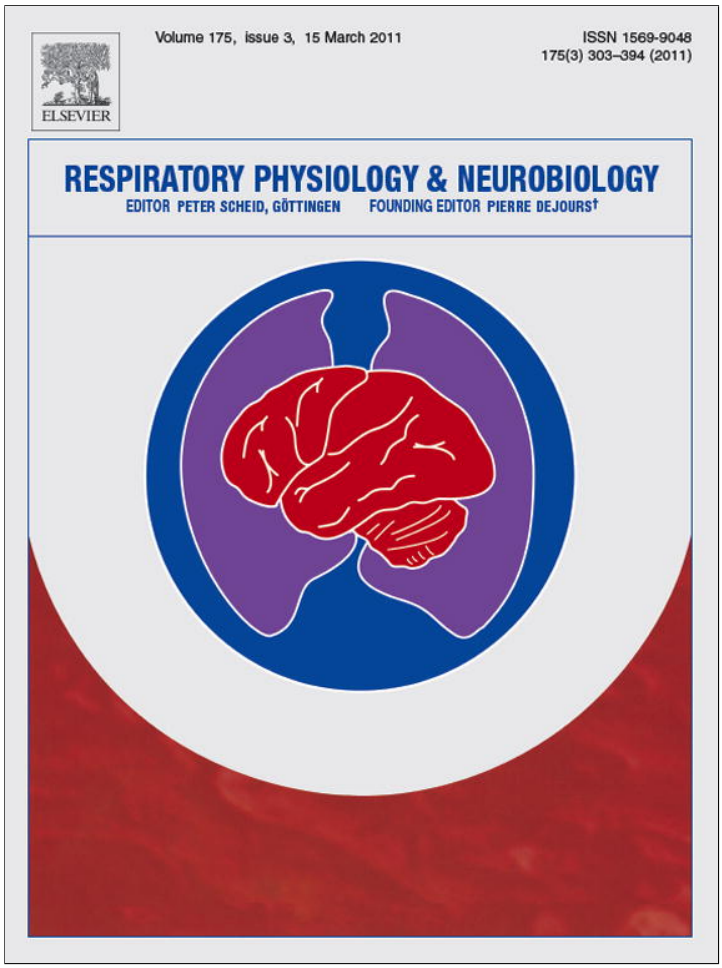

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Influence of serum protein and albumin addition on the structure and activity of an exogenous pulmonary surfactant

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ABSTRACT

The comparative analysis of the deleterious action of albumin and total serum proteins (SP) might help to understand the nature of the interaction surfactant – SP.

This study evaluated the effects of serum proteins and albumin on bulk shear viscosity, surface tension, surface area reduction, and the ratio between the light and heavy subtypes of surfactant suspensions. Our results showed a correlation between the bulk viscosity and aggregation degree of surfactant suspensions. The addition of albumin or SP induced the transformation from the heavy to the light subtype, reducing the viscosity. SP caused disaggregation and inactivation, whereas albumin caused only disaggregation without loss of surface activity. When SP were removed, the heavy fraction obtained recovered its surface activity. We conclude that the disaggregation may not be the primary cause for the loss of surface activity. Surfactant inactivation by a serum component, different from albumin, would be probably due to a physical interaction, a phenomenon that is reversed when SP are removed.

1. Introduction

Lung surfactant deficiency has been established as the primary cause of respiratory failure in infant respiratory distress syndrome (IRDS), and transbronchial application of surfactant preparations has become the gold standard for the treatment of this disorder (Clements and Avery, 1998). Such surfactants often provide immediate relief from symptoms and improved oxygenation and gas exchange (Günther et al., 2001; Poulain and Clements, 1995; Veldhuizen et al., 1996). However, in some cases such as that of meconium aspiration syndrome, substances that not normally present in the alveolar fluid inactivate the surfactant, leading to a less effective surfactant therapy (Dargaville and Mills, 2005; Finer, 2004; Poulain and Clements, 1995).

The bronchial fluid extracted from patients with acute respiratory distress syndrome (ARDS), presents increased levels of serum and inflammatory proteins. The concentration ratio of soluble proteins/surfactant in lung lavages correlates with the severity and outcome in ARDS (Lauer et al., 2006; Taut et al., 2008). Lung lavages from ARDS patients also have a decreased surface activity in terms both of the lower speed with which they adsorb to an exposed air–water interface and of the minimum surface tension at a given compression (Creuwels et al., 1997). Biophysical studies of lung surfactant mixed with serum proteins have shown that, at sufficiently high protein concentrations, the activity of the surfactant decreases (Lu et al., 2000; Nag et al., 2007; Seeger et al., 1993).

Upon the inspiratory stretch of the alveolar cell layer, alveolar type II pneumocytes secrete lamellar bodies containing surfactant in the alveolar hypophase, which are then reorganized into the highly surface-active tubular myelin and large multilamellar vesicles. Lamellar bodies, tubular myelin, and large multilamellar vesicles are called large surfactant aggregates (Günther et al., 2001). The pulmonary surfactant obtained from lung lavages can be separated by differential centrifugation into two distinct subfractions known as the active heavy subtype (large surfactant aggregates) and the light subtype (small surfactant aggregates with less surface activity) (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., 1993). When serum proteins (SP) are added to the heavy subtype surfactant, the conversion rate from heavy to light subtypes is accelerated. Light subtypes obtained in vitro do not have surfactant activity and are similar to in vivo light forms (Gross et al., 2000).

Numerous studies using different trademarks of surfactant and bovine lung extract surfactant (BLES) have been performed to assess the effect of proteins on surfactant activity. The results cover a wide range of conclusions, depending on the inactivation model proposed and the methods used to analyze the inactivation. Because of the heterogeneity of results, it is not possible to extrapolate the conclusions (Ainsworth and Milligan, 2002; Blanco and

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Pérez-Gil, 2007; Braun et al., 2007; Playfor and Nootigattu, 2006; Ramanathan, 2009a; Taeusch et al., 2005).

Albumin is the most abundant serum protein and its interaction with lung surfactant has been the subject of many studies. Larsson et al. (2006) have found that albumin disrupts the bilayers and induces a significant decrease of the bilayer thickness, but on the other hand Braun et al. (2007) have concluded that inactivation due to albumin is not caused by alterations in surfactant microstructure. Furthermore, Otsubo and Takei (2002) have found that the surface activity of synthetic lung surfactant (SLS) was only slightly influenced by albumin. More recently, Fernsler and Zasadzinski (2009) proposed a competitive adsorption model for lung surfactant inactivation.

As mentioned above, different theories have been proposed to explain the mechanism of lung surfactant inactivation by SP, but much remains unknown on how to rationally construct appropriate surfactant formulations for therapeutic uses in diseases in which the exogenous surfactants are currently ineffective.

The comparative analysis of the deleterious action of albumin and total serum proteins might help to understand the interaction between surfactant and proteins, and to clarify whether albumin is the main responsible for surfactant inactivation. The present study focuses on evaluating the in vitro effects of human serum and bovine serum albumin (BSA) on bulk shear viscosity, dynamic surface properties and the ratio between the light and heavy subtypes of surfactant suspensions.

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 S.A. and Richet S.A. Laboratories) for the elaboration of therapeutic surfactants. Prosurf® is a sterile chloroform solution that contains lipids and proteins extracted by means of bronchioalveolar lavage from bovine lungs, with slightly hypertonic solution (Hager and De Paoli, 2001). Prosurf composition is: phospholipids 94.8%; DPPC 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).

2.2. Exogenous proteins

Bovine serum albumin (BSA) was purchased from Sigma. Human serum (HS) was obtained from healthy adult donors. A pool of these HS was used (total proteins 6.8 g/dl, albumin 3.7 g/dl, cholesterol 180 mg/dl).

2.3. Samples

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

2.4. Heavy and light subtypes

2.4.1. Isolation

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).

2.4.2. Quantification

The percentage of each subtype was estimated as: (PL concentration in the fraction/PL concentration in the non fractionated EPS) × 100.

2.4.3. Mixtures

Different aliquots of fractions obtained in 2.4.1 were mixed in order to obtain suspensions with an equal final PL concentration (8, 15 or 20 mg/ml) but with a different proportion of subtypes.

2.5. Viscosimetry

Bulk shear viscosity was measured with a Brookfield cone-plate microviscometer (DV-II+; Brookfield Engineering Laboratories, Stoughton, MA). The share rate was varied using a CP 42 spindle (0.0–384 s^{-1}) and the temperature was regulated at 37 °C. One millilitre of each sample was placed in the sample cup. Viscosity measurements were determined during a single set of experiments, proceeding from low to high shear rates to minimize variations from shear-induced aggregate changes. At fixed shear rate, the viscosity values were assumed to be at steady state after at least 10 s with no significant changes in magnitude.

The viscosity of each sample (η) was obtained at a fixed speed gradient of 384 s^{-1}.

The relative viscosity was calculated as η/η_o, where η is the viscosity of the sample and η_o is the viscosity of the solvent.

2.6. Surface tension measurements

Surface activity measurements were made with a pulsating bubble surfactometer (Electronetics, Buffalo, NY), 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 was 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.

2.6.1. 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.

2.6.2. Percentage reduction in bubble surface area (ΔA_{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, $\Delta A_{10} = [(\max SA - SA\ 10\ mN/m)/\max SA] \times 100\%$. ΔA_{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

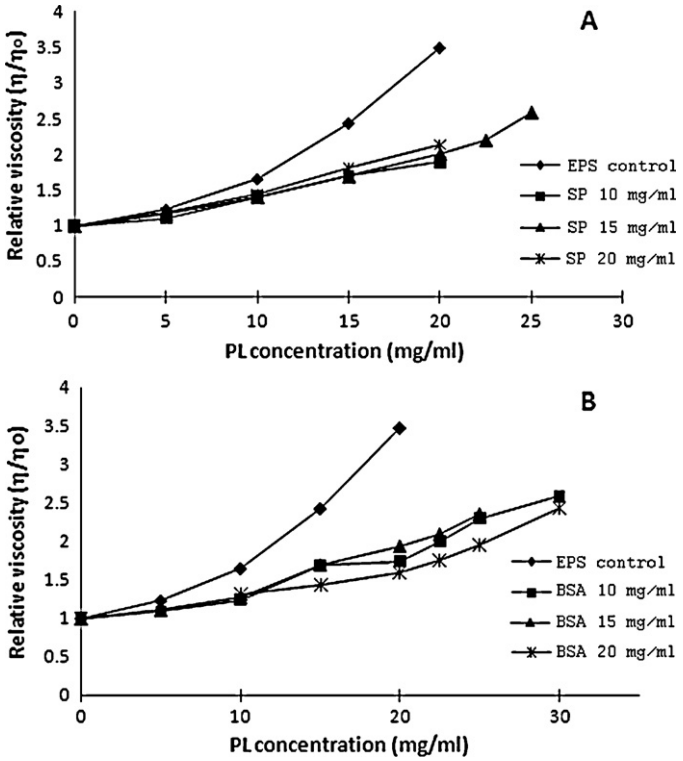

Fig. 1. Relative viscosity (η/η_o) of Prosurf suspensions in the function of phospholipids concentration. The relative viscosity was calculated as η/η_o, where η is the viscosity of the sample and η_o is the viscosity of the solvent The surfactant was mixed with serum (A) or BSA (B) in order to obtain different final protein concentrations. Viscosities were measured with a Brookfield cone-plate microviscometer at 37 °C and at a fixed speed gradient of 384 s^{-1}. This is one of the five experiments; the same pattern was found in all of them. Lines are visual guides.

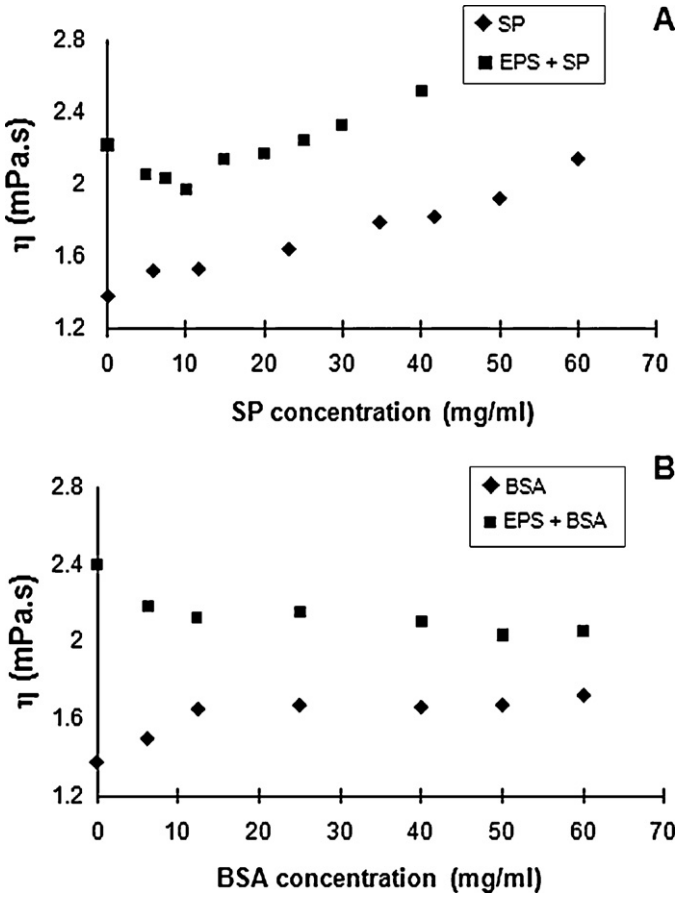

Fig. 2. Viscosity (η) of Prosurf suspension (10 mg/ml of PL) added with serum (A) or albumin (B) at different protein concentrations. The viscosities of serum and albumin solutions without surfactant were used as references. This is one of the twelve experiments; the same pattern was found in all of them.

unmeasured ΔA_{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.

2.7. Chemical determinations

Phospholipids and proteins concentrations were measured by the Stewart (1980) and Lowry et al. (1951) methods, respectively. Cholesterol was determinate by enzymatic method.

2.8. Experimental data acquisition and statistical analysis

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

The viscosity results shown are representative experiments, where samples from the same surfactant batch are compared. The results obtained with different batches showed the same profile.

The results of the percentage of active heavy fraction and tension shown are the average of 10 experiments. Data are expressed as the mean ± SD.

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

3. Results

3.1. Effects of proteins on the viscosity

Prosurf is similar to Infasurf, since both have the same animal origin and SP-B and SP-C. The quantitative composition of EPS is: 94.8% phospholipids (PL), 4.4% cholesterol and 0.8% proteins (SP-B, SP-C).

Prosurf suspensions presented non-Newtonian pseudoplastic behavior (data not shown), similar to Exosurf, Survanta and Infasurf (King et al., 2002). The viscosity of EPS was dependent on PL concentration (Fig. 1). The addition of serum (Fig. 1A) or BSA (Fig. 1B) clearly decreased the relative viscosity at all PL concentrations tested ($p < 0.01$). At each PL concentration tested, the decrease in surfactant viscosity was similar for the different protein concentrations added.

Fig. 2 shows the viscosity of EPS (10 mg/ml) in function of protein concentrations. When either serum or BSA was added, between 0 and 10 mg/ml, the viscosity of the suspension decreased as protein concentration increased, reaching a minimum value at 10 mg/ml; however, at higher concentrations, viscosity increased showing a profile parallel to that obtained for protein solutions without surfactant.

In order to evaluate the influence of the proportion of the light and heavy subtypes on the viscosity of surfactant suspensions, different amounts of these fractions were mixed and the viscosity was measured. The results, for two different total PL concentrations, are shown in Table 1. At PL concentrations below 10 mg/ml, the viscosity of the different mixtures was only slightly higher than that of the saline solution alone, without any significant difference between them (data not shown). At PL concentrations higher than 10 mg/ml, the relative viscosity of EPS containing only the heavy or the light fraction was higher than the viscosity of suspensions

Table 1
Relative viscosity of EPS with different subtype ratios.

PL concentration of EPS (mg/ml)	Subtypes ratio (light/heavy)	Relative viscosity (η/η_o)
8	0/100	1.4 ± 0.1
	15/85	1.4 ± 0.1
	30/70	1.3 ± 0.1
	100/0	1.3 ± 0.1
15	0/100	2.1 ± 0.1
	15/85	1.8 ± 0.1
	30/70	1.6 ± 0.1
	100/0	2.2 ± 0.1
20	0/100	2.6 ± 0.1
	15/85	1.9 ± 0.1
	30/70	1.7 ± 0.1
	100/0	–[a]

The relative viscosity was calculated as η/η_o, where η is the viscosity of the sample and η_o is the viscosity of the solvent.

[a] The value of viscosity is missing because it was impossible to obtain 100% of the light fraction at this.

containing both fractions. The viscosity of mixtures also decreased as the light subtype proportion increased.

3.2. Heavy and light subtypes in the presence of serum and BSA

Fig. 3 shows that the amount of the heavy subtype obtained in the presence of serum or albumin decreased as the concentration of added proteins increased. Significant differences were found between controls and each SP or BSA concentration tested ($p < 0.01$). The statistical analysis also showed significant 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. Since the added protein, in all cases, remained in the supernatant (data not shown), the heavy fractions obtained were free of added proteins.

Lipids like cholesterol that take part of lipoproteins, could be potentially transferred into surfactant complexes. In order to probe if this transference occurs we have determined the proportion of cholesterol in surfactant exposure to serum. Our results showed that the cholesterol/phospholipids rate in the heavy fraction of samples added with serum did not increased significantly regard to the heavy subtype control (data not shown).

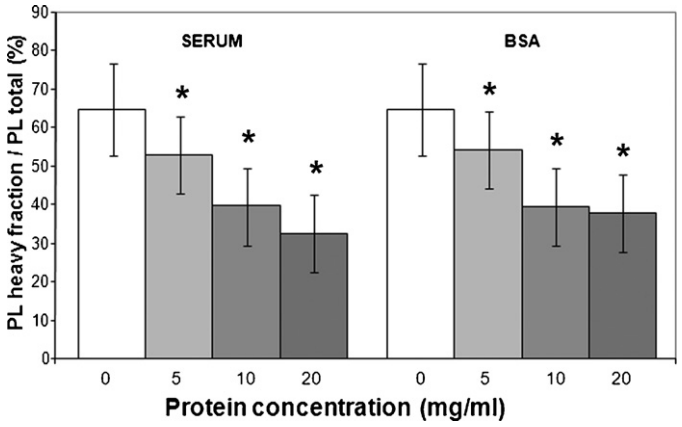

Fig. 3. Amount of the heavy subtype obtained in function of the added protein concentration, expressed as % phospholipids. Prosurf suspension (10 mg/ml of PL) was mixed with serum or BSA, reaching a final protein concentration of 5, 10 or 20 mg/ml. The surfactant without added protein was used as control. Data are represented as the mean ± SD. Statistically significant decrease compare to control $^*p < 0.01$.

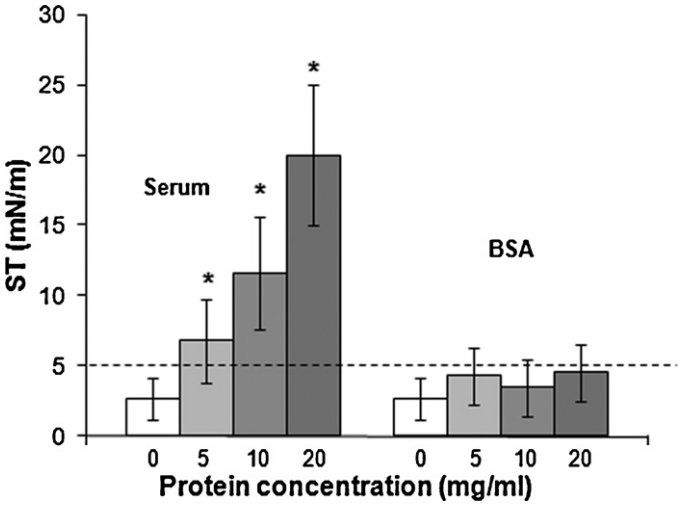

Fig. 4. Surface tension coefficient (ST) of Prosurf suspension in function of added protein concentration. The ST was measured with a pulsating bubble surfactometer at 37 °C. The surfactant (10 mg/ml of PL) was mixed with serum or BSA reaching a final protein concentration of 5, 10 or 20 mg/ml. Surfactant without added protein was used as control. Data are represented as the mean ± SD. Statistically significant increase compared to control $^*p < 0.01$. The dotted line represents the upper limit value of surface tension for a proper activity of the Prosurf suspensions.

3.3. Effects of proteins on EPS surface tension

Surface tensions of EPS added with different concentrations of SP or BSA are shown in Fig. 4. The addition of SP increased ST as compared to the control, at all the concentrations tested ($p < 0.01$), reaching values higher than 5 mN/m at 10 and 20 mg/ml of SP. The surface tension of EPS added with 5 mg/ml of SP was not significantly different from 5 mN/m. By contrast, there were no significant changes in surface tension by the addition of BSA at any of the concentrations tested.

In order to evaluate if the heavy subtype obtained after incubation with serum or BSA retained its surface activity, ST and ΔA_{10} of this fraction were measured (Table 2).

When serum was added to Prosurf suspensions, the ΔA_{10} values increased as SP concentrations increased. At 5 mg/ml of SP, ΔA_{10} was higher than control ($p < 0.01$), but remained lower than 47%. At 10 and 20 mg/ml, the ΔA_{10} values were higher than 47%. These results are in agreement with the values of ST obtained. For the heavy fractions of samples added with serum, the ΔA_{10} and ST were always lower than the ΔA_{10} and ST of their corresponding non fractionated sample ($p < 0.01$). Furthermore, these heavy subtypes showed values of ΔA_{10} below 47% and ST near to 5 mN/m and did not show significant differences as compared to the heavy subtype control. 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. On the other hand, 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.

4. Discussion

Many papers have reported that serum proteins inactivate lung surfactant, although the results differed according to the different surfactants and conditions used (Blanco and Pérez-Gil, 2007; Braun et al., 2007; Nag et al., 2007; Ramanathan, 2009a; Taeusch et al., 2005).

The surface tension value is commonly used as a single parameter to estimate the quality of a surfactant, but its biological activity also depends on other properties such as its ability to spread and its viscosity (King et al., 2001). Rheological behavior is important

Table 2
Surface tension and percentage reduction in bubble surface area (ΔA_{10}) of the surfactant and its subtypes. Prosurf suspension (10 mg/ml of PL) was mixed with serum or BSA, reaching a final protein concentration of 5, 10 or 20 mg/ml, and the 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*

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

for the surfactant activity and for an efficient administration of the pharmaceutical products (do Campo et al., 1994; Ramanathan, 2009b). Viscosity studies may also give information about particle size and aggregation–disaggregation processes.

EPS can be considered as complex colloids of a solid-like phase with different sized particle structure and aggregate-free liquid volume. At low concentrations, the rheological behavior of EPS is dominated by the free liquid phase, and the viscosity approaches that of the saline solution. At higher concentrations, viscosity increases exponentially as the solid volume fraction increases above a certain threshold, in association with maximum particle packing (Lu et al., 2009). A theoretical work has shown that viscosity also depends on the polydispersivity of spheres in a suspension; it has been found that viscosity is reduced when the ratio of maximum to minimum radii was increased (Wagner and Woutersen, 1994). The results shown in Table 1 are in concordance with that since surfactant suspensions containing very similar sized particles (only one fraction) had higher viscosity than those with wider particle size distribution (mixtures of the two fractions). The decrease in surfactant viscosity obtained with increasing amounts of light fraction can be understood as a consequence of an increase in the maximum packing of surfactant particles as the particle size distribution becomes wider.

The addition of serum or albumin to EPS might increase the viscosity of the suspension. However, our results showed that the addition of either serum or albumin significantly decreased the viscosity of EPS. This decrease could be explained as a consequence of the greater amount of light fraction present in surfactant suspensions supplemented with serum or BSA. The addition of serum or albumin to the surfactant facilitates the transformation of the active heavy subtype to the light one with lower surfactant capacity. This finding is consistent with the hypothesis that the disaggregation of the surfactant structure occurs in the presence of SP or BSA leading to a decrease in the viscosity of the suspension.

The minimum value of viscosity obtained at 10 mg/ml of added protein (Fig. 2) and the similar amount of heavy fraction obtained at 10 and 20 mg/ml of SP or BSA (Fig. 3) seem to imply that 10 mg/ml is the protein threshold concentration for the observed effects, at least at the PL concentration of EPS used in these experiments. In addition, the active fraction obtained in the presence of SP or albumin at equal concentrations did not differ significantly, so it is possible to suppose that the disaggregation of the surfactant depends mainly on the concentration of the added protein regardless of the protein type itself (Fig. 3).

Undoubtedly, the surface-active behavior and the composition of lung surfactant are the most important properties for its physiological function. In this study, serum affected the surface tension, causing the total loss of surfactant activity at protein concentrations higher than 5 mg/ml. A priori, the deleterious effect of serum on the surface tension might be explained by the increase in the inactive light fraction obtained with increasing SP concentrations. However, the presence of BSA did not affect the surface tension at any of the concentrations tested (0–20 mg/ml), although it caused an increased quantity of the inactive subtype (Fig. 4 and Table 2). Considering these results, we may conclude that the presence of proteins causes disaggregation of the surfactant structures, but this effect would not be enough to produce the EPS inactivation. It is possible to suppose that a serum component different from albumin, may be interacting and producing the loss of surfactant 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 could be think that these serum components could be potentially transferred into surfactant complexes and originate part of the alterations described, but in this study this transference could not be demonstrated.

When serum was present, EPS showed high values of ST, but when the serum proteins were removed (by centrifugation and lavages), the heavy fraction obtained recovered its surface activity, reaching values of ST and ΔA_{10} similar to those of their control (Table 2). Consequently, it could be concluded 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 suggest that EPS inactivation by serum is probably due to a physical interaction between the surfactant and one or more serum components, and that this is reversed when they are removed.

5. Conclusions

In summary our results show that (a) a correlation may exist between bulk viscosity of surfactant suspensions and their aggregation degree; (b) the addition of albumin or serum proteins to Prosurf induces the transformation of the active to the inactive subtype, an effect that depends on the total added protein concentration, regardless of the type of added protein; (c) albumin produces disaggregation of the surfactant without the loss of surface activity; (d) serum proteins cause disaggregation and inactivation of EPS; (e) the disaggregation would not be the primary cause of the surfactant inactivation; and (f) a serum component, different from albumin, would be responsible for the loss of surfactant activity.

Further studies will be carried out to find out which serum fractions (proteinaceous or non proteinaceous) different from albumin, are responsible for the inactivation of EPS suspensions. This knowledge will help to rationally construct new surfactant formulations appropriate for therapeutic uses.

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