Effect of natural porcine surfactant in *Staphylococcus aureus* induced pro-inflammatory cytokines and reactive oxygen species generation in monocytes and neutrophils from human blood

Yuliannis Lugones Ladrón de Guevara [a], Odalys Blanco Hidalgo [a], Sidnéia Sousa Santos [b], Milena Karina Colo Brunialti [b], Roberto Faure [a], Reinaldo Salomao [b,*]

[a] Centro Nacional de Sanidad Agropecuaria (CENSA), San José de las Lajas, Mayabeque, Cuba
[b] Division of Infectious Diseases, Hospital São Paulo, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil

ABSTRACT

Surfacen® is a clinical surfactant preparation of porcine origin. In the present study, we have evaluated the effect of Surfacen® in the modulation of oxidative burst in monocytes and neutrophils in human blood and pro-inflammatory cytokine production in peripheral blood mononuclear cells (PBMC). Reactive oxygen species (ROS) level was measured in monocytes and neutrophils by flow cytometry using 2,7-dichlorofluorescein diacetate (DCFH-DA) as substrate, while, tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels were estimated in PBMC supernatant by enzyme-linked immunosorbent assays (ELISA). Our results show that *Staphylococcus aureus*-induced ROS level was slightly affected by Surfacen® added to whole blood monocytes and neutrophils. The time course experiments of pre-incubation with Surfacen® showed no significant increase of ROS level at 2 h; however, the ROS level decreased when pre incubated for 4 h and 6 h with Surfacen®. Pre-incubation of PBMC cells with Surfacen® at 0.125 and 0.5 mg/mL showed a dose-dependent suppression of TNF-α levels measured after 4 h of *S. aureus* stimulation, an effect less impressive when cells were stimulated for 24 h. A similar behavior was observed in IL-6 release. In summary, the present study provides experimental evidence supporting an anti-inflammatory role of Surfacen® in human monocytes and neutrophils in vitro.

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1. Introduction

The pulmonary surfactant is a complex mixture of lipids and proteins. It is synthesized by alveolar type II epithelial cells into alveolar space. The main functions of pulmonary surfactant include (i) reducing the surface tension at the air–liquid interface of the alveolus, (ii) avoiding the alveolar collapse, and, (iii) reducing the work of breathing [1]. Besides these properties, pulmonary surfactant also play a major role in the pulmonary defense through preventing the access of pathogens at the large alveolar surface exposed to the environment; there is growing evidence that pulmonary surfactant has a potential role in modulating inflammation in normal and injured lungs [2]. Pulmonary surfactant has been used to treat Neonatal Respiratory Distress Syndrome (NRDS), with improvement in morbidity and mortality in very low birth weight babies [3,4]. Surfacen® is a clinical surfactant preparation of porcine origin which is widely used in Cuba to treat pre-term babies at risk or already suffering Neonatal Respiratory Distress [5,6].

The potential use of surfactant preparations in other diseases such as Acute Lung Injury (ALI) and its severe form Acute Respiratory Distress Syndrome (ARDS) demands the evaluation of its immunomodulatory properties. ALI or ARDS is a life-threatening condition that is characterized by increased inflammatory cytokine levels and cell infiltration into the lungs, non-cardiogenic pulmonary edema, and diffuse alveolar damage that culminates in respiratory failure [7,8]. Neutrophils play a key role in the immune defense against invading microbes. These cells can readily move to the inflammatory sites by chemotaxis, phagocytize the microbes and release reactive oxygen metabolites or enzymes for bacterial death [9]. *Staphylococcus aureus* (*S. aureus*) remains a major cause of human infections, and the arising of highly virulent, drug-resistant strains has made treatment more difficult [10]. It is also known to be one of the pathogens responsible for sepsis-induced ALI/ARDS [11].

The inflammatory response is fundamental for the control of infection, but also underscores the pathophysiologic events of organ dysfunction in sepsis [12]. The regulation of mediators released from cells within the alveolar space would represent a desirable effect of surfactants. Within the alveolar space, resident macrophages are constantly bathed in the phospholipid-rich surfactant; regulatory effects are

* Corresponding author at: Laboratório de Imunologia, EPM, UNIFESP, Rua Pedro de Toledo, 669, 10º andar, 04039-032 São Paulo, SP, Brazil.
E-mail address: rsalomao@unifesp.br (R. Salomao).

important in protecting these delicate gas exchange regions from inflammatory damage. In the present study, we evaluated the effects of a clinical surfactant preparation, Surfacen®, in the modulation of oxidative burst and pro-inflammatory cytokine production induced by a bacterial stimulus in monocytes and neutrophils in peripheral human blood.

2. Materials and methods

2.1. Drug

The Surfacen® preparation was provided by the Centro Nacional de Sanidad Agropecuaria (CENSA, Mayabeque, Cuba). Surfacen® is obtained from organic extracts of porcine bronchoalveolar lavages, which are subjected to acetone precipitation to reduce their content of neutral lipids. This surfactant contains about 95% polar lipids, mainly dipalmitoylphosphatidylcholine (DPPC), and 1% hydrophobic proteins (SP-B and SP-C). It is provided as a sterile white lyophilized powder, in 50 mg phospholipids vials [13]. To reconstitute Surfacen®, the appropriate amount of the surfactant is weighted and resuspended in sterile distilled water. For experiment of oxidative burst and cytokines modulation, surfactant was used at the concentrations from 0.125 to 0.5 mg/mL.

2.2. Healthy volunteers

Blood samples from 13 healthy volunteers (mean age, 37.4 ± 10.2 years; 69% females) were drawn into heparin-treated vacuum tubes (Becton Dickinson, UK). All volunteers agreed to participate and reported being healthy without medication.

2.3. Analysis of oxidative burst production in whole blood after stimulation with S. aureus

The oxidative burst production was quantified in monocytes and neutrophils in whole blood by measuring the oxidation of 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma, St. Louis, MO) as described previously [14]. In brief, ROS were measured constitutively and after stimulation with S. aureus (ATCC 25923; Difco, Detroit, MI) at 2.4×10^8 colonies/mL [15]. The whole blood was pre-incubated with Surfacen® for 0, 60, 120, 240 and 360 min at 37 °C and 5% CO_2 before adding S. aureus. Samples were further incubated in the presence of 0.3 mM DCFH-DA in a 37 °C shaking water bath for 30 min. After the incubation, 2 mL of 3 mM EDTA (Sigma) was added to each tube and the mixture was then centrifuged (652 ×g for 5 min at 4 °C). Hypotonic lyses in 0.2% saline was followed by the addition of 1.6% saline and centrifugation (652 ×g for 5 min at 4 °C). The supernatants were discarded, and the pellets were incubated with 5 μL of CD14-peridinin-chlorophyll-protein monoclonal antibody (PerCP;) and 5 μL of CD15 allophycocyanin (APC; both antibodies from BD Biosciences, San Jose, CA) at room temperature for 15 min in the dark. Two milliliters of PBS was then added to each tube, and the mixture was centrifuged (652 ×g for 5 min at 4 °C). The supernatants were discarded, and the pellets were resuspended in 250 μL of 3 mM EDTA in PBS for ROS determination.

Neutrophils were characterized in whole blood by side-scatter and forward scatter parameters, and positive staining for CD15; monocytes were characterized by side-scatter and forward-scatter parameters and positive staining for CD14. ROS generation is expressed as the geometric mean of fluorescence intensity (GMFI).

2.4. Induction of cytokines in peripheral blood mononuclear cells (PBMC)

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) supplemented with 10%

fetal calf serum, 10 IU/mL penicillin, 10 μg/mL streptomycin (Gibco, Gaisthersburg, MD), and 200 mM L-glutamine (Sigma), adjusted to a concentration of 2×10^6 cells/mL. The cell viability and count were made with trypan blue dye using a hemocytometer chamber. After preliminary dose–response experiments (data not shown), cells were pre-incubated with Surfacen® for 0, 60, 120, 240 and 360 min and then stimulated with 4.8×10^7 colonies/mL of S. aureus for 4 and 24 h at 37 °C with 5% CO_2. Cells without stimulus were used to measure the unspecific stimulation. Supernatants were collected after 4 or 24 h of incubation, by centrifugation the samples at 805 ×g for 5 min at 4 °C. Cell-free supernatants were stored in aliquots at −80 °C until used for cytokine determination.

2.5. Measurement of cytokines

The TNF-α and IL-6 were measured by capture enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Antibody pairs and reagents (OptEIA sets) were obtained from BD Biosciencies. Samples were tested in duplicates, and a standard curve with human recombinant cytokine was prepared in each plate. Sensitivity was 10 pg/mL for both cytokines measured.

2.6. 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.

3. Results

3.1. Effect of pulmonary surfactant on ROS production in monocytes and neutrophils of human blood

Un-stimulated monocytes and neutrophils did not show any significant changes in ROS levels with the concentrations of 0.125, 0.25 and
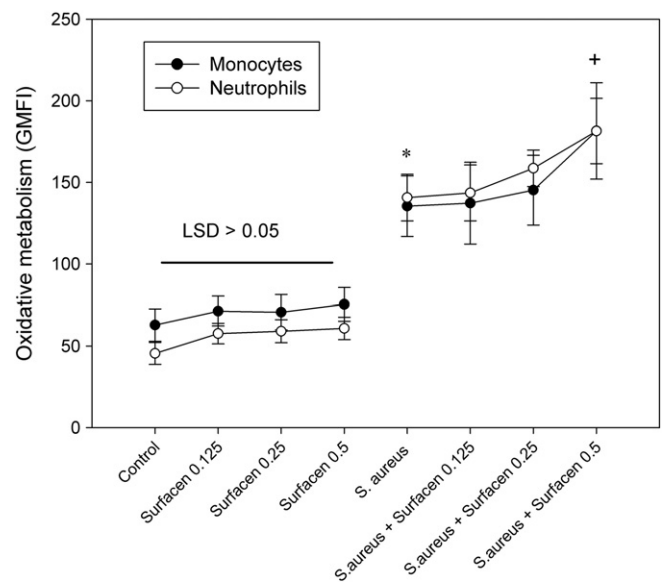
Fig. 1. Production of ROS by whole blood monocytes and neutrophils from healthy volunteers ($n = 7$) in different conditions: control and after S. aureus stimuli without or with simultaneously added Surfacen® (0.125, 0.25 and 0.5 mg/mL). The monocytes and neutrophils were characterized by side-scatter and forward scatter parameters and positive staining for CD14 and CD15 respectively. ROS generations are expressed as the geometric mean of fluorescence intensity (GMFI) ± standard error. * $p \leq 0.05$ S. aureus compared to control in monocytes and neutrophils; + $p \leq 0.05$ S. aureus + Surfacen® 0.5 mg/mL compared to S. aureus in neutrophils.

0.5 mg/mL of Surfacen® (Fig. 1). In contrast, monocytes and neutrophils showed significant increase of ROS levels (135.5 ± 48.3 and 140.7 ± 37.0 GMFI respectively) after stimulation of *S. aureus*. It is also observed that Surfacen® did not induce ROS in the *S. aureus* stimulated cells in low concentrations (0.125 and 0.25 mg/mL), while, neutrophils showed significant changes in ROS level with 0.5 mg/mL of Surfacen® (Fig. 1).

The time course experiments of pre-incubation with Surfacen® showed a biphasic effect on *S. aureus*-induced ROS generation in monocytes (Fig. 2A), with a slight and non significant increase until 2 h of pre-incubation, followed by a decreased ROS generation after 4 and 6 h of Surfacen® pre-incubation (Fig. 2A). The same trend was found in neutrophils (Fig. 2B).

3.2. 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 fourfold (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 pre-incubated with different concentrations of Surfacen® and challenged with *S. aureus* for 4 h (Fig. 3A). The level of TNF-α decreased up to 60% in *S. aureus*-induced cells when pre incubated with Surfacen® for 4 h (Fig. 3A). 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® (Fig. 3B).

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 ($p \leq 0.05$) (Fig. 3C). When cells were stimulated for 4 h, Surfacen®, at a concentration of 0.5 mg/mL, induced about 40%
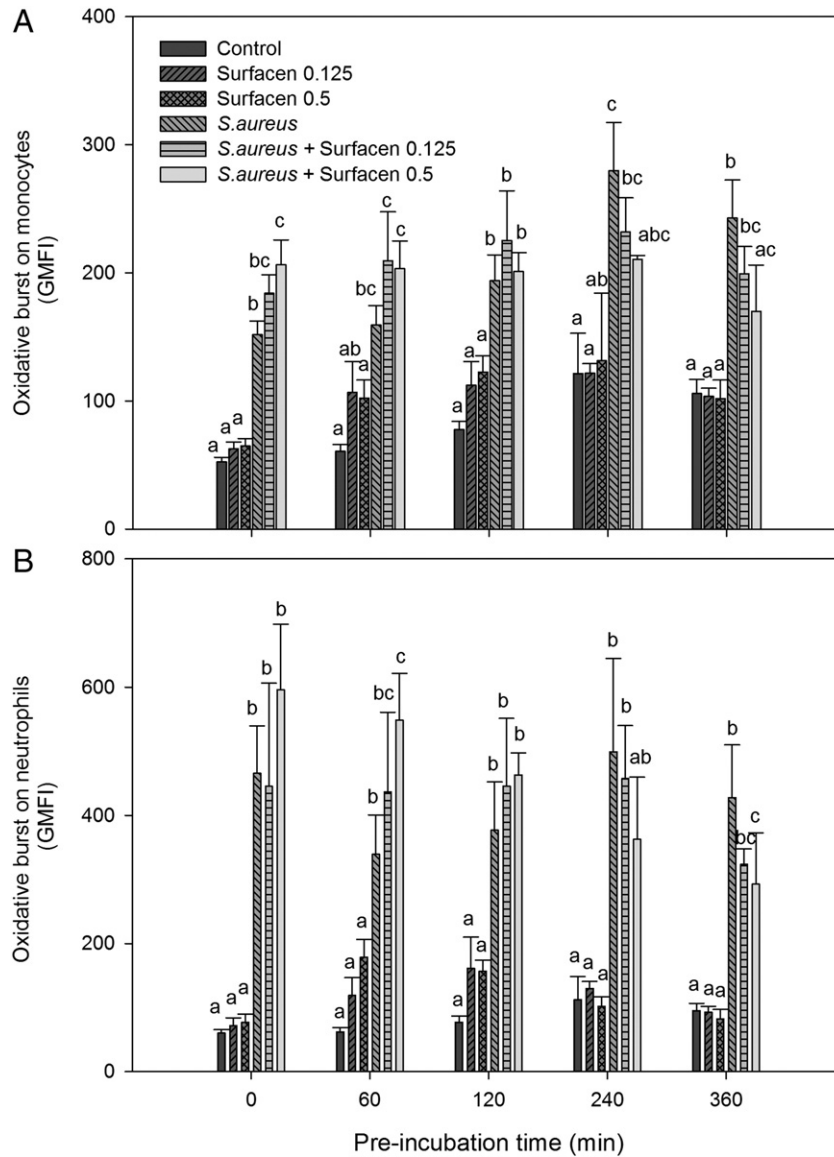

Fig. 2. Time course of ROS production by monocytes (A) and neutrophils (B) in response to pre-incubation with Surfacen® in whole blood. The monocytes and neutrophils were characterized by side-scatter and forward scatter parameters and positive staining for CD14 and CD15 respectively. ROS generation is expressed as the geometric mean of fluorescence intensity (GMFI) $\pm$ SE. Samples from 3 to 12 healthy volunteers were used in each time point. Different letters means significant difference between the groups ($p \leq 0.05$) in each time point.
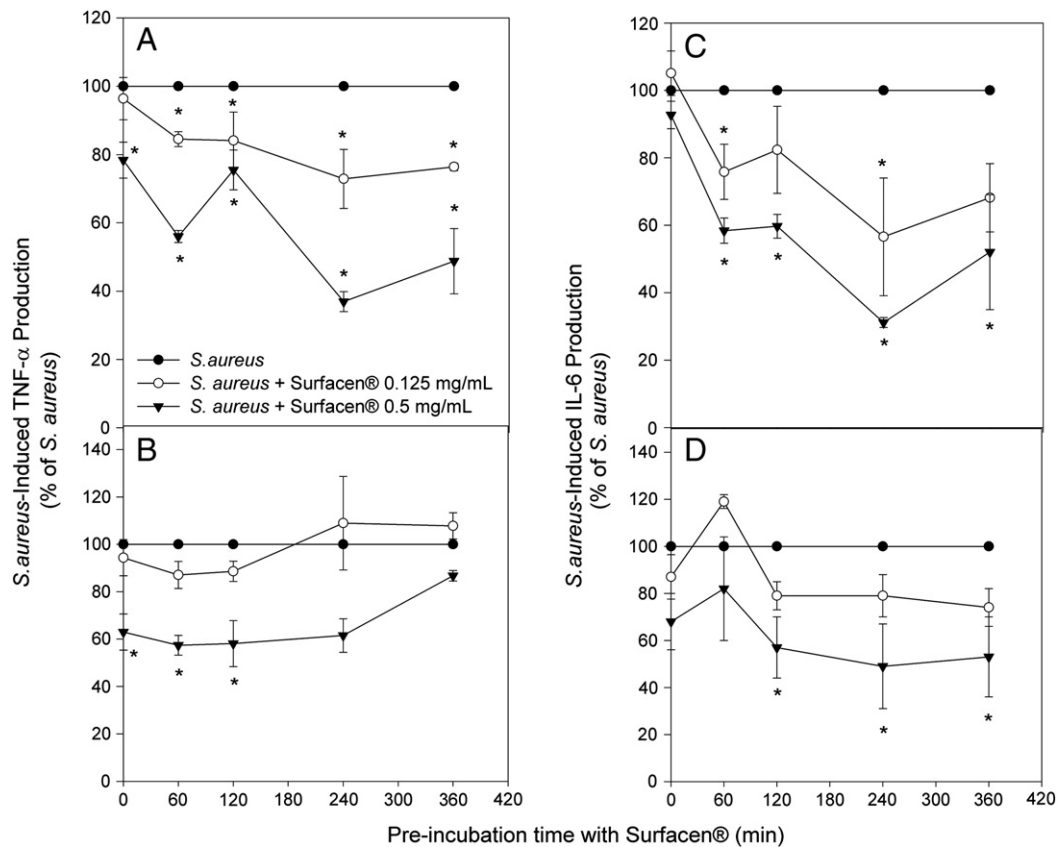
Fig. 3. Surfacen® modulation of *S. aureus*-induced TNF-α and IL-6 production by PBMC. PBMCs were pre-incubated with Surfacen® for different time points and stimulated with *S. aureus* for 4 and 24 h. Values of TNF-α (A and B) and IL-6 (C and D) for 4 health volunteer are expressed as the percentage of cytokines levels produced in presence of *S. aureus* alone (%*S. aureus*) in function of time of pre-incubation. *$p \leq 0.05$ cells pre-incubated with Surfacen® and stimulated with *S. aureus* compared to cell stimulated with *S. aureus* alone, +$p \leq 0.05$ between *S. aureus* + Surfacen® 0.5 mg/mL after 60 minutes compared to 240 min.

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 Surfacen® 0.5 mg/mL after 24 h of stimulation (Fig. 3D).

4. Discussion

Phagocytes, particularly macrophages and polymorphonuclear (PMN) cells, are recognized as the main components of the cellular immune and inflammatory response in the lung [16,17]. *S. aureus* stimulates alveolar macrophages to produce inflammatory cytokines and free oxygen radicals, which can damage the lungs. *S. aureus* has been isolated from bronchoalveolar lavage in patients with ARDS [11]. Our results showed that surfactant inhibits TNF-α and IL-6 induced by *S. aureus* in mononuclear cells in vitro. The modulation of ROS generation was less impressive, yet a biphasic effect might be envisaged with increasing pre-incubation periods.

The effect of pulmonary surfactant preparations in the production of ROS has been studied and conflicting results have been obtained [18–20]. The surfactant effect in the production of ROS is determined, among other factors, by the dose and type of surfactant, type of stimulus, variations in interspecies or cell type, or differences in experimental conditions [21]. Our results using whole blood stimulated with *S. aureus* to induce ROS production suggest that Surfacen® has little effect in the production of ROS, when added simultaneously with the stimulus. This finding is in agreement with previously reported studies showing no surfactant modulation of PMN stimulated by opsonized group B streptococci [22] or monocytes stimulated by phorbol 12-myristate 13-acetate (PMA) and opsonized zymosan [19]. Individual surfactant

phospholipids differently modulate priming of macrophages for oxidative responses, and the effect of individual phospholipids does not account for the effect of complete pulmonary surfactant on priming of macrophages. Also, the effects of phospholipids on oxidative responses of macrophages are different when elicited with PMA or Op-Zym [23]. Curosurf® (Chiesi Farmaceutici, Parma, Italy) did not affect the PMA-induced superoxide anion production; however, pre-incubation with Curosurf® caused a significant inhibition of the OM-85-induced superoxide anion production [21]. Survanta®(Abbott Laboratories, Abbott Park, IL) and Alveofact® (Boehringer Ingelheim Co., Ingelheim, Germany), other clinical surfactant preparations, stimulated the oxygen metabolite release from PMN incubated with *E. coli* and *S. aureus* respectively; however, these effects did not reach statistical significance [24].

More recently, three clinical surfactants were evaluated against ROS production. At low concentrations these surfactants did not influence the production of ROS stimulated by PMA, while at higher concentrations, all surfactant preparations tested significantly reduced ROS production. These authors also studied the effect of DPPC on ROS production, and revealed an inhibitory effect [25]. In general some of the observed differences mentioned above might be explained by the use of different stimulants (e.g. PMA or bacteria), as it has been demonstrated that PMA and bacterial induced oxygen metabolite release are regulated by different mechanisms [9].

Furthermore, the dose of phospholipids and the time of incubation have a critical impact on oxidative functions. Phosphatidylcholine arachidonoyl-palmitoyl (PAPC) showed a stimulatory or inhibitory response in the respiratory burst which depends on lipid dose and time of incubation [26]. Our results also suggested a time dependent changes in ROS generation with pre-incubated surfactant. The clinical

implications of the modulating effects of surfactants in ROS generation are not clear, contrasting with the potential benefits of inhibiting inflammatory cytokines.

Although the effect of surfactant preparations modulating ROS production has been controversial, their inhibitory effect on proinflammatory cytokines seems to be consensus.

Our results confirmed previous reports with other clinical surfactants, that is, Surfacen® is also able to inhibit the production of TNF-α and IL-6. Studies have been conducted to investigate the kinetics of cytokines in animal models of acute lung injury [27–29]. TNF-α and IL-6, among other mediators, were demonstrated in lung tissues as well as in alveolar macrophages and polymorphonuclear cells from bronchoalveolar lavage of animals with acute lung inflammation induced by lipopolysaccharide or bacteria.

Several of the surfactants used as replacement therapy showed an inhibitory effect on cytokine release in patients with NRDS. Curosurf® inhibited the secretion of TNF-α in monocytes [19], while Survanta® and Exosurf® (Burroughs Wellcome, Research Triangle Park, NC) inhibited the production of IL-1 β, pro-IL-1 β and TNF α in alveolar macrophages stimulated with LPS [30]. These results showed that a synthetic surfactant (Exosurf®) as well as an exogenous bovine natural surfactant (Survanta®), containing hydrophobic proteins (SP-B and SP-C) has a cytokine inhibitory effect.

Both Survanta® and Exosurf® exert inhibitory effect by decreasing activation of the nuclear factor (NF)-κB in one human monocytic cell line [31]. It has also been demonstrated that exogenous porcine natural surfactant, Curosurf® affects TNF-α release of LPS-stimulated monocytes at a pretranslational level by down-regulating both mRNA for TNF-α and TNF-α-Receptor II, therefore acting as an anti-inflammatory agent within alveolar space [32]. Other studies have demonstrated that surfactant suppressed NF-κB transcriptional activation and translocation to the nucleus and significantly inhibited IκB phosphorylation and degradation by blocking IKKa kinase expression in the lung tissue [33].

Several studies revealed the mechanism and effects of phospholipids and their function in host defense. Total phosphatidylcholine (tPC; heterogeneous species of PC from egg) and DPPC, but not the PC 16/20:4 inhibit the release of TNF α in a monocyte cell line, suggesting that the mechanism by which this effect is exerted is by altering the fluidity of the membrane [26]. Also, the DPPC can inhibit LPS-stimulated PAF production via perturbation of the cell membrane, which inhibits the activity of specific membrane-associated enzymes involved in PAF biosynthesis [34]. Studies have demonstrated that the SP-C, when combined with DPPC vesicles, is capable of inhibiting the binding of LPS on RAW 264.7 macrophages and therefore inhibited both nitric oxide production and TNF-α in peritoneal and alveolar macrophages [35]. The surfactant anionic phospholipids have an important role in suppressing inflammation within the alveolar compartment in the lung [36]. It significantly inhibits the production of TNF α and nitric oxide induced by LPS in alveolar macrophages and reduces phosphorylation of multiple intracellular protein kinases. At the same time the anionic phospholipids were also effective in attenuating inflammation when administered intratracheally to mice challenged with LPS. Palmitoyl oleoyl phosphatidylglycerol (POPG) has a direct action on the attenuation of the LPS-induced inflammatory response by inhibiting the production of IL-6 and IL-8 in respiratory tract infections such as Respiratory Syncytial Virus, indicating that the administration of specific phospholipids can be utilized in the prevention and treatment of respiratory disease [37]. Surfacen® is characterized by the highest content of PG and also by the higher content of anionic phospholipids in their biochemical composition in comparison with other clinical surfactants [38]. This feature allows a preparation Surfacen® to be a successful candidate in the treatment of ALI/ARDS.

In summary, the present study provides experimental evidence in favor of an anti-inflammatory role of Surfacen® in human monocytes and neutrophils in vitro.

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