REPORT

Biophysical and immunomodulatory basis for treatment of the acute respiratory distress syndrome with exogenous pulmonary surfactant and the SP-A protein

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ABSTRACT

No properly evaluated surfactants are currently available in Cuba for the treatment of the acute respiratory distress syndrome (ARDS), and it is not known whether SURFACEN© and the SP-A protein are suitable for the formulation of this type of product. This study evaluated the biophysical, anti-inflammatory and antimicrobial properties of SURFACEN®, as well as the antioxidant and antibacterial activities of SP-A, to assess the potential for their use as the pharmacological basis of this new therapeutic product. Results proved that the biophysical properties of the Cuban surfactant are similar to those of the native surfactant, with superior interfacial characteristics when compared to CUROSURF® and thermotropic characteristic which are qualitatively different to those of homologous preparations. Similarly, *in vivo* models, showed that its anti-inflammatory activity decreases pulmonary edema, myeloperoxidase activity, malondialdehyde concentrations and the total counts of inflammatory cells, restoring the normal values of the macrophage-polymorphonuclear ratio as well. A dose of 100 mg/kg, in a pleurisy model, decreased pleural exudation to levels comparable to those achieved by indomethacin. During *in vitro* assays, it also inhibited the production of tumor necrosis factor a (TNF α) in activated monocytes, as well as the expression of the ICAM-1 adhesion molecule in endothelial cells. Studies in five pathogenic strains proved that it exhibits antibiotic activity against Gram-positive and Gram-negative bacteria usually associated to respiratory infections. On the other hand, it is shown that pig or porcine SP-A can scavenger hydroxyl radicals and hypochlorous acid, can chelate iron, and has a protective effect in lung mitochondria and microsomes exposed to hydroxyl radicals. SP-A also protected SURFACEN® from the oxidative action of hypochlorous acid and hydroxyl radicals, as demonstrated by the inhibition of lipid peroxidation and a lower modification of the tensoactive properties of the surfactant when treated with these chemicals, as well as by the protection of the polyunsaturated fatty acids present in SURFACEN®. These new biophysical and immunomodulatory properties are the pharmacological supports for a prospective pharmaceutical formulation based on this surfactant and this protein, which can be used for the treatment of acute respiratory distress syndrome. This study contributes to the search and endorsement of a pharmaceutical formulation that may become the choice therapy for a problem that currently remains unsolved.

Keywords: pulmonary surfactant, acute respiratory distress syndrome, SURFACEN®, surfactant protein A, biophysical properties, anti-inflammatory properties, antioxidant properties, antibacterial properties

*I*ntroduction

The pulmonary surfactant is a complex mixture of lipids and specific proteins known as SP-A, SP-B, SP-C and SP-D, which is usually found covering the alveolar epithelium. This surfactant decreases surface tension in the air-liquid interface, preventing the collapse of the alveoli enabling breathing [1]. Additionally, it is known to play an important role in host defenses in the lungs [2]. Pulmonary surfactant is essential, since any deficiency or inactivation causes severe lung disorders.

Neonatal respiratory distress syndrome (NRDS) is characterized by an insufficient synthesis and secretion of the pulmonary surfactant, due to the immaturity of type II pneumocytes. It has been shown that the therapeutic delivery of an pulmonary surfactant from animal source known as SURVANTA® can be effective for the treatment of neonates with RDS [3]. This pioneering work has prompted the development of a number of pharmaceutical preparations known as modified or exogenous natural surfactants, such as SURFACTANTTA® (Tokoyo Tanabe, Japan), ALVEO FACT® (Boehringer Ingelheim, Germany), CUROSU RF® (Chiesi Pharmaceuticals, Italy), INFASURF® (Forest Laboratories, USA) and BLES® (BLES Biochem, Canada), all of which have proven to be effective in the treatment of this syndrome [4, 5]. These preparations differ in their source and production method, which influences their tensoactive [6] and defensive [7] properties.

Acute respiratory distress syndrome is characterized by a damaged pulmonary parenchyma, which in

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turn compromises the normal respiratory function of the patients. It is a complex disorder, with inflammatory, infectious and oxidative etiological agents. ARDS remains an unsolved, life-threatening health problem with a 40% mortality rate, even in highly developed countries 8]. The pulmonary surfactant is seriously affected from a biochemical and biophysical point of view in ARDS patients [9, 10], which in turn compromises the pulmonary defensive response. It is caused by the presence of edema-associated proinflammatory mediators, plasma proteins and oxidative products, which are released to the alveolar space and severely inhibit the surfactant system. Obviously, one potential solution for its treatment would be by giving the patient an exogenous surfactant. However, several preparations of exogenous surfactants under clinical trials have failed to show any efficacy [8], which is explained by the fact that many of them are markedly more sensitive to inhibition by plasmatic components than the natural surfactant [11]. Since the suboptimal nature of these surfactants is a consequence of underestimating the importance of certain components and their interactions, it is assumed that a preparation that is more similar to the natural surfactant stands a higher chance of success for treating ARDS.

A natural surfactant available in Cuba, under the commercial name of SURFACEN®, has been shown to be effective for the treatment of NRDS [12]. This surfactant is a good candidate for ARDS treatment, due to its biochemical composition compared to similar products. However, the biophysical properties of SURFACEN® have not been characterized in detail, especially at the limiting concentrations found in the alveolar space under conditions of inactivation; and its anti-inflammatory and antibacterial activities, which are vitally important for its potential use in the treatment of ARDS have not been evaluated. Additionally, the effect of the inclusion of the SP-A surfactant protein. A to the preparations of this surfactant must be taken into account, since this molecule is not present in any of the commercially available surfactants preparations in spite of its prominent role in pulmonary defenses: it opsonizes, increases phagocytosis, and modulates many mediators of the inflammatory response [5]. The antioxidant properties of this protein are especially relevant for ARDS, although there is contradictory information on its effect in oxygen reactive species [2]. Furthermore, the activity of SP-A obtained from porcine lung is unknown. The purpose of this study is to determine the scientific basis that substantiates the use of SURFACEN® as a good therapeutic candidate for ARDS, since it has very promising pharmacological features according to the information available on the physiopathological characteristics of this disease.

Materials and methods

The definition of the *in vitro* models to study biophysical activity includes the evaluation of interfacial and morphologic characteristics, as well as the thermotropic behavior of SURFACEN® compared to homologous surfactant preparations. The selection of *in vitro* and *in vivo* models for the study of anti-inflammatory and antioxidant activities is based on their relationship to some of the most potentially damaging pro-inflammatory and pro-oxidant mediators.

Biophysical properties

The interfacial properties were analyzed using pressure-area isotherms (*p*-A) in a Wilhelmy plate, in both organic and aqueous media [13]. The interfacial-morphological characteristics were analyzed by compression *p*-A isotherms and the acquisition of phase transition images by epifluorescence microscopy [14]; the thermotropic characteristics (phase transition temperature, Tm) were measured by differential scanning calorimetry. The following products were used: natural porcine pulmonary surfactant (NPPS), organic extracts from swine pulmonary surfactant (ENPPS), SURFACEN® and CUROSURF®.

Anti-inflammatory, antibacterial and antioxidant effects of SURFACEN®

The anti-inflammatory properties were determined in a septic shock model by lipopolysaccharide (LPS) in Balb/c mice, measuring pulmonary edema by gravimetry. An LPS-induced acute lung injury model in rats was also used, evaluating the following damage parameter: total cell counts, cellular differential [15], myeloperoxidase activity (MPO) [16] and malon-dialdehyde concentrations (MDA) [17]. Additionally, the concentration of TNFα in activated monocytes was measured using a cytotoxicity assay in the L 929 cell line [18], as well as the expression of the ICAM-1 adhesion molecule, using a colorimetric assay with 3-(4,5-dimethylthiazol-2-il) -2,5-diphenyltetrazolium (MTT) [19]. The antibacterial activity was determined by measuring bacterial growth with the quantitative suspension method by dilution and counting 20, 21]. Antioxidant activity determinations were made by measuring the scavenger of the superoxide anion, generated by the xanthine/xanthine oxidase system [22].

Antioxidant and antibacterial effects of SP-A

The determinations for the antioxidant effect were performed by the 2-deoxy-D-ribose assay [23], and the lipid peroxidation of mitochondria, microsomes and SURFACEN® was measured using the Fenton system [23], followed by lipid extraction, analysis of fatty acids by gas chromatography, and scavenger by hypochlorous acid (HOCl) [25]. A pulsating bubble surfactometer was used to measure tensoactive activi-ty [26]. An inhibitory halo test for microbial growth was used to assess antibacterial activity [27].

Statistical analysis

The results were expressed as the mean and its standard error (SEM). The data were evaluated statistically with a non-parametric test (Kruskal-Wallis), with statistically significant differences for *p* values lower than 0.05 (Statistical software application STATGRAPHICS, version 3.1 for Windows). The Origin software, version 7, was used for the calorimetric experiment. A General Linear Model was used for the analysis of pulmonary edema, based on the formula $DW_{(1-28)} = HW_{(1-28)} + T_{(1-5)} + E$, where T is the treatment, HW is the humid weight, DW is the

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dry weight and E is the error, using SAS version 8.02 TS level 02MO (from 1999 to 2001).

Results and discussion

Biophysical properties

The results of the evaluation of SURFACEN® showed that its *p*-A isotherms are characterized by reproducible curves, with long exclusion (compression) and reinsertion (expansion) plateaus, similar to those of the organic extract (ENPPS) and the natural surfactant (NPPS), and clearly superior to those of CUROSURF® (figure 1). Its maximum surface pressure is close to 70 mN/m, like in NPPS. When comparing the maximum and minimum *p* for each cycle in the case of these preparations, there was a statistically significant superiority of SURFACEN® over ENPPS ($p = 0.002$), even though the qualitative behavior of the cyclic isotherms is very similar, or even better, in the latter preparation. The data showed the superiority of SURFACEN® compared to CUROSURF® ($p = 0.001$). It must also be highlighted that at the end of the expansion, a sustained minimal *p* was obtained in SURFACEN®. Qualitatively, this preparation showed a dynamic behavior during compression that is very similar to that of ENPPS (figure 2). From a structural point of view, we verified that SURFACEN® has condensed domains that start at $p =$ 22 mN/m and disappear at 40 mN/m, indicating the presence of dipalmitoylphosphatidylcholine (DPPC) coexisting with a fluid phase. Its crystalline gel-liquid coexistence phase behavior is similar to that of the homologous preparations analyzed here. The thermotropic characteristics of SURFACEN® include a broad phase transition phase between 15 and 50 ºC and a Tm of 28.05 ºC. These results explain why SURFACEN® films show lateral phase separation processes similar to those of the native surfactant, and thermotropic characteristics consistent in complex phase transitions. Overall, these functional and structural properties closely mimic the behavior of the native surfactant system.

Anti-inflammatory, antibacterial and antioxidant effects of SURFACEN®

The results of the evaluation of SURFACEN® in the septic shock model by LPS in Balb/c mice showed that this treatment significantly decreased the wet weight of the lungs [28]. On the other hand, the evaluation of SURFACEN® in an acute lung injury model in rats indicated that the treatment with this preparation, one hour after the administration of LPS, decreased the number of inflammatory cells, restored the macrophage-polymorphonuclear ratio, inhibited MPO activity and, decreased MDA concentration (Table 1). The evaluation of SURFACEN® in the classic systemic local acute inflammation model (pleurisy), indicates that this preparation, at a dose of 100 mg/kg, decreased the amount of pleural exudates in rats, to a degree comparable to that of indomethacin. This result is the first demonstration of the anti-inflammatory effect of the pulmonary surfactant in outside the lungs [29]. SURFA CEN® inhibited the production of TNF α in human monocytes activated with LPS, and decreased the expression of ICAM-1, a decisive adhesion molecule that plays an important role in the interaction of leukocytes with vascular endothelial cells, which is an essential step in the inflammatory process [28]. The incubation of both Gram-positive (*Streptococcus agalactiae*, *Streptococcus pneumoniae* and *Staphylococcus aureus*) and Gram-negative (*Klebsiella pneumoniae* and *Escherichia coli*) bacteria with SURFACEN® resulted in a dose-dependent decrease of the number of colony-forming units, expressed as an increase in the reduction of microbial growth [30]. SURFACEN®, at a concentration of 2.5 mg/mL, also inhibited the production of superoxide anions by 61%.

Antioxidant and antibacterial effect of SP-A

Pig SP-A displays a dose-dependent inhibition on the formation of reactive species in the 2-deoxy-D-ribose assay, reaching levels of 75% at 1 mg/mL, and it was able to scavenger hydroxyl radicals (OH) [31]. It had a significant effect in reducing the ion-dependent generation of OH^- from H_2O_2, probably through iron chelation [31]. Furthermore, it reverted the damage mediated by hypochlorous acid on SURFACEN®, restoring its normal surface tension values [32]. These results contribute to an understanding of the mechanisms of action of SP-A, suggesting that this protein acts by sequestering hydroxyl and hypochlorous radicals, and that it may chelate iron ions, althought the inhibition mechanism of lipid peroxidation by SP-A is not well understood.

Swine SP-A inhibits the oxidation of polyunsaturated fatty acids (lipid peroxidation) present in SURFACEN®, mainly that of arachidonic acid, in a

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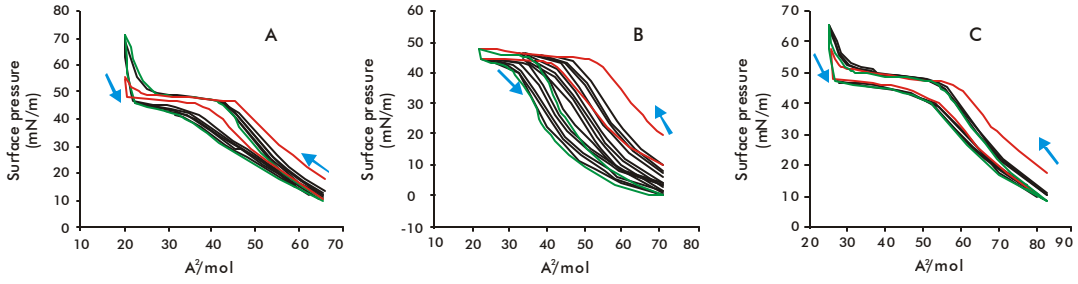

Figure 1. Cyclical compression-expansion isotherms of SURFACEN® (A), CUROSURF® (B) and ENPPS (C) monolayers in organic medium. The monolayers were compressed and expanded at constant temperature (25°C) during 10 consecutive cycles, registering the change in surface pressure as a function of area. The upward or downward arrows indicate the start of compression or expansion, respectively. The red curve represents the first cycle, and the green represents the tenth. The curves are an average of 3 repeated measurements.

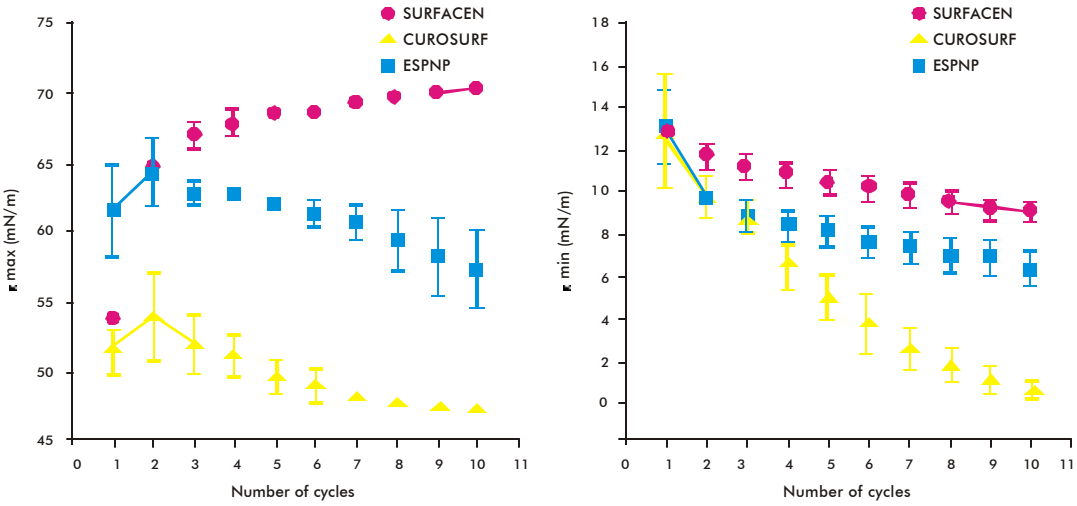

Figure 2. Maximum and minimum surface pressure (p) values for ENPPS, SURFACEN® and CUROSURF® in an organic solution, obtained from the p-A isotherms as a function of cycle number. The data are expressed as the mean ± SEM from three independent experiments. Different letters indicate the presence of statistically significant differences ($p < 0.05$).

dose-dependent manner, as evidenced in the inhibition values of 60% and 73.6% at concentrations of 2.0 µg/mL and 4.0 µg/mL, respectively, upon measuring the inhibition of light emission (figure 3) and analyzing fatty acid composition [33]. These results proved that swine SP-A protects surfactant phospholipids from oxidative damage; exerting a dose-dependent inhibitory effect on the peroxidation of cellular membranes (mitochondria and microsomes). This effect is highest in the former, where 5 mg provide 100% protection, compared to an inhibition value of 51.2 ± 3.48% in microsomes using 7.5 mg of SP-A 34]. In general, the data suggests that swine SP-A is protective not only for the surfactant system, but also for the mitochondria and microsomes of lung cells.

Swine SP-A at concentrations of 0.25, 0.5 and 1 mg/mL produced inhibitory haloes of 22, 26 and 31 mm, respectively, for *Escherichia coli.*

Conclusions

The basic studies on the Cuban product SURFACEN® give a detailed characterization of its biophysical properties and demonstrate, for the first time, its anti-inflammatory activities. It should be highlighted that this is the first international report on the anti-inflammatory activity of lung surfactant in extra-pulmonary areas, as well as on the wide antibacterial spectrum of SURFACEN® when compared to other surfactants. Furthermore, this study provides data on the antioxidant properties of swine SP-A which shed light on its mechanism of action. The results, concerning the biophysical, anti-inflammatory and antibacterial characteristics of SURFACEN®, suggest that this surfactant may be potentially useful for the treatment of ARDS, and predict a very efficient pharmacological preparation through its enrichment with SP-A. The importance of this work is underlined by the fact that there is no satisfactory therapy for ARDS at the international level, placing Cuba among a select group of countries which have access to this essential pharmaceutical preparation.

Table 1. Total cell counts, MPO activity and MDA concentration in the model of acute lung injury. Effect of SURFACEN ®

	Total cells • 10^4/mL	MPO U/mL	MDA, nmol/L
Saline solution	36.67 ± 4.56[a]	0.006 ± 0.001[a]	0.045 ± 0.006[a]
LPS	938.10 ± 211.60[b]	0.412 ± 0.120[b]	0.070 ± 0.005[b]
LPS+ SURFACEN®	388.96 ± 101.44[c]	0.134 ± 0.020[c]	0.060 ± 0.003[c]

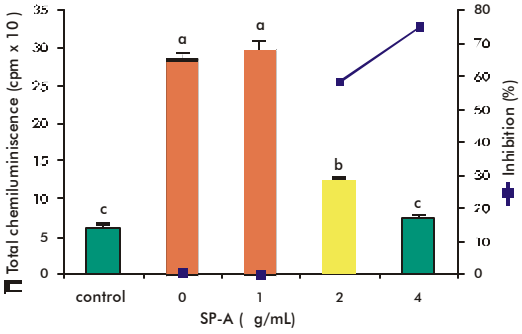

Figure 3. Total chemoluminiscence produced by SURFACEN® (3 mg/mL) on the Fe^{+++}-ascorbate system and inhibition percentage, as a function of SP-A concentration. The data are expressed as the mean ± SEM from 3 independent experiments. Different letters indicate statistically significant differences at $p < 0.05$.

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