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

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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.

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

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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

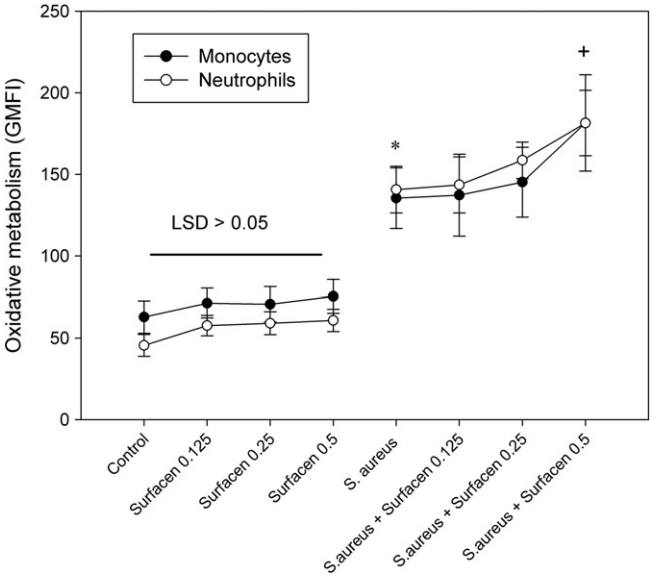

Fig. 1. Production of ROS by whole blood monocytes and neutrophils from healthy volunteers ($n = 7$) in different conditions: control and after *S. aureus* stimuli without or with simultaneously added Surfacen® (0.125, 0.25 and 0.5 mg/mL). The monocytes and neutrophils were characterized by side-scatter 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.