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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-