REVISTA ESPAÑOLA DE FISIOLOGÍA, 48 (3), 203-210, 1992

Effect of Centrifugation on Amniotic Fluid Phospholipid Composition

J. Ruiz-Budría[1], E. Fabre[2], M. T. Higueras[2] and J. L. Serrano[1]

Departamentos de Química Orgánica[1]
Facultad de Ciencias y de Obstetricia y Ginecología[2]
Facultad de Medicina
Universidad de Zaragoza
50009 Zaragoza (Spain)

(Received on February 3, 1992)

J. RUIZ-BUDRÍA, E. FABRE, M. T. HIGUERAS and J. L. SERRANO. *Effect of Centrifugation on Amniotic Fluid Phospholipid Composition.* Rev. esp. Fisiol., 48 (3), 203-210, 1992.

The first step in the determination of phospholipid in amniotic fluid is generally the removal of cells and debris from the fluid by centrifugation. Low-speed centrifugation of the supernatant is reported to have the same phospholipidic profile and L/S, PG/S and PI/S ratios similar to those of the uncentrifuged amniotic fluid sample. With high-speed centrifugation almost all the pulmonary surfactant seems to be recovered in pellet with the same characteristics as the uncentrifuged amniotic fluid.

Key words: Amniotic fluid, Lecithin/Sphingomyelin ratio, Centrifugation, Phospholipids.

It is generally accepted that surfactant deficiency is an important factor in the pathogenesis of the respiratory distress syndrome (RDS) (6). Pulmonary surfactant largely consists of lipids, while containing small amounts of proteins (14). It is extruded into amniotic fluid as intact lamellar body structures (7). A sudden increase in the concentration of surfactant or lamellar body phospholipids in amniotic fluid indicates that the lung has reached a stage of maturity compatible with its normal function.

* To whom all correspondence should be addressed.

In most methods for the assessment of fetal lung maturity, amniotic fluid is subjected to preliminary low-speed centrifugation (15) in an attempt to separate whole cells and cell debris from lung-derived surfactant phospholipid. However, because lamellar body phospholipid is present in amniotic fluid in a membranous form or in particles, it is also partly sedimented by this procedure (3, 8). Some of the false-negative predictions based on amniotic fluid analyses could be attributed to centrifugation of the samples, but without this centrifugation the results might also be misleading. The number of cells in amniotic fluid tend to increase substan-

tially in the last trimester of pregnancy (10).

Studies of the influence of centrifugation on phospholipid content in supernatant amniotic fluid show that an increase in g forces results in a variable loss of lecithin (9, 18), and sphingomyelin (9) and a decrease in the lecithin/sphingomyelin ratio (L/S) (1, 18).

LINDBACK and FRANTZ (9) suggest that centrifugation at 900 × g for 10 min does not lead to variations in the L/S ratio obtained from amniotic fluid of gestational ages between 26 and 35 weeks. However, centrifugation at 12,800 × g 10 min reduces the L/S ratio 40-60 %.

CHERAYIL *et al.* (1) show that the most significant variations in the L/S ratio related to the g-force occur in a range between 350 and 2800 × g. These authors recommend a preparation of amniotic fluid using 3,000 × g for 10 min at 4 °C. However, OULTON (11) concluded that the L/S ratio was not affected by centrifugal forces in amniotic fluid with gestational ages of less than 33 weeks or over 38 weeks. The L/S ratio in samples between 34 and 37 weeks increased in the pellet obtained at 10,000 × g.

Although it has been generally observed that increasing centrifugal forces results in increased removal of amniotic fluid phospholipids, the nature of the phospholipids removed has never been fully clarified. The present research has been carried out in order to study the effect of centrifugation on phospholipid content in both pellet and supernatant of amniotic fluid obtained in human gestation at term.

Materials and Methods

The patients involved in this study were cared for by the Zaragoza University Clinical Hospital Perinatal Services. Amniotic fluid samples (15 to 20 ml) from thirty term human pregnancies (37 to 41 weeks of gestation) obtained by transvaginal needle aspiration through a 4-6 cm dilated cervix were analysed. All the patients had a disease-free prenatal course. Samples which were blood and/or meconium stained were excluded from the study. When samples could not be analysed immediately they were frozen and stored at −20 °C.

The amniotic fluid was centrifuged at 130 × g for 5 minutes in order to remove cellular debris. The supernatant fluid was decanted without disturbing the sediment and divided into seven 2 ml aliquots.

In order to determine the effect of different rates of centrifugation, the aliquots were tested uncentrifuged, centrifuged at 500 × g for 5 minutes, 1,400 × g 5 min, 2,500 × g 5 min, 10,000 × g 20 min, 32,000 × g 60 min and 65,000 × g 60 min. Fractions were centrifuged in a Hettich-Rotanta/K (Tuttlingen, Germany) centrifuge or a Sorvall OTD 65 high-speed centrifuge using rotor T-865 (DuPont Instruments, Newton, Connecticut). After each aliquot had been centrifuged, supernatants were removed from the pellets, which were resuspended in 2 ml, 85 % NaCl. In both fractions, the areas corresponding to phospholipids were determined by a previously reported method (5). All the aliquots of the same sample were analysed simultaneously.

Data are expressed as mean ± standard deviations ($\bar{X} \pm SD$). The analysis of variance (ANOVA) was used to detect possible differences between the groups; a $p < 0.05$ was considered significant. As significant differences were found, the Student-Newman-Keuls test (SNK test) was used in order to identify the homogeneous groups (16).

Results

Differential sedimentation of phospholipid classes. — The differential sedimentation of each phospholipid for each centrifugal force used was calculated accord-

Table I. *Sedimentation percentage (mean ± SD) of phospholipids at different centrifugal forces.*

	Centrifugation force (1000 × g)/centrifugation time (min)					
	0.5/5 min	1.4/5 min	2.5/5 min	10/20 min	32/60 min	65/60 min
PC	26.1 ± 7.4	34.8 ± 8.4	40.4 ± 8.2	54.2 ± 7.8	62.6 ± 6.2	69.4 ± 7.2
Sph	17.1 ± 5.6	24.4 ± 7.7	30.5 ± 9.1	42.7 ± 8.1	59.5 ± 8.1	72.5 ± 7.7
PG	19.3 ± 8.9	32.3 ± 11.4	40.8 ± 11.5	62.5 ± 11.0	75.6 ± 7.8	81.0 ± 7.7
PI	16.6 ± 8.9	28.1 ± 11.7	37.8 ± 12.4	65.1 ± 13.5	77.8 ± 9.6	86.1 ± 8.3
PE	11.9 ± 10	24.7 ± 12.3	32.4 ± 14.4	59.7 ± 17.5	80.6 ± 10.6	91.7 ± 9.1
PS	9.2 ± 9.3	21.3 ± 12.0	30.3 ± 12.9	61.1 ± 15.8	84.5 ± 8.6	94.9 ± 6.9

Abbreviations: PC, Phosphatidylcholine or lecithin; Sph, Sphingomyelin; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PE, Phosphatidylethanolamine; and PS, Phosphatidylserine.

Table II. *Percentage distribution (means ± SD) of phospholipids at different centrifugal forces in supernatant fraction.*

	Centrifugation force (1000 × g)/centrifugation time (min)						
	0	0.5/5 min	1.4/5 min	2.5/5 min	10/20 min	32/60 min	65/60 min
PC	46.4 ± 7.7	47.4 ± 7.5	48.1 ± 7.3	47.7 ± 7.4	55.1 ± 7.4	61.3 ± 6.3	66.7 ± 7.7
Sph	7.7 ± 2.7	8.2 ± 3.1	7.8 ± 3.0	8.3 ± 3.1	10.8 ± 3.8	10.5 ± 3.7	9.6 ± 3.9
PG	5.9 ± 4.9	15.4 ± 5.0	15.7 ± 4.5	15.6 ± 4.7	12.4 ± 4.8	12.1 ± 4.8	13.4 ± 4.7
PI	13.2 ± 2.7	13.2 ± 3.7	12.8 ± 3.2	12.6 ± 3.0	9.8 ± 4.4	8.3 ± 4.0	6.5 ± 3.7
PE	10.8 ± 4.1	9.7 ± 4.2	9.4 ± 3.6	9.8 ± 3.6	7.4 ± 4.0	5.5 ± 2.4	2.7 ± 2.8
PS	6.0 ± 1.8	6.0 ± 2.0	6.1 ± 2.2	6.1 ± 2.1	4.6 ± 2.2	2.3 ± 1.3	1.0 ± 1.5

Abbreviations as in table I.

ing to the following expression: % = phospholipid densitometric area × 100/phospholipid densitometric area in uncentrifuged fraction.

The mean sedimentation percentage values for each phospholipid and for each procedure applied are shown in table I.

It was observed that the increase in relative force and time of centrifugation increased the differential sedimentation of phospholipid classes. A recovery in pellet of between 69.4 ± 7.2 (mean ± SD) for lecithin and 94.9 ± 6.9 for phosphatidylserine was obtained when a centrifugal force of 65,000 × *g* 60 min was used. The ANOVA applied to each phospholipid indicated significant differences ($p < 0.001$) between the different procedures applied.

Distribution percentages of phospholipids in supernatant and pellet fractions. — The values obtained with each *g* force were expressed as a distribution percentage of each phospholipid, this percentage being the contribution of each phospholipid to the total amount of phospholipid in the same aliquot. The distribution percentage was obtained in both supernatant and pellet fractions obtained after centrifugation.

The mean percentage values for each phospholipid and each procedure applied are shown in tables II (supernatant fraction) and III (pellet fraction).

In the supernatant fraction (table II), we observed an increase in the distribution percentage for lecithin and sphingomyelin from the aliquots processed at high *g* forces (10,000 to 65,000 × *g*), whereas the distribution percentage for the rest of the phospholipids from the same aliquots decreased. The ANOVA applied to each phospholipid showed significant differences between the different centrifugation processes used. Likewise, the SNK test (to identify homogeneous subgroups) showed that the distribution percentage of each phospholipid was not different for each *g* force applied.

On the other hand, the pellet fraction (table III) behaved contrariwise to the supernatant fraction. Lecithin showed a distribution percentage decrease ranging from (63.0 ± 7.7 for the fraction centrifuged at 500 × *g* to 44.9 ± 6.7 for the fraction centrifuged at 65,000 × *g*). With the rest of the phospholipids an increase in the distribution percentage value was observed, values similar to those of the control fraction being obtained at high speed centrifugation (65,000 × *g*).

The ANOVA applied to each phospholipid showed significant differences ($p < 0.001$) between the different procedures used. On the oder hand, the homogeneous subgroups identified by means of the SNK test showed that the distribution percentage of each phospholipid studied is not different for each treatment applied.

L/S ratio. — The mean values and standard deviation of L/S ratio for the different *g*-forces analysed are shown in table IV. When the *g*-force increases, a considerable rise in the L/S ratio obtained in the supernatant fraction is not observed. However in the pellet fraction, this L/S ratio shows a decrease from 12.1 ± 6.1, when it is centrifuged at 500 × *g* for 5 min, to 6.9 ± 2.7 when it is centrifuged at 65,000 × *g* for 60 min. The ANOVA applied to the L/S ratio of the supernatant fractions show that there are no significant differences between the treatments applied.

On the other hand, in the pellet fraction there are significant differences between the uncentrifuged and centrifuged fractions (from 500 × *g* for 5 min to 10,000 × *g* 20 min). Likewise, the L/S ratios obtained in the pellet of high speed centrifugation (10,000 to 65,000 × *g*) differ considerably from the L/S ratios obtained at low speeds (500 to 2,500 × *g*).

Finally, the L/S ratios obtained at low speed centrifugation in supernatant and pellet fractions are significantly different.

Table III. *Percentage distribution (mean ± SD) of phospholipids at different centrifugal forces in pellet fraction.*

	Centrifugation force (1000 × *g*)/centrifugation time (min)					
	0.5/5 min	1.4/5 min	2.5/5 min	10/20 min	32/60 min	65/60 min
PC	63.0 ± 7.7	57.3 ± 8.7	52.8 ± 8.8	50.5 ± 8.4	46.5 ± 7.0	44.9 ± 6.7
Sph	6.8 ± 4.3	5.6 ± 2.4	5.8 ± 2.5	6.1 ± 2.0	6.8 ± 2.3	7.3 ± 2.6
PG	14.6 ± 5.9	16.3 ± 5.2	17.3 ± 5.5	15.8 ± 5.2	16.7 ± 4.5	17.4 ± 4.6
PI	9.3 ± 3.6	10.8 ± 3.6	12.3 ± 3.8	14.1 ± 4.0	13.3 ± 3.8	12.7 ± 2.7
PE	4.2 ± 4.2	6.6 ± 3.4	7.4 ± 3.5	8.4 ± 4.0	10.9 ± 3.5	11.2 ± 3.2
PS	2.0 ± 2.1	3.5 ± 1.8	4.3 ± 1.8	5.2 ± 1.7	5.9 ± 1.7	6.3 ± 1.7

Abbreviations as in table I.

Table IV. *Effect of* g-*force (x*g*) on the L/S, PG/S and PI/S ratios obtained in the supernatant and pellet fraction of the centrifugation.*

	Supernatant			Pellet		
g-force	L/S	PG/S	PI/S	L/S	PG/S	PI/S
0	6.8 ± 3.2	2.3 ± 1.2	1.9 ± 0.7			
500	6.6 ± 2.7	2.2 ± 1.1	1.8 ± 0.8	12.1 ± 6.1	3.1 ± 2.2	1.8 ± 1.1
1,400	7.2 ± 3.7	2.4 ± 1.2	1.9 ± 1.2	12.3 ± 6.1	3.6 ± 2.2	2.3 ± 1.4
2,500	7.0 ± 5.0	2.3 ± 1.6	1.9 ± 1.5	10.7 ± 5.4	3.6 ± 1.9	2.6 ± 1.9
10,000	5.7 ± 2.4	1.3 ± 0.7	1.0 ± 0.8	9.2 ± 4.3	3.0 ± 1.7	2.6 ± 1.5
32,000	7.2 ± 4.5	1.4 ± 0.8	1.1 ± 1.7	8.2 ± 4.9	2.9 ± 1.7	2.5 ± 2.5
65,000	8.8 ± 6.9	1.7 ± 1.5	1.0 ± 1.7	6.9 ± 2.7	2.7 ± 1.2	1.9 ± 0.6

PG/S ratio. — The results of PG/S ratio in the supernatant fraction indicate that an increase in the *g*-force causes a slight decrease in the values of this ratio (table IV).

In the supernatant fraction, there are significant differences between the uncentrifuged and high-speed centrifuged fractions. Likewise, the PG/S ratio values obtained after both high-speed and low-speed centrifugation are significantly different.

In the pellet fraction, the PG/S ratios obtained at moderately low-speed centrifugation (1,400 to 2,500 × *g*) are signifi cantly different from the PG/S ratios obtained in the uncentrifuged fraction.

In conclusion, a comparison between the PG/S ratios obtained in supernatant and pellet fractions indicates significant differences between all of them.

PI/S ratio. — The results obtained with PI/S ratios, both in supernatant and pellet fractions (table IV) show similar behaviour to the PG/S ratio. So, significant differences exist between the PI/S ratio values obtained in the uncentrifuged aliquot and the supernatant of high speed centrifugation. Significant differences between the supernatant of high-speed centrifugation and the low-speed centrifugation fractions were also observed.

On the other hand, the comparison between the PI/S ratios obtained from uncentrifuged and pellet fractions show no significant differences, with the exception of the uncentrifuged fraction and the pellet of centrifugation at 10,000 × *g* 20 min.

Finally, a comparison between the PI/S ratios obtained from the supernatant and pellet fractions shows that there are significant differences between the high speed centrifugations.

Discussion

As far as phospholipid levels are concerned, the present results show that the relative *g*-forces applied to the amniotic fluid samples has an effect on phospholipid elimination in the supernatant obtained after centrifugation. These results are in agreement with those described by other authors (9, 17), except for the amount of phospholipid removed. LINDBACK and FRANTZ (9) suggest that centrifugation at 900 × *g* for 10 minutes removes about 50 % of the lecithin in amniotic fluid, whereas the same results were obtained in the present experiment when the amniotic fluid sample was centrifuged at 10,000 × *g* for 20 minutes.

Other authors report that towards the end of a pregnancy, the pellets obtained by centrifugation at 9,200 × *g* for 10 min (7) and 10,000 × *g* for 20 min respectively (4, 12) contain about 75 % of all the lecithin, phosphatidylglycerol being the second phospholipid. The lecithin contained in the sample was only about 50 % of the total phospholipid.

The presence of homogeneous subgroups in both supernatant and pellet fractions indicates that the distribution percentages of phospholipids behave differently according to the relative *g*-force used. Thus, in the supernatant fraction the phospholipid distribution percentage obtained by centrifugation at low speeds and with the uncentrifuged fraction hardly differs at all. This suggests that low-speed centrifugation removes cells and debris and shows that after centrifugation the supernatant has the same phospholipidic profile as the uncentrifuged amniotic fluid sample.

On the other hand, in the pellet fraction, the phospholipid distribution percentages of the aliquots centrifuged at high speed and the uncentrifuged aliquots only show slight differences. Therefore, the high-speed centrifugation of the amniotic fluid recovers in pellet almost all the pulmonary surfactant, with the same characteristics as the uncentrifuged amniotic fluid.

Finally, the comparison between the

phospholipid distribution percentages in the supernatant and pellet fraction is in agreement with the results obtained by EGBERTS and SOEDERHUIZEN (4).

With regard to the L/S, PG/S and PI/S ratios, it is shown that the three ratios behave differently because of the *g*-force. Whereas, LINDBACK and FRANTZ (9) observed that near the end of gestation significant variations in the L/S ratio obtained from supernatant fraction are produced no significant variations in the L/S ratio obtained from the supernatant fraction were observed in the present study. However, significant variations were observed with uncentrifuged and pellet fractions obtained at low centrifugation speeds. When a *g*-force of more than 10,000 × *g* was used, the results obtained for the L/S ratio were in agreement with those reported by OULTON (11) for a sample group with a gestational age of over 38 weeks. Unlike OULTON (11), a significant decrease in the L/S obtained from pellet fraction when low centrifugation speeds were used, was observed.

Centrifugation can also be used as a quick method for isolating surfactant from amniotic fluid. As HOOK *et al.* (7) and others (2, 13) suggest, the lamellar bodies originated by alveolar type II cells can be isolated by means of high speed centrifugation or by means of density gradient centrifugation. In this paper, we observe that the high-speed centrifugation pellet shows values of L/S, PG/S and PI/S ratios similar to those obtained from the uncentrifuged fraction. Although this does not mean that all the pulmonary surfactant has been sedimented, it does indicate a similarity in the features between the pellet and uncentrifuged amniotic fluid.

Resumen

El primer paso en la determinación de los fosfolípidos en el líquido amniótico es, generalmente, la eliminación por centrifugación de células y desechos. Se muestra que el sobrenadante obtenido tras centrifugación a baja velocidad presenta el mismo perfil fosfolipídico y valores similares de los índices L/S, PG/S y PI/S que la muestra de líquido amniótico sin centrifugar. También se indica que con la centrifugación a alta velocidad, parece recuperarse en el sedimento casi todo el surfactante pulmonar, manteniendo las mismas características que el líquido amniótico sin centrifugar.

Palabras clave: Líquido amniótico, Cociente lecitina/esfingomielina, Centrifugación, Fosfolípidos.

References

1. Cherayil, G. D., Wilkinson, E. J. and Burkow, H. I.: *Obstet. Gynecol.*, 50, 682-688, 1977.
2. Duck-Chong, C. G.: *Life Sci.*, 22, 2025-2030, 1978.
3. Duck-Chong, C. G., Brown, L. M. and Hensley, W. J.: *Clin. Chem.*, 27, 1424-1426, 1981.
4. Egberts, H. and Soederhuizen, P.: *Am. J. Obstet. Gynecol.*, 145, 889-890, 1983.
5. Fabre, E., Martínez, M. A., Ruiz, J., *et al.*: *Rev. esp. Fisiol.*, 42, 171-178, 1986.
6. Hallman, M., Merritt, T. A., Schneider, H. *et al.*: *Pediatrics*, 71, 473,482, 1983.
7. Hook, G., Gilmore, L., Trombopoulos, E., *et al.*: *Am. Rev. Resp. Dis.*, 117, 541-550, 1978.
8. Ivie, W. M., Novy, M. J., Reynolds, J. W. *et al.*: *Clin. Chem.*, 33, 24-31, 1987.
9. Lindback, T. and Frantz, T.: *Acta Obstet. Ginecol. Scand.*, 54, 101-103, 1975.
10. Lohninger, A., Salzer, H., Simbruner, G. *et al.*: *Clin. Chem.*, 29, 650-655, 1983.
11. Oulton, M.: *Am. J. Obstet. Gynecol.*, 135, 337-343, 1979.
12. Oulton, M., Martin, T. R., Faulkner, G. T., *et al.*: *Pediat. Res.*, 14, 722-728, 1980.
13. Oulton, M., Bent, A. E., Gray, J. H., *et al.*: *Am. J. Obstet. Gynecol.*, 142, 684-691, 1982.
14. Post, M., Batenburg, J. J., Schuurmans, E. A., *et al.*: *Exp. Lung. Res.*, 3, 17-28, 1982.
15. Ruiz, J.: Estudio de la concentración de los fosfolípidos en el líquido amniótico mediante HPTLC. Validación de algunos índices de predicción de la madurez pulmonar fetal. Tesis

doctoral. Facultad de Ciencias. Universidad de Zaragoza. Zaragoza, 1987.
16. Sokal, R. R. and Rohlf, F. J.: «Biometría, Principios y métodos estadísticos en la investigación biológica» H. Blume. Madrid, 1979.
17. Van Voorst tot Voorst, E. J.: *Clin. Chem.*, 26, 232-234, 1980.
18. Wastaff, T. T., Whyley, G. A. and Freedman, G.: *J. Obstet. Gynaecol. Br. Commonw.*, 81, 264-277, 1974.