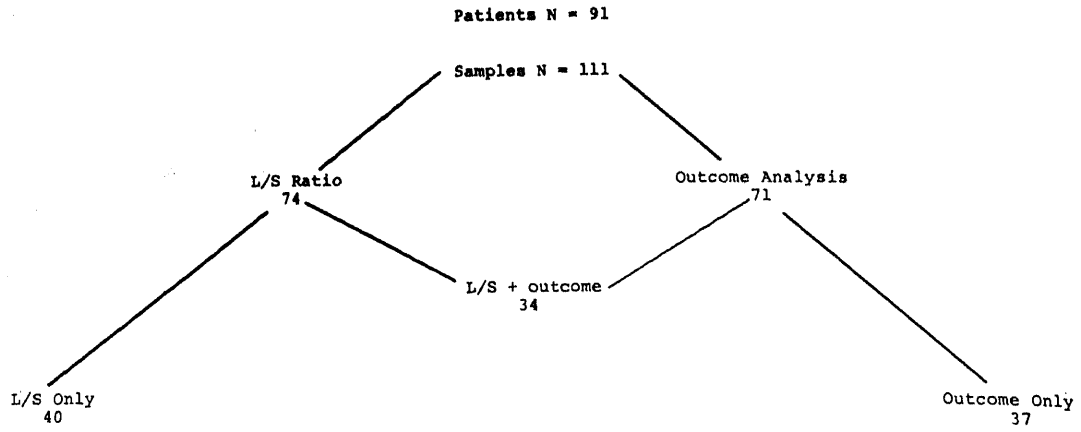


Fig. 1. Distribution of the evaluation of 111 amniotic fluid samples from the patient population.

Klaus and co-workers[17] characterized a surface-active compound as a phospholipid which was like dipalmitoyl lecithin and had two hydrophobic fatty acid side chains and a hydrophilic choline group. Scarpelli,[2] in 1967, studying amniotic fluid and tracheal aspirates, concluded that ". . . with the onset of breathing and establishment of the alveolar lining layer, the concentration of surfactant will determine the functional stability of the alveoli." Gluck and his associates,[4–7] in a series of experiments in the rabbit and then in the human subject, were able to isolate surface-active lecithin (a primary component of surfactant) and not only demonstrated its intracellular storage in the type II pneumocyte but also correlated increased lecithin with an increase in the number of osmiophilic inclusion bodies within these cells as gestation progressed. Two synthetic pathways of lecithin were found: (1) methylation, which is the major path up to 36 weeks and yields an α-palmitic/β-myristic lecithin, and (2) cystidine diphosphate–choline incorporation, which is predominant after 36 weeks and yields an α-palmitic/β-palmitic lecithin. Gluck and associates[18] utilized this research to establish the clinical test on amniotic fluid, the L/S ratio. This work and that of others[19–22] confirmed the ability and reliability of the L/S ratio to predict antenatally the likelihood of neonatal RDS: L/S $\geq 2:1$, mature—no RDS; 1.0 to 1.9, transitional—RDS possible; $<1:1$, immature—RDS probable. In an effort to provide a more rapid test, Clements and associates[23] returned to the study of amniotic fluid surface tension and bubble stability and developed the so-called "shake test" to predict pulmonary maturity. Boehm and associates[24] confirmed the validity of foam stability but showed it to be somewhat less sensitive than the L/S ratio. The present work describes the use of surface tension to assess rapidly and accurately fetal pulmonary maturity.

Materials and methods

Patient population. Patients were selected from the private and clinic Obstetrical Services at the University of Nebraska Medical Center. For the purposes of statistical analysis, only patients of 28 weeks' gestation or greater were included. Fig. 1 describes the study of 111 samples of amniotic fluid from 91 patients.

The amniotic fluid was obtained by four methods: (1) by sterile transabdominal amniocentesis, (2) via a transcervical intra-amniotic pressure catheter during labor, (3) at the time of amniotomy with the use of transcervical amniocentesis, and (4) amniocentesis at the time of cesarean section. All amniocenteses performed prior to the onset of labor were for obstetrically indicated reasons. The conduct of labor, amniotomy, and mode of delivery were not influenced by this study, and accepted obstetric practices were utilized. Age, parity, race, and underlying coexistent medical problems were beyond the scope of this paper.

Laboratory procedure. Samples were centrifuged at 3,000 × g for 15 minutes (RC-2B Ivan Sorvall, Inc., Norwalk, Connecticut), and the supernatant was divided into two parts for analysis of both surface tension and L/S ratio. The L/S ratio of amniotic fluid samples was determined by the modified method of Gluck and colleagues.[18]

Amniotic fluid samples for surface tension analysis were stored at 4° C. in clean glass screw-capped vials.

A 1 to 2 ml. aliquot of the amniotic fluid was transferred to a 15 ml. glass-stoppered conical centrifuge tube for extraction of the lipids; an equal volume of absolute methanol (certified by the American Chemical Society) was added, and the tube was mixed on a vortex mixer for 30 seconds. Next, a volume of chloroform (certified by the American Chemical Society) equal to the total volume in the tube (sample plus methanol) was added and mixed on a vortex mixer for 30 sec-

onds. The addition of the chloroform caused the formation of an emulsion which was broken by centrifugation at 1,000 × g for 15 minutes (PR-2 centrifuge, IEC). Three layers were formed in the tube: an upper aqueous layer, an interfacial protein layer, and the lower chloroform layer, which contained the lipids. A Pasteur pipet was introduced into the lower layer, and this layer was immediately transferred to a 15 ml. glass-stoppered tube. Caution was taken not to remove any of the aqueous or protein layer, as contamination with these layers affected surface tension measurements.

Extracts were analyzed on a DuNouy tensiometer (Model No. 70535, Cenco) with the use of a platinum ring of known circumference (5.992 cm.). The instrument was calibrated with a 500 mg. standard weight. Calibration was checked daily by measuring the surface tension of twice-distilled water (72.0 dynes per centimeter).

The cover of a standard 60 by 15 mm. glass Petri dish (inside diameter 57 mm., surface area 98.5 sq. cm.) was filled with 15 ml. of twice-distilled water and used for surface tension measurements. The platinum ring was submerged in the water, and 20 μl of the lipid extract was layered on the surface of the water with a glass micropipet (20 μl microdispenser, Drummond Scientific Co., Broomall, Pennsylvania). After all visible droplets of extract spread over the water surface, the surface tension was measured by withdrawing the ring. For subsequent volume additions, the ring was submerged and the above procedure was repeated. The platinum ring was cleaned between patient samples in benzene and methyl ethyl ketone and then flamed.

It was necessary for the size of the dish and treatment of the extract to remain constant to allow comparison of data from different samples at specific volumes. Changing the size of the dish (surface area) or concentrating the extract by evaporation affected the slope of the midportion of the surface tension–volume curve but not the beginning or the final plateau values. It was this midportion of the curve which was analyzed to establish group differences.

The volume of water in the dish had no effect on surface tension measurements. A volume of 15 ml. was chosen and used throughout the study. There was no difference between saline or double-distilled water used as the medium in the dish. Filling the dish with unextracted amniotic fluid or layering it on the water in the dish produced no meaningful results.

Study of blood and serum contamination of the amniotic fluid.

Serum. The surface tension of unextracted serum was determined to be 50.4 dynes per centimeter for an adult and 54.6 dynes per centimeter for serum from the cord blood of a mature infant. Lipid extracts of each serum sample were made, and surface tension was determined by the previously described method. Both reached a plateau of 40 μl of extract with values of 36 dynes per centimeter for the adult and 32.8 dynes per centimeter for the mature infant.

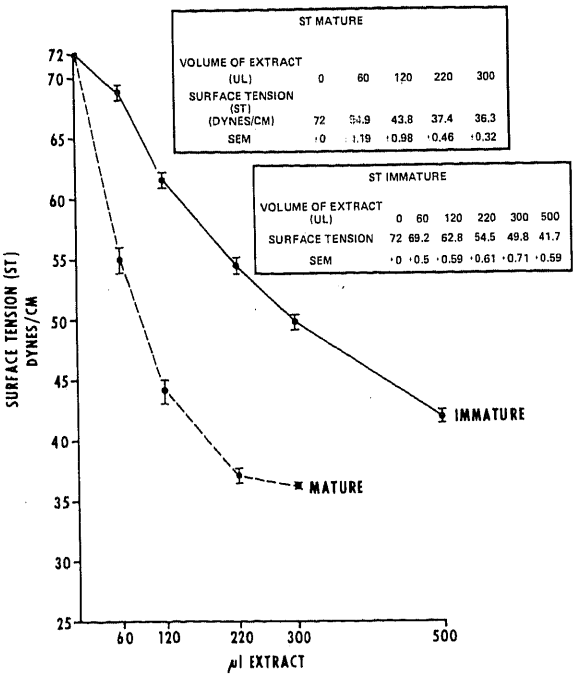


Fig. 2. Graphic representation of mean surface tension values ± standard error of the mean *(SEM)* for amniotic fluid lipid extracts from babies with pulmonary maturity and immaturity.

Blood. Blood contamination of amniotic fluid was studied by the addition of fresh whole blood, in concentrations ranging from 0.33 to 5.0 per cent, to aliquots of pooled amniotic fluid from babies with pulmonary maturity and immaturity. Specimens were allowed to remain at room temperature for one hour, after which they were centrifuged at 3,000 × g for 15 minutes and then submitted to the standard surface tension–analysis procedure. In a second set of experiments, whole blood was added to pooled immature and mature amniotic fluid, and hemolysis of the blood was accomplished by twice freezing (−70° C.) and thawing (in a +37° C. water bath). Blood concentrations were 0.25 to 5.0 per cent. These samples were then centrifuged and processed by our surface tension–analysis procedure. Notation was made of the visual discoloration of the samples before and after centrifugation in both sets of experiments. In each case, the uncontaminated pooled amniotic fluids were used as the controls.

Study of meconium contamination of the amniotic

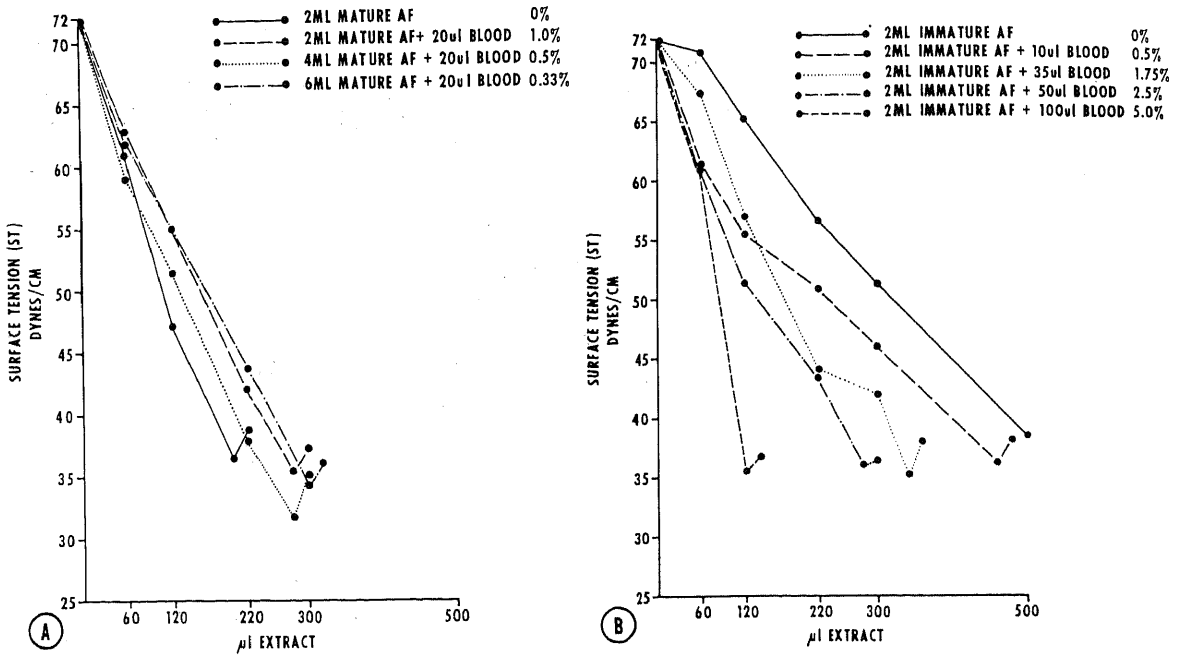


Fig. 3. Effect of nonhemolyzed whole blood on the surface tension of amniotic fluid from babies with pulmonary maturity and immaturity. *A*, Mature amniotic fluid. *B*, Immature amniotic fluid.

fluid. Fresh meconium was obtained from a normal newborn infant and refrigerated. Then 0.5 Gm. of meconium was mixed with 9.5 ml. of pooled amniotic fluid from babies with pulmonary maturity and immaturity. This was mixed with a vortex mixer for five to 10 minutes. To the pooled mature fluid was added 0.01, 0.12, and 0.25 Gm. of meconium, and to the pooled immature fluid was added 0.01, 0.025, 0.12, and 0.25 Gm. of meconium. The final volume in each sample was 6 ml. This fluid, with samples from each pool used as a control, was then analyzed for surface tension. Notation was made of sample color before and after the extraction procedure. The experiment was rerun with meconium from a premature neonate.

Stability studies. Amniotic fluid samples centrifuged and stored at room temperature, 4° C, and 20° C. for up to two weeks were stable with no effect on the surface tension measurements of the extracts.

Neonatal evaluation. Clinical evaluation of the baby as to the presence, absence, or degree of RDS was made by the pediatricians without knowledge of the surface tension results.

For definition, Type I RDS leads to hyaline membrane disease. Type II or transient tachypnea of the neonate was not included in the RDS group but rather with the normal cases.

Statistics. Statistical differences between groups were analyzed by Student's t test and the chi-square test.

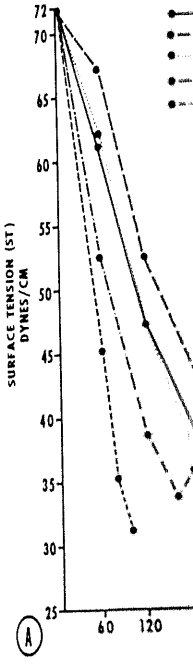
Results

General. Pulmonary maturity by surface tension was defined in this study as a surface tension less than 56 dynes per centimeter at 120 μl of extract and less than 46 dynes per centimeter at 220 μl of extract. Values exceeding these limits at each extract volume were classified as immature. For purposes of definition, a value of greater than 56 dynes per centimeter at 120 μl and less than 46 dynes per centimeter at 220 μl was called transitional and required a second surface tension analysis before pulmonary maturity could be predicted accurately. The defined limits were based on our previous work[9] and included mature and premature babies, outcome, and clearly defined L/S ratios.

Graphic display of the surface tension results of the mean values at 0, 60, 120, 220, and 300 μl of extract was plotted for each population (Fig. 2). Use of Student's t test at each volume yielded a highly significant difference ($p < 0.001$).

Effect of amniotic fluid contamination by blood and meconium.

Blood. As seen in Fig. 3, the addition of whole blood to mature amniotic fluid produced essentially no change in the surface tension values, but the values from immature fluid appeared more mature as the concentration of blood increased. All samples, prior to centrifugation, appeared to be at least pink tinged. Contamination of amniotic fluid by hemolyzed blood (Fig. 4) produced surface tension values that were



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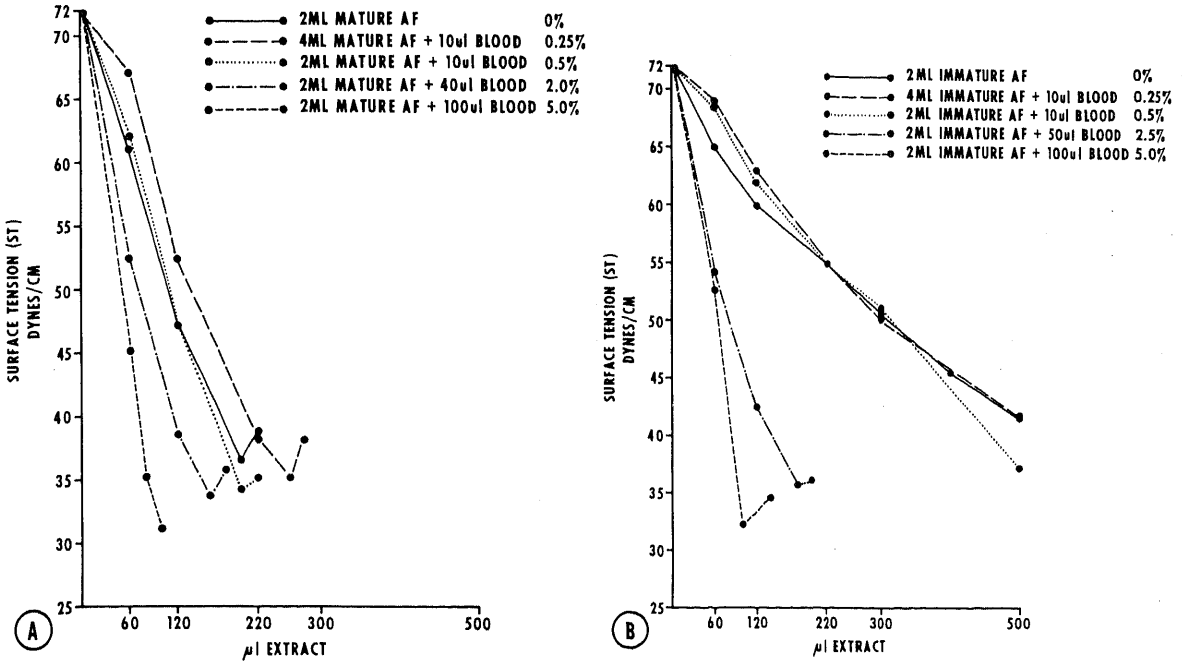

Fig. 4. Effect of hemolyzed whole blood on the surface tension of amniotic fluid for babies with pulmonary maturity and immaturity. *A,* Mature amniotic fluid. *B,* Immature amniotic fluid.

progressively more mature with the pooled immature fluid as the amount of contamination increased. Also, hemolyzed blood in pooled mature amniotic fluid lowered the surface tension even more than that in the control. All samples appeared to be pink, at the minimum, both before and after centrifugation.

Meconium. With increasing amounts of meconium contamination of the amniotic fluid (Fig. 5) from babies with pulmonary immaturity and maturity, there was a progressive lowering of surface tension values. As little contamination as 1.7 mg. of meconium per milliliter of amniotic fluid yielded values that would correlate with maturity. All contaminated samples had some visible greenish discoloration.

Outcome. Seventy-one patients were delivered within 48 hours (the majority within 24 hours) of the amniotic fluid collection (Table I). Forty-nine of these patients had amniotic fluid with a mature surface tension and no babies developed RDS. Twenty-two patients had immature amniotic fluid surface tension, and seven babies (31.8 per cent) developed Type I RDS. Three of these babies died. In no case in which the surface tension predicted maturity did a baby develop Type I RDS. Nine babies had diagnoses of Type II RDS or transient tachypnea, but there was no significant morbidity in any of these cases and they were grouped with the normal babies. In one set of identical twins, born at 33 to 34 weeks, in Twin A (2059 grams) amniotic fluid surface tension was immature,

Table I. Outcome of surface tension analysis and the presence or absence of RDS

	RDS	
Surface tension analysis	*Present*	*Absent*
Mature	0	49
Immature	7	15

$\chi^2 = 17.296$. $p < 0.001$.

Table II. Comparison of surface tension analysis and the L/ S ratio

	L/S ratio		
Surface tension analysis	*Mature (>2.0)*	*Transitional (1.0-1.9)*	*Immature (<1.0)*
Mature	22	1	2
Immature	14	20	15

χ^2 Yields $p < 0.001$.

Type I RDS was present, while in Twin B (1814 grams amniotic fluid surface tension was mature and RDS did not occur.

L/S ratio. In 74 cases, L/S ratios as well as surface tension were determined (Table II). In 22 of these, when the surface tension was mature, the L/S was mature (>2.0). One patient with a transitional L/S ratio and one with an immature L/S ratio had mature surface tension, and the infants did not develop RDS, while no outcome was available in one patient with an

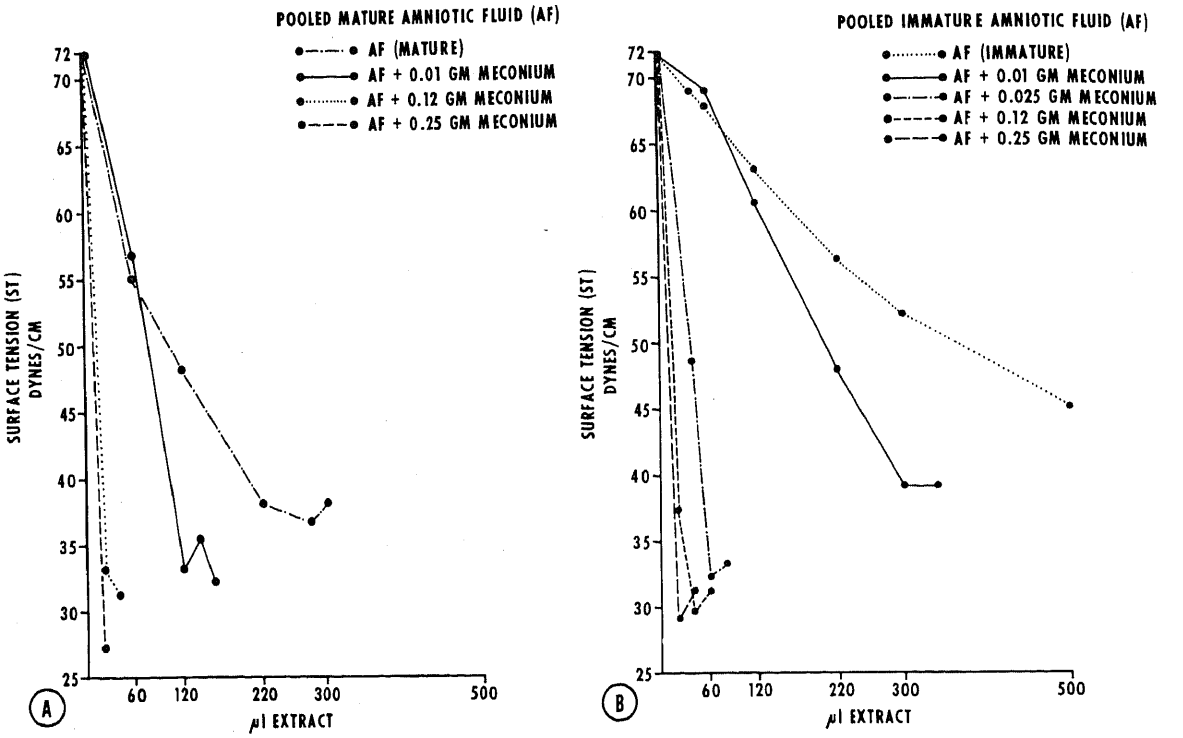

Fig. 5. Effect on surface tension of meconium contamination of amniotic fluid. *A*, Mature. *B*, Immature.

immature L/S and mature surface tension. In 49 samples with an immature surface tension, the L/S ratio on the same samples was immature (≤1.0) in 15 (30.6 per cent), transitional (1.1 to 1.9) in 20 (40.8 per cent), and mature (>2.0) in 14 (28.6 per cent). Thirty-four patients had fluid analysis within 48 hours of delivery. Twenty-eight had a mature L/S ratio, and no babies developed Type I RDS, while three of six with an immature L/S ratio developed Type I RDS.

Surface tension maturation. In several patients, there was opportunity for serial sampling of the amniotic fluid as pregnancy progressed. The surface tension was noted to change from immature to mature either gradually or abruptly. Fig. 6 displays two patients and each pattern of surface tension maturation.

Observation of 22 to 24 weeks' gestation. Five patients had amniotic fluid sampled at 22 to 24 weeks. Surprisingly, in three of the five with apparently mature surface tension, the infants were born the day the fluid was obtained and died of severe pulmonary immaturity. One of the five had a transitional type of pattern. In one of these patients, who was followed by repeated amniocenteses for suspected Rh sensitization, the analysis at 22 weeks indicated maturity but developed an immature pattern by 26 weeks, which persisted until 36 weeks. The mature pattern then reappeared, and a normal baby weighing 3,200 grams was delivered and did not develop RDS.

Comments

This paper is the first successful attempt to utilize the direct assessment of the surface tension–lowering properties of an amniotic fluid lipid extract to predict pulmonary maturity and the likelihood of neonatal RDS. Our studies of outcome yielded no false positive result, which is a mandatory determination in this type of analysis. Of the 22 samples which were immature by surface tension, there were seven babies with and 15 without RDS. This compares extremely well to previous work with the L/S ratio. Hobbins and associates[19] had eight babies delivered with an immature L/S ratio, and two developed RDS. In the study of Spellacy and Buhi,[20] 18 babies were delivered within one week of an immature L/S ratio, and six of these had RDS. Our previous work[9] and this study provide data that there is a good correlation between the surface tension and the L/S ratio. Significantly, the surface tension results define more clearly mature and immature values than does the L/S ratio (Tables I and II). This makes easier the clinical decision of whether or not to deliver a baby with no resulting risk of RDS. If the test errs at all, it is on the side of conservatism; this would require a brief

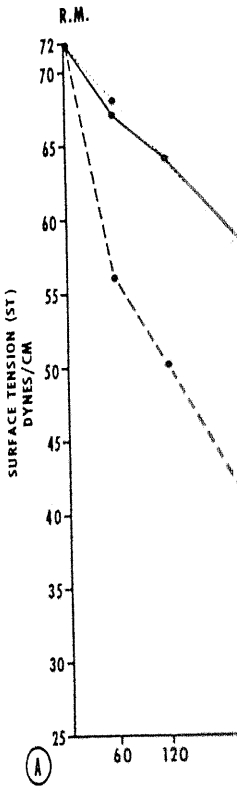

Fig. 6. Patterns
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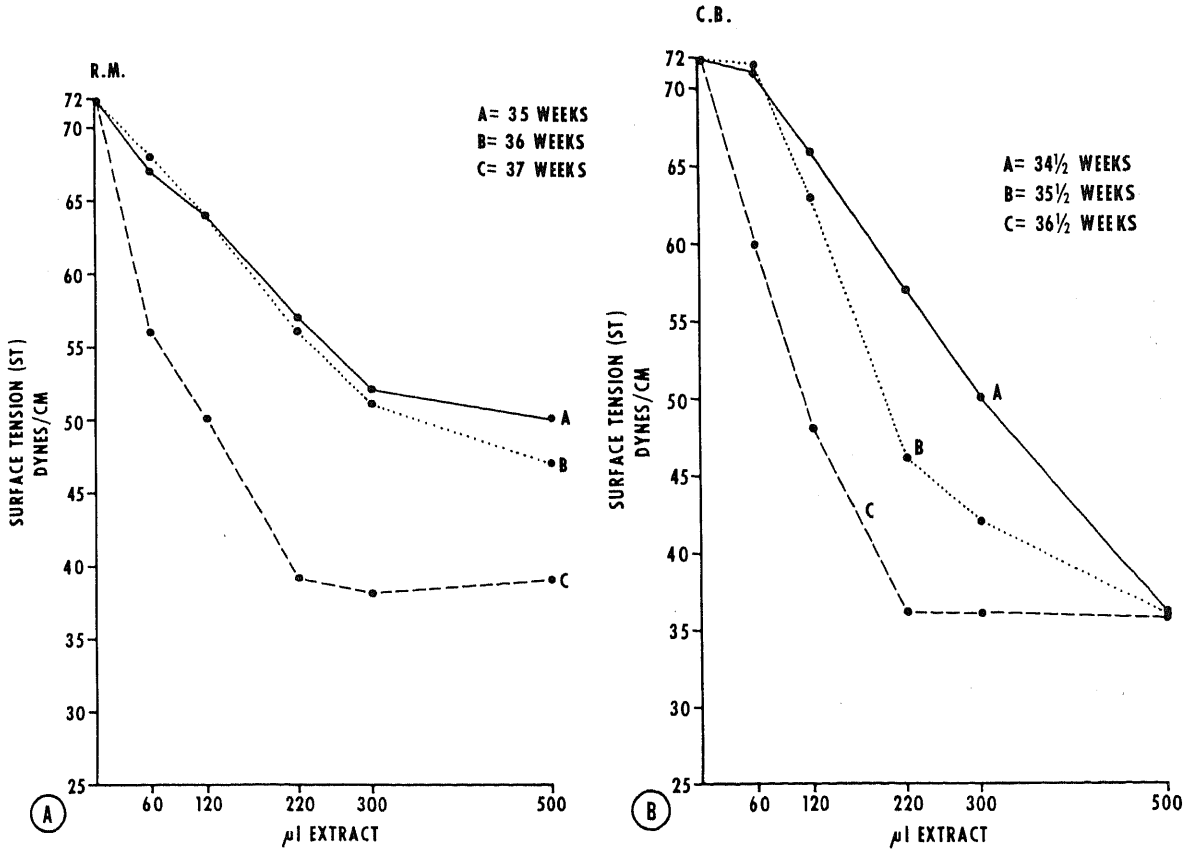

Fig. 6. Patterns of pulmonary maturation as seen by surface tension analysis of an amniotic fluid lipid extract in two patients (R. M. and C. B.) followed by serial samples for Rh-sensitized pregnancies.

waiting period prior to repeating the test and obtaining a mature surface tension level.

We analyzed the effect of blood and meconium contamination of amniotic fluid. Whole-blood contamination, which mainly represents serum, gave apparently mature surface tension values to the contaminated immature amniotic fluid. A greater response was seen with hemolyzed blood contamination, which represents the phospholipids in both the red blood cells and the plasma. This agrees with several reports[23, 25, 26] yet disagrees with some others[27, 28] that blood contamination of amniotic fluid will give falsely mature L/S ratios. Our initial work[9] showed less obvious changes toward maturity of the immature fluid. We believe that the present study, reported here, is correct and that our previous results were the product of a less sensitive system. These discrepancies may be expressing a variability in the phospholipids that affect surface activity, but which may or may not affect the L/S ratio. This probably depends on the amount of contamination and whether or not there is hemolysis present. Meconium contamination of the fluid produced lower surface tension values and yielded a false mature profile from the immature fluid. This agrees with previously published reports.[23, 25, 26] Therefore, with either serum, hemolyzed blood, or meconium contamination, a mature surface tension curve may be unreliable in predicting pulmonary maturity and the risk of neonatal RDS. However, if the values correspond with pulmonary immaturity, they may be considered reliable and utilizable. Visual examination of the color of the amniotic fluid, prior to being sent to the laboratory, should establish the presence or absence of contamination.

The serial samples from individual patients provide an interesting demonstration of how the surface tension characteristics of amniotic fluid change during the course of the latter half of pregnancy.

The method utilized provides a rapid analysis with a relatively simple lipid extraction and tensiometer reading. This should allow surface tension measurements to be performed in most community hospitals, as well as at referral centers. When required, an answer can be available within an hour or so of obtaining the amniotic fluid. This would be valuable in evaluating a patient in

premature labor. The reliability of the surface tension testing provides an essential clinical tool in any situation where the status of fetal pulmonary maturity is required. Our studies on the stability (in relation to time and temperature) of the surface-active compounds in the amniotic fluid should be invaluable to clinicians who must send away their amniotic fluid samples to be analyzed.

Some of the clinical correlations of our laboratory results with clinical outcome raise interesting questions. Why should the identical twins have different degrees of pulmonary maturity? The group of babies at 22 to 24 weeks' gestation, with the apparently mature surface tension readings, had poor clinical outcomes. This probably implies that there are other surface-active compounds present in the amniotic fluid which do not relate to pulmonary maturity. This would make the test unreliable, except for the fact that with delivery at 22 to 24 weeks fetal viability is not clinically anticipated or sought after diagnostically. Further work needs to be done to explain this observation.

The present study has (1) delineated a simplified method for the analysis of the surface tension of amniotic fluid lipid extract, (2) demonstrated statistically and clinically the reliable predictability of pulmonary maturity, based on the surface tension values, (3) compared the new method with the currently utilized L/S ratio, and (4) provided a basis for future investigation.

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