REPORT

Organization of the protein-lipid nanostructures Surfacen® in correspondence with its effectiveness as agent for stabilizing the air-liquid interface respiratory and first report as antileishmanial agent

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ABSTRACT

The understanding of the correlation between the biophysical processes mediating the activity of clinically introduced surfactants is essential for their clinical efficacy, together with their simultaneous impact on coexistent respiratory infections. Therefore, this study was aimed to characterize the interfacial and structural properties of the films formed by Surfacen® and how they could mediate the drug efficacy of this surfactant as a stabilizer of the respiratory air-liquid interface. Moreover, the rheological properties and particle size were also studied, and the product's anti-inflammatory effect against the respiratory pathogen *Staphylococcus aureus* and also *Leishmania amazonensis* were assessed in pharmacological studies in vitro. Surfacen® displayed similar properties as those of an endogenous surfactant and an organic extract, by forming stable and efficient film-active surfaces in the air-liquid interface. Its effect was characterized by a much higher segregation of lipid condensed phases, with significantly larger and more stable ordered domains when subjected to compression, its increased mechanical stability relevant for respiratory dynamics. All these results are relevant for the optimization of surfactants by stabilizing its film-forming properties. Significantly, Surfacen® showed a low viscosity, which is attractive for the intratracheal administration, and simultaneously inhibited the release of pro-inflammatory cytokines (TNF-α and IL-6) from monocytes and neutrophils stimulated with *S. aureus*. Moreover, Surfacen® and SP-A were active against *L. amazonensis*, this response enhanced by combining both, constituting the first report of the antileishmanial activity of a pulmonary surfactant-SP-A combination, as a potential therapeutic strategy. This research granted the 2015 Award of the Cuban National Academy of Sciences.

Keywords: pulmonary surfactant, surface tension, Surfacen®, inflammation, *Leishmania amazonensis, Staphylococcus aureus*

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RESUMEN

Organización de las nanoestructuras proteico-lipídicas del Surfacen® en correspondencia con la eficacia como agente de estabilización de la interfase aire-líquido respiratoria y primer reporte como agente antileshmanial. La comprensión de la correlación entre los procesos biofísicos que median la actividad clínica de los surfactantes clínicamente disponibles es esencial para potenciar su eficacia clínica, con un impacto simultáneo en las infecciones respiratorias coexistentes. Por tales razones, el objetivo del presente estudio fue caracterizar las propiedades interfaciales y estructurales de las películas formadas por Surfacen® y relacionarlas con su eficacia farmacológica como agente de estabilización de la interfase respiratoria aire-líquido, así como estudiar sus propiedades reológicas y el tamaño de partícula. Estas investigaciones se complementaron con nuevos estudios farmacológicos in vitro mediante el reto del producto contra el patógeno respiratorio *S. aureus*, para conocer su efecto anti-inflamatorio, y además contra *Leishmania amazonensis*. Se demostró que Surfacen® exhibió propiedades similares a las del surfactante endógeno y su extracto orgánico, al formar películas de superficies activas estables y eficientes en la interfase aire-líquido, con una segregación de las fases lipídicas condensadas mucho mayores, con dominios ordenados significativamente más grandes y estables cuando son comprimidas. El surfactante se caracterizó por una baja viscosidad, aspecto atractivo para su suministro por vía intratraqueal, e inhibió la liberación de citoquinas pro-inflamatorias (TNF-α e IL-6) en monocitos y neutrófilos estimulados con *Staphylococcus aureus*. Surfacen® y la SP-A mostraron actividad contra *Leishmania amazonensis* y la combinación de ambos potencia dicho efecto, lo cual fue el primer informe de la actividad antileishmanial de un surfactante pulmonar y de la SP-A, con nuevas potencialidades terapéuticas contra la Leishmania. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2015.

Palabras clave: surfactante pulmonar, tensión superficial, Surfacen®, inflamación, *Leishmania amazonensis, Staphylococcus aureus*

Introduction

Clinical pulmonary surfactant (PS) preparations are commonly used to treat premature infants with respiratory distress syndrome. They show great potential in the treatment of a number of complex respiratory diseases with inflammatory and infectious basis, in infants, children and adults. Despite the extensive study of their chemical composition, surface activity and clinical effectiveness of various PS preparations, a direct comparison of PS films with its source material remain to be characterized at the interfacial and structural levels, and its relation with biochemical composition and clinical efficacy.

Noteworthy, the application of microscopic techniques and surface biophysics to the study of PS has revolutionized the understanding of these preparations in the last decade. These methodologies have revealed phase separation of phospholipids, phospholipid-protein interaction and local chemical composition of PS films [1]. In fact, they are relevant for the conventional evaluation in vitro of clinically available PS preparations and to elucidate their mechanism of action.

At the same time, PS preparations are also key modulators of pulmonary innate and acquired immunity, regulating lung inflammatory processes. Because the inhibition of local immune response may decrease lung injury, the PS therapeutic efficacy may be related not only to its biophysical characteristics but, at least in part, to its anti-inflammatory features [2]. However, there is a need to identify which surfactant preparation ensures the highest anti-inflammatory activity, and, thereby, potentially decrease the inflammatory process driving the respiratory distress syndrome.

SP-A (lung collectin not present in clinical preparations surfactant) is a versatile preparation capable of interacting with a variety of pathogens, such as bacteria, viruses and fungi [3], as well as it is also expressed outside of lung. On the other hand, the SP-B protein is present in clinical preparations of surfactants and has structural homology with the dermaserpin antimicrobial peptide, this last property subjected to further research on the potential antibacterial properties of SP-B [4]. It was recently reported that dermaserpin has activity against *Leishmania* [5], these findings encouraging the evaluation of PS preparations, as well as the SP-A, against parasitic protozoa.

Therefore, the aim of this series of experiments was to characterize the interfacial and structural properties of the films formed by Surfacen®, to relate them to its pharmacological efficacy as a stabilizer of the respiratory air-liquid interface, and to study its rheological properties and particle size. These investigations were supplemented with new pharmacological studies in vitro, challenging this product against *Staphylococcus aureus*, a significant respiratory pathogen, to characterize the Surfacen®'s anti-inflammatory effect and also against *Leishmania amazonensis*.

Materials and methods

Surfactants

Preparations of Surfacen®, a clinical surfactant used in the Neonatal Respiratory Distress Syndrome therapy in Cuban hospitals, were obtained from the Centro Nacional de Sanidad Agropecuaria (CENSA, Mayabeque, Cuba). Native porcine lung surfactant (NPLS) was obtained from bronchoalveolar lavage of porcine adult fresh lungs obtained from the slaughterhouse. The organic extract of native porcine lung surfactant (ENPLS) was obtained by chloroform/methanol extraction of NPLS. To prepare ENPLS aqueous suspensions, the required volume of the extract was first dried under nitrogen and then hydrated in buffer 5mM Tris pH 7, 150 mM NaCl.

Biochemical studies

Analysis of protein content in Surfacen® and other surfactant samples was carried out by electrophoresis under reducing conditions. Protein bands were then transferred onto nitrocellulose membranes and analyzed by Western blot. For SP-B analysis, membranes were first incubated with SP-B-antibody 1:5000 (rabbit antihuman SP-B, Seven Hills Bioreagents, USA), and then with peroxidase conjugated goat Ig-G anti rabbit (SC-2004 from Santa Cruz Biotechnology). The same procedure was followed to detect and analyze SP-C content (primary antiSP-C antibody W RAB-MSPC, from Seven Hills Bioreagents).

Biophysical studies by *captive bubble surfactometer (CBS)*

The surface activity of the different surfactant samples was evaluated using a fully computer-controlled CBS. Bubble volume, interfacial area and surface tension were calculated using height and diameter of the bubble as previously described [6].

Structural analysis *of surfactant films*

Epifluorescence microscopy

This technique allows transfer and characterization of films compressed at different surface pressures, in the glass support. Films of NPLS, reconstituted ENPLS, or Surfacen® were prepared by spreading the suspensions at 5 mg/mL onto the buffered subphase of a modified Langmuir balance (LB; Nima Technology, Coventry, UK), thermostated at 25 °C. The suspensions were previously doped with 1 mol percent of the fluorescent lipid. Alternatively, monolayers were also prepared from ENPLS or Surfacen® extracts (1 mg/mL) in chloroform/methanol (2:1). The films were compressed to the required pressure at a compression rate of 25 cm^2/min. the films were transferred to glass coverslips previously immersed in the subphase, to obtain continuously varying surface pressure (COVASP) films. Epifluorescence images of the supported LB films were acquired in a Zeiss Axioplan II fluorescence microscope (CarlZeiss, Jena, Germany).

Atomic force microscopy (AFM) of surfactant films

Samples for AFM were prepared upon transfer of surfactant films onto freshly cleaved mica surfaces using the classic Langmuir–Blodgett method. NPLS or Surfacen monolayers were prepared by spreading organic extract solutions (1 mg/mL) onto the buffer subphase of the surface balance. The films were then compressed until reaching the required surface pressure (37 mN/m) and transferred onto mica supports.

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The AFM images were obtained with a Multimode Nanoscope IIIA equipped with a type J scanner(Veeco Instruments, Santa Barbara, CA), operated in tapping mode.

Antiinflammatory studies on the induction of cytokines in peripheral blood mononuclear cells (PBMCs)

PBMCs were collected by the Ficoll density gradient method (Ficoll-paque plus, Amersham Bioscience GE Healthcare, Uppsala, Sweden) and suspended in RPMI 1640 medium (Sigma, Germany) supplemented with 10 %. The TNF-α and IL-6 were measured by capture enzyme-linked immunosorbentassays (ELISA) according to the manufacturer's instructions. Antibody pairs and reagents (OptEIA sets) were obtained from BD Biosciences (USA). Samples were tested in duplicates, and a standard curve with human recombinant cytokine was prepared in each plate.

Antiamastigote activity

In the case of combination for the drug interaction against intracellular amastigotes of L. amazonensis, 5 µL of Surfacen® and SP-A in combination were added at concentrations ranging 6.25-50 µg/mL to each product, in duplicates, for 48 h. The cultures were then fixed with absolute methanol, stained with Giemsa, and examined under light microscopy. In parallel, the activity of the products was controlled, where infected macrophages were exposed to each product alone using the same methodology. The fractional inhibitory concentration (FIC) index was calculated by the equation: FIC = [A]/IC_{50} A + [B]/IC_{50} B, where: IC_{50} A and IC_{50} B are the IC_{50} of each compound alone and [A] and [B] are the IC_{50} of Surfacen® and SP-A used in combination. An FIC index lower than or equal to 0.5 indicates synergy, higher than 4 indicates antagonism, and between 0.5 and 4 indicates indifference. The fixed-ratio method was used to analyze the combined drug effects at different Surfacen®/SP-A ratios: 4:1; 3:2; 2:3 and1:4. The IC_{50} was determined and the FIC index was calculated.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software. Data were expressed as mean ± SEM and analyzed with one way ANOVA with a LSD *post hoc* test. A probability value of $p \leq 0.05$ was considered statistically significant.

Results

Biochemical studies

Surfacen® contains well-detectable proportions of both proteins, in the order of 0.7 % SP-C and 0.4 % SP-B with respect to phospholipid by mass, comparable in broad terms to the amounts of SP-B and SP-C present in a well known clinical surfactant like Curosurf®.

Biophysical studies

Adsorption and quasi-static and dynamic cycling isotherms were obtained in a captive bubble surfactometer, under more physiologically relevant conditions of high surfactant concentrations and physiological temperature. Surfacen properties were similar to that of native porcine surfactant or the organic extract, forming films of effectively active and stable surfaces at the air-liquid interface. In fact, they were able to reach surface tensions below 5 mN/m in repetitive compression-expansion cycles [7].

Structure of surfactant films

Compositional differences between NPLS, ENPLS and Surfacen® would anticipate differences in the structure of the different films, in spite of their comparably efficient behavior at the interface. Epifluorescence images of films formed by spreading NPLS, ENPLS or Surfacen® aqueous suspensions are shown in Figure 2 A. The three types of films exhibited segregated dark domains, indicative of the existence of ordered regions with packing excluding the bulky fluorescent probe, but these condensed domains showed very different morphology in the materials compared. In contrast to domains in NSPL or ENPSL films, domains segregated upon compression of Surfacen® films showed a high contrast, which was maintained at all the pressures analyzed. Furthermore, Surfacen® domains were larger and occupied a larger fraction of area than condensed domains in NPLS or ENPLS. At pressures above 45 mN/m, bright spots of structures presumable protruding in the Z-axis were also present in Surfacen® films, although much smaller and more diffusely distributed than those seen in ENPLS layers.

To explore the nature of the marked differences between the lateral structure of compressed ENPLS and Surfacen® films, their organization was analyzed at the submicrometer and nanometer scales by AFM. At large scale, AFM images confirm the presence of more numerous and larger condensed domains in Surfacen® films than observed in films formed by ENPLS. Therefore the illustrative topology and phase AFM images of ENPLS and Surfacen® films compressed to 37 mN/m were compared [7], this surface pressure the one at which maximal segregation of condensed phase was observed at the microscopic scale. At the largest scale scanned, AFM images showed the presence of well-defined micron-size domains, presumably constituted by DPPC-enriched condensed phase, as they showed size and distribution fully comparable to those of the dark domains observed under epifluorescence microscopy. The higher resolution of AFM showed clear differences in size and morphology between the segregated domains in Surfacen® films and those in ENPLS layers.

These findings were an important contribution to the knowledge of pulmonary surfactant and particularly for the design of new surfactants, since they provided the basis to optimize the stability of the films of surfactants, among other properties relevant for respiratory dynamics. This work was one of the few studies in which the structural and functional properties of a clinical surfactant preparation were compared in detail with the source material from which it originates. Hence, brought a setting adequate to analyze to what extent the procedures involved in the extraction, handling, production and storage of a clinical surfactant preserves the functional properties of native.

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Effect of Surfacen® on TNF-α and IL-6 release by PBMCs stimulated with *S. aureus*

TNF-α secretions were measured after 4 and 24 h in unstimulated and *S. aureus*-stimulated PBMCs with Surfacen®. In the absence of stimulus low levels of TNF-α were detectable, which were not affected by surfactant. Stimulation with 4.8×10^7 colonies/mL of *S. aureus* resulted in TNF-α release after 4 h that was four-fold (1167 ± 539.2 pg/mL) the basal production (273.9 ± 106 pg/mL) of TNF-α, with sustained levels until 24 h (1064.2 ± 929.4 pg/mL) of incubation. A dose-dependent suppression of TNF-α release was observed when PBMCs were preincubated with different concentrations of Surfacen® and challenged with *S. aureus* for 4 h [8]. The level of TNF-α decreased up to 60% in *S. aureus*-induced cells when pre incubated with Surfacen® for 4 h [8].

When cells were stimulated for 24 h, the reduction of TNF-α supernatant's levels was less impressive, with a significant decrease observed only with the higher concentration of Surfacen® [8]. Similarly, the level of IL- 6 was minimal with and without surfactant in unstimulated cells. In contrast, the level of IL 6 increased up to two fold (16399 ± 9719.3 pg/mL) after 2 h and fivefold (51180.8 ± 36075.9 pg/mL) after 24 h in *S. aureus* stimulated cells (4.8×10^7 colonies/mL) when compared with normal (8684.4 ± 5122.7 pg/mL). A dose dependent suppression of IL-6 levels detected after 4 h of *S. aureus* stimulation was observed with pre incubation of Surfacen® in PBMCs ($p \leq 0.05$) [8]. When cells were stimulated for 4 h, Surfacen®, at a concentration of 0.5 mg/mL, induced about 40 % inhibition of *S. aureus*-induced IL-6, an effect that was sustained from 1 to 6 h of pre-incubation, with the higher inhibition observed at 4 h. Similar to TNF-α, no significant changes were observed in IL-6 production with 0.125 mg/mL of Surfacen®; however, significant reduction observed with 0.5 mg/mL Surfacen® after 24 h of stimulation [8].

Antiamastigote activity of Surfacen®

Surfacen® showed activity against intracellular amastigotes forms, inhibiting the percent of infected macrophage and the average number of amastigotes per macrophage. The IC_{50} value against parasite was of 17.9 ± 3.0 μg/mL; while no toxic effect on mice macrophage was observed at the highest concentration evaluated (200 μg/mL). The product showed a similar activity ($p > 0.05$) to a drug used as first line, glucantime, which showed an IC_{50} value of 11.0 ± 3.4 μg/mL [9]. Surfacen® did not cause inhibitory activity on promastigote forms of the parasite. SP-A showed activity against the promastigote and amastigote forms of *L. amazonensis* and moderate cytotoxicity against host cells [10], requiring 5-fold higher concentrations in the latter case to reach the levels of cell death it exhibited against parasites. SP-A was less active than glucantime. It should be noted, however, that the antiparasite toxicity of pentostam, another first-line option derivative from pentavalent antimonial, is similar to that of SP-A (IC_{50} = 30 μg/mL).

The results obtained by the fixed-ratio method identified the most effective dose combination against *L. amazonensis*. The greatest activity was obtained with a 4: 1 ratio of Surfacen/SP-A [11]. This combination caused a lower FIC index of 0.287, demonstrating synergism. This result indicates that the Surfacen® contributed more than the protein to the antileishmanial activity.

Relevance of the study

The scientific impact of these results revealed an aggregate value of Surfacen®. They were fully expressed on its biophysical, rheological, biochemical and pharmaceutical properties, demonstrating the relevance of the pharmaceutical formulation and the freeze-drying process (unique natural clinical surfactant in the form of lyophilized). This last contributed to the low viscosity of the formulation, rendering it suitable for drug delivery and as attractive for future pharmaceutical formulations by the addition of new active ingredients. Moreover, biophysical studies showed segregation of sustained lipid phases depending on the pressure surface, what explains the observed high mechanical stability of Surfacen®, being related to the lyophilization process and its biochemical composition

Moreover, these were the first results on the anti-inflammatory effects of Surfacen® against a pathogen that remains as the leading cause of human respiratory infections, with special relevance in the lower respiratory tract. And it provided the first international report on the antileishmanial activity Surfacen® and SP-A. This also encourages the study on the inclusion of these products for the design of new therapeutic formulations against *Leishmania*.

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