Chapter 12
Antioxidant Properties of Surfactant

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List of Abbreviations

BPD	Bronchopulmonary dysplasia
CAT	Catalase
DPPC	Dipalmitoylphosphatidylcholine
FRs	Free radicals
PUFAs	Polyunsaturated fatty acids
PUPLs	Polyunsaturated phospholipids
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SP-A	Surfactant protein A
SP-D	Surfactant protein D

Introduction

Preterm birth affects about 5–9 % of gestations in European countries and 12–13 % in the USA [1] and is at present the leading cause of overall infant death in developed countries [2]. Despite general improvement in perinatal care, preterm birth rates are still increasing [3]. Respiratory distress syndrome (RDS), the major acute complication of prematurity, occurs in about 50 % of preterm neonates born before 30 weeks of gestation [4] ranging from 100 % at 23 weeks to 50 % at 29 weeks [5].

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RDS remains at present the leading cause of death in extremely preterm infants and often requires surfactant replacement therapy and respiratory support [5].

Endotracheal surfactant administration has affirmed as one of the milestones of RDS treatment, as it is associated with reduced mortality, air leaks, and need for mechanical ventilation, although it has been proved ineffective in preventing bronchopulmonary dysplasia (BPD) [6].

Surfactant is produced by mammalians type II alveolar cells and is a complex mixture of 90 % lipids and 10 % proteins. The most abundant phospholipid in surfactant is dipalmitoylphosphatidylcholine (DPPC), which is the main agent responsible for surface tension lowering at the air-liquid interface, probably with the contribution of other lipid compounds, as phosphatidylglycerol [7]. Surfactant proteins (SPs) constitute a mixture of hydrophilic and hydrophobic components of the surfactant, which contribute to surface tension lowering through specific interactions with DPPC and involvement in surfactant turnover process. Because of its well-known tensioactive properties, exogenous surfactant administration in RDS results in reduction of alveolar opening pressure, increase of lung volume at a certain distending pressure, and stabilization of lung volume during the deflation phases, thus reducing the work of breathing [8, 9].

However, surfactant is a pleiotropic compound. In fact, besides its chemicophysical properties, surfactant was also demonstrated to exert consistent antiinflammatory and antioxidant activities, for which nonenzymatic and enzymatic proteins and some minor lipid components appear mainly responsible [10–12]. Although less extensively investigated, these functions may contribute to the efficacy of exogenous surfactant administration in preterm neonates with RDS.

Oxidative Stress in Preterm Newborn Lungs

Oxidative stress occurs when a relative unbalance exists between pro-oxidant agents and antioxidant pathways, leading to accumulation of reactive oxygen species (ROS) and other reactive components within different tissues, resulting in cascade damages of lipids, proteins, polysaccharides, and DNA strains [13, 14]. Antioxidant defense consists of a complex web of interacting molecules which exert scavenging activity both by enzymatic pathways, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase, and nitric oxide synthase, and nonenzymatic pathways, such as vitamins C and E and uric acid [15, 16].

Oxidative stress was demonstrated to play a significant role in the development of several complications of preterm birth, as RDS and BPD, but also retinopathy of prematurity and intraventricular hemorrhage, which are all grouped together as “free radical-related diseases” [13, 14].

Preterm newborns have to deal with a consistent load of ROS and other reactive species, as a consequence of several perinatal conditions related to preterm birth determining activation of pro-oxidant pathways, as hyperoxia, tissue perfusion impairment and reperfusion, infections, and rapidly growing energy demand.

However, antioxidant defenses show impaired activity because of developmental arrest of both placental transfer and endogenous production of enzymatic and nonenzymatic compounds, thus leading to the occurrence of a pro-oxidant status [13–16]. In fact, when compared to term newborns, preterm newborns present significantly reduced activity of glutathione peroxidase and SOD for several weeks after birth [17] and lower circulating levels of vitamin E and ascorbic acid [13, 14]. Moreover, susceptibility to oxidative stress may be further increased by high concentration of polyunsaturated fatty acids (PUFAs) which are highly sensitive to peroxidation injuries, particularly in the neuronal membranes, and relatively increased free iron release favoring free radicals (FRs) formation [13, 14]. It was also demonstrated that preterm newborns lack the capacity to induce a proper antioxidant enzymatic activity in response to oxidative challenges [18].

Lung tissues of preterm newborns are particularly exposed to ROS activity, as the main treatment options for RDS, oxygen supplementation, and mechanical ventilation are known to promote oxidative stress and local proinflammatory responses [19, 20]. Such detrimental effects of RDS therapy lead in turn to endogenous surfactant inactivation, lung tissue damage, and further aggravation of respiratory failure [19, 20]. Moreover, although BPD is definitely a multifactorial disease, a close relationship has been established between BPD and oxidative and inflammatory stress, as ROS and inflammatory cytokines production as a consequence of hyperoxia, volutrauma, chorionamnionitis, and postnatal infections are clearly related to the risk of BPD [21]. Moreover, several single nucleotide polymorphisms of genes involved in the inflammatory response, oxidative stress, and antioxidant enzymes show a definite relation with the risk of BPD [22–24], thus reinforcing the importance of FRs and their clearance in determining such complications of prematurity.

Antioxidant Effects of Surfactant

Endotracheal surfactant administration prevents oxidative alveolar injury firstly by the replacement of preexisting surfactant partially damaged by ROS [11]. However, the main antioxidant effects of surfactant probably relay on different mechanisms, since surfactant itself shows a consistent antioxidant activity attributable both to enzymatic and nonenzymatic scavenger molecules naturally contained in the mixture. Consequently, exogenous surfactant would be able to abate the local ROS concentration in the alveolar areas, thus directly preventing both local damage and ROS diffusion to the surrounding tissues and vessels [11, 12, 25, 26].

It is well known that antioxidant enzymes and other antioxidant molecules are commonly found in the epithelial lining fluid of the normal human lower airways [27]. Basing on the observation that bronchoalveolar lavage of preterm neonates treated with surfactant presented lower levels of pro-oxidant markers than untreated neonates [28], it was demonstrated in the animal model that natural calf lung surfactant contains a measurable amount of SOD and CAT, which have been also demonstrated to exert consistent scavenging activity when incubated with a definite amount of H_2O_2 [11].

Moreover, endotracheal administration of surfactant also induced a significant increase of SOD content of alveolar type II cells, demonstrating the occurrence of enzyme uptake via liposome during the surfactant recycle process [11]. Therefore, the positive effects of surfactant administration in preterm neonates may be related not only to direct improvement of lung mechanics but also to local antioxidant activity of surfactant and to increased antioxidant potential of alveolar cells. Interestingly, the comparison between natural lung surfactant and a lung surfactant extract containing only 1 % of proteins showed that the latter contains no significant amount of antioxidant enzymes and does not exert any scavenging activity against H_2O_2 [11]. These data reinforce the concept that surfactant mixtures not containing proper amount of protein compounds, as synthetic surfactants, are inadequate for clinical purpose at present, probably not only because of suboptimal tensoactive properties but also for the lack of enzymes naturally present in lung surfactant.

A recent study confirmed that natural porcine and bovine surfactants contain definite amounts of SOD and CAT, which have been measured in four different natural surfactants, namely, Poractant (Curosurf), Beractant (Survanata), Calfactant (infasurf), and Bovactant (Alveofact) [12]. Comparing these mixtures, Beractant roughly showed the highest content of SOD per mg of phosholipids, while Calfactant showed the highest content of CAT, but when enzymatic activities were expressed per mL of surfactant, Poractant provided the highest content of SOD while Calfactant was confirmed to contain the highest amount of CAT. Moreover, considering the recommended doses for clinical use, Poractant was shown to provide the major amount of SOD, while Calfactant was proved to contain the major amount of CAT (Table 12.1). After incubation with different amounts of H_2O_2, Curosurf showed the

Table 12.1 Antioxidants content in natural surfactants

	Poractant	Beractant	Bovactant	Calfactant
Doses				
mg of PLs/Kg	200	100	100	100
mL of surfactant/Kg	2.5	4	2.2	2.86
SOD				
U/mg of PLs	0.396*	0.474	0.027#	0.383§
U/mL of surfactant	31.7	11.9	1.21	13.4
U/dose per Kg	73.3	47.6	2.6	38.3
CAT				
nmol/min/mL of PLs	0.81°	2.60	1.58	3.23
nmol/min/mL of surfactant	64.80	65.00	71.10	113.10
U/dose per Kg	149.80	260.00	157.80	323.50
Plasmalogens				
mol % of total PLs	3.8 ± 0.1	1.5 ± 0.2	0.9 ± 0.3	n.a.
PUPLs				
mol % of total PLs	26 ± 1	6 ± 1	11 ± 1	n.a.

Modified from Refs. [12, 25]

PLs phospholipids, *PUPLs* polyunsaturated phospholipids, *n.a.* not available

*$p = 0.019$ vs. Survanta; #$p < 0.0001$ vs. Survanta; §$p = 0.003$ vs. Survanta; °$p < 0.0001$ vs. Infasurf

Table 12.2 Scavenger activity of natural surfactants

	Poractant	Beractant	Bovactant	Calfactant
25 μM	29 ± 8*	17 ± 4	47 ± 6 * # §	36 ± 5 * #
50 μM	30 ± 6	32 ± 4	75 ± 8 * # §	56 ± 9 * #
100 μM	66 ± 8	64 ± 9	113 ± 11 * #	117 ± 10 * #
250 μM	201 ± 15	214 ± 20	278 ± 23 * #	332 ± 18 * #

H_2O_2 concentration[a] after incubation with 25, 50,100 and 250 μM of H_2O_2 (mean ± SD) (Modified from Dani et al. [12])

*$p < 0.05$ vs. Survanta; #$p < 0.05$ vs. Curosurf; $p < 0.05$ vs. Infasurf

[a]Data report the difference between H_2O_2 concentration at time 0 and at the end of the experiment

highest scavenger activity, except that at the lowest studied concentrations of H_2O_2 when Beractant exerted the highest scavenger activity. For any concentration of H_2O_2, Calfactant and Bovactant showed significantly lower scavenger activity vs. Poractant and Beractant (Table 12.2) [12].

However, not only SOD and CAT but also different antioxidant enzymes, as glutathione peroxidase and reductase, may be involved in determining the net antioxidant effect of surfactant, but unfortunately their activity in natural surfactants have not been detailed yet.

Moreover, the complex mixture of natural surfactants also contains nonenzymatic antioxidant molecules which might further contribute to the overall antioxidant activity of the mixture. Even if these aspects have been poorly investigated as far as now, plasmalogens and polyunsaturated phospholipids (PUPLs) are putatively the main responsible molecules for nonenzymatic antioxidant activity of natural surfactants.

Plasmalogens, a subgroup of phospholipids normally contained in cellular membranes, present definite scavenger activity against FRs due to the presence of a specific reductant group and are also minor components of natural surfactants [25]. Plasmalogens were recently considered of great importance to the proper biophysical function of the surfactant. According to the "surface-associated reservoir" model, large areas of surfactant layer are folded during the expiration phase, but remain adherent to the monolayer present at the air-liquid interface and extend during inspiration. Plasmalogens, together with cholesterol, were proved to work synergistically with the hydrophobic SP-B and SP-C for the proper formation of the reservoir and the spreading of DPPC and, in the experimental model, plasmalogens addition to surfactant achieved further reduction of surface tension and viscosity in comparison to surfactant alone [8, 25, 29]. Besides surface-active properties, plasmalogens were recently demonstrated to confer consistent antioxidant protection against ultraviolet light-induced lipid peroxidation within cell membranes [30] and also to exert antioxidant functions in low density lipoproteins [31]. The content of plasmalogens has been detailed for Poractant, Beractant, and Bovactant, but not for Calfactant [8, 25], and resulted consistently higher for Poractant than Beractant and Bovactant (Table 12.1). These results could partially explain the different scavenger performances against H_2O_2 observed for the natural surfactants, together with the differences in the content of antioxidant enzymes [12].

Moreover, along with plasmalogens, PUFAs are considered one of the main substrates for lipid peroxidation in lung surfactant and they were also recently demonstrated to exert consistent scavenger activity against ROS in cellular models [32]. Peroxidation of PUPLs induced by ROS at the air-liquid interface would then initiate a chain reaction, increasing in turn the availability of ROS in the alveolar spaces [25]. Interestingly, Poractant presents the highest concentration of PUPLs among natural surfactants [25], and, because of the highest concentration in the recommended dose, Poractant putatively has also the highest scavenger activity due to PULPs per treatment dose [12].

Plasmalogens and PUFAs content were detailed in tracheal aspirates collected at birth in a cohort of preterm neonates. Plasmalogens and PUFAs content resulted significantly higher in patients who developed BPD in comparison to controls of the same gestational age who did not develop BPD, suggesting a general protective role toward lung parenchyma, probably due to an overall antioxidant effects and not only to the surface tension-lowering properties [26].

Therefore, the importance of some protein and lipid components of surfactant, which are usually believed to be minor elements, should be properly considered in the setting of new synthetic surfactant preparations as beneficial effects of surfactant probably relay not only on its biophysical properties at the air-liquid interface but also on its complex antioxidant effects in the alveolar environment, which is burdened with high load of ROS in case of preterm birth.

Antioxidants Addition to Surfactant

SOD, CAT, and other antioxidants are physiologically present in measurable concentrations in natural surfactants and lung epithelial lining fluid and take part in the regulation of postnatal lung vascular development [33] and in the protection of microvasculature from ROS-induced injury [34].

Since exogenous surfactant is rapidly taken up by type II alveolar cells, it appears reasonable to supplement surfactant with antioxidant molecules in order to enhance its antioxidant potential, as both surfactant and lung antioxidant enzymes are deficient until the final 10–15 % of gestation [35].

Beractant was firstly studied as a vehicle for antioxidant enzymes to be delivered to the alveolar epithelium [36]. Incubation of lung epithelial cells with an emulsion of Beractant plus SOD and CAT resulted in significantly higher SOD and CAT activity in comparison with incubation with CAT and SOD alone or surfactant alone. These results were confirmed in lung homogenates obtained by animal models after endotracheal administration of Beractant plus SOD and CAT, suggesting that surfactant supplementation with antioxidant enzymes is an efficacious way to increase antioxidant defenses in the alveolar environment. Moreover, since intracellular localization of antioxidant enzymes may be a crucial point for their functionality, liposome encapsulation of SOD and CAT was studied, in order to enhance cellular delivery of the enzymes. Such method was proved effective in increasing

the antioxidant enzymes activity in the alveolar cells in the animal model of hyperoxia-induced lung injury of prematurity [37].

The addition of SOD and CAT to four different natural surfactants was recently studied in vitro [12]. As expected, the addition of SOD to Poractant, Beractant, and Bovactant resulted in increased scavenger activities in comparison to the surfactants alone, while the addition of SOD to Calfactant resulted in a paradoxical reduction of its scavenger activity, which was putatively attributed to other mechanisms induced by SOD overexpression, as increased H_2O_2 production and hydroxyl radical formation. CAT addition to the four surfactants resulted in increased scavenger activity of the surfactants even if it did not reach significance in the case of Beractant. The addition of both SOD and CAT induced a further increase in comparison with SOD or CAT addition alone, suggesting a synergic activity of the two enzymes.

On the other hand, previous studies by Davies et al. demonstrated the safety of intratracheal administration of rhSOD in preterm infants [38] and its effectiveness in decreasing the need of asthma medications and emergency department visits in preterm infants during the first year of life [39].

Basing on the available data, the addition of antioxidant enzymes to surfactant appears as a possible future strategy in order to improve the lung antioxidant defenses during the phase of RDS treatment. However, clinical studies are mandatory in order to confirm these preliminary experimental results.

Anti-inflammatory and Antibacterial Effects of Surfactant

Inflammation and oxidative stress are strictly related processes, since infectious and inflammatory stimuli are the main triggers of pro-oxidant pathways in biological systems. In addition to specific antioxidant properties, surfactant has been shown to posses anti-inflammatory and immune-modulating activities, which indirectly contribute to the antioxidant effects, because of the interactions between inflammatory and oxidative stress.

The modulation of inflammatory processes by surfactant is mainly ruled by protein compounds, specifically SP-A and SP-D [10, 40]. SP-A and SP-D are two hydrophilic proteins which belong to the family of collectins and were shown to affect several steps of the immune response to several pathogens both in vivo and in vitro [41, 42]. These proteins have to property to bind and aggregate to virus, bacteria, fungi, and endotoxins present in the alveolar spaces, favoring their phagocytosis and killing by cells of the innate immune response, namely, the alveolar macrophages and neutrophil granulocytes. [10, 40]. As a consequence, the transgenic mice model not expressing SP-A and SP-D presents increased susceptibility to lung infections and to the development of severe lung lesions from several pathogens [41, 42]. Moreover, it was recently demonstrated that SP-A downregulate inflammation in presence of LPS suppressing the proinflammatory pathways mediated by NF-kB [43]. On the other hand, SP-A and SP-D downregulate the specific immune response to pathogens, through the inhibition of proinflammatory cytokines secretion, as TNF-alpha, and the modulation of lymphocytes proliferation [40].

The combined effects of enhanced pathogens clearance from the alveolar spaces and reduced specific immune response activation, together with the maintenance of a proper surfactant layer, result in an overall reduction of oxidative stress in the alveoli and the surrounding tissues.

It is noteworthy that oxidative modifications of SP-A by reactive oxygen/nitrogen intermediates interfere with their ability to enhance killing of pathogens [44]. Furthermore, nitration of SP-A that has been described in vivo in the lungs of patients with ARDS [45] decreased its ability to act synergistically with SP-B and SP-C to lower surface tension [46].

However, SP-A and SP-D probably play a more complex role in lung immune-modulation. In fact, these proteins present different behavior in the presence of different stimuli, exerting proinflammatory or anti-inflammatory activity according to different conditions. Particularly, SP-A was proved to suppress NO production by LPS-activated macrophages [47], but increases NO production in the presence of other pathogens such as Mycoplasma [48], thus suggesting differential activities in response to different triggers.

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