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Analysis of the structure and surfactant activity of novel formulations containing exogenous pulmonary surfactant and glucocorticoids

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ABSTRACT

Exogenous pulmonary surfactant (EPS) could be used as carrier of glucocorticoids (GCs) in therapy for respiratory diseases. We formulated novel combination drug products containing bovine EPS and one GC (10 wt%): beclomethasone (Be), budesonide (Bu) or fluticasone (Flu), and studied the GCs action on the surface activity and biophysical properties of EPS.

Subtype ratio was evaluated by phospholipid determination; surface tension (ST) with a pulsating bubble surfactometer and conformational changes by Electron Spin Resonance (ESR).

GCs were incorporated into EPS in more than 80%. None of them generated disaggregation of surfactant, only Bu was found in the light subtype. Bu and Be caused minimal changes in fluidity on polar region of bilayers, but these changes were not enough to inactivate the surfactant. Flu did not significantly alter any biophysical properties or surface activity.

These novel combination EPS-GC products might be a promising strategy in the therapy of pulmonary diseases as the incorporation of the GCs tested did not cause detrimental effects on EPS functionality.

1. Introduction

Chronic lung disease can be caused by several alterations that damage the lung tissue and are often associated with inflammatory processes that alter the normal function of pulmonary surfactant (PS). PS is a lipid–protein material that coats the entire mammalian respiratory surface. It forms a surface-active film at the air-water interface of alveoli, capable of reducing surface tension to near 0 mN/m to prevent pulmonary collapse during expiration and to minimize the work required for inhalation (Creuwels et al., 1997). Although PS composition varies among different species and environmental conditions, it is mainly made up of phospholipids (80–90%), mostly saturated dipalmitoyl-phosphatidylcholine (DPPC), neutral lipids (6–10%) of which cholesterol (Cho) is the most abundant; and at least four specific proteins (5–10%), two hydrophilic (SP-A and SP-D), with immune function, and two hydrophobic (SP-B and SP-C), which contribute to the mechanical stability of the interfacial film and are essential for surfactant activity.

The administration of exogenous pulmonary surfactants (EPS) often provide immediate relief of symptoms and improve oxygenation and gas exchange in some of chronic lung diseases (Günther et al., 2001; Poulain and Clements, 1995; Veldhuizen et al., 1996). At physiological temperature, two phases coexist in monolayers and bilayers of almost all lung surfactants: a semi-crystalline liquid-ordered phase (Lo) and a liquid-disordered phase (Ld) (Alonso et al., 2004; de la Serna et al., 2004). This coexistence of phases is crucial for the surfactant activity, and the role of Cho in this lateral organization has been extensively researched. Several studies have revealed that Cho at 5–10 wt% has no negative effects on EPS function, but that supra-physiological levels of this compound are detrimental (Gunasekara et al., 2005; Palmer et al., 2000; Zhang et al., 2012; Zuo et al., 2008). Although the mechanism of Cho-induced inhibition has not been fully elucidated, many groups, including our own, have demonstrated that the presence of this compound causes alterations of the microstructure of EPS films with the subsequent inactivation of the surfactant (Keating et al., 2007; Leonenko et al., 2007; Malcharek et al., 2005; Martínez Sarrasague et al., 2013). For this reason some authors recommend avoiding the presence of cholesterol in EPS formulations (Yu and Possmayer, 1994).

Glucocorticoids (GCs), due to their anti-inflammatory actions, have been commonly used to modify the course of chronic lung

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disease (Jobe, 2009; Shah et al., 2012). Over the years, the use of systemic corticosteroids has been replaced for topical administration of GCs directly to the lungs, because of their serious side effects (Cole, 2000). Two different pulmonary drug-delivery methods have been clinically tested: inhalation delivery of steroid aerosols and, most recently, intra-tracheal instillation of steroids using EPS as a carrier (Nimmo et al., 2002; Wang et al., 2012; Yang et al., 2010). Many authors have demonstrated that the intratracheal co-administration of EPS and GCs such as budesonide (Bu) or beclomethasone (Be) significantly improved pulmonary outcome in meconium aspiration or respiratory distress syndromes, showing that these substances have a certain therapeutic synergism: the surfactant improves respiratory mechanics and the GCs reduce the inflammation (Dani et al., 2009, 2011; Kuo et al., 2010; Mikolka et al., 2013; Yang et al., 2010). However, despite their low risk-benefit ratio, the pulmonary co-administration of GCs and EPS requires a comprehensive understanding of the molecular interaction between them.

All corticosteroids are biochemically derived from Cho and hence share a close structural similarity with it (Ghosh et al., 1996). For this reason GCs might modify the properties of the surfactant and lead to its inactivation, similarly to Cho. Only few studies have assessed the GC-EPS interaction and there is disagreement about the possible deleterious effect of these compounds on the surfactant, depending on the EPS studied and on the amount of GCs added. Yeh et al. (2008) reported that the dynamic surface activity of the suspension was minimally affected when Bu was added to Survanta at 2 wt%, but when it was added at 25 wt%, this GC blocked the ability of Survanta to reduce the surface tension (ST). On the other hand, Palmer et al. (2000) found that the addition of Bu at low (0.6 wt%) and high (20 wt%) concentration adversely affected the surface-tension properties of two Cho-containing surfactant preparations (Survanta and BLES), meanwhile Zhang et al. (2012) demonstrated that Bu (10 wt%) had no deleterious effect on a Cho-free surfactant preparation (Curosurf).

It is known that the local lung delivery efficiency for drugs in general and GCs in particular, is very low (e.g. between 4.4% and 26.6% for budesonide aerosolized), and depends on the system utilized (Berlinksi and Waldrep, 1997).

As Hidalgo et al. (2015) discussed in their review, diverse strategies have to be developed to improve the delivery of active molecules into airways. Among them, the pulmonary surfactant could be considered a promising strategy for transporting drugs efficiently. It provides advantages because it can dissolve and transport poorly water-soluble drugs along the entire respiratory surface, while avoiding the physiological barriers of the air pathway. Since the GCs are highly lipophilic, it could be thought that GCs and EPS formulated together, with the GC incorporated into the membranes of the EPS, could be a novel alternative for pulmonary drug-delivery. This combination drug product would allow a more efficient delivery of the GCs in the alveolar region of the lung, due to its lipid composition and spreading capabilities. We have not found studies with this type of formulation in the literature. In such preparations, EPS not only act as a carrier but could be expected to have therapeutic effect by itself. Therefore, it is critical that the GCs incorporated to EPS do not impair its biophysical properties and allow the desired synergistic effect.

In order to investigate whether GCs incorporated into surfactant membranes modify the structure of EPS and alter the surfactant activity we carried out the present study. To achieve these objectives, we prepared a combination drug product containing an exogenous surfactant (Prosurf) and one of the GCs commonly used in pulmonary therapy: beclometasone, budesonide and fluticasone (Flu) and analyzed the surfactant macrostructure, the bilayer organization and their relation with the surfactant activity.

2. Materials and methods

2.1. Samples

2.1.1. 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 containing surfactant lipids and lipophylic proteins from broncho alveolar lavage fluid of bovine lungs (Hager and De Paoli, 2001). Prosurf is composed of: phospholipids (PL) 94.8%; DPPC46% of total PL; Cho 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 PL concentration of 30 mg/ml. This final suspension, fractionated in sterile vials, constitutes the exogenous pulmonary surfactant (EPS). EPS was diluted with saline solution (0.9% NaCl) to a final PL concentration of 10 mg/ml and pH5.8–6.0, and this diluted EPS was used as control.

2.1.2. Combination drug product (EPS-GC)

EPS with the different GCs (EPS-Be, EPS-Bu and EPS-Flu) was performed as follows: an appropriate amount of each GC in chloroform solution was added to Prosurf in order to obtain a GC/PL weight ratio 1:10. Then, chloroform was evaporated and the preparation of EPS continued as is detailed above (2.1.1).

Adequate aliquots of cholesterol chloroform solution were added to Prosurf (before solvent was evaporated) in order to obtain EPS with extra Cho (10 wt%) in equivalent proportions to GCs. This sample (EPS-Cho) was used as positive control.

All samples were diluted with saline solution to a final PL concentration of 10 mg/ml and final pH5.8–6.0.

2.2. Chemicals

Budesonide and cholesterol were purchased from Sigma. Fluticasone propionate (Sigma) was donated by Casasco Laboratory, and Beclomethasone dipropionate was purchased from Saporiti SACIFIA. The spin derivatives of stearic acid, 5- and 16-doxyl stearic acids (5DSA and 16DSA respectively) were purchased from Sigma. All the reagents were of analytical grade.

2.3. Chemical determinations

Phospholipid concentrations were measured by the Stewart (1980) method. Cholesterol was determined by the enzymatic method (Allain et al., 1974).

To determine the concentration of GC in the EPS-GC and in the surfactant sub-fractions, an aliquot of each sample was dissolved in chloroform – methanol (2:1) and its absorbance was measured at 250 nm using a Shimadzu double beam spectrophotometer. The concentration of each GC was calculated using the extinction coefficient from the respective calibration curve.

2.4. Heavy and light subtypes

2.4.1. 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).

2.4.2. 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 as described above.

2.5. Electronic spin resonance spectroscopy (ESR)

The use of hydrophobic spin probes in the study of membranes is well known. The ESR spectrum of the nitroxyl ring in 5DSA and 16DSA is sensitive to the local host environment (Nusair et al., 2012). Thus, the ESR spectroscopy allows the investigation of structural and dynamic aspects of the bilayers.

2.5.1. 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.

2.5.2. 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 100Gauss, center field 4380 G, time constant 5.12 ms, conversion time 5.12 ms, modulation amplitude 0.75 G, modulation frequency 50 kHz, resolution 1024 points, microwave power 10 mW and microwave frequency 9.7 GHz.

2.5.3. Determination of the order parameter (S)

Samples were labeled with 5DSA. For 5DSA spectra, A// and A⊥, 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//) and innermost (2A⊥) peaks of the ESR spectra (Fig. 1A and Eq. (1)). These are indicators of the rotational motional freedom of the phospholipids acyl chain 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|| − A⊥) 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)

$$S = \frac{A_{//} - A_{\perp}}{A_{zz} - 0.5\left(A_{xx} + A_{yy}\right)} \times \frac{a_0'}{a_o} \quad (1)$$

where A⊥ and A|| are the true hyperfine constant values. The polarity correction term $aí_0/a_0$is introduced to take into account the hyperfine splitting dependence on the polarity of the label environment, where: $a_0 = (A_{||} + 2A_{\perp})/3$ and $aí_0 = (A_{xx} + A_{yy} + A_{zz})/3$. The value Aí obtained from the spectrum has to be corrected to give the true A value. The correction is given for S < 0.45 by A = Aí + 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.

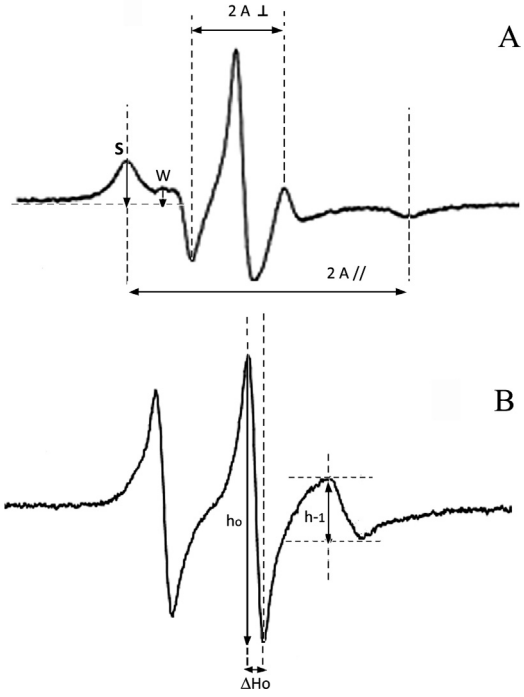

Fig. 1. ESR spectra of EPS labeled with 5DSA (A) or 16DSA (B): Fig. A shows the separation, in Gauss, of the outermost (2A//) and innermost (2A⊥) peaks of the ESR spectra signal. The arrows show the amplitude of two spectral components of the low-field peaks. These components are commonly called strongly (S) and weakly (W) immobilized. Figure B shows the width of the central peak (ΔH_0), the amplitude of the central peak h_0, and the high field peak h_{-1} of the ESR spectra signal.

2.5.4. Determination of the rotational correlation time (τ)

The dynamic properties of the probe 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 phospholipids acyl chains. In this case, τis the parameter that can be used to measure the motion of the phospholipids 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)):

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

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 τvalue 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 S, τ increases as the environment fluidity decreases.

2.5.5. ESR determination of the S/W ratio

The ESR spectra of spin-labeled phospholipids 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, 1973).

2.6. 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.

2.6.1. Surface tension (ST)

This parameter represents the tendency of liquids to reduce their exposed surface to the smallest possible area. To determine ST, the pressure across the bubble was measured by a pressure transducer and the ST calculated using the La Place equation: $P = 2\ ST/r$, 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 results, 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 (ΔA_{10}) was calculated after 100 cycles of bubble cycling. ΔA_{10} is an indicator of dynamic film compressibility. Films with low compressibility cause a large decrease in ST with a relatively small decrease in SA. If the ST of the surfactant suspension does not reach 10 mN/m, then the actual, although unmeasured, ΔA_{10} values should be >47% because that is the difference in SA between the maximum and the minimum bubble areas in the Electronetics pulsating bubble surfactometer.

2.7. Experimental data acquisition and statistical analysis

All measurements were repeated with several independent surfactant batches that showed similar qualitative behavior. The ESR results were expressed as ratios between samples and the control from the same surfactant batch. The results obtained with different batches showed the same profile.

The other results shown (percentage of heavy fraction, the proportion of GC incorporated to surfactant and surfactant activity) are the average of at least eight 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. Surfactant macrostructure

3.1.1. Heavy and light subtypes

It is known that, in vivo, two surfactant subtypes with different physiological capabilities, called heavy subtype (active) and light subtype (inactive), may coexist (Gross et al., 2000; Ueda et al., 1994; Veldhuizen et al., 1993). We have previously found that the presence of serum or albumin causes disaggregation of the surfactant structures of an EPS, inducing the transformation of the active into the inactive subtype, while the incorporation of Cho or serum lipoproteins to the surfactant do not significantly change the heavy/light subtype ratio (Martínez Sarrasague et al., 2013). To evaluate whether the incorporation of each GC cause disaggregation of the EPS macro-structure, the amount of heavy and light subtypes obtained in the final formulation was measured by the chemical method. The heavy fraction of the EPS used for these assays contains on average 75% of total PL. No significant difference was found between the amounts of heavy and light fraction in the EPS-GC or EPS-Cho, regarding the EPS controls (data not shown).

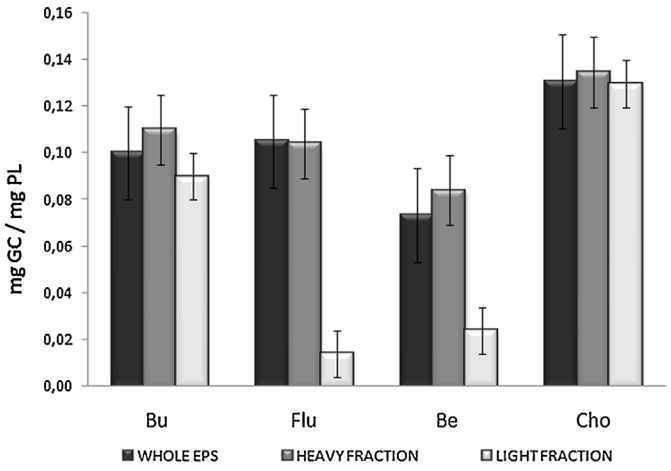

Fig. 2. Amount of GCs or Cho incorporated into the whole EPS and in its sub-fractions. EPS (10 mg/ml PL) was added with each GC or Cho in a 10:1mass ratio. The sub-fractions of each sample were separated by centrifugation at 12,000 rpm. The amount of GCs was measured by UV absorption, and PL and Cho were determined by the chemical method. The results are expressed as mg GC/mg PL. Data are represented as the mean ± SD.

3.1.2. Incorporation of GC and Cho in whole EPS and in its subtypes

In the combination EPS-GC products, it is important to know the real amount of GC incorporated into the membranes. To evaluate whether all the GCs added to EPS were totally incorporated, and to analyze their distribution in the surfactant sub-fractions, the concentrations of Bu, Be, Flu and Cho in whole EPS and in its sub-fractions were determined. Fig. 2 shows that the GC/PL weight ratio in EPS-Flu and EPS-Bu was close to 0.1, meaning that Bu and Flu were totally incorporated to EPS, while EPS-Be contained approximately 80% of the total Be added. The Cho/PL weight ratio obtained in EPS-Cho was 1.4:10. This result indicates that Cho was almost fully incorporated into EPS, adding to the endogenous Cho of the original surfactant. In terms of distribution in the different fractions, the results showed that Bu and Cho were incorporated equally in the whole EPS and in both sub-fractions, while Flu and Be were mainly found in the heavy active subtype. Only about 10% of last two GCs were incorporated into the light subtype.

3.2. Surfactant micro-structure: ESR spectral analysis

Since the main components of EPS are PL (90–95%), the use of doxyl-stearic acid spin probes with along hydrophobic tail 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 surfactant PL. The ESR spectrum yields information about the molecular environment of the spin probe. The correlation time τ calculated from the ESR spectrum of 16DSA incorporated into the EPS reflects the motion of the PL acyl chain near the hydrophobic zone. Fig. 3 shows the correlation time obtained from the spectra of EPS-Cho and EPS-GC labeled with 16DSA. High values of this parameter can be understood as a slower movement of the probe

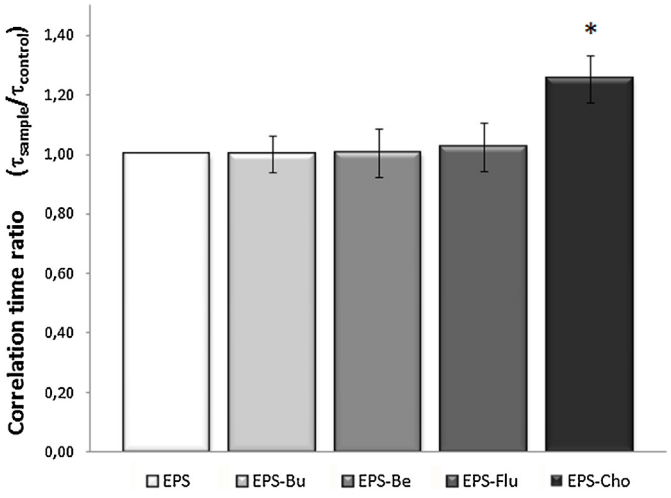

Fig. 3. Correlation time (τ) obtained from the spectra of EPS-Cho and EPS-GC labeled with 16DSA. The surfactant without added Cho or GCs was used as control. Due to the variability of the batches, the results obtained are expressed as a ratio of each sample relative to the respective control. This allows better visualization of the profile of samples with different GC or Cho. Data are represented as the mean ± SD. Statistically significant increase compared to the control (*) $p < 0.01$.

in the hydrophobic core due to a decrease in the fluidity of the environment. The correlation time increased in the presence of Cho ($p < 0.01$). In contrast, the addition of Bu, Be or Flu did not significantly change this parameter.

The ESR spectra of 5DSA incorporated into the EPS bilayer showed an anisotropic motion, indicating that the probe movement is highly restrained. The order parameter S calculated from these spectra reflects the rotational freedom of PL close to the polar head groups in the layer. An increased S, due to an increase in 2A//, can be associated with a decrease in bilayer fluidity (2.5.3). Fig. 4A shows the spectra of EPS samples and Fig. 4 B shows the order parameter obtained from these spectra. In concordance with our previous results (Martínez Sarrasague et al., 2012), EPS-Cho showed an increased value of this parameter ($p < 0.01$). Among the GCs tested, the presence of Bu or Be caused an increase in this value ($p < 0.01$), but Flu did not change it significantly. The increase caused by Cho was higher ($p < 0.05$) than that caused by Bu or Be, while no significant differences were observed between the increasing order parameter in EPS-Bu and EPS-Be.

A common feature of almost all lung surfactants and model mixtures is the coexistence of a semi-crystalline Lo phase and a Ld phase. The spin probe incorporated into these phases has different rotational motion and consequently yields a spectrum with differences at the low-field peak (Fig. 1). 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 liquid-ordered phase where the spin probe is more immobilized. Fig. 5A shows the S/W ratio obtained from the 5DSA spectra of EPS with and without GCs or Cho. Only the presence of Flu (Fig. 5B) in the bilayers increased the S/W ratio ($p < 0.05$), whereas the presence of Be, Bu or Cho did not significantly change this parameter.

3.3. EPS surface properties: ST and ΔA10

The ST coefficient 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 compress (King et al., 2001). A low coefficient value corresponds to liquids capable of reducing the interfacial tension with low energy cost, while high ST solutions are associated with systems with high

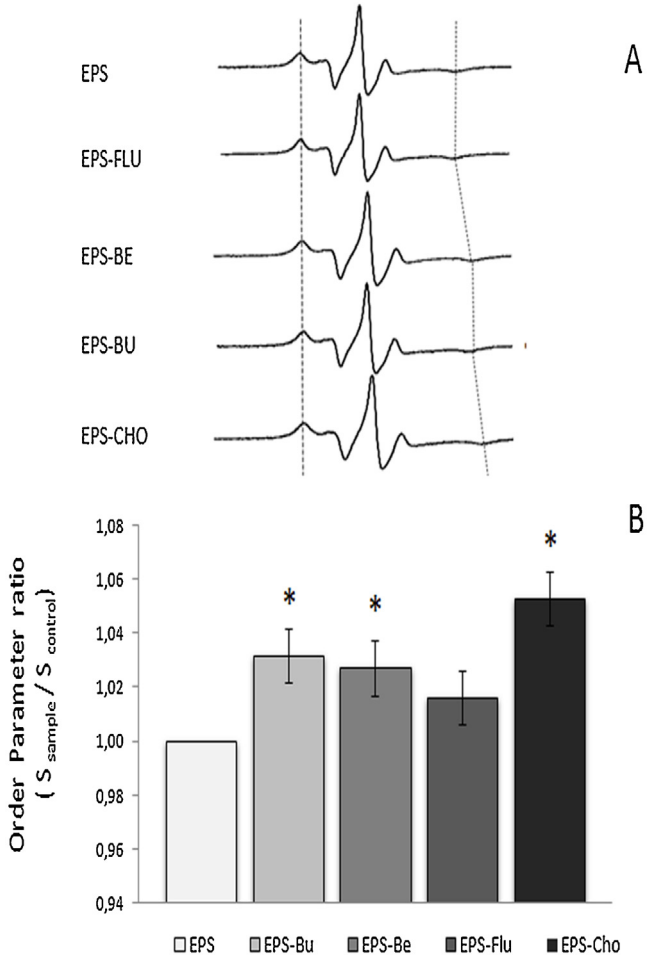

Fig. 4. (A) 5DSA Spectra obtained from EPS, EPS-GC and EPS-Cho. The vertical dotted lines indicate maximum 2A//. (B) Order parameter (S) obtained from the spectra of EPS-Cho and EPS-GC labeled with 5DSA. The surfactant without added Cho or GCs was used as control. Due to the variability of the batches, the results obtained are expressed as a ratio of each sample relative to the respective control. This allows better visualization of the profile of samples with different GCs or Cho. Data are represented as the mean ± SD. Statistically significant increase compared to the control (*) $p < 0.01$.

surface energy. On the other hand, ΔA_{10} is a parameter associated with the property of compressibility film surfactant. Films with low compressibility cause a large decrease in ST with a relatively small decrease in the area (Lu et al., 2005).

Fig. 6 shows that the addition of Cho to EPS at a concentration equal to that of the GCs tested increased ST and ΔA_{10}, reaching values slightly above the upper limit accepted (5 mN/m) for this surfactant ($p < 0.01$). Among the different EPS-GCs, EPS-Bu and EPS-Be also showed increased values of ST and ΔA_{10} but still within acceptable limits of surface activity ($p < 0.05$). In contrast, the addition of Flu to EPS caused no significant change in the ST coefficient.

3.4. Statistical correlations

Linear regression studies between the spectroscopic parameters evaluated and surfactant activity were carried out for the different EPS-GCs, EPS-Cho and EPS control. The increase in the order parameter S was associated directly with an increase in ST ($r = 0.966$, $p < 0.01$) and with the increase in ΔA_{10} ($r = 0.992$, $p < 0.01$). On the other hand, the correlation time, the S/W ratio and the amount of heavy subtype did not correlate with the ST parameters.

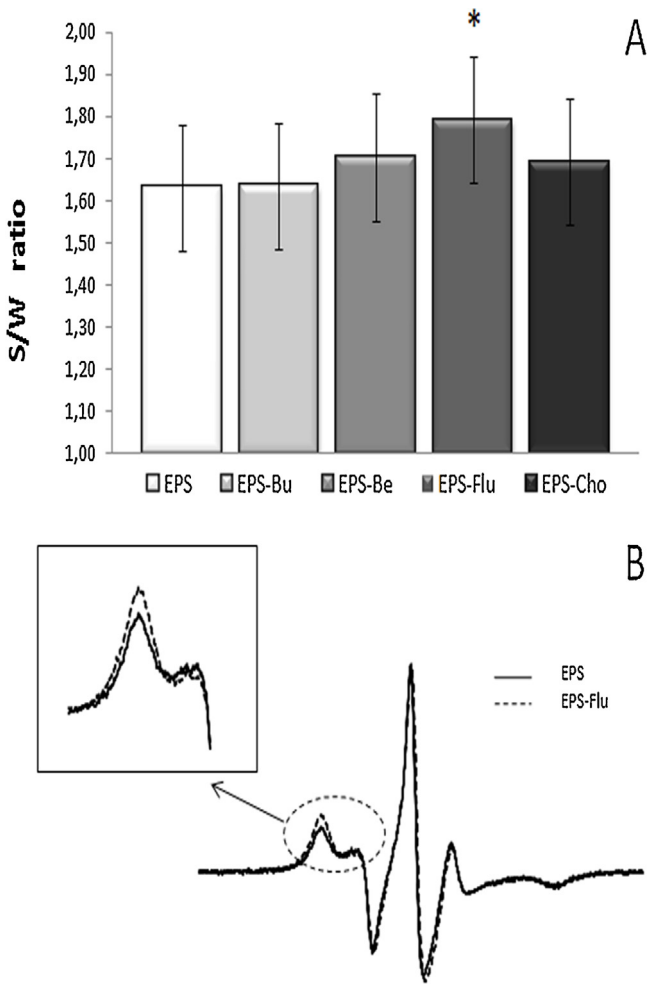

Fig. 5. (A) S/W ratio obtained from the spectra of EPS-Cho and EPS-GC labeled with 5DSA. (B) Spectra obtained from EPS and EPS-Flu. In the insert it can appreciate the difference in height of the peaks at low field. The surfactant without added Cho or GC was used as control. Data are represented as the mean ± SD. Statistically significant increase compared to the control (*) $p < 0.01$.

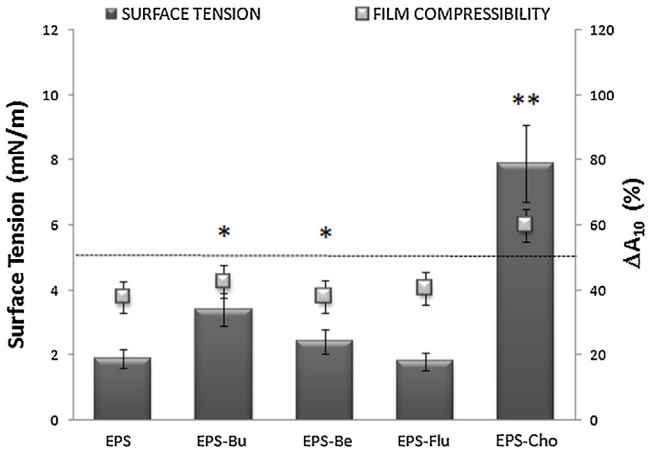

Fig. 6. Surface tension (ST) and percentage reduction in bubble surface area (ΔA_{10}) of the EPS added with the different GC or Cho. The surfactant without added Cho or GC was used as control. ST was measured with a pulsating bubble surfactometer at 37 °C. ΔA_{10} is an indicator of dynamic film compressibility. Data are represented as the mean ± SD. The bars represent the ST data and the points represent ΔA_{10} values. The dotted line represents the upper limit value of ST (5 mN/m) and ΔA_{10} (47%) for a proper activity of the surfactant. Statistically significant differences compared to the control (**) $p < 0.01$ and (*) $p < 0.05$.

4. Discussion

The idea of using EPS as a transporter started to be developed years ago and some initial experiments proved its potential in the late 1990s. Delivering drugs in combination with EPS increased the local effectiveness while minimizing systemic side effects (Hidalgo et al., 2015). In recent years, the intra-tracheal co-administration of GCs and EPS has been probed as therapeutic strategy for chronic lung diseases. However, the possible deleterious effect of GCs on EPS surfactant activity still remains unclear. The present study was designed to establish whether the presence of these compounds in the surfactant bilayers generates changes in the biophysical properties of EPS when formulated together.

The ST 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 the ability to spread and the viscosity (King et al., 2001). Rheological behavior is important not only for the surfactant activity of EPS formulations, but also for an efficient administration and distribution of pharmaceutical products (do Campo et al., 1994; Ramanathan, 2009). EPS can be considered as complex colloids of a solid-like phase with different sized particle structure and aggregate-free liquid volume. The presence of GCs could alter the proportion of light and heavy subtypes formed during the manufacturing process and consequently obtain end products with different macrostructure. We have previously demonstrated a correlation between the bulk viscosity and aggregation degree of surfactant suspensions (Martínez Sarrasague et al., 2011). In this study we found that the incorporation of GCs into the surfactant structures did not change the proportion of heavy and light subtypes of the EPS. In this way, the three GCs tested, at the assayed concentration, showed a behavior similar to that of Cho. Since the proportion of heavy/light subtypes affects the rheological properties of EPS, we conclude that the EPS-GC formulations would have similar rheological properties of pure EPS, and consequently be properly managed similarly to EPS used daily in therapy of pulmonary diseases.

Based on their lipophilicity, it was expected that the GCs were easily incorporated into the surfactant membranes, making EPS an ideal vehicle for this type of drugs. Our results confirmed this hypothesis because all the GCs tested were almost completely incorporated into the surfactant membranes. However, differences were observed in the distribution of the GCs in the surfactant sub-fractions, perhaps due to their molecular structures or their different affinity to the PL of the surfactant.

It is known that compression-expansion cycling leads to progressive conversion of the surface active fractions of surfactant into much less active lipid/protein complexes. Maintenance of a fully functional surfactant film requires continual film refinement through efficient removal of spent surfactant and incorporation of newly secreted complexes. The alveolar surfactant pool is continuously depleted through cellular uptake by type II epithelial cells and alveolar macrophages. Most of the PS internalized is recycled and the remaining material, which is known as surfactant light fraction, consisting of small, less active aggregates go through degradation pathways (Perez-Gil and Weaver, 2010). Based on this physiological process it could be expected that GCs incorporated to EPS were finally excluded from the films after some compression/expansion cycles and be progressively released into the lung tissue or reach the blood stream, in a way similar to some of the natural surfactant components, such as unsaturated lipids or Cho as described Hidalgo et al. in their review (2015). Corticosteroids located in the light fraction could be internalized faster than those located only in the heavy fraction and in consequence may potentially increase the rate of absorption. In this way the difference observed in the distribution of these GCs in the surfactant sub-fractions could have

implications on the pharmacokinetics and on the bioavailability of these substances once they reach the alveolar surface.

Bilayer fluidity reflects the order and dynamics of phospholipid alkyl chains in the layer and is mainly dependent on its composition. Cho is widely recognized as a substance that alters the structure and surface activity of PS. We have previously demonstrated that the addition of extra Cho to EPS causes an alteration in the polar zone order and the surfactant inactivation (Martínez Sarrasague et al., 2013). Since the molecular structure of GCs is similar to that of Cho, these compounds could have a similar behavior. The results obtained in the present study partially agree with this hypothesis. The analysis of the ESR spectra obtained with EPS added with Bu or Be showed that the GCs interact with PL, generating an increased rigidity of the bilayer in the proximity to the polar zone. In contrast, Flu did not modify the fluidity in this zone. As we have already demonstrated, the fluidity in the polar area of the PL bilayer is critical for a proper EPS surfactant activity (Martínez Sarrasague et al., 2012). In this study, we confirmed this statement since the addition of Bu, Be or Cho increased the rigidity in the polar zone and modified the surface properties of EPS, altering the dynamic ST and compressibility (ΔA_{10}). Furthermore, Cho was the substance that generated the highest increase in viscosity in the polar zone and the only one that caused total inactivation of EPS. Be and Bu induced an increase in ST that did not reach the inactivation threshold. On the other hand, Flu did not generate changes in ST or in the order parameter, confirming the correlation between both parameters.

It is known that the presence of Cho modulates the thermodynamic properties and packing of lipids in surfactant bilayers and has a profound impact on the lateral structure and dynamics of these membranes (de la Serna et al., 2004; Fidorra et al., 2009; Mouritsen and Jorgensen, 1994). In their work, Zhang et al. have reported that 10 wt% of Cho completely inhibits Curosurf surface-tension lowering ability and alters the "solid–fluid" phase coexistence at the monolayer. However, our results showed that the addition of 10 wt% extra Cho to Prosurf affected its surface properties but did not modify the lateral structure since the Lo/Ld proportion was preserved. The reason for this discrepancy is currently unclear, but it may be related to the methods employed or differences in the surfactants studied. Our results did not show correlation between the Lo/Ld ratio and the dynamic surface tension or compressibility. Among the GCs tested, only Flu increased the proportion of Lo phase but without altering the surface properties.

In ESR analysis, the difference in location of 5DSA and 16DSA provides information about the environment surrounding each spin probe. Changes in the mobility of the probes in EPS-GCs membranes may allow hypothesizing about the location of the GCs and to speculate on the disturbances that these substances generated in the conformation of the bilayers.

It is known that the Cho is transversely located in most of the lipid bilayers (Smith and Butler, 1976). The changes in the fluidity detected with both probes in the formulation containing extra Cho, allow us to speculate that this compound would have a similar transversal position in the membranes of Prosurf. The GCs studied did not produce the same effects that cholesterol in the microstructure. Bu and Be changed spin probe motility in the polar region without changing the Lo/Ld phases while Flu increased Lo/Ld ratio, but did not alter the motility of the probes in any region. In consequence, it could be thought that these GCs would have different locations in EPS membranes and/or different interaction with the PL, due to the different side chain at C-17 of these molecules.

In this study, we found that the presence of each GC into the EPS membranes induced different effects on the biophysical properties of Prosurf but none of them inactivated the surfactant. Nevertheless, since Be and Bu slightly increased the surface tension and alter the dynamic film compressibility, they could be potentially harmful to the EPS activity at higher concentrations. Most interestingly, our results showed that Flu did not affect the structure and surfactant activity of EPS and thus EPS-Flu would be the most promising system for therapeutic use.

5. Conclusions

In summary, we formulated a novel combination drug products containing EPS (Prosurf) and GC (Bu, Be or Flu) and demonstrated differential effects of these compounds on the biophysical properties of EPS:

- GCs were easily incorporated into the surfactant membranes, making EPS an ideal vehicle for this type of drugs.
- None GCs generated the disaggregation of the macrostructure of the surfactant retaining the rheological properties of the formulations.
- The distribution of these GCs in the surfactant sub-fractions was different, only Bu was found in the light subtype. This could have implications on the pharmacokinetics and on the bioavailability of these substances once they reach the alveolar surface.
- Bu and Be caused minimal changes in the fluidity of the polar region, which were not enough to inactivate the surfactant.
- Among the GCs tested, Flu was the only one that did not significantly alter the biophysical properties of Prosurf.

The novel combination EPS-GC products employed in this study might be a promising strategy in the therapy of pulmonary diseases as the incorporation of the GCs tested did not cause detrimental effects on EPS functionality.

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