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# Hyaluronan Decreases Surfactant Inactivation *In Vitro*

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## ABSTRACT

Hyaluronan (HA) is an anionic polymer and a constituent of alveolar fluid that can bind proteins, phospholipids, and water. Previous studies have established that nonionic polymers improve the surface activity of pulmonary surfactants by decreasing inactivation of surfactant. In this work, we investigate whether HA can also have beneficial effects when added to surfactants. We used a modified pulsating bubble surfactometer to measure mixtures of several commercially available pulmonary surfactants or native calf surfactant with and without serum inactivation. Surface properties such as equilibrium surface tension, minimum and maximum surface tensions on compression and expansion of a surface film, and degree of surface area reduction required to reach a surface tension of 10 mN/m were measured. In the presence of serum, addition of HA dramatically improved the surface activities of all four surfactants and in some cases in the absence of serum as well. These results indicate that HA reduces inactivation of surfactants caused by serum and add evidence that endogenous HAs may interact with alveolar surfactant under normal and abnormal conditions. **(*Pediatr Res* 57: 237–241, 2005)**

**Abbreviations**

**HA,** hyaluronan
**PEG,** polyethylene glycol
**SA,** surface area
**$\Delta A_{10}$,** decrease in surface area from the maximum to reach a surface tension of 10 mN/m

Over the past decade, pulmonary surfactant replacement has revolutionized the therapy of respiratory distress syndrome of premature infants (1–3). However, effects of surfactant therapy are less dramatic when used to treat lung diseases associated with acute lung injury and acute respiratory distress syndrome (4–7). The less successful clinical response in these diseases may be due in part to surfactant inactivation caused by leakage of plasma and inflammatory products into the alveoli. In the presence of inactivating substances, surface activity of alveolar surfactant is adversely affected, worsening overall lung function (8,9).

Variation in susceptibility to inactivation exists among therapeutic surfactants. Many studies have reported that surfactant-associated proteins help resist inactivation caused by a variety of factors (6). We and others have found that the addition of nonionic polymers [principally polyethylene glycol (PEG), or dextran] further reduces surfactant inactivation by serum, meconium, or other substances *in vitro* (10–12). *In vivo* studies also demonstrate that the addition of nonionic polymers to therapeutic surfactants improves pulmonary function after lung injury caused by meconium, albumin, hydrochloric acid, milk acid, or endotoxin (11,13–16).

The successful experiments with nonionic polymers led us to consider using ionic polymers. In this study, we investigated whether hyaluronan (HA) has effects on pulmonary surfactants that are similar to those described for dextran and PEG. HA is a nonsulfated, polymeric glycosaminoglycan (mucopolysaccharide) of *N*-acetyl-glucosamine linked $\beta$-1, 4 to glucuronic acid, which is linked $\beta$-1, 3 to *N*-acetylglucosamine, etc. Unlike PEG or dextran, HA, an anionic polymer, is a natural, ubiquitous substance in the body. However, HA (like PEG and dextran) shares the ability to bind water but has much greater binding capacity. In addition, HA can interact with phospholipids to form various complexes that depend on the molecular weight of HA (17). HA and other glycosaminoglycans are secreted by alveolar epithelial cells (18,19), and early papers already speculated that complex carbohydrates may interact in important ways with pulmonary surfactant under normal conditions in the alveoli (20–22). More recently, Bray (23) suggested that HA has important extracellular functions that affect

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lung surfactant. To study the effects of HA on samples of surfactants that are derived from several animal species, we compared the surface activity of three commercial pulmonary surfactants approved for human use (Survanta, Curosurf, Infasurf) and native calf surfactant, with and without HA, and also with and without serum used as an inhibitor of surfactant function.

## METHODS

***Materials.*** HA of 250 and 100 kD were a gift from GlycoMed Research (Hastings-on-Hudson, NY). Molecular weight determinations were carried out by the manufacturer using extrapolation of viscosity measurements to zero concentration, a method that gives estimates within 10% of direct measures. These HA preparations contain <0.01% protein and a ratio of hexuronic acid to hexosamine of 1:1 (characteristic of HA). HA of 1240 kD was purchased from Sigma Chemical Co. (St. Louis, MO) with a protein content of 0.07%. Lipopolysaccharide concentrations in samples of HA used in these studies were 0.015–0.025 EU/mg of HA determined by chromogenic *Limulus* amoebocyte assay (QLC-1000; BioWhittaker, Walkersville, MD). The three HA preparations were isolates from *Streptococcus* fermentation and were used as supplied.

Survanta was obtained from our neonatal intensive care unit. Curosurf was purchased from Dey Laboratory (Napa, CA). Infasurf was a gift from Forest Laboratories (New York, NY). Native surfactant was obtained by saline bronchoalveolar lavage of freshly obtained calf lung. The recovered lavage fluid was centrifuged at 500 × *g* for 10 min to remove cellular debris. The supernatants then were centrifuged at 17,000 × *g* for 2 h at 10°C. The pelleted material was lyophilized, stored at −80°C, and reconstituted just before use with distilled water. The concentration of phospholipids in native calf surfactant was determined by measuring lipid phosphorus after Bligh-Dyer extraction (24,25). Reported protein content of native calf surfactant is 10% of the total weight with SP-A 4% and SP-B and C each 1% (26). All of the surfactants were diluted with a buffered solution of 0.9% NaCl and 5 mM of HEPES at a pH of 6.5. The final concentrations of Survanta, Curosurf, and Infasurf were 1.25 mg weight phospholipid/mL, and native surfactant was 1.0 mg/mL. HA in dry powder form was added to the surfactant and mixed by Vortex for ~60 s at room temperature to a final concentration of 0.25% (wt/vol). This concentration was chosen from pilot experiments. Serum obtained from normal adult laboratory volunteers was used as a nonspecific inhibitor of surfactant activity. The serum was frozen at −20°C until use, then thawed and added to the surfactant mixtures and mixed by Vortex for 20 s before testing (within 30 min). The smallest volume of serum that when added to 1 mL of surfactant suspension was able to raise the minimum surface tension to >14 mN/m was used for inactivation of each surfactant (5 μL of serum/mL for Curosurf and Infasurf and 10 μL for Survanta and native surfactant, equivalent to 350–700 μg of serum protein).

***Surface activity.*** Surface activity was measured in a modified pulsating bubble surfactometer (Electronetics, Buffalo, NY) using a technique to prevent wetting of the capillary tube (27). The temperature of the 25-μL sample chamber was maintained at 37°C throughout. Pressure measurements for the device were calibrated electronically according to the manufacturer's instructions and checked with a water manometer. Surface tension measurements were calibrated using pure fluids with known surface tensions.

Equilibrium surface tension was defined as the value of surface tension ~30 s after the formation of a static microbubble of 0.40 mm radius. We used this measure as an approximate index of the amount of surface active material that reached the air–water interface before cycling (inflation-deflation) of the microbubble. Thereafter, the microbubble was cycled at 20/min with radii changing between 0.40 and 0.55 mm. Minimum and maximum surface tensions were recorded at the 10th cycle (60 s) and after 5 min. The surface tension was calculated by use of the Laplace formula P = 2ST/r, where ST is the surface tension, P is the inflating pressure, and r is the radius of the assumed spherical bubble.

We also calculated the percentage reduction in bubble surface area (SA) from its maximum value to that required for the surface tension to reach a value of 10 mN/m. This measurement ($\Delta A_{10}$) was made 5 min after the start of bubble cycling. The parameter $\Delta A_{10}$ reflects film compressibility: films with low compressibility (a low $\Delta A_{10}$) produce a large decrease in surface tension with a relatively small decrease in SA. $\Delta A_{10}$ also indicates the ability of preformed films to respread after repeated expansions and reductions of SA. The formula is $\Delta A_{10} = [(\text{max SA} - \text{SA@ 10 mN/m})/\text{max SA}] \times 100\%$. If the surface tension did not reach 10 mN/m, then the actual $\Delta A_{10}$ values would have had to be greater than 47% because that is the difference in SA between the maximum and minimum bubble areas in our pulsating bubble surfactometer. In all, there were six measures of surface activity: minimum and maximum surface tensions at the 10th cycle and after five minutes, $\Delta A_{10}$, and equilibrium surface tension.

***Analysis.*** The data are presented as means ± SEM. Measurements were analyzed by one-way ANOVA using SigmaStat software (SPSS Science, Chicago, IL). Comparisons between pairs of groups were done using the Tukey test or the Kruskal-Wallis test when necessary to correct for multiple comparisons. A $p \leq 0.05$ was taken to indicate statistical significance.

## RESULTS

The six measures of surface activity previously defined were obtained for four surfactants, with and without the addition of HAs of three different molecular weights, in the presence or absence of serum. In general, surface activity of Survanta, Infasurf, Curosurf, and native surfactant was affected by the addition of HA before and (most dramatically) after serum inactivation. Equilibrium surface tension (surface tension after 30 s of incubating before cycling) is least affected by HA, and $\Delta A_{10}$ is affected the most. Overall, the changes caused by the addition of HA improved surface activity.

***Surface activity in the absence of serum or HA.*** Mean equilibrium surface tension values for the four surfactants ranged from 22 ± 1 to 25 ± 1 mN/m, with no significant differences among them. After 10 cycles (total elapsed time of 60 s from bubble formation), the average minimum surface tension for Survanta was 3 mN/m, whereas the other surfactants had values >10 mN/m. All surfactants reached a minimum surface tension <4 mN/m after 5 min of cycling. Survanta had the highest maximum surface tension, which was 40 ± 1 mN/m after the 10th cycle and 41 ± 2 mN/m at 5 min. Curosurf had the lowest maximum surface tension, which was 30 ± 1 mN/m after the 10th cycle and 35 mN/m after 5 min of cycling. SA reduction of 17–27% was required for the surfactants to reach a surface tension of 10 mN/m ($\Delta A_{10}$).

***Addition of HA.*** In general, the addition of HA improved surface activity. Considering all combinations of surfactants and molecular weights of HA, approximately one third of the minimum surface tensions and $\Delta A_{10}$s were significantly improved (Figs. 1*A* and 2*A*). However, the maximum surface tension with 1240-kD HA was significantly elevated for Survanta and native surfactant (Table 1). There was little effect associated with the addition of HA on the mean equilibrium surface tension.

***Addition of serum.*** We adjusted the amount of serum to ensure that minimum surface tensions remained above 14 mN/m, that is, that inactivation occurred. When serum was added, the equilibrium surface tension was typically ~10 mN/m above the normal values absent serum. The maximum surface tension was slightly higher than without serum inactivation (Table 1). The value for $\Delta A_{10}$ was >47% because minimum surface tensions for all surfactants remained >10 mN/m by design.

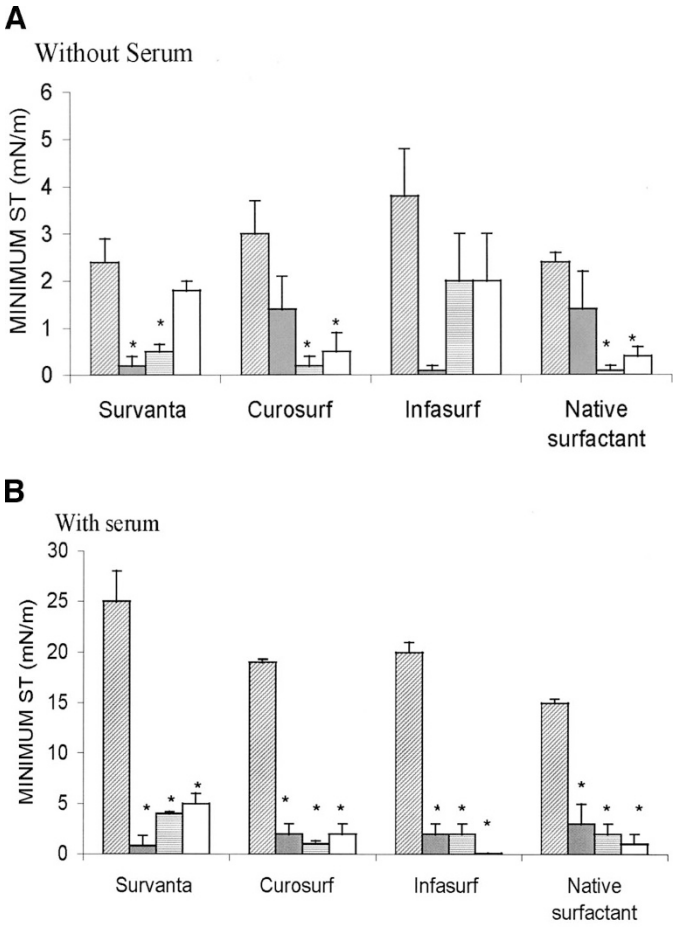


**Figure 1.** Minimum surface tension after 5 min of cycling is shown for four surfactants without (*A*) and with (*B*) serum inactivation with or without HA 1240 kD, 250 kD, or 100 kD ($n = 5$). ▨, surfactant without HA; ■, surfactant with HA 1240 kD; ▤, surfactant with HA 250 kD; □, surfactant with HA 100 kD; $*p \leq 0.05$ for comparisons of HA-treated *vs* untreated samples (by ANOVA corrected for multiple comparisons).

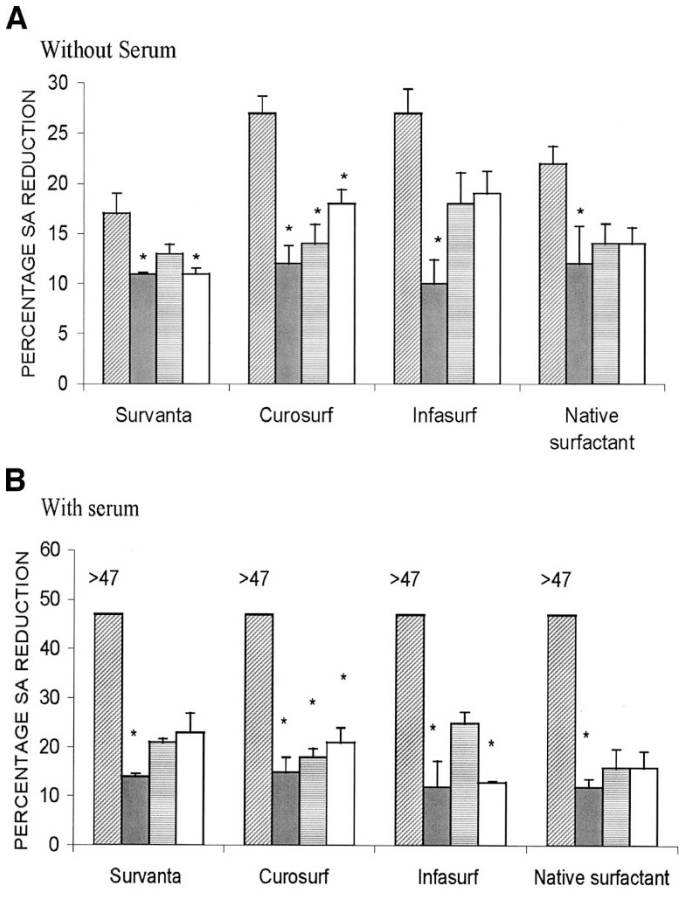


**Figure 2.** $\Delta A_{10}$ values are shown for four surfactants without (*A*) and with (*B*) serum inactivation with or without HA 1240 kD, 250 kD, or 100 kD ($n = 5$). Bars are the same as in Fig. 1. $*p \leq 0.05$ for comparisons of HA-treated *vs* untreated samples (by ANOVA corrected for multiple comparisons). For $\Delta A_{10}$ values that are >47%, we used a value of 47% to carry out the statistical analysis, a substitution that increases *p* values relative to what they would be if $\Delta A_{10}$ values >47% were measurable.

***Addition of both serum and HA.*** Minimum surface tension was dramatically improved (compared with mixtures that did not contain HA). Values obtained were comparable to those without serum inactivation (Fig. 1). Also, values of $\Delta A_{10}$ seemed similar to those for surfactants alone (no HA and no serum inactivation; Fig. 2).

***Effects of HA on different surfactants.*** For Survanta, all six measures of surface activity were affected significantly with the addition of at least one of the HAs in the absence of serum, and all measures were affected except for maximum surface tension at the 10th cycle with serum inactivation. For Curosurf, all measures except equilibrium surface tension and maximum surface tension at 5 min were significantly altered by HA in the absence of serum, and all six measures were affected significantly by one or more HA additives after addition of serum. For Infasurf, $\Delta A_{10}$ was the only measure of surface activity that was markedly affected by HA in the absence of serum; however, all but the equilibrium surface tension were affected by HA in the presence of serum. For native surfactant, all measures except for equilibrium surface tension were improved by the addition of one or more of the HAs with and without serum.

***General effects of HA molecular weight.*** Overall, 1240 kD improved measures of surface activity more than the two lower molecular weight HAs. For example, with Survanta in the absence of serum, 1240-kD HA improved three of six measures of surface activity to a significantly greater degree than 250-kD HA and five of six measures to a significantly greater degree than 100-kD HA. The differences associated with different molecular weight HAs was least apparent with Curosurf. For Curosurf, only four surface activity measures out of 12 (six with and six without serum inactivation) were improved to a greater degree with 1240-kD HA than with lower molecular weight HAs. With serum, the maximum surface tension was significantly increased both at the 10th cycle and after 5 min in the presence of 1240-kD HA for Survanta, Infasurf, and native surfactant. Whereas the maximum surface tensions for those surfactants with 1240-kD HA mixtures were significantly increased, the minimum surface tensions were significantly decreased to a much greater degree. Lower molecular weight HA was associated in only one case with significantly elevated maximum surface tension (Survanta, serum, 100-kD HA), but in most cases, there was a trend (nonsignificant) toward lower

**Table 1.** *Maximum surface tensions are shown for four surfactants with three molecular weights of HA in the presence or absence of serum**

| | Survanta + | | | | Curosurf + | | | |
|---|---|---|---|---|---|---|---|---|
| | No HA | HA 1240 kD | HA 250 kD | HA 100 kD | No HA | HA 1240 kD | HA 250 kD | HA 100 kD |
| No serum | 41 ± 2 | 47 ± 1† | 38 ± 1 | 39 ± 1 | 35 ± 2 | 35 ± 0.4 | 33 ± 1 | 33 ± 0.5 |
| With serum | 43 ± 1 | 49 ± 2† | 46 ± 1 | 50 ± 1† | 42 ± 2 | 38 ± 2 | 36 ± 1 | 32 ± 1† |
| | Infasurf + | | | | Native surfactant + | | | |
| | No HA | HA 1240 kD | HA 250 kD | HA 100 kD | No HA | HA 1240 kD | HA 250 kD | HA 100 kD |
| No serum | 36 ± 1 | 44 ± 3 | 33 ± 1 | 31 ± 1 | 35 ± 0.4 | 44 ± 1† | 33 ± 0.3 | 33 ± 0.4† |
| With serum | 39 ± 3 | 52 ± 3† | 36 ± 1 | 33 ± 1 | 35 ± 1 | 46 ± 2† | 36 ± 1 | 34 ± 1 |

* Values obtained after 5 min of cycling. Values represent means ±SEM ($n = 5$).

† $p \leq 0.05$ for comparisons of HA-supplemented samples with HA-free samples using ANOVA and corrected for multiple comparisons by Tukey or Kruskal Wallis tests.

maximum surface tensions with the lower molecular weight HAs (Table 1).

## DISCUSSION

In this study, we found that HA over a range of molecular weights is able to substantially improve most measures of surface activity of Survanta, Curosurf, Infasurf, and native calf surfactant especially after inactivation with serum. The finding that HA is effective with each of the surfactants tested is in contrast to results with nonionic polymers in which dextran was found to be significantly more effective with Curosurf than PEG, and PEG was more effective with Survanta than dextran (28). These results suggest that ionic and nonionic polymers may affect surfactants by different mechanisms.

Until some years ago, the main role of HA was thought to be one of the inactive fillers ("ground substance") of the interstitial space. It is present in high concentrations in the vitreous humor, synovial fluid, and umbilical cord. More recently, HA has been found to interact with a number of receptor proteins, such as CD44 and RHAMM, thereby acting to modulate inflammatory reactions, cell growth, and migration during embryo- and tumorigenesis. In alveolar fluid, HA is reported to have molecular weights of ~220 kD with a concentration of ~4 $\mu$g/mL, based on the amount retrieved from bronchoalveolar lavage matched with calculations of the alveolar aqueous subphase volume (29). This may be an underestimation because of the inefficiency of removal of protein-bound HA and because of difficulties in ascertaining subphase volumes. The alveolar concentrations of surfactant under normal and abnormal conditions are also difficult to measure accurately.

How does HA interact with pulmonary surfactant to cause these alterations in surface activity? The properties of HA include ability to self-aggregate, interactions with proteins, and ability to bind up to one thousand times its own weight of water (23,30). By binding water molecules, HA (like PEG and dextran) can increase the concentration of some molecules in the nonbound water and by this mechanism may act to segregate surfactant from serum constituents and thus mitigate inactivation. HA forms a three-dimensional macromolecular mesh in water that may also act to separate inactivating substances from surfactant by acting as a size-discriminating filter (31). Phospholipids in the presence of HA tend to form lipid aggregates (17). HA may also stabilize surfactant phospholipids at the air–water interface and/or increase surface adsorption of subphase lipids. HA in addition to its hydrophilic properties has hydrophobic regions created by its physical shape that could potentially serve as interaction sites for the hydrophobic surfactant proteins B and C. Several of these attributes and effects of HA are shared by SP-A, such as prevention of surfactant inactivation, improvement of surface activity under some conditions, binding of phospholipids, and synergy with SP-B.

The possibility of HA's binding albumin has been addressed by a number of investigators. Gramling *et al.* (32) inferred from electrophoretic mobility studies that HA forms stable complexes with albumin. They also found that some free HA exists even with an albumin excess. Gold *et al.* (33) found binding of HA and other glycosaminoglycans to albumin-agarose columns. However, they also found that this binding was not observed unless the salt concentration was far below physiologic ranges that we used.

The reason for the increased maximum surface tension with high molecular weight HA is not clear. This finding is not present with the two HAs that have molecular weights closer to those reported for alveolar lavage.

Although HA has beneficial *in vitro* interactions with surfactant, it is difficult to predict what the *in vivo* effects would be, granted the largely unknown subphase concentrations of surfactant, HA and other endogenous polymers, and serum under normal and abnormal conditions. Various lung injuries result in either increased or decreased levels of HA (34,35). Inflammatory processes have been found to be affected *in vivo* by HA in part dependent on molecular weight (36–38). With repeated aerosolization of 100-kD HA into lungs of guinea pigs that were injured with elastase, Cantor *et al.* (39) found a decrease in elastic fiber injury and no adverse morphologic changes in the lungs attributable to HA. The existing evidence suggests that the administration of HA into alveoli will not be harmful and may prove to be beneficial.

## CONCLUSION

In summary, HA improves the surface activity of four different pulmonary surfactants, especially in the presence of

serum inactivation. Therefore, HA may be a useful additive to current formulations of surfactants for therapeutic use if animal studies find both effectiveness and safety. Although dextran, PEG, and HA all have been approved by the Food and Drug Administration for purposes in humans, use of an endogenous substance (HA) as a surfactant additive seems attractive. These results also add *in vitro* evidence in support of the hypothesis that HA may have a normal role in the function of surfactant at the alveolar surface.

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