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Effect of serum proteins on an exogenous pulmonary surfactant: ESR analysis of structural changes and their relation with surfactant activity

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ABSTRACT

The study of the structural changes in surfactant microviscosity and bilayer organization would help to understand the mechanisms by which surfactant could be inactivated by serum components. The in vitro effects of human serum, albumin and gamma-globulin on dynamic and structural properties of surfactant suspensions and their heavy fractions were evaluated by electronic spin resonance and surface tension measurements. Our results showed that albumin and serum modified the aggregation state, transforming the active into inactive subtype, but only serum decreased the fluidity in the polar region and inactivated surfactant. In contrast, albumin and gamma-globulin generated a greater proportion of fluid-like disordered phase, without loss of surface activity. Statistical analysis showed that surface activity correlated with the fluidity in the polar area but not with that in the hydrophobic region. We concluded that one or more serum components different from albumin or gamma-globulin cause a structural change in the surfactant bilayer, increasing the rigidity in the polar area, which would be critical for proper physiological activity.

1. Introduction

Pulmonary surfactant is a complex mixture of phospholipids (80–90%), neutral lipids (5–10%) and at least four specific proteins (5–10%) (SP-A, SP-B, SP-C and SP-D), which is synthesized and secreted by alveolar type II cells (Creuwels et al., 1997). Pulmonary surfactant forms a thin film on the liquid layer lining the alveolar air spaces. Rapid adsorption of pulmonary surfactant in the air–liquid interface is essential for normal breathing. Surfactant deficiency is the leading cause of acute infant respiratory distress syndrome (IRDS), and thus transbronchial surfactant application has become the gold standard for treating this disease (Clements and Avery, 1998). Exogenous surfactants often provide immediate relief of symptoms and improve oxygenation and gas exchange (Günther et al., 2001; Poulain and Clements, 1995; Veldhuizen et al., 1996). However, in certain diseases such as meconium aspiration syndrome and respiratory distress syndrome, substances not normally present in the alveolar fluid make the replacement therapy ineffective. Plasma proteins leaking into the airspaces inhibit the surfactant and raise the alveolar surface tension, a mechanism that might be of pathophysiological importance in adult respiratory distress syndrome (Dargaville and Mills, 2005; Finer, 2004; Poulain and Clements, 1995).

Different theories have been proposed to explain the mechanism of lung surfactant inactivation by serum proteins, but much remains unknown and efforts to rationally construct surfactant formulations appropriate for therapeutic uses in diseases in which the exogenous surfactants are currently ineffective have thus far been frustrating (Ochs et al., 2006; Matthay and Zemans, 2011).

It is known that, in vivo, two surfactant subtypes, with different physiological capabilities, may coexist. They can be separated by centrifugation and are known as subtype heavy or active (present in the pellet) and subtype light or inactive (present in the supernatant) (Gross et al., 2000; Ueda et al., 1994; Veldhuizen et al., 1993). The ratio between small and large surfactant aggregates increases in several types of lung injury (Maitra et al., 2002; Veldhuizen et al., 1996). We have previously found that although the presence of proteins causes disaggregation of the surfactant structures (i.e. induces the transformation of the active into the inactive subtype), this effect was not enough to inactivate the exogenous surfactant. Our results also indicate that surfactant inactivation by serum is probably due to a physical interaction between the surfactant and one or more serum components different from albumin (Martínez Sarrasague et al., 2011).

In the last years, several studies have provided evidence that a particular lateral structure occurs in native membranes of surfactant. Lipid phases, such as gel/fluid and fluid ordered/fluid disordered, coexist in these membranes, a coexistence that allows explaining specific physicochemical properties of the membrane (Nag et al., 2002; Scherfeld et al., 2003). de la Serna et al. (2004) have stated that even though lung surfactant has cholesterol

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concentration of up to 20–22 mol%, there is no clear understanding of how this molecule impacts the lateral structure of the native material (Hook et al., 1984; Lange and Steck, 1985).

Although the mechanism through which pulmonary surfactant spreads at the air–cell interface and the factors that influence that process are not yet resolved, it is known that fluidity is a major factor determining its ability to spread. The study of the structural changes in surfactant microviscosity and bilayer organization would help to understand the mechanisms by which surfactant is absorbed at the air–liquid interface and could be inactivated by serum components (Budai et al., 2003). Among spectroscopic techniques, electron spin resonance (ESR) has proved to be one of the most useful tools applicable in this field. Its dynamic sensitivity is optimally matched to the timescale of the rotational motions of the lipids in biological membranes (Ahlin et al., 2000; Budai et al., 2004; de Sousa Neto et al., 2009; Moser et al., 1989). ESR analysis allows quantifying both the stoichiometry and the selectivity of the interactions of different spin-labeled lipids with the protein and to study the dynamics of the protein-associated lipids. It also reflects changes in the layer mobility. Spin probes produce an ESR spectrum that yields information about the molecular environment of the label (Budai et al., 2003, 2004; Lange and Steck, 1985; Marsh and Horvath, 1998). In the present study, 5-doxyl stearic acid (5DSA) and 16-doxyl stearic acid (16DSA) were chosen as spin probes, which are oriented like the lipids in the surfactant layers. The radical in the 5- or 16-position of the alkyl chain can thus determine local motional profiles near the polar head group (5DSA) or at the end of the hydrophobic chain of the lipid (16DSA) (Nusair et al., 2012).

The analysis by ESR of the deleterious action of serum proteins might help to understand the nature of the *surfactant–protein* interaction, and to establish *structure–function* relations To achieve these objectives, the present study focused on evaluating the in vitro effects of human serum, bovine serum albumin (BSA) and gamma globulin on dynamic and structural properties of surfactant suspensions and their respective heavy subtypes.

2. Materials and methods

2.1. Exogenous pulmonary surfactant (EPS)

Prosurf is an active pharmaceutical ingredient (API) produced at industrial scale in Argentina (Nialtec S.A., Buenos Aires, Argentina). This API has been used by the pharmaceutical industry (GeMePe SA and Richet SA Laboratories) for the elaboration of therapeutic surfactants. Prosurf is a sterile chloroform solution that contains lipids and proteins extracted by means of bronchoalveolar lavage from bovine lungs, with slightly hypertonic solution (Hager and De Paoli, 2001). Prosurf is composed of phospholipids (PL) 94.8%; DPPC (dipalmitoylphosphatidylcholine) 46% of total phospholipids; cholesterol 4.4%; and proteins (SP-B, SP-C) 0.8%.

Chloroform was evaporated at low pressure and below 40 °C; the pellet was resuspended in sterile saline solution (0.9% NaCl) at 50 °C obtaining a final phospholipid concentration of 30 mg/ml. This final suspension, fractionated in sterile vials, constitutes the exogenous pulmonary surfactant (EPS).

To obtain EPS with different cholesterol concentrations, adequate aliquots of cholesterol chloroform solution were added to Prosurf before solvent was evaporated.

2.2. Exogenous proteins

Human serum was obtained from healthy adult donors. A pool of these human serum was used and the concentration of its main components were: total proteins 6.8 g/dl, albumin 3.7 g/dl, gamma globulin 0.9 g/dl, cholesterol 182 mg/dl, triglycerides 80 mg/dl and phospholipids 175 mg/dl.

Bovine serum albumin (BSA) and cholesterol were purchased from Sigma. Gamma globulin was purchased from Spectrum Chemical Mfg. Corp.

2.3. Chemicals

The spin derivatives of stearic acid, 5- and 16-doxyl stearic acids (5DSA and 16DSA, respectively), were purchased from Sigma.

2.4. Chemical determinations

Phospholipid and protein concentrations were measured by the Stewart (1980) and Lowry (1951) methods, respectively. Cholesterol was determined by the enzymatic method (Allain et al., 1974).

All the reagents were of analytical grade.

2.5. Samples

EPS was diluted with saline solution (0.9% NaCl) to a final PL concentration of 10 mg/ml. Samples added with proteins were incubated for 20 min at 37 °C with either BSA, gamma globulin or serum to obtain a final protein concentration of 5, 10 or 20 mg/ml. EPS (10 mg/ml of PL) without added proteins was used as control.

Serum, pure solutions of gamma globulin or albumin (4–60 mg/ml) and EPS (5–30 mg of PL/ml) were also used as controls in ESR analysis.

2.6. Heavy and light subtypes

2.6.1. Isolation

The surfactant subtypes were obtained by centrifugation at 10,000 × *g* for 20 min at room temperature. The supernatants containing the light subtype were separated, and the pellets with the heavy subtype were washed and resuspended to initial volume with saline solution (0.9% NaCl).

2.6.2. Quantification

The percentage of each subtype was estimated as: (PL concentration in the fraction/PL concentration in the non-fractionated EPS) × 100, measured by chemical determination, and by ESR, as described below.

2.7. Electronic spin resonance spectroscopy

The use of hydrophobic spin probes in the study of membranes is well known; however, the method has been little used to study EPS. ESR spectroscopy allows the investigation of structural and dynamic aspects.

The ESR spectrum of the nitroxyl ring in 5DSA and 16DSA is sensitive to the local host environment (Nusair et al., 2012).

2.7.1. ESR samples

An adequate quantity of the spin probe in ethanolic solution was dried onto the sides of the incubation tubes under a stream of N_2 gas. Samples were added and incubated with the spin probe for 10 min at room temperature. The final concentration of the spin probe was 1.74 μM. Each sample was then placed into a capillary tube, and each capillary was placed into a quartz ESR sample tube and centered in a rectangular microwave cavity for ESR measurement.

2.7.2. ESR measurements

ESR measurements were performed using a Bruker EMX-Plus, X-band spectrometer (Germany).

All ESR experiments were performed at 20 °C. Instrumental parameters were as follows: sweep width 100 Gauss, center field 4380 Gauss, time constant 5.12 ms, conversion time 5.12 ms, modulation amplitude 0.75 Gauss, modulation frequency 50 kHz, resolution 1024 points, microwave power 10 mW and microwave frequency 9.7 GHz.

2.7.3. ESR determination of the percentage of light and heavy subtypes

The intensity of the ESR spectrum was quantified by double integration (DI) of the signal of each sample. With the DI values, we estimated the proportion of phospholipids in the different EPS fractions. The percentage of each EPS subtype was estimated as: (DI in the fraction/DI in the non-fractionated EPS) × 100.

2.7.4. Determination of the order parameter (S)

Samples were labeled with 5DSA. For 5DSA spectra, $A_{\parallel}$ and $A_{\perp}$, the hyperfine splitting tensors parallel and perpendicular with respect to the perpendicular direction of the membrane plane, were estimated by the separation in gauss of the outermost ($2A_{\parallel}$) and innermost ($2A_{\perp}$) peaks of the ESR spectra (Fig. 1A and equation1). These are indicators of the rotational motional freedom of the phospholipid acyl chains parallel and perpendicular to the external magnetic field. The order parameter (S) represents the time averaged angular deviation of the stearic acid chain from its average orientation in the lipid bilayers. S is given by the ratio between the spectral anisotropy in the membranes ($A_{\parallel} - A_{\perp}$) and the maximum anisotropy obtained in a rigidly oriented system (defined by A_{xx}, A_{yy} and A_{zz} the principal values of the spin label tensor) and can be calculated from the ESR spectrum by the following expressions (Costanzo et al., 1994; Gaffney, 1976; Hubbell and McConnell, 1971).

$$S = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - 0.5(A_{xx} - A_{yy})} \times \frac{a'_0}{a_o} \quad (1)$$

where $A_{\perp}$ and $A_{\parallel}$ are the true hyperfine constant values. The polarity correction term a'_0/a_0 is introduced to take into account the hyperfine splitting dependence on the polarity of the label environment, where: $a_0 = (A_{\parallel} + 2A)/3$ and $a'_0 = (A_{xx} + A_{yy} + A_{zz})/3$. The value A' obtained from the spectrum has to be corrected to give the true A value. The correction is given for $S < 0.45$ by $A = A' + 0.8$.

Ordered phases, such as the gel or ordered liquid crystal phase, are characterized by values of S that approach 1, while the more fluid phases are characterized by S values that are significantly lower than 1. An increase in the S value is understood as a decrease in membrane fluidity.

When it is not possible to measure the hyperfine constant $A_{\perp}$, the value $A_{\parallel}$ is used instead of S to evaluate the rigidity of the environment. Although $2A_{\parallel}$ is less sensitive than S, an increase in $A_{\parallel}$ is also understood as an increase in the structural order in the membrane.

2.7.5. Determination of the rotational correlation time (τ)

The dynamic properties are related to the signal width (ΔH_0) and were estimated by the calculation of the rotation correlation time (τ). The ESR spectrum of 16DSA incorporated into the EPS reflects the motion of the phospholipid acyl chains. In this case, τ is the parameter that can be used to measure the motion of the phospholipid acyl chains near the hydrophobic end. This empirical parameter can be calculated by the equation from the classic formula given by Keith and Snipes (1974), and Morse et al. (1979) (Fig. 1B and Eq. (2)):

$$\tau = 6.5 \times 10^{10} \cdot \Delta H_0 \left(\left(\sqrt{\frac{h_0}{h_{-1}}} \right) - 1 \right) seg \quad (2)$$

where ΔH_0 is the width of the central peak (in Gauss) and h_0, and h_{-1} are the amplitude of the central and high field peaks, respectively. An increase in the τ value is understood as a decrease in the motional freedom of the probe in the hydrophobic region, due to an increase in the microviscosity of the environment. As in the case of $2A_{\parallel}$, τ increases as the environment fluidity decreases.

2.7.6. ESR determination of the S/W ratio

The ESR spectra of spin-labeled phospholipid bilayers are generally characterized by the coexistence of two spectral components with very different states of probe mobility. These components are commonly called strongly (S) and weakly (W) immobilized ones, and are associated with restricted and less restricted nitroxide motion. The S/W ratio of the low-field peaks represents the population ratio of the spin label in the two motional states (Fig. 1A). Although this ratio is empirical, it provides a convenient method for the comparison of the 5DSA spectra in different environments (Hayes and Jost, 1976).

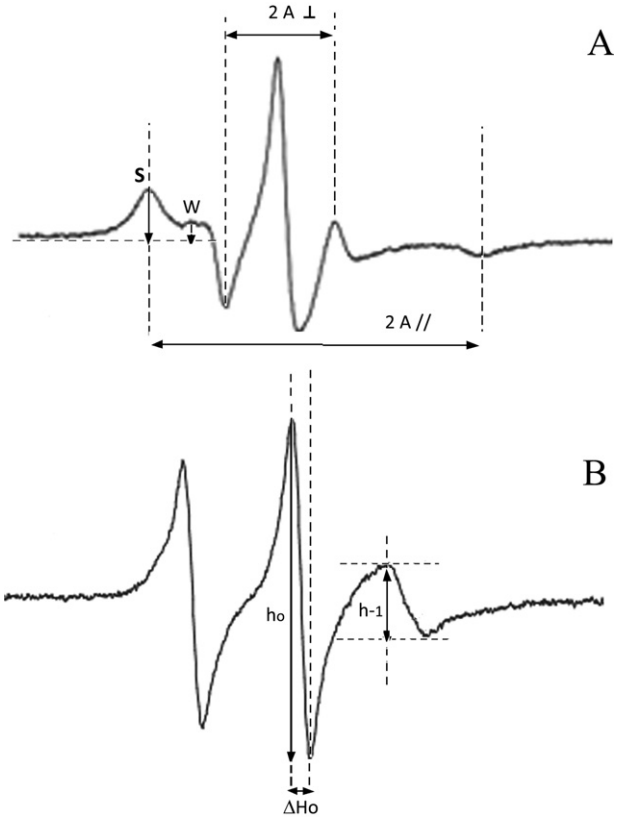

Fig. 1. ESR spectra of whole native surfactant (EPS) labeled with 5DSA (A) and 16DSA (B).

2.8. Surface tension measurements

Surface activity was measured with a pulsating bubble surfactometer (Electronetics, Buffalo, NY, USA), as described by Enhorning (1977). Pressure measurements were calibrated electronically according to the manufacturer's instructions and also checked with a water manometer. Briefly, 36 μl of EPS suspension were instilled into the sample chamber of the surfactometer at 37 °C. A bubble communicating with ambient air was created in the surfactant suspension and the surfactant was allowed to adsorb to the air/liquid interface for 10 s. After this time the bubble was pulsated

at 20 oscillations per min between a minimum radius of 0.4 mm and a maximum radius of 0.55 mm.

2.8.1. Surface tension (ST)

This parameter represents the tendency of liquids to reduce their exposed surface to the smallest possible area. For its determination, with the pulsating bubble surfactometer, the pressure across the bubble was measured by a pressure transducer and the ST calculated using the La Place equation: $P = 2ST/r$, where P is the inflating pressure, and r is the radius of the bubble. The minimum value of ST at 200 cycles was determined. Each sample was measured five times, and the results are expressed as the mean ± SD. For the analysis of the result, a ST limit value of 5 mN/m was considered for a proper surfactant activity.

2.8.2. Percentage reduction in bubble surface area (A_{10})

The percentage reduction in bubble surface area (SA) from its maximum value to that required for the surface tension to reach a value of 10 mN/m was calculated after 100 cycles of bubble cycling. Then, $A_{10} = [(\text{max SA} - \text{SA } 10\,\text{mN/m})/\text{max SA}] \times 100\%$. A_{10} is an indicator of dynamic film compressibility. Films with low compressibility cause a large decrease in surface tension with a relatively small decrease in SA. If the surface tension of the surfactant suspension did not reach 10 mN/m, then the actual, although unmeasured, A_{10} values should have been >47% because that is the difference in SA between the maximum and the minimum bubble areas in the electronetics pulsating bubble surfactometer.

2.9. Experimental data acquisition and statistical analysis

All measurements were repeated with several independent surfactant batches that showed similar qualitative behavior.

The results shown are the average of at least five separated experiments. Data are expressed as the mean ± SD.

Statistical analyses were performed using analysis by one-way repeated measures of variance (ANOVA), and comparisons between pairs of groups were made using the Bonferroni test.

The linear relation between two variables was determined using the Pearson correlation test.

3. Results

3.1. ESR spectral parameters for EPS, serum and protein solutions

To evaluate the ESR spectral parameters of the whole native surfactant and pure proteins, the samples were labeled with 5DSA and 16DSA. Since the main components of EPS are phospholipids (90–95%), the long hydrophobic tail of doxyl stearic acids favors the intercalation of the molecule into the hydrophobic regions of EPS, with the spin probe alignment similar to that of the fatty acid chains of the EPS phospholipids. The ESR spectrum yields information about the molecular environment of the spin probe.

Fig. 2 shows the spectra of BSA, serum, and whole native surfactant labeled with 5DSA and 16DSA. The ESR spectra of 5DSA incorporated into the EPS lipid layers, BSA and serum proteins show an anisotropic motion, indicating that the probe movement is highly restrained.

The ESR spectrum of 16DSA incorporated into the EPS reflects an isotropic motion of the PL acyl chain. In this case, the rotational correlation time (τ) is the parameter that can be used to measure the motion of the PL acyl chains near the hydrophobic end. On the other hand, the spectra of 16DSA incorporated into BSA and serum proteins were characteristic of a highly immobilized spin label. The spectra of gamma pure solution labeled with 5DSA and 16DSA corresponded to a free spin label, because this type of proteins cannot be labeled with these spin probes.

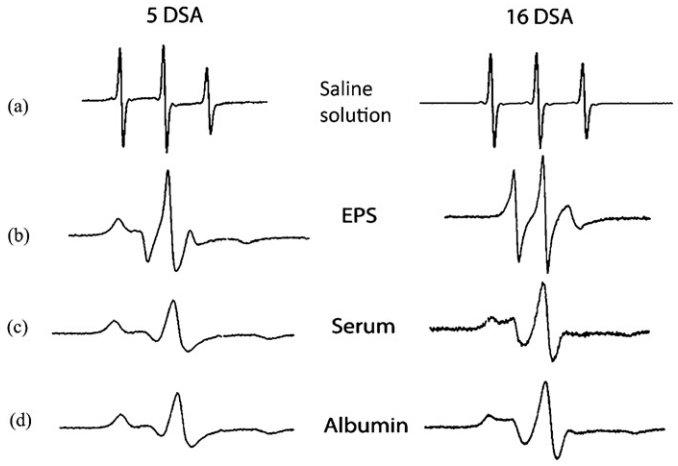

Fig. 2. ESR spectra of (a) 5DSA and 16DSA in saline solution, and (b–d) whole native surfactant (EPS), serum and pure BSA solution labeled with 5DSA and 16DSA.

Table 1 shows the $2A_{\parallel}$ and S/W calculated from the 5DSA spectra and τ calculated from the 16DSA spectra of whole native surfactant and pure solutions of serum or BSA. In samples of pure serum or BSA, it was not possible to measure the values of $2A$, and thus $2A_{\parallel}$ is shown instead of S (as explained in Section 2.7.4). This parameter reflects the rotational freedom of PL close to the polar head groups in the layer. An increase in $2A_{\parallel}$ can be associated with a decrease in bilayer fluidity. The $2A_{\parallel}$ values shown in Table 1 indicate that the probe incorporated into BSA and/or other serum component has a more restricted anisotropic movement than the probe incorporated into the surfactant bilayer. Furthermore, the S/W ratio of protein solutions was almost 100% higher than the S/W ratio of EPS. The increase in this ratio in protein solutions can be understood as a large population of the probe strongly immobilized. No significant changes were found in $2A_{\parallel}$ and S/W between BSA and serum solutions. The rotational correlation time of serum and BSA solutions could not be calculated due to the anisotropic characteristics of the spectra. The $2A_{\parallel}$, S/W and τ values of gamma globulin pure solution could not be calculated because the 5DSA and 16DSA spectra in this solution corresponded to the free spin label. All the spectroscopic parameters were neither dependent on the concentration of phospholipids (5–30 mg of PL/ml) for EPS nor dependent on the concentration of protein (4–60 mg/ml) for pure protein solutions (data not shown).

3.2. EPS added with proteins

3.2.1. Heavy and light subtypes in the presence of proteins

Since the spin probe binds to both PL and serum proteins, it might be expected that the spectrum obtained from a mixture of surfactant with serum, in the absence of interaction, would be the sum of the individual spectra probe incorporated in each of the components of the mixture. Although the probe was originally incorporated into the surfactant, it shares the possibility that it would migrate to the protein fraction by a partition process. In order to verify or discard the probe partition between PL and proteins, the percentage of heavy fractions obtained in the presence of BSA, serum or gamma globulins was analyzed by ESR and by PL chemical determination (Fig. 3A and B).

The amount of the heavy subtype calculated by the chemical method decreased as the concentration of added serum proteins or BSA increased, but no significant changes were found in the presence of gamma globulin. Significant differences were found between controls and each SP or BSA concentration tested ($p < 0.01$). The statistical analysis also showed significant

Table 1
$2A_{\parallel}$ and S/W calculated from 5DSA spectra and rotational correlation time (τ) calculated from 16DSA spectra of whole native surfactant (PL 10 mg/ml), and serum (20 mg/ml of total proteins) and BSA solution (20 mg/ml).

	EPS (PL 10 mg/ml) (N = 25)	Serum (SP 20 mg/ml) (N = 8)	BSA (20 mg/ml) (N = 8)
5 DSA			
$2A_{\parallel}$ (Gauss)	53 ± 1	65 ± 1	64 ± 1
S/W	2.4 ± 0.2	4.2 ± 0.5	4.5 ± 0.5
16 DSA			
τ (10^{-10} s)	0.39 ± 0.03	–[a]	–[a]

[a] The τ values could not be calculated due to the anisotropic characteristics of the spectrum.

differences between 5 and 10 mg/ml of added protein ($p < 0.01$) but not between 10 and 20 mg/ml for both SP and BSA. When comparing the amount of heavy fraction obtained in the presence of equal concentrations of SP or BSA, no significant differences were found between them. Similar profiles were obtained when the amount of heavy fractions was calculated by ESR. This fact is relevant to discard the probe partition. If there is such a partition, the spin probe/PL ratio in the supernatant, where almost the totality of the added protein is (Martínez Sarrasague et al., 2011), should increase as the total added protein increases. An opposite approach would be valid for the heavy fraction. If the spin probe migrated to the added protein, the percentage of precipitated fraction calculated from the integrals of the spectra would be lower (Fig. 3A, ESR) than that estimated by the PL concentration measurements (Fig. 3B, Stewart method). However, the percentage of heavy fractions obtained with the different protein concentrations tested, evaluated by measuring the concentration of the spin probe or the concentration of PL, was practically the same. Therefore, we can say that the spin probe incorporated into the EPS does not migrate to the added protein. This fact is very important in the interpretation of the spectra; taking this into account, any spectral change may allow us to infer a structural change in the surfactant.

Furthermore, the results shown in Fig. 3 indicate that both serum and BSA can induce the transformation of the active into the inactive subtype, causing the disaggregation of the macro-structure of the surfactant.

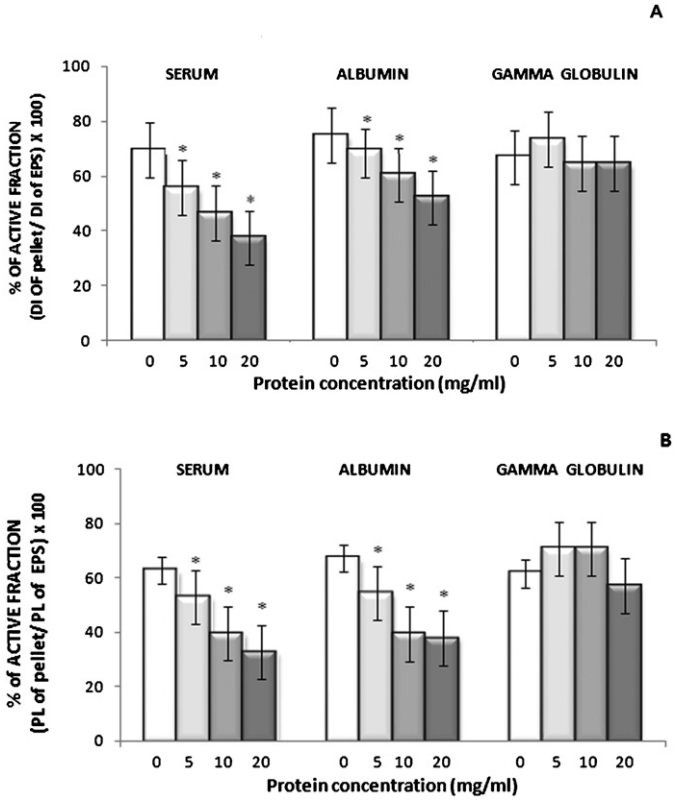

Fig. 3. (A and B) Amount of the heavy subtype obtained in function of the added protein concentration. (A) Percentage of active fraction calculated by ESR, expressed as % of double integration of the spectrum of pellet/double integration of the spectrum of whole EPS. (B) Percentage of active fraction calculated by chemical method, expressed as % of PL of pellet/PL of whole EPS. EPS (10 mg/ml of PL) was mixed with serum, BSA or gamma globulin, reaching a final protein concentration of 5, 10 or 20 mg/ml. The surfactant without added protein was used as control. Data are represented as the mean ± SD. Statistically significant compare to control: $^{*}p < 0.01$.

3.2.2. *ESR parameters of EPS added with serum, BSA or gamma globulin*

Fig. 4 shows the rotational correlation time (τ) obtained from the spectra of EPS labeled with 16DSA, with and without exogenous proteins. The rotational correlation time increased in the presence of serum or albumin ($p < 0.01$) and this increase was directly dependent on the concentration of added protein. This increase in the correlation time caused by the presence of albumin or serum can be understood as a slower movement of the probe in the hydrophobic core due to a decrease in the fluidity of the environment. In contrast, the addition of gamma globulins did not change this parameter significantly (Fig. 4C). In all cases, the correlation time of the heavy fractions obtained from the samples added with protein did not show significant differences respect to the heavy fraction control. Further, the spectra of these fractions showed values of τ similar to those of the whole native surfactant.

Fig. 5 shows the order parameter (S) obtained from the spectra of EPS labeled with 5DSA, with and without exogenous proteins. As mentioned above, the value of S reflects the rotational motional freedom of the phospholipids close to the polar head groups in the bilayer. When serum was added to EPS, S increased as serum proteins increased (Fig. 5A) ($p < 0.01$) but did not change significantly with the addition of albumin or gamma globulin for all protein concentrations tested (Fig. 5B and C).

Similarly to that observed with the correlation time, the S of the heavy fractions obtained from the samples added with serum did not show significant differences respect to the heavy fraction control or the whole native control surfactant (data not shown).

A common feature of almost all lung surfactants and model mixtures is the coexistence between a semi-crystalline, fluid-ordered phase and a fluid disordered phase (Alonso et al., 2004). The spin probe incorporated into these phases has different rotational motion and consequently yields a spectrum with differences at the low-field peak. The S/W ratio of the low-field peaks represents the population ratio of the spin label in the strong and weak motional states (Hayes and Jost, 1976). The increase in this ratio could be understood as an increase in the fluid-ordered phase where the spin probe is more immobilized. Fig. 6A shows the S/W ratio obtained from the 5DSA spectra of whole native surfactant, and Fig. 6B shows the ratio of their respective heavy fractions. When serum was added to whole EPS, the S/W ratio did not change significantly at any concentration of serum protein tested. However, the addition of BSA decreased the S/W ratio at all the concentrations tested ($p < 0.05$). No significant differences were found between 5, 10, and 20 mg/ml

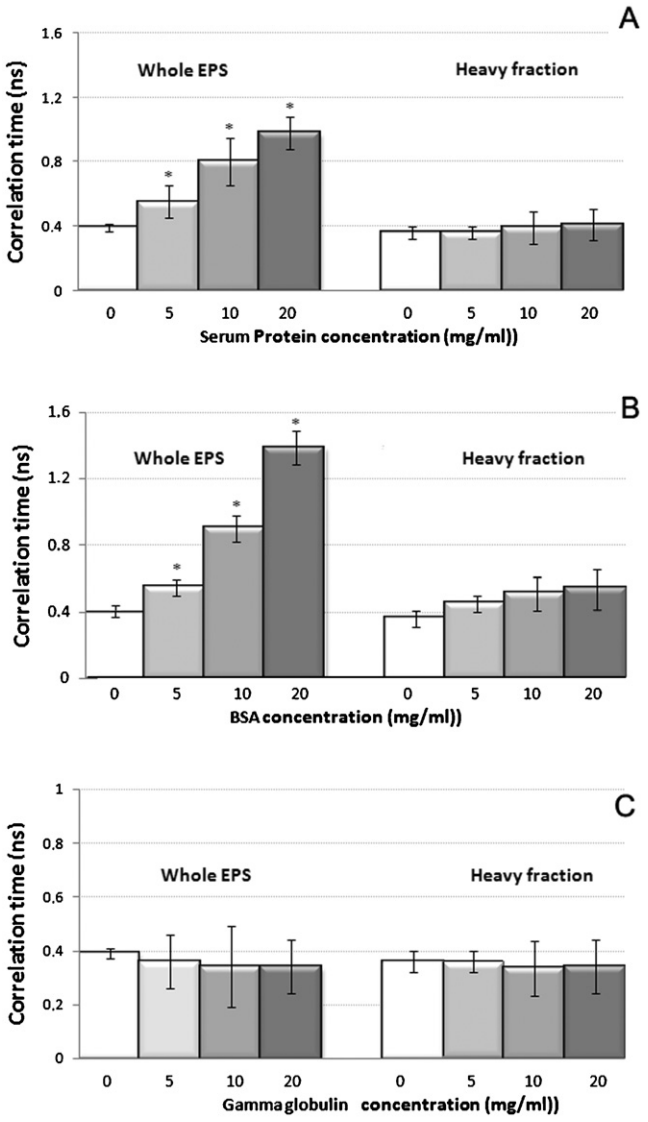

Fig. 4. Rotational correlation time (τ) obtained from the spectra of EPS (10 mg/ml of PL) labeled with 16DSA (1.74 μM) in presence and absence of exogenous proteins and their respective heavy fractions. (A) EPS added with serum; (B) EPS added with BSA; (C) EPS added with gamma globulin. The surfactant without added protein was used as control. Data are represented as the mean ± SD. Statistically significant increase compare to control: $^{*}p < 0.01$.

of added BSA. In the presence of gamma globulin, this ratio also decreased and significant differences were found between control and 10 and 20 mg/ml of this added protein ($p < 0.05$).

In all the cases, the S/W ratio of heavy fractions obtained from samples added with proteins did not show significant differences respect to the heavy fraction control.

It is known that total serum is much more than just proteins. However, plasma proteins act as carriers of most non-hydrophilic serum components. Among these components, cholesterol is widely recognized as a substance that alters the structure and surface activity of surfactant (Blanco and Pérez-Gil, 2007; Daniels and Orgeig, 2003). Although cholesterol accounts for the main fraction of neutral lipids in pulmonary surfactant, its role in the surfactant remains unknown (Diemel et al., 2007; Discher et al., 2002; Keating et al., 2007; Markart et al., 2007).

In order to evaluate if the serum cholesterol was responsible for the changes in the spectral parameters of EPS added with serum, whole native surfactants with different final cholesterol concentrations (0.7–2.5 mg/ml) were prepared. The order parameter S and the correlation time of these samples were evaluated. The correlation time of all samples showed no significant differences between them and the control. Order parameter S increased with cholesterol concentration, reaching a maximum value of 0.62 ± 0.01 for a cholesterol final concentration of 2.5 mg/ml (Fig. 5D). This value was lower than the S value (0.66 ± 0.01) obtained for the surfactant with the maximum amount of serum added (protein concentration of 20 mg/ml and final cholesterol concentration of 1.3 mg/ml).

Furthermore, to evaluate if serum transfers cholesterol to the surfactant, the amount of cholesterol was evaluated in heavy fractions. The heavy fraction obtained from EPS incubated with serum (20 mg/ml of proteins) had about 5% more cholesterol than the heavy fraction of the whole native surfactant (data not shown).

These results demonstrate that cholesterol was not responsible for the changes induced by serum in the spectral parameters, because (a) surfactant preparations with an amount of cholesterol equivalent to the one that would be obtained if the serum transferred all its cholesterol to the surfactant showed a lower decrease in the fluidity and (b) the heavy fractions obtained from surfactant added with serum did not show a significant increase in the cholesterol contained or in the order parameter S.

3.2.3. Effects of proteins on EPS surface properties

Table 2 shows that the addition of serum to EPS increased ST and ΔA_{10} as serum protein concentrations increased, reaching values higher than those compatible with biological activity at 10 and 20 mg/ml of protein. The heavy fractions of samples added with serum did not show ΔA_{10} and ST values significantly different from those of the heavy subtype control.

On the other hand, when BSA was added to EPS, ΔA_{10} and ST of EPS and their respective heavy fractions did not change significantly, at any protein concentration. The addition of gamma globulin to EPS neither changed these parameters significantly at any of the concentrations tested.

Furthermore, all the supernatants showed ΔA_{10} values higher than 47% and ST higher than 18 mN/m, confirming that the light fraction is devoid of surfactant activity.

3.2.4. Statistical correlations

To determine possible relations between the spectroscopic parameters evaluated and surfactant activity, linear regression studies were carried out for EPS added with proteins and EPS control. The increase of the order parameter S was associated directly with the increase in the surface tension ($r = 0.937$; $p < 0.01$), and with the increase in ΔA_{10} ($r = 0.891$; $p < 0.01$). On the other hand, neither the fluidity in the hydrophobic region nor the S/W ratio correlated with the surface tension parameters.

The increase in the bilayer order and the increase in the surface tension correlated directly with the amount of protein added in EPS added with serum ($r = 0.963$; $p < 0.05$), but not in those added with albumin or gamma globulin.

The decrease in the fluidity in the hydrophobic region was associated directly with the amount of protein added for serum ($r = 0.920$; $p < 0.01$) and BSA ($r = 0.981$; $p < 0.01$).

4. Discussion

The composition of lung surfactant and its surface-active behavior are the most important properties for its physiological function.

In a previous study, we have reported that serum affected the surface tension, causing the total loss of surfactant activity at protein concentrations higher than 5 mg/ml. Further, both serum and albumin produce the disaggregation of the surfactant structures, increasing the quantity of the inactive subtype. A priori, it could

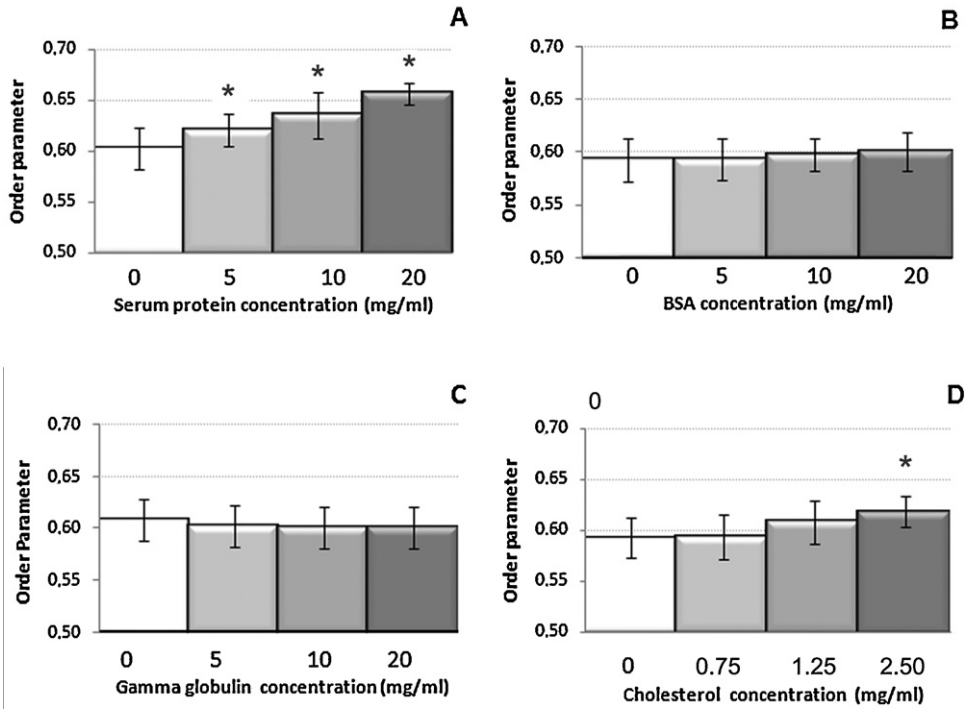

Fig. 5. Order parameter (S) obtained from the spectra of EPS (10 mg/ml of PL) labeled with 5DSA (1.74 μM), in presence and absence of exogenous proteins (A) EPS added with serum; (B) EPS added with BSA; (C) EPS added with gamma globulin; (D) EPS manufactured with different final cholesterol concentration. The surfactant without added protein or cholesterol was used as control. Data are represented as the mean ± SD. Statistically significant increase compare to control: $^{*}p < 0.01$.

be thought that the deleterious effect of proteins on the surface tension might be explained by the result of the increase in the inactive light fraction, but the presence of albumin did not affect the surfactant activity, although it induced the transformation of the active into the inactive form. Considering these results, we conclude that the disaggregation would not be the primary cause of the surfactant inactivation and the EPS inactivation is probably due to a physical interaction between the surfactant and one or more serum components, different from albumin (Martínez Sarrasague et al., 2011). Taking these findings into account, we designed the present study with the aim to highlight the existence of possible structural changes due to the physical interaction between surfactant and serum components, and their relation with the surfactant activity.

The study of the structural changes in surfactant microviscosity and bilayer organization would help to understand the mechanisms by which surfactant is adsorbed at the air–liquid interface and could be inactivated by serum components (Alonso et al., 2004; Pérez-Gil, 2008). Bilayer fluidity reflects the order and dynamics of phospholipid alkyl changes in the layer and is mainly dependent on its composition. It has been demonstrated that the proteins–phospholipids interaction modifies the order and mobility of phospholipids (Jost et al., 1973). The ESR techniques are used to monitor the molecular dynamics of lipids and have been largely used to assess the mobility and conformational changes of the bilayer structure.

In this study, the analysis of the spectra obtained with surfactant added with serum showed an increase in the correlation time and in the order parameter, showing that one or more serum components interacting with phospholipids would generate an increased rigidity of the bilayer in both the hydrophobic core and in the proximity to the polar zone. The addition of albumin to the surfactant modified only the fluidity in the hydrophobic zone, but did not cause alteration in the polar zone order, so it could be argued that the increased rigidity in the hydrophobic area observed in the presence of serum could be due to the albumin present in it, while another

Table 2
Surface tension (ST) and percentage reduction in bubble surface area (ΔA_{10}) of the surfactant and its subtypes. EPS (10 mg/ml of PL) was mixed with serum BSA, or gamma globulin (GG), reaching a final protein concentration of 5, 10 or 20 mg/ml. Subtypes were obtained by centrifugation. ST was measured with a pulsating bubble surfactometer at 37 °C.

	EPS		Heavy subtype		Light subtype	
	ΔA_{10} (%)	ST (mN/m)	ΔA_{10} (%)	ST (mN/m)	ΔA_{10} (%)	ST (mN/m)
Control EPS	34.5 ± 3.3	2.7 ± 1.8	34.5 ± 3.0	4.5 ± 2.2	>47	18.4 ± 3.4
EPS + SP 5 mg/ml	41.7 ± 4.3	6.8 ± 3.8	28.3 ± 4.0*	5.5 ± 4.0*	>47	20.3 ± 4.7
EPS + SP 10 mg/ml	>47	11.6 ± 4.4	31.8 ± 4.1*	5.3 ± 3.2*	>47	20.4 ± 3.4
EPS + SP 20 mg/ml	>47	20.0 ± 5.7	41.7 ± 5.8*	9.5 ± 5.4*	>47	20.2 ± 2.6
EPS + BSA 5 mg/ml	30.6 ± 4.1	4.3 ± 2.2	33.6 ± 3.8	4.2 ± 2.0	>47	20.7 ± 2.6
EPS + BSA 10 mg/ml	37.7 ± 3.9	3.5 ± 1.9	34.8 ± 4.5	4.4 ± 1.9	>47	20.7 ± 4.1
EPS + BSA 20 mg/ml	34.4 ± 4.3	4.5 ± 2.0	34.2 ± 3.9	5.1 ± 2.2	>47	19.5 ± 4.5
EPS + GG 5 mg/ml	32.0 ± 4.1	5.3 ± 1.7	34.5 ± 3.8	5.0 ± 2.0	>47	22.7 ± 2.6
EPS + GG 10 mg/ml	39.4 ± 3.9	4.5 ± 1.2	41.2 ± 4.5	4.1 ± 1.2	>47	23.7 ± 3.8
EPS + GG 20 mg/ml	37.1 ± 4.3	4.8 ± 2.1	40.5 ± 3.9	5.1 ± 2.1	>47	20.5 ± 4.8

* Statistically significant different from the corresponding non-fractionated sample: $p < 0.01$.

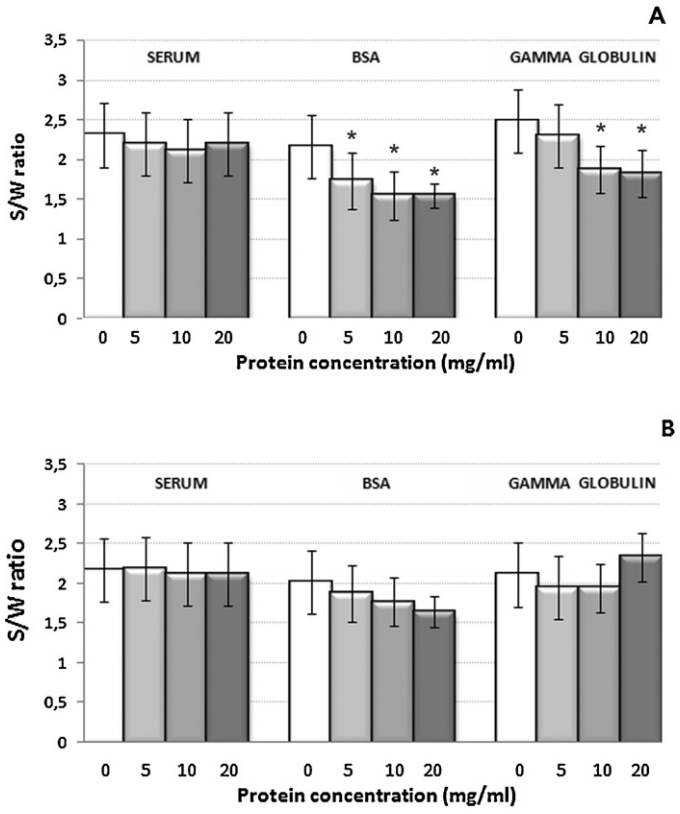

Fig. 6. (A) S/W ratio obtained from 5DSA spectra of whole native surfactant added with serum, albumin or gamma globulin; and (B) S/W ratio of their respective heavy fraction. The surfactant without added protein was used as control. Data are represented as the mean ± SD. Statistically significant differences: $^{*}p < 0.05$.

serum component would be responsible for the decrease in fluidity in the polar area.

Furthermore, considering that albumin did not alter the dynamic surface tension and compressibility (ΔA_{10}) at any of the concentrations tested, but that the serum did, it might be that the increased rigidity in the hydrophobic region does not affect surfactant activity, while the fluidity in the polar area would be critical for proper physiological activity. This last assumption is supported by the correlation between the results of the order parameter and the tension activity obtained by statistical analysis. In addition, neither albumin nor gamma globulin generated changes in surface tension or in the order parameter *S*, confirming the link between the fluidity of the polar zone of the lipid bilayer and the surfactant activity.

It has been reported that bilayer membranes made of native pulmonary surfactant could be organized heterogeneously with the coexistence of two distinct fluid phases (fluid ordered and fluid disordered-like phases). The particular lateral organization of lipids and proteins in surfactant membranes would be essential to support not only a rapid interfacial adsorption to equilibrium surface pressures but for the formation of well-defined surface-associated structures in order to support a proper dynamic surface behavior when the surfactant material is subjected to rapid dynamic cycling. Experiments using fluorescent labeled proteins SP-B and SP-C have shown that these proteins are located exclusively in the fluid disordered-like phase (Korlach et al., 1999). These proteins catalyze the transfer of the surfactant molecules from the bilayers to the interface, stabilize the monolayer formed, and favor the spreading process. It is thus possible that a change in the lipids order in the area where these proteins are anchored may result in a change in their structure, thus affecting the important role that they have in surfactant activity. Although this assumption still has to be explored in detail, our results clearly indicate that the change in fluidity in the polar region affects surfactant activity and that less important would be the fluidity in the hydrophobic zone since the surfactant does not act as a bilayer but becomes a monolayer to expand-positioned at the air–liquid interface.

The decrease in the S/W ratio on the EPS added with albumin or gamma globulin indicates that these proteins interact with the surfactant, generating a larger proportion of fluid-like disordered phase. This is further evidence that neither albumin nor gamma globulin would be responsible for the inactivation of the surfactant, because the increase in fluid-disordered-like phases would allow better spreading and proper dynamic surface behavior. The serum, by contrast, did not change this relationship, although it contains albumin and gamma globulin. It is possible that some other serum component generates an increased proportion of regions with the spin probe more immobilized (fluid ordered phase), so as to offset the decrease in S/W caused by albumin and gamma globulin. This assumption is supported by the increase in the order in the polar area observed by the addition of serum to the surfactant. These evidences strengthen the hypothesis that some serum component is responsible for the increased rigidity of the bilayer, which would trigger the loss of the biological activity.

It is known that total serum is much more than just proteins; however, plasma proteins act as carriers of most non-hydrophilic serum components. The contribution of other serum non-proteinaceous components (lipids and particularly cholesterol) to the aggregation state and the surface activity of surfactant has not been completely elucidated. It may be that these serum components are potentially transferred into surfactant complexes and originate part of the alterations described. Among these components, cholesterol is widely recognized as a substance that alters the structure and surface activity of surfactant. However, the conclusions about the role of cholesterol in surfactant function are contradictory.

In our experiments, surfactant prepared with increasing amounts of cholesterol showed a decrease in the fluidity in the polar region of the bilayer, an effect also observed with the addition of serum. A priori, we may hypothesize that this change in the fluidity obtained with serum could be due to a transfer of cholesterol from serum to surfactant membranes. However, this hypothesis must be discarded since the decrease in the fluidity of the surfactant, caused by cholesterol concentrations even higher than those which could be transferred from serum, was lower than that due to the serum itself. Thus, it could be concluded that the decreased fluidity cannot be related only to cholesterol in serum, and that another serum component would be responsible for this effect. Furthermore, if a significant amount of cholesterol was transferred from serum, the heavy fractions obtained post-incubation with serum should be enriched in this neutral lipid and would show a greater rigidity. Here, we found clear evidence that this transfer is not significant.

The spectral parameter *S*, the rotational correlation time τ and the S/W ratio of all heavy fractions obtained from surfactant added with different concentrations of serum, albumin and gamma globulin showed no significant differences regarding the respective heavy fraction control or the whole EPS. This indicates that the effect generated by the exogenous proteins on the order and fluidity of the surfactant disappeared when the added proteins were removed. Moreover, when serum was present, EPS showed high values of surface tension, but after the lavages, the heavy fraction obtained recovered the surface activity. We obtained evidence that although the addition of serum to EPS induces the conversion of the heavy to the light fraction, the smaller amount of the active subtype obtained in this condition has not been permanently modified. These findings confirm that surfactant inactivation by serum is due to a physical interaction between the surfactant and one or

more serum components, and that this is reversed when they are removed.

It has been proposed that the mechanism of inactivation by serum does not involve alterations in the surfactant bilayer bulk phase, and that it may come from a competition between serum proteins and surfactant for the available air-water interface. Albumin and other serum proteins diffuse much faster than aggregates of surfactant in the race to an uncoated interface (Nag et al., 2010; Zasadzinski et al., 2010). In this work, we obtained unambiguous evidence that one or more serum components different from albumin, gamma globulin and cholesterol cause a structural change in the surfactant bilayer phase. We believe that, regardless of this competition, the serum leads to increased structural rigidity in the polar area of the PL, which compromises the surfactant performance.

5. Conclusions

In summary, our results show that (a) the addition of albumin or serum to EPS induces the transformation of the active into the inactive subtype, but that this effect is not caused by gamma globulin; (b) albumin and gamma globulin generate greater proportion of fluid-like disordered phase, without the loss of surface activity; (c) the serum modified aggregation state increases the inactive subtype, decreases the fluidity in the polar region and inactivates EPS; (d) a serum component, different from albumin, gamma globulin and cholesterol, may be responsible for the loss of surfactant activity; (e) there is a correlation between the fluidity in the polar region of the surfactant bilayer and the surface activity.

Our results contribute to a better understanding of the structure-function relations in an exogenous pulmonary surfactant which may help in the design and production of more efficient clinical surfactants. Further studies will be carried out to find out which serum fractions (proteinaceous or non-proteinaceous) different from albumin, gamma globulin and cholesterol are responsible for the inactivation of EPS suspensions.

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