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Effects of Antioxidants on Surfactant Peroxidation by Stimulated Human Polymorphonuclear Leukocytes

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Production of oxygen radicals by stimulated phagocytes followed by surfactant lipid peroxidation (LPO) and loss of surfactant function have all been implicated in the pathogenesis of acute lung injury.

We studied the interactions between natural lung surfactant (Curosurf) and neutrophils *in vitro*, and compared various antioxidants; (superoxide dismutase (SOD), vitamin E, vitamin C, ebselen and melatonin), or combinations of them in duplicate and triplicate regarding their ability to decrease superoxide production and the peroxidation level of surfactant caused by activated phagocytes. The superoxide production of neutrophils activated by *Candida albicans* was measured with the nitroblue tetrazolium (NBT) test. The subsequent LPO was estimated as the content of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE).

We found that lung surfactant decreased the superoxide production by activated neutrophils (29.7%) and that Curosurf was peroxidized with elevated MDA/4-HNE values. With supplements of antioxidants (except vitamin C), superoxide radical production and the surfactant LPO level fell in a dose-dependent manner. The protective effect of the antioxidants differed in each test. SOD had a slight effect in both tests. The findings with vitamin E, melatonin and ebselen were similar. The best combination was that of a natural and a synthetic antioxidant (melatonin–ebselen) with a 60% decrease in comparison to the corresponding control.

These findings suggest that antioxidants, particularly in combination, prevent LPO of lung surfactant.

Keywords: Surfactant; Antioxidants; Reactive oxygen species; Lipid peroxidation

INTRODUCTION

Pulmonary surfactant, a mixture of lipids, proteins, and carbohydrates, synthesized and secreted by alveolar type II cells, lines the alveolar surfaces of the lung. One of its main functions is to prevent lung collapse at end-expiration.[1] Exogenous surfactant improves lung function in premature infants born with inadequate pools of endogenous surfactant and improves gas exchange in infants with established infant respiratory distress syndrome (IRDS).[2] Pilot studies of exogenous administration of surfactant to adults with respiratory distress syndrome (ARDS) have also been done with some preliminary success.[3,4]

ARDS and IRDS are characterized by intense inflammation, marked influx of neutrophils into the lung, and increased numbers of neutrophils in broncho-alveolar lavage fluid.[5] Oxidative lung damage is present in chronic lung disease and surfactant dysfunction follows phagocyte activation.[6–8] We have previously shown that the metabolic products from live bacteria, isolated from the lungs during the neonatal period, induce LPO of lung surfactant.[9] Further, phagocytes, neutrophils and alveolar macrophages, cause LPO of surfactant and adjacent cells when stimulated by bacteria or fungi to increase their oxidative metabolism.[10] We also found that surfactant LPO may injure lung cells[11] and that the function of peroxidized surfactant is decreased.[12] We have previously for this purpose tested vitamin E[10] and are in this study testing further antioxidants/antioxidants

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combinations for an optimal protection of a surfactant preparation against oxidation. Thus, the prevention of excess ROS formation, using antioxidants, is a vital step in improving the quality of exogenous surfactant.

Antioxidant mechanisms can be defined as any cellular process that prevents the formation of free radicals, converts oxidants to less toxic species, or repairs molecular injury induced by ROS.[13]

In this study, we compared antioxidants or combinations of them regarding their ability to reduce the peroxidation level of surfactant caused by ROS released from activated phagocytes. We believe that exogenous surfactant can be improved by adding antioxidants. We mainly compared the effect of the natural antioxidants, vitamin E, vitamin C, SOD, melatonin, and synthetic ebselen.

MATERIALS AND METHODS

Surfactant

Curosurf (Serono Nordic AB, Solna, Sweden) is a modified porcine surfactant isolated from minced lungs and suspended in saline in a concentration of 80 mg/ml. This surfactant contains about 99% polar lipids and 1% hydrophobic proteins (SP-B and SP-C).[1]

Microorganism

A strain of *Candida albicans* (ATCC 10231; American Type Culture Collection) was used as a stimulus. It was grown for 24 h at 30°C in Sabouraud's dextrose broth. After centrifugation, the pellet containing the yeast cells was diluted with 10 mM sodium phosphate buffer (PBS) and the cells were counted. Then, the yeast cells were heat-killed at 80°C in a water bath for 1 h.

Antioxidants

All the antioxidants used in this study were purchased from Sigma, St Louis, USA. Vitamin E was dissolved in a small volume of ethanol and ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) was dissolved in dimethyl-sulfoxide (DMSO). PBS was then used to make various dilutions. Superoxide dismutase (SOD), vitamin C and melatonin (*N*-acetyl-5-methoxytryptamine) dissolved rapidly in solution. The choice of each antioxidant's concentration used in this study was based mainly on earlier investigations[10,14–16] and adjusted to our *in vitro* system and methods after preliminary experiments.

Separation of Polymorphonuclear Neutrophils (PMN)

Human venous blood was collected in vacutainer tubes containing heparin from healthy adult donors. The erythrocytes were sedimented with a 3% solution of dextran for 30 min at 37°C. The leukocyte-rich supernatant was then removed and centrifuged for 30 min at 110*g*. The sedimented leukocytes were washed twice in PBS, concentrated by centrifugation, and suspended in PBS in a concentration of 5×10^7 PMN/ml.

Nitroblue Tetrazolium (NBT) Reduction Test

The ability of phagocytes to reduce the almost colorless compound NBT to dark blue formazan is due to their superoxide production and reflects the oxidative metabolism of the cells which is increased during phagocytosis. The NBT activity of neutrophils was stimulated by *C. albicans* and measured quantitatively with a spectrophotometric method.[10] The neutrophil suspension (0.1 ml) in a final concentration of 5×10^6 cells/ml was mixed with a *C. albicans* suspension (10 μl) to a concentration of 3×10^8 organisms/ml and then with 30 μl of various concentrations of antioxidants: vitamin E in 5, 10, 20 μM (final concentrations), SOD (12.5, 25, 50 U), vitamin C (30, 60, 120, 240 μM), ebselen or melatonin (0.01, 0.1, 1 mM) on a microtiter plate. The experiments were done with or without surfactant, 10 μl, in a final concentration of 0.4 mg/ml.

In a second step, each antioxidant and various combinations of these four antioxidants (in duplicate and triplicate) were tested at the maximal concentration of each one. The experiments were performed, with or without surfactant (0.4 mg/ml), in the presence of heat-killed *C. albicans* (3×10^8) as the stimulus.

In both steps, resting and stimulated neutrophils without antioxidants were used as controls.

Then 50 μl NBT was added and the trays were covered with adhesive tape and incubated for 60 min at 37°C in an incubator-shaker system (Stat Fax-2200). The reaction was then stopped by adding 100 μl HCl. The plates were centrifuged at 1500 rpm, the supernatant was removed, and 200 μl of DMSO was added to each well. The trays were again covered and placed on a sonicator for 10 min, to accelerate the extraction of formazan. Finally, the optical densities (OD) were spectrophotometrically recorded at A570 on ELISA PLUS (Meddata, Inc., NY, USA).

Measurement of Lipid Peroxidation

Neutrophils together with *C. albicans*, as neutrophil stimulators, were incubated with surfactant,

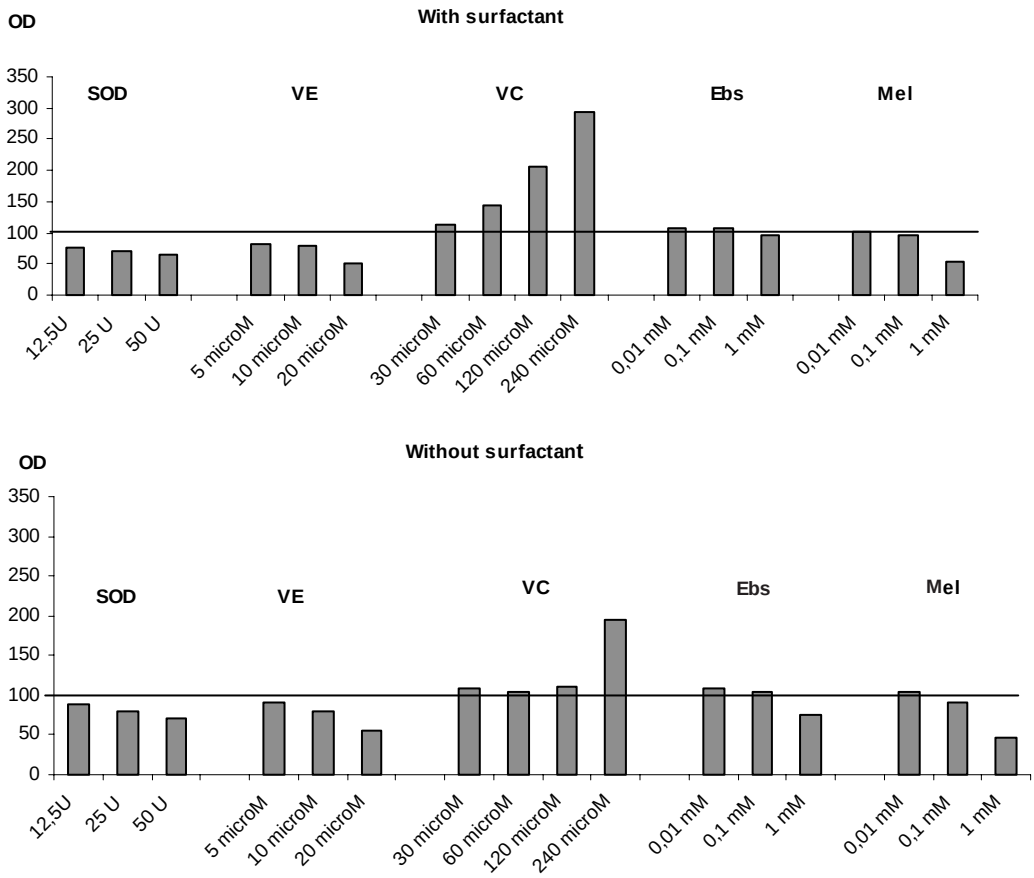

FIGURE 1 NBT reduction of *C. Albicans*-stimulated PMN incubated with or without surfactant and with SOD 12.5, 25 or 50 U, vitamin E (VE) 5, 10 or 20 μM, vitamin C (VC) 30, 60, 120 or 240 μM, ebselen (Ebs) or melatonin (Mel) 0.01, 0.1 or 1 mM. The values are given in percentages (%) of the corresponding values without antioxidant. In optical densities, these values were 0.72 ± 0.21 with surfactant and 0.91 ± 0.21 without it. Corresponding values without stimulation were 0.44 ± 0.14 with surfactant and 0.55 ± 0.11 without it. The values represent % of means of 8–10 experiments.

0.4 mg/ml and antioxidants for 5 h at 37°C in the above-mentioned incubator-shaker system.

First, vitamin E (5, 10, 20, 40 μM), SOD (12.5, 25, 50 U), vitamin C (30, 60, 120, 240, 480, 960 μM), ebselen (0.01, 0.1, 1 mM), and melatonin (0.01, 0.1, 1 mM) were used separately in various concentrations.

Secondly, we tested various combinations of these antioxidants (each one at its maximal concentration). Resting and stimulated neutrophils with surfactant or without antioxidants were used as controls.

At the end of the incubation period, the lipid peroxidation (LPO) in the samples was measured. This assay, LPO 586 test, is based on the reaction of a chromogenic reagent (R1: 10.3 mM *N*-methyl-2-phenylindole in acetonitrile) with malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) to yield a stable chromophore, with maximal absorbency at 586 nm.

Samples of 200 μl were well mixed with 650 μl of R1 and 150 μl of R2 (15.4 M methane sulfonic acid), the tubes were closed and then incubated for 40 min in a water-bath at 45°C. The samples were cooled on ice and their absorbencies were measured with an Ultrospec-3000 spectrophotometer (Pharmacia Biotech, Stockholm, Sweden).

In each series of assays, we included a blank ([aldehyde] = 0) by replacing sample with buffer. Blank absorbency was then subtracted from sample absorbency for calculations.

Statistics

NBT data was analyzed using a two-way ANOVA, with repeated measures on two factors. LPO data was analyzed using a one-way ANOVA, with repeated measures.

RESULTS

Dose-response Effect of Antioxidants on NBT Values of Stimulated Neutrophils

The NBT reduction of *Candida*-stimulated neutrophils was about 170% of the unstimulated value. In

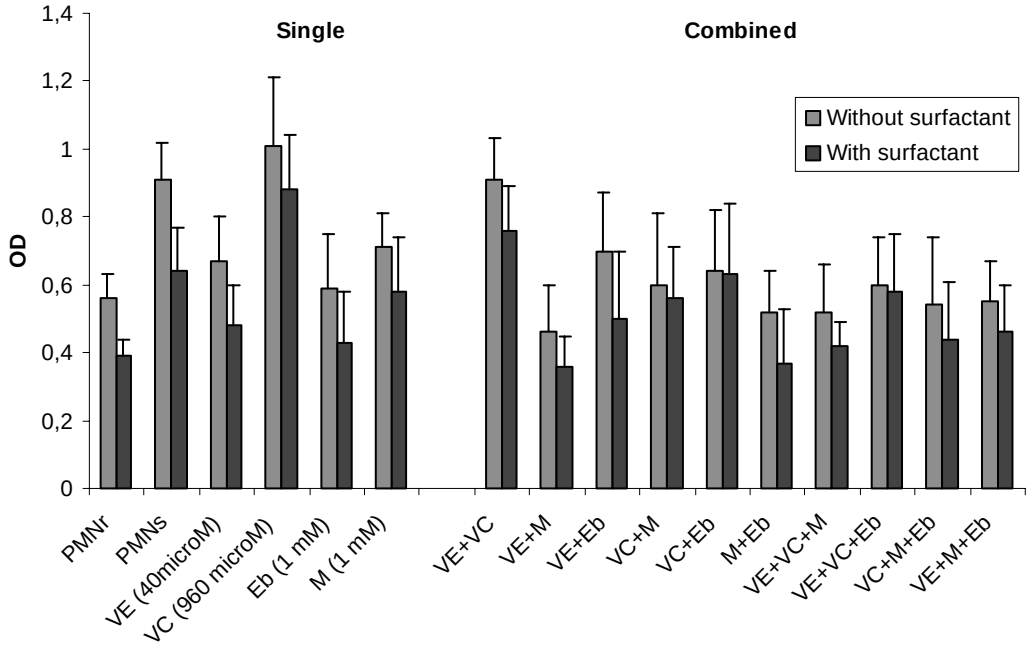

FIGURE 2 NBT reduction of stimulated PMN incubated with single or combined antioxidants (in their maximal concentrations, see Fig. 1) in the presence or absence of surfactant. Controls were resting PMN (PMNr) and stimulated PMN (PMNs) without antioxidant. The diagram shows the mean ± SD of seven experiments.

general, the extent of NBT reduction was lower in the presence than in the absence of lung surfactant.

SOD and vitamin E significantly decreased ($p < 0.001$) the NBT reduction in a dose-dependent manner. However, vitamin C increased the NBT reduction by neutrophils which was even greater in the presence of surfactant ($p < 0.001$, Fig. 1). When ebselen was tested, only the highest concentration decreased the NBT reduction significantly ($p < 0.05$, Fig. 1). In all cases, the addition of surfactant decreased the NBT reduction in comparison to samples without surfactant. However, this decrease was not significant compared to the control containing stimulated neutrophils without ebselen. Melatonin decreased the NBT reduction of stimulated neutrophils with or without addition of surfactant, and the decrease was significant at the highest concentration used ($p < 0.001$, Fig. 1).

When the various antioxidants were mixed, the NBT reduction of the cells decreased in most cases, but not in the vitamin E–vitamin C mixture. The addition of surfactant decreased the superoxide production by the neutrophils in all cases (Fig. 2).

Effect of Antioxidant Preparations on Surfactant Peroxidation by Stimulated Neutrophils

SOD had no effect on the LPO of surfactant; vitamin E significantly decreased the LPO level in a dose-dependent manner ($p < 0.01$, Fig. 3).

The addition of vitamin C decreased the LPO rate in a dose-dependent way, but was significant only in the three first concentrations used ($p < 0.001$, Fig. 3). Ebselen or melatonin reduced the rate of surfactant LPO in a dose-dependent manner and was significant at 1 mg/ml ($p < 0.001$, Fig. 3).

In most cases, when the antioxidants were combined in pairs or in triplicate, the surfactant LPO was lower than in the corresponding control, i.e. stimulated neutrophils with or without surfactant. The effect was not observed when vitamins E and C were combined (Fig. 4). Melatonin and ebselen had the best effect on the decrease in LPO.

DISCUSSION

In this study, the superoxide production of the neutrophils increased considerably when these cells were stimulated by *C. albicans*. This increase in oxidative metabolism was inhibited by lung surfactant. The effect of Curosurf is similar to that reported by our group earlier.[17] Surfactant or surfactant components have been variably reported to induce, increase[18,19] or suppress[20,21] macrophage or monocyte oxidative responses. Reports also conflict as to the effect of surfactant on human neutrophil function. Some have suggested that most of these functions, including oxidative responses, were unchanged by pre-exposure to bovine surfactant or phospholipid preparations,[22] but others have found that human neutrophil oxidative responses were inhibited by bovine surfactant[23] or suspensions of unsaturated phospholipid.[24] Surfactant may change subsequent steps in the assemblage of respiratory burst oxidase.[25] We suggest that

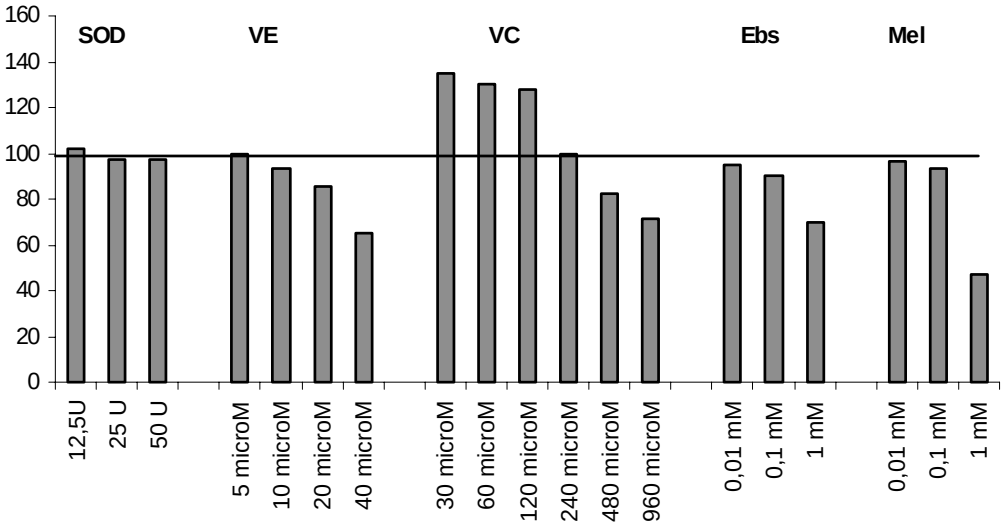

FIGURE 3 Various antioxidants were evaluated in several concentrations for their ability to reduce the LPO level of lung surfactant incubated with stimulated PMN. The concentrations of the antioxidants were as follow: SOD 12.5, 25 or 50 U, vitamin E (VE) 5, 10 20 or 40 μM, vitamin C (VC) 30, 60, 120, 240, 480 or 960 μM, ebselen (Ebs) or melatonin (Mel) 0.01, 0.1 or 1 mM. The diagram summarizes the values given in percentages (%) of the corresponding values without antioxidants. Resting PMN with a value of 7.84 ± 3.27 were negative controls. The cells were incubated in the presence of surfactant and calculated as the mean ± SD of 8–10 experiments.

surfactant acts mainly as a scavenger for free radicals and thus it is peroxidized. A further effect of surfactant on lung phagocytes, however, can not be excluded.

Unsaturated fatty acids are substrates for the peroxidative attack by reactive oxygen species which may cause aldehydic products of LPO with significant chemotactic or cytotoxic activity.[26] Only a few unsaturated phospholipid acyl side-chains of surfactant have been found and therefore only minor changes in lung surfactant structure due to LPO can occur. However, even these small changes in surfactant cause significant changes in the ability to lower surface tension which has been shown with incubation of surfactant with LPS[27] and GBS-stimulated neutrophils.[12]

We found that addition of antioxidants to stimulated neutrophils lowered NBT reduction—i.e. superoxide release—and the LPO level of lung surfactant. The various antioxidants affected NBT reduction of the neutrophils and peroxidation of surfactant differently.

Vitamin E or α-tocopherol is the principal defense against oxidant-induced membrane injury in human tissue.[28] Thanks to its hydrophobicity and lipid solubility, vitamin E divides into lipid membranes, thus being placed optimally for maximal antioxidant effectiveness.[29,30]

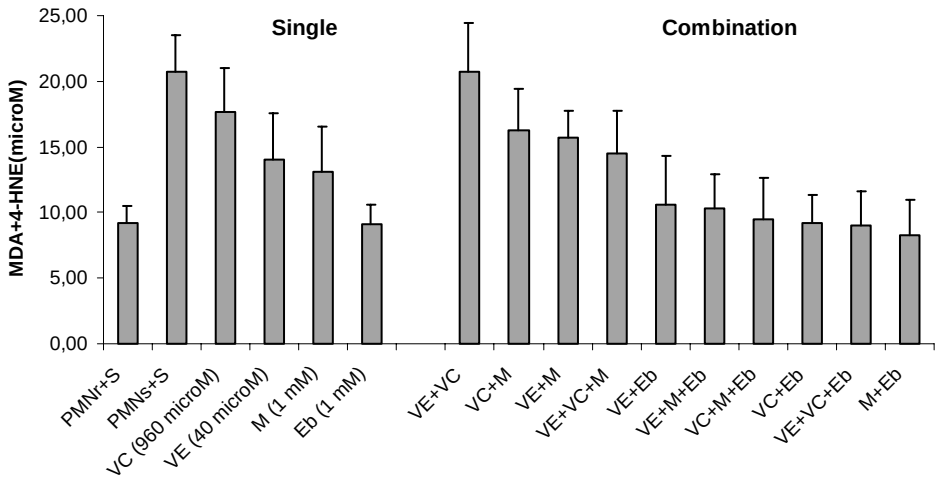

FIGURE 4 The effects of various single and combined antioxidants on the LPO level of lung surfactant incubated with stimulated PMN (PMNs). The antioxidants were used in their maximal concentrations (see Fig. 3). Controls were resting PMN (PMNr) and stimulated PMN (PMNs) with surfactant and without antioxidants. The values correspond to the MDA + 4-HNE concentrations in micromole. The mean values ± SD of seven experiments were calculated.

As in our earlier studies,[10,12] the use of vitamin E decreased superoxide production of activated neutrophils and significantly reduced the LPO level of surfactant. As compared to the other antioxidants used, vitamin E has a moderate activity.

Vitamin C, a water-soluble antioxidant, can take part in redox reactions and contribute to antioxidant activity via several mechanisms.[31,32] Our findings, however, showed that it further increased the release of superoxide by activated PMN in a dose-dependent manner. Evidently vitamin C added with *C. albicans* to phagocytes is unable to reduce their powerful respiratory burst. An antioxidant[33] as well as a prooxidant effect of vitamin C[34,35] have previously been found. In the present study, in contrast to chemical and enzymatic systems[14] vitamin C did not increase, but even slightly reduced the LPO level of surfactant. Combinations of vitamin C with other antioxidants decreased the LPO level considerably.

The combined effect of vitamins E and C against LPO may be expected. The main effect can then be attributed to vitamin E, which scavenges the peroxy radicals more readily than ascorbic acid does.[36] Vitamin C promotes the regeneration of membrane-bound oxidized vitamin E, allowing it to function again as a chain-breaking antioxidant.[32] However, in our study, the combination of vitamins E and C had no effect on reduction of NBT or on that of the LPO level.

SOD is an enzyme system that appears to have evolved specifically to deal with superoxide radicals as a substrate,[37] and provides a second barrier of defense after glutathione peroxidase and catalase against free radical injury.[38] The enzyme may therefore be expected to interact in the LPO chain reaction. Our results with SOD, however, showed that it is much more efficient in quenching superoxide radicals (lessened NBT reduction), than in interacting in the LPO reaction.

We made several experiments with various concentrations of selenomethionine or sodium selenite (data not shown), but such compounds mainly had a pro-oxidant effect. We therefore chose ebselen (2-phenyl-1,2-benzoisoselenazol-3-(2H)-one), a synthetic selenium-containing heterocycle. Ebselen has a glutathione peroxidase-like activity[39,40] and a protective effect on several models of inflammation.[41,42] It reduces lipid hydroperoxides to their corresponding hydroxy compounds, thereby suppressing the formation of free radicals.[43] Hitherto, LPO products in the presence of ebselen have been analyzed only occasionally. For instance, Thomas and Jackson[44] studied its effect on the oxidation of low-density lipoproteins (LDL). In our study, ebselen lessened the NBT reduction of the neutrophils and the LPO rate of surfactant.

Our study also showed that melatonin, as compared to the other antioxidants used, was best in quenching the superoxide radicals and the second after ebselen in reducing the LPO level of lung surfactant. The best combination of antioxidants tested was the ebselen–melatonin mixture.

Melatonin (*N*-acetyl-5-methoxy-tryptamine) is a highly lipid-soluble agent and a potent scavenger of the highly toxic hydroxyl radical and other oxygen-derived radicals. Melatonin has been shown to decrease both *in vitro* and *in vivo* MDA and 4-HNE levels in rats in a dose-dependent manner.[45] Melatonin also seemed to be more effective than other antioxidants (e.g. mannitol, glutathione, and vitamin E) in protecting against oxidative damage.[46]

Our main intention in this study was to find a tool to optimize the exogenous surfactant intended for newborn infants, especially those with RDS and pneumonia. Preliminary experiments done by our group on premature newborn rabbits with induced bacterial pneumonia showed that exogenous surfactant is peroxidized after incubation for 5 h. However, the LPO decreased and their survival and respiration improved after vitamin E was added (data not shown). Administration of antioxidants together with surfactant would thus be a way to protect surfactant effectively.

Oxidation of endogenous surfactant, however, may also occur *in vivo*. Since ROS are released into the alveolar space during ARDS, and pulmonary surfactant function changes in this syndrome, it seems likely that the lipid components of surfactant are specific targets for ROS. Ward P.A. *et al.*[47] found that oxygen radical-mediated acute lung injury occurring after complement activation was closely associated with the appearance of LPO products in plasma and lung.

Endogenous surfactant LPO may also be found in cystic fibrosis. Many of these patients are colonized in the respiratory tract by bacteria, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia* as well as fungi—most commonly *C. albicans* and *Aspergillus fumigatus*. We previously found, *in vitro*, surfactant LPO by AM challenged with these organisms.[11] Evidence is now accumulating that ROS may be important mediators of tissue damage in CF patients, especially when abnormalities in protective antioxidants[48,49] are present. Thus, indices of oxidative damage were detected in some patients with CF, even in the presence of relatively normal concentrations of antioxidants.[50] Therefore, patients with CF may benefit from exogenous administration of antioxidants.

As mentioned above, pulmonary surfactant phospholipids contain only a few polyunsaturated fatty acid and side-chains (~1.5% of the total) and thus

should be relatively resistant to peroxidation. Moreover, surfactant is produced in an environment rich in antioxidants—chiefly catalase—and to a lesser extent SOD and glutathione.[51] Moreover, alveolar lining fluid contains, in addition to glutathione,[51,52] vitamin C[53] and ceruloplasmin,[54] which can potentially quench free radicals, protect α_1-protease inhibitor(α_1-PI) and prevent LPO.[55] Nevertheless, two factors may promote oxidation of surfactant lipids in the lungs of patients with acute lung inflammation. First, antioxidant defenses may be exhausted by an excess of reactive oxygen products and may fail to provide protection. Secondly, the major antioxidant species in the alveoli may be excluded from the micro-environment in which LPO occurs. Furthermore, the extracted porcine lung surfactant (Curosurf) is not likely to contain any antioxidants.

In conclusion, the experimental results reported here suggest that exogenous surfactant supplemented with antioxidants in mixtures may play an important role, not only in improving surfactant functions but also in preventing neutrophils from further activation in injured lungs, thus reducing the possible development of lung injury in both ARDS and CF patient-groups.

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