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Developing an exogenous pulmonary surfactant-glucocorticoids association: Effect of corticoid concentration on the biophysical properties of the surfactant

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ABSTRACT

Glucocorticoids (GCs) are used to treat lung disease. GCs incorporated in an exogenous pulmonary surfactant (EPS) could be an alternative management to improve drug delivery avoiding side effects. In the development of these pharmaceutical products, it is important to know the maximum amount of GC that can be incorporated and if increasing quantities of GCs alter EPS biophysical properties. Formulations containing EPS and beclomethasone, budesonide or fluticasone were analyzed (PL 10 mg/ml; GC 1–2 mg/ml). The microstructure was evaluated by electron paramagnetic resonance spectroscopy, GCs incorporated were determined by UV absorption and polarized light microscopy and surfactant activity with pulsating bubble surfactometer. We found that GCs have a ceiling of incorporation of around 10 wt%, and that the GC not incorporated remains as crystals in the aqueous phase without altering the biophysical properties of the surfactant. This fact is important, because the greater the proportion of GC that EPS can carry, the better the efficiency of this pulmonary GC system.

1. Introduction

Pulmonary surfactant is a complex mixture of lipids and at least four specific proteins, that form a surface-active film at the air-water interface of alveoli, capable of reducing surface tension to near 0 mN/m (Creuwels et al., 1997). It has been proved that the administration of exogenous pulmonary surfactants (EPS) often provide immediate relief of symptoms and improve oxygenation and gas exchange in some lung diseases like infant respiratory distress syndrome (IRDS), pneumothorax, and pulmonary interstitial emphysema. This therapy may also be effective in other lung diseases such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS), meconium aspiration syndrome (MAS) and pulmonary edema (Dushianthan et al., 2012; El-Gendy et al., 2013; Raghavendran et al., 2011; Willson and Notter, 2011; Zhang et al., 2013)

On the other hand, glucocorticoids (GCs), due to their anti-inflammatory actions, have been commonly used to modify the course of chronic lung disease (Jobe, 2009; Shah et al., 2012). Topical administration of GCs into airways is nowadays the most frequently used method because it avoids the serious side effects of the systemic administration of these substances (Cole, 2000). The local lung delivery efficiency for drugs in general and GCs in particular, is very low and depends on the system used, i.e. delivery of aerosolized budesonide ranges from 4.4% to 26.6% while for dry-powder inhaler (DPI) it reaches a maximum of 38% (Berlinksi and Waldrep, 1997; Tang et al., 2009). It has been demonstrated that the deposition of the drug in the lungs have some type of predictive role for the efficacy of drugs given by the inhaled route. Improving lung deposition, clinical response is enhanced (Newman et al., 2000; Patil and Sarasija, 2012; Thorsson et al., 1994). Thus, it arises the challenge of developing new technologies to achieve a better alveolar deposition of these substances. Exogenous pulmonary surfactant can incorporate and transport drugs poorly soluble in water along the entire respiratory surface, avoiding the physiological barriers of the airway (Hidalgo et al., 2015). These properties make EPS a promising strategy for efficient GCs transport by improving the supply of active molecules in the airways. In addition, EPS could be expected not only to act as a carrier, but also to have a therapeutic effect by itself contributing to the overcoming of the lung disease. Therefore, it is critical that the GCs incorporated to EPS do not impair its biophysical properties and allow the desired synergistic effect.

It is known that the presence of cholesterol (Cho) 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). All corticosteroids are biochemically derived from Cho and hence share a close structural

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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. In our previous study, we evaluated the GCs action on the surface activity and biophysical properties of a bovine EPS (Prosurf) in a novel combination drug product containing EPS and one GC (EPS-GC). We assayed three GCs: beclomethasone (Be), budesonide (Bu) or fluticasone (Flu) and found that the incorporation of each GC in the EPS membranes induced different effects on the surfactant properties: Flu did not significantly alter any biophysical properties while Bu and Be caused minimal changes in the fluidity of the polar region of the bilayers and induced a slightly increase in the surface tension, but these changes were not enough to inactivate the surfactant (Cimato et al., 2016). Taking into account our results, we considered that Be and Bu could be potentially harmful to the EPS activity at higher concentrations. On the other hand, in the process of developing a pharmaceutical product combining an EPS and a GC it is important to know the maximum amount of GC that can be incorporated into the membranes and in thus, maximize the efficiency of this pulmonary GC delivery system.

The aim of the present study was to evaluate if the presence of increasing quantities of GCs in the EPS bilayers alters the structure and/or the activity of the surfactant and to determinate the maximum amount of GC that can be incorporated into the membranes without affecting their biophysical properties. To achieve these objectives, we prepared formulations containing an exogenous surfactant (Prosurf) and different amounts of the GCs commonly used in pulmonary therapy: beclomethasone, budesonide or fluticasone, and analyzed the amount of GC incorporated into surfactant membranes, the bilayer organization, and their relationship 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 different GC/PL weight ratio (PL = 10 mg/ml; GC 1.0; 1.5 and 2.0 mg/ml). 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 (10–20 wt%) in equivalent proportions to GCs. These samples (EPS-Cho) were used as positive control.

All EPS-GC samples were diluted with saline solution to a final PL concentration of 10 mg/ml and final pH 5.8–6.0.

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. Percoll and 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 determinate the GC concentration, 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 amount of each GC was calculated using the extinction coefficient from the respective calibration curve.

2.4. Incorporation of GCs into EPS membranes

The amount of each GC incorporated in the EPS membranes was qualitatively and quantitatively determined by polarized light microscopy and UV absorption respectively.

2.4.1. Polarized light microscopy

The EPS-GC samples were observed using a polarizing light microscope (Zeiss Axioscope 2 *plus,* Germany). A pin-tip amount of each EPS-GC formulation was smeared onto a microscope glass slide using a micro syringe dispenser and then quickly covered with a cover slip. The micro syringe containing the EPS-GC formulation was slowly pressed over the glass slide to make it as thin as possible. A 40× objective lens and a 10× eyepiece lens were used with semi-cross polarizers in the bright field to detect birefringence. Micrographs was taken using the polarizing microscope.

2.4.2. Percoll density centrifugation

To determine the concentration of GC incorporated into the EPS membranes, unincorporated GC crystals were separated by Percoll density centrifugation. In brief, this involved mixing 200 microliters of each sample with 150 microliters of Percoll 40% and centrifuging the mixture at 10,000*g* for 20 min at room temperature. The supernatants containing the EPS-GC membranes were separated and the pellets (with the unincorporated crystals) were discarded. The amount of each GC incorporated into the EPS membranes was measured by chemical determination as described above and expressed as GC/PL wt. ratio.

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 ethanol 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-

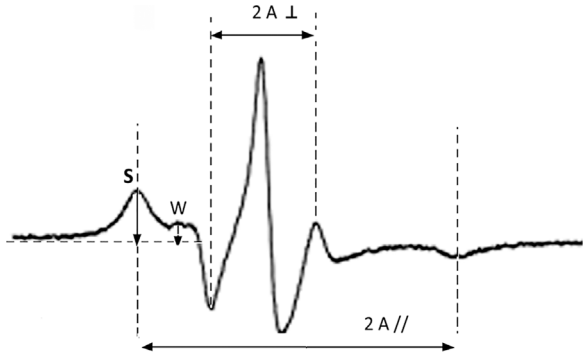

Fig. 1. ESR spectra of EPS labeled with 5DSA. The figure shows the separation, in Gauss, of the outermost (2A//) and innermost (2A⊥) peaks of the ESR spectra signal. The arrows show the amplitude of two spectral components of the low-field peaks. These components are commonly called strongly (S) and weakly (W) immobilized.

band spectrometer (Germany). All ESR experiments were performed at 20 °C. Instrumental parameters were as follows: sweep width 100Gauss, center field 4380 G, time constant5.12 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; the hyperfine splitting tensors parallel (A//)and perpendicular (A⊥) 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. 1 and Eq. (1)). These parameters 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)

$$S = \frac{A_{//} \quad A_{\perp}}{A_{zz} \quad 0.5(A_{xx} \quad A_{yy})} \times \frac{a_0}{a_o} \tag{1}$$

where A⊥ and A|| are the true hyperfine constant values. The polarity correction term $á_0/a_0$is introduced to take into account the hyperfine splitting dependence on the polarity of the label environment, where: $a_0 = (A_{||} + 2\,A_{\perp})/3$ and $á_0 = (A_{xx} + A_{yy} + A_{zz})/3$. The value Á obtained from the spectrum has to be corrected to give the true A value. The correction is given for $S < 0.45$ by $A = Á + 0.8$.

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.

2.5.4. 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. 1). 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 10mN/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 10mN/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 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. Incorporation of GCs and cho into EPS membranes

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 quantity of the GC added to EPS were totally incorporated, each sample was observed using a polarized light microscope and the concentrations of Bu, Be, Flu and Cho in the EPS membranes were determined (Mat. Met. 2.4). Fig. 2 shows the photos of the EPS-GC samples with increasing amounts of each GC, obtained by polarized light microscopy. Bu and Flu crystals were not observed in formulations containing 10 wt% and only a very small amount of crystals per field were observed in the sample of EPS-Be (10 wt%), indicating that this GC is incorporated into EPS membranes in a GC/PL ratio lower than 0.1. All EPS-GC formulations with 15 wt% and 20 wt% showed crystals in the aqueous phase. The amount of crystals observed per field increased with the concentration of GC originally added to the formulation. The EPS-Cho samples did not show the presence of crystals

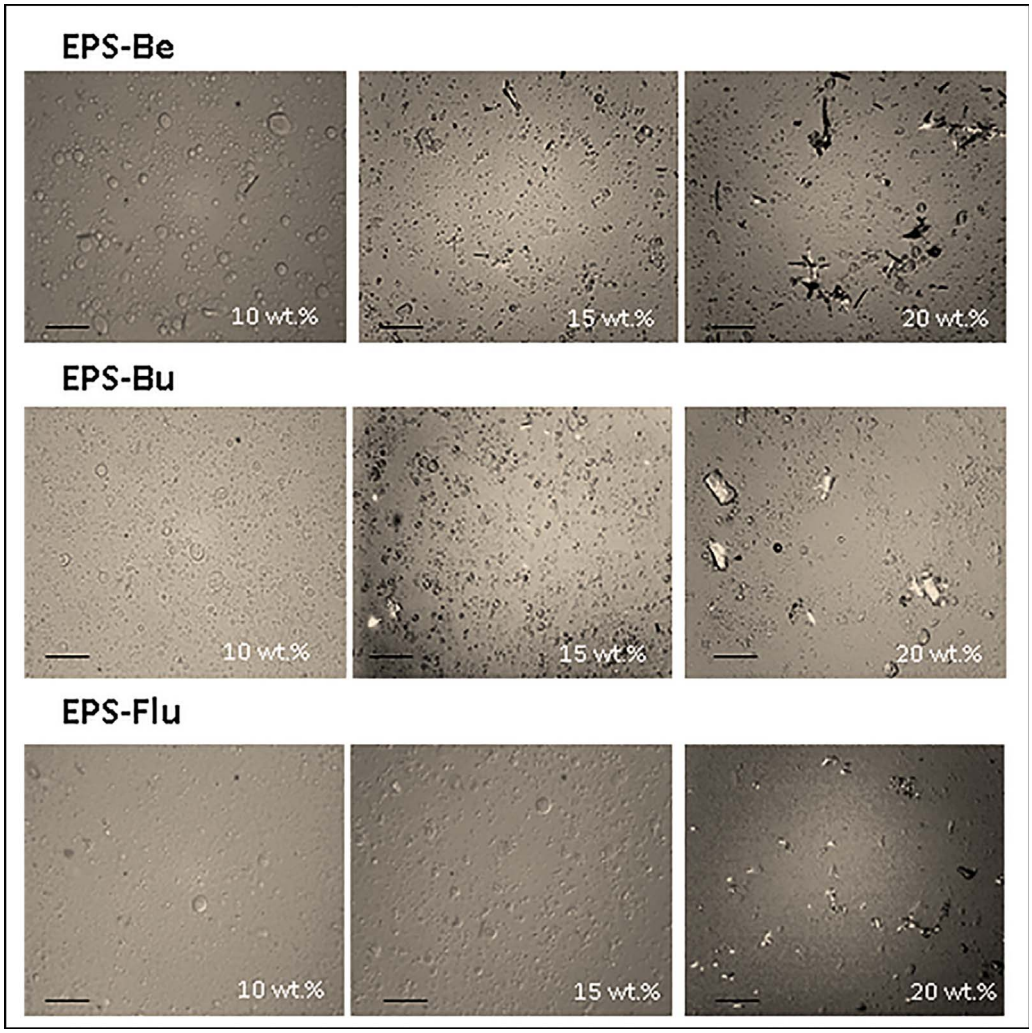

Fig. 2. Representative images of EPS-GC membranes formulated with increasing amounts of each GC, under polarized light microscope, at room temperature. The scale bars correspond to 10 μm.The optical anisotropy confirming the presence of GCs crystals in the formulations.

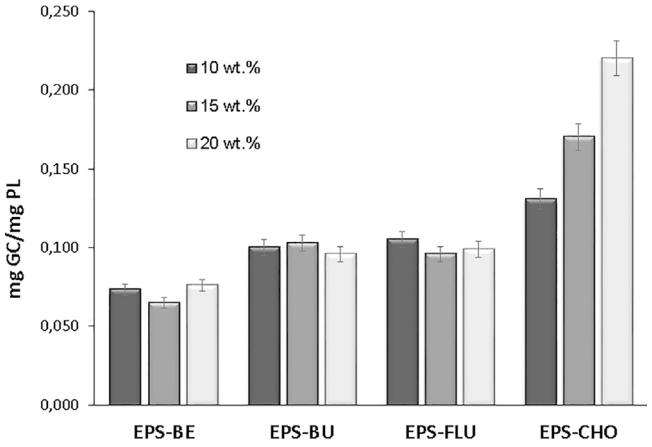

Fig. 3. Amount of GCs or Cho incorporated into the whole EPS. EPS-GC and EPS-Cho with different GC/PL or Cho/PL ratios were added with 150 micro liters of Percoll 40%. Mixtures were centrifuged at 10,000*g* for 20 min at room temperature. The supernatants containing the EPS-GC membranes were separated and the pellets (with the unincorporated crystals) were discarded. 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.

for any of the concentrations of Cho tested (photo not shown). In order to quantify the maximum amount of GC that was effectively incorporated into the EPS membranes, unincorporated crystals were separated by Percoll density centrifugation and the concentrations of Bu, Be, Flu and Cho were determined in the supernatant containing the whole EPS, Fig. 3 shows the results obtained. The maximum GC/PL wt. ratio in the EPS-Flu and EPS-Bu was close to 0.1 regardless of the amount of GC added originally in the preparation, meaning that Bu and Flu were totally incorporated to EPS up to 1 mg/ml, while the maximum GC/PL wt. ratio reached in the EPS-Be was only 0.07. Cho was almost fully incorporated into EPS at all the concentration assayed. The Cho/PL wt. ratio obtained in each case showed that the extra cholesterol in the formulations was added to the endogenous Cho of the original surfactant.

3.2. Surfactant micro-structure: ESR spectral analysis

The ESR spectrum yields information about the molecular environment of the spin probe. The order parameter (S) calculated from the ESR spectra of 5DSA incorporated into the EPS bilayer 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. 4 shows the order parameter obtained from the spectra of EPS-GC and EPS-Cho with increasing amount of each GC or Cho respectively. In concordance with our previous results (Cimato et al., 2016), the addition of 10 wt% of Bu or Be caused a slight increase in the value of the order parameter ($p < 0.01$), but the addition of increasing amount of these GCs to the formulations did not cause a higher increase of this parameter. On the other hand, Cho increased the bilayer rigidity as a function of the added amount. This increase was higher ($p < 0.05$) than that caused by Bu or Be at all

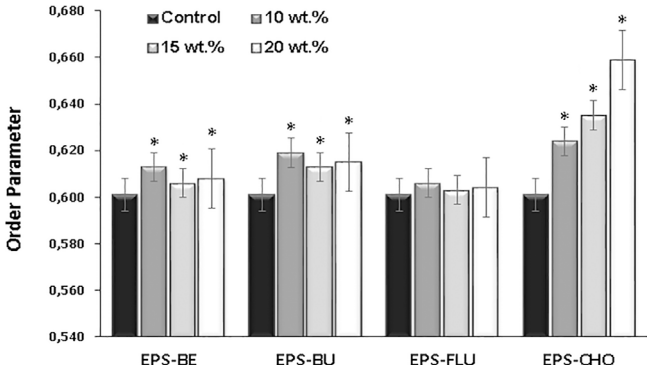

Fig. 4. Order parameter (S) obtained from the 5DSA spectra of EPS-Cho or EPS-GC prepared with increasing amounts of each GC or Cho. Surfactant without added Cho or GCs was used as control. Data are represented as the mean ± SD. Statistically significant increase compared to the control (*) $p < 0.01$.

concentrations tested, but no significant differences were observed between the order parameter of EPS-Bu and EPS-Be. For EPS-Flu formulations, no changes in the order parameter were detected at any Flu concentration.

A common feature of almost all lung surfactants and model mixtures is the coexistence of a semi-crystalline liquid ordered (Lo) and a liquid disordered (Ld) phases. 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 proportion of PL in Lo phase where the spin probe is more immobilized. Fig. 5 shows the S/W ratio obtained from the 5DSA spectra of EPS with increasing amount of each GC or Cho respectively. The addition of Bu, in contrast to what happens with the order parameter, did not significantly change the S/W ratio at any concentration whereas the presence of the other two GCs and Cho increased this parameter as a function of the concentration. However, among all formulations at 10 wt%, only EPS-Flu showed a S/W value higher than control ($p < 0.05$), the remaining EPS-GC or EPS-Cho formulations showed no significant differences at this concentration.

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

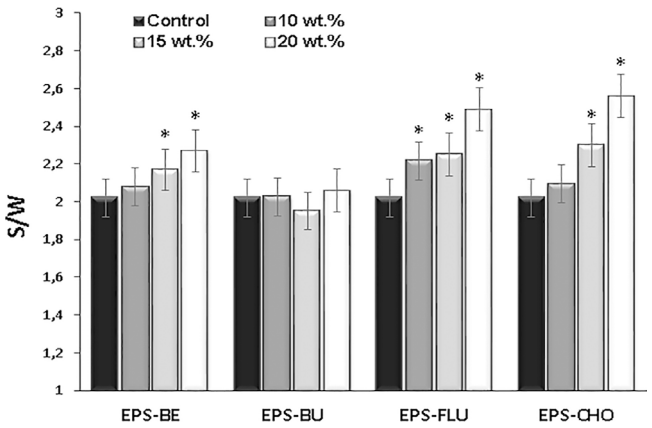

Fig. 5. S/W ratio obtained from the 5DSA spectra of EPS-Cho or EPS-GC prepared with increasing amounts of each GC or Cho. Surfactant without added Cho or GC was used as control. Data are represented as the mean ± SD. Statistically significant increase compared to the control (*) $p < 0.01$.

(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 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 10 or 20 wt% of none of the GCs tested to EPS caused a significant increase in the ST and ΔA_{10} values, so all EPS-GC formulations showed values of surface tension and spreading ability within acceptable limits for this surfactant (lower than 5 mN/m and 47% respectively). In contrast, the addition of Cho affected the surfactant properties as a function of the concentration. The EPS-Cho formulations showed ST and ΔA_{10} values greater than the upper limit accepted ($p < 0.01$) at both concentrations.

4. Discussion

The idea of using EPS as a carrier started to be developed years ago and some initial experiments proved its potential in the late 1990s (Herting et al., 1999; Katkin et al., 1997; Wang et al., 2012). EPS 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. We have previously discussed 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 (Cimato et al., 2016). Since the GCs are highly lipophilic, it was expected that they were easily incorporated into the surfactant membranes, making EPS an ideal vehicle for this type of drugs. It has usually been taken for granted that all the lipids would be equally incorporated into the bilayers, i.e. that the liposome lipid composition would be the same as that of the original mixture from which the vesicles were formed. However, it has been demonstrated that particularly in mixtures with high cholesterol contents, this might not be the case. Cho does not become quantitatively incorporated into lipid bilayers, it has a ceiling of incorporation in the bilayers depending on their lipid composition. For example, in bilayers containing Cho and DPPC, Cho appeared to be incorporated only partially, with a maximum at 60 mol % irrespective of the original proportion of lipids (Ibarguren et al., 2010). The same is expected to happen with the GCs, that is, these compounds would also have a ceiling of incorporation in the surfactant bilayers. Our results confirmed this hypothesis since Bu and Flu were incorporated into the surfactant membranes up to 10 wt%, and Be did so in slightly lower proportion. Since lipophilicity depends on the molecular structure, it could be thought that the maximum GC proportion that becomes incorporated into the EPS bilayers are due to the different side chains that these molecules possess (Takegami et al., 2008). It is very important to know what is the maximum amount of GC that can be incorporated into the EPS membranes when formulating these pharmaceutical products, because the greater the proportion of GC that can be incorporated to the surfactant membranes more efficient would be the delivery of these substances in the lung. In this case, we found that GCs can be incorporated into the membrane up to 10 wt% and that the rest of the GC not incorporated into the surfactant bilayers remains in crystalline form in the aqueous phase.

As we said above, in these combination drug products, EPS not only acts as a carrier but could be expected to have therapeutic effect by itself. Therefore, it is critical that the presence of the GC do not impair the biophysical properties of the EPS and allow the desired synergistic effect. It is known that the fluidity in the polar zone of the PL bilayer is critical for a proper EPS surfactant activity (Martínez Sarrasague et al., 2012). The bilayer fluidity reflects the order and dynamic of phospholipids alkyl chains in the layer and is mainly dependent on its composition. In our previous study we demonstrated that the addition of Flu to EPS (10 wt%) did not alter the biophysical properties of the membranes, but Bu and Be, at the same concentration, caused a

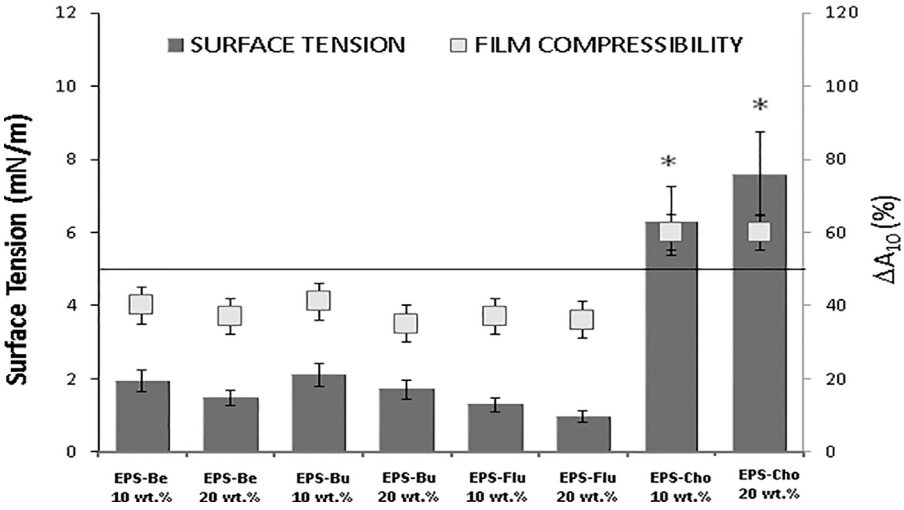

Fig. 6. Surface tension (ST) and percentage reduction in bubble surface area (ΔA_{10}) of the EPS added with 10 wt.% or 20 wt.% of each GC or Cho. ST was measured with a pulsating bubble surfactometer at 37 °C. ΔA_{10} is an indicator of dynamic film compressibility. Data are represented as the mean ± SD. The bars represent the ST data and the points represent ΔA_{10} values. The 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 upper limit values (*) $p < 0.01$.

disturbance of the order in the polar zone of the membranes and slightly altered the surface properties (Cimato et al., 2016). For this reason, although none of them inactivated the surfactant, we raised the possibility that higher concentrations of Bu and Be could be potentially harmful to the EPS activity. However, the results obtained in the present study showed that a greater amount of these GCs added to the preparation did not generate greater alteration of membrane fluidity. This could be because GCs have a ceiling of incorporation of around 10 wt%, while Cho, incorporated completely to the membranes at all concentrations tested, generated an increase in stiffness as a function of the concentration. Therefore, we concluded that there is a correlation between the concentration of these substances within the surfactant membranes and the increase of rigidity in the polar zone, and that the unincorporated GCs present in the preparation in crystalline form have no effect on this property of the membrane.

The particular lipid composition of surfactant, including a high proportion of DPPC, induces segregation of Lo and Ld phases in surfactant membranes and films at physiological temperatures. The segregation of DPPC-enriched ordered phase has been related with the ability of surfactant films to produce very low tensions (Bernardino de la Serna et al., 2013). In our previous study, we have already shown that among the GCs tested, only Flu at 10 wt% altered the Lo/Ld ratio of the surfactant membranes but this modification did not correlate with surfactant activity. In the present study, our results showed that increasing amounts of Flu, even when not incorporated into the membrane, generated a greater increase of the proportion of Lo phase. The same happened in EPS-Be formulations. The fact that Flu and Be affected the Lo/Ld ratio but Bu did not, remains unclear under the perspective of this study. However, since increasing amounts of Flu and Be increased the proportion of Lo phase but without altering the surface properties, we concluded that the change in this parameter did not reflect an alteration in the surfactant properties that has implications in the functionality of the EPS.

We have already discussed that only the fluidity in the polar zone of the PL bilayer is critical for a proper EPS surfactant activity and, in this study, we found that the change in bilayer fluidity depends on the amount of GC incorporated into the membranes. As all GCs were incorporated to about 10 wt%, we can say that this amount of GC is not enough to modify the membrane fluidity and, consequently, the surfactant properties. On the other hand, the excess of GC present in the formulations in crystalline form did not alter the surfactant properties either, so it can be concluded that the effect on the surface tension depends on the amount of corticoid effectively incorporated into the membrane. Furthermore, Cho, fully incorporated into EPS membranes at all concentrations tested, increased stiffness in the polar zone and modified the surface properties of EPS.

As we said above, in the process of developing a pharmaceutical product combining an EPS and a GC t is important to know the maximum amount of GC that can be incorporated into the membranes and that the GCs incorporated to EPS do not impair its biophysical properties and allow the desired synergistic effect. The relevance of this study is that we have been able to establish the maximum amount of each GC that can be incorporated into the EPS membranes and that both the GC incorporated and the remaining crystalline form in the aqueous phase do not alter the biophysical properties of the surfactant. This fact is important, because the greater the proportion of GC that EPS can carry, the better the efficiency of this pulmonary GC delivery system and the desired synergistic effect between surfactant and GC could exist.

It remains to be studied whether these EPS-GC combination products allow a better delivery of GC in the lung and to establish whether the formulations that have unincorporated crystals reach to the lung in the same way adding therapeutic effect.

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