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# Hyaluronan Production *in vitro* by Fetal Lung Fibroblasts and Epithelial Cells Exposed to Surfactants of N-acetylcysteine

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## ABSTRACT

Fetal human lung fibroblasts and feline lung epithelial cells were exposed to either a surfactant or N-acetylcysteine in various concentrations for 24-48 hours, after which the hyaluronan concentration in the culture medium was determined. Most of the experiments showed no stimulatory effect of either artificial or natural surfactant on hyaluronan synthesis. N-acetylcysteine 5-100 mg/mL induced progressive stimulation of hyaluronan synthesis by human fetal lung fibroblasts, resulting in a maximum hyaluronan concentration six times that released by unexposed cells. A slight increase in hyaluronan synthesis was also observed after exposure of feline fetal lung epithelial cells to N-acetylcysteine 50-100 μg/mL.

## INTRODUCTION

The hyaluronan concentration in the lung decreases during the last fifth of normal fetal development, with the lowest value immediately before term (1). The production of hyaluronan increases during pulmonary disease in infants and adults, and as an early response to lung injury in animal models (2, 3, 4). It also increases as a reaction to hyperoxia (5,6). The *in vitro* hyaluronan production is known to be enhanced by several inflammatory mediators (7).

In adult patients with inflammatory diseases of the lungs, increased hyaluronan concentrations have been observed both in interstitial lung tissue and in bronchoalveolar lavage fluid (8). In the adult respiratory distress syndrome (ARDS), characterized by non-cardiogenic pulmonary edema and inactivation of surfactant as a result of leakage of plasma proteins into the air spaces from areas of epithelial disruption (9), accumulation of hyaluronan parallels the development of edema (10,11).

In infant respiratory distress syndrome (IRDS), a frequent complication of preterm birth, there is a reduction of surfactant activity, due to a combination of surfactant deficiency and surfactant inactivation by plasma proteins (9, 12). This results in increased permeability of the alveolar wall, hyaline membrane formation, and increased interstitial lung water (13). The hyaluronan concentration in lung extracts has been shown to increase with increasing severity of IRDS in premature monkeys, and to decrease with surfactant treatment (2).

Surfactants, which are used to treat infants with IRDS by direct instillation into the airways, must not be assumed to be inert substances with a singular capacity to alter surface tension and gas exchange properties of the lung (14). Arnon *et al* (15) reported that surfactant treatment of newborns with IRDS increased the total white cell count and the number of macrophages in bronchoalveolar lavage fluid, while Gerdes *et al* (16) found no increase in neutrophil elastase in tracheal aspirates after surfactant treatment.

The effects of surfactants in cellular cultures have mostly been studied in macrophages and monocytes, where inhibition of effects of inflammatory mediators has generally been found (17,18). A decrease in the phagocytic function of these cells has also been noted in some studies (19,20). Fewer reports have dealt with the effects on fibroblasts, but surfactants have been shown *in vitro* to both stimulate and inhibit fibroblast proliferation (21), to have effects on the surfactant metabolism in exposed cells (22), and to inhibit synthesis of DNA and inflammatory mediators in normal human lung fibroblasts (23). Surfactants may also affect tracheal epithelial cells by increasing the membrane potential, and by slightly (<10%) increasing the ciliary beat frequency in a dose-dependent manner (18).

Several surfactants are in clinical use, and these are either animal lung extracts (*e.g.* Curosurf®; 20) or artificial products (*e.g.* Exosurf®; 24). Apart from the surfactant itself, surfactant preparations may contain other components that may have effects of their own. Some preparations from animal sources have contained platelet-activation factor (PAF), which has been associated with neonatal disease and increased leukotriene production (25). Tyloxapol and cetyl alcohol (detergent and spreading agent, respectively), which are used in Exosurf, can function as antioxidants *in vitro*, and their *in vivo* instillation is associated with reduction of lung edema, of oxidized tissue products, and of mortality after hyperoxia (14).

N-acetylcysteine is frequently used in ventilator-treated infants and applied directly into the airways, usually by nebulization. It is primarily used for its mucolytic effect (26), but it may also act as a free oxygen radical scavenger (27), prevent the production of cytokines by stimulated fibroblasts (28), and prolong the human fibroblast life span (29).

In the airway, any instilled drug primarily affects the epithelial lining cells, but epithelial cells and underlying fibroblasts closely interact. For example it is proposed that the prenatal steroid-induced production of surfactant in preterm infants may be due to a direct effect of steroids on fibroblasts, with subsequent release of a stimulatory factor that influences the lung epithelial cells (30). In addition, fetal fibroblasts differ in some respects from adult cells; for instance the inhibition of normal hyaluronan synthesis seen after a cell culture has grown to confluence is less pronounced in fetal than in adult cells (31). Fetal cells, therefore, may yield *in vitro* results that more closely reflect the events in the perinatal period *in vivo*.

The aim of the present study was to determine whether addition of surfactant or N-acetylcysteine to cultures of fetal lung cells would affect their hyaluronan production. Two brief reports have been presented, one preliminary communication based on the first two experiments (32), and a report on the results of surfactant exposure (33).

## METHODS

Three drugs were tested: a) Exosurf®, a synthetic surfactant composed of dipalmitoyl-phosphatidyl-choline (DPPC, colfosceril palmitate) in cetyl alcohol (1-hexadecanol) and tyloxapol [4-(1,1,3,3-tetramethylbutyl) phenol polymerized with formaldehyde and oxirane], from Glaxo Wellcome, Göteborg, Sweden, b) Curosurf ®(a porcine lung extract containing lipids and surfactant-associated proteins B and C 80 mg/mL in DPPC), from Serono, Geneva, Switzerland, and c) Acetylcystein NM Pharma® (N-acetylcysteine 200 mg/mL in 0.5 mg/mL EDTA, and ascorbic acid and sodium hydroxide *q.s. ad* pH 7) from NM Pharma, Stockholm, Sweden. All preparations are in clinical use.

Two types of cells, obtained from the European Collection of Cell Cultures, Salisbury, UK, were used. Human fetal fibroblasts (WI -38, from approximately the 12th gestational week, passage (P) 14-17) were cultured in Dulbecco's modified Eagle medium (DMEM), and feline fetal lung epithelial cells (AKD, P 24) were cultured in Ham's F12 medium with 1% non-essential amino acids. To both culture media 4 mM L-gluthamine, penicillin 120 IU/mL, and streptomycin 100 μg/mL were added. Cells were grown in incubators in 5% $CO_2$ at 37°C in a humidified atmosphere.

Fibroblasts (30-40 000) and epithelial cells (200 000) were first grown in 12-well dishes in 0.5 mL culture medium containing 10% fetal bovine serum (FBS) for 24 hours. Following one wash with medium containing 0.1or 0.25% FBS (starvation medium), the cells were cultured in 0.5 mL "starvation medium" for 48 hours, in order to obtain basal conditions. Then 0.5 ml of fresh starvation medium, containing one of the three tested drugs at indicated concentrations, was added to duplicate or quadruplicate wells. The tested concentrations were: Exosurf 5 μg - 40 mg/mL, Curosurf 5 μg - 20 mg/mL, and N-acetylcysteine 5 μg - 120 mg/mL. Plain starvation medium served as control and medium containing 10% FBS as reference. The cultures were then incubated for another 24 or 48 hours. The hyaluronan concentrations in the culture media were determined with a radiometric assay kit (HA 50, Pharmacia, Uppsala, Sweden) (34). Mean hyaluronan values ± SEM were calculated for each drug concentration. The values were then divided by the number of cells and the drug exposure time. Since the absolute hyaluronan values differed substantially between the different experiments, the results were finally expressed in percent of the control value (starvation medium only). The *coefficients of variation* (the standard deviation divided by the mean) (35) within groups were calculated.

## RESULTS

Cells were tested in six instances between December 1993 and April 1995, with some variation in drug concentration, duration of exposure, and % FBS added to the starvation media (see method). In one experiment fetal feline epithelial cells were used, and in the other five experiments only fetal human fibroblasts.

The following letters are used to denote the different experiments:

A. Fibroblasts, P14, drug exposure 48 hours, 30 000 cells/well.

B. Fibroblasts, P16, drug exposure 24 hours, 25 000 cells/well.

C. Fibroblasts, P16-18, drug exposure 24 hours, 30 000 cells/well.

D. Fibroblasts, P17, drug exposure 24 hours, 30 000 cells/well.

E. Epithelial cells, P24, drug exposure 48 hours, 200 000 cells/well, also indicated by*.)

F. Fibroblasts, P17, drug exposure 48 hours, 30 000 cells/well.

For each experiment, the mean hyaluronan concentration/cell/24 hour drug exposure was calculated and expressed as percent of the control (starvation medium alone). As an example, the result of the first experiment with exposure to Exosurf is shown in *Table 1*. The rest of the results are summarized in *Figures 1-3*.

**Table 1.** Hyaluronan (HA) concentration in cell culture medium. Results from experiment A with human fetal lung fibroblasts, passage 14, exposed to Exosurf or fetal bovine serum (FBS) only for 48 hours, 30 000 cells/well.

| **Exosurf mg/ml** | **FBS** | **n** | **HA/well** mean ± SEM **ng/ml** | **HA/cell/24h** mean **pg** | **HA/cell/24h** mean **% of control** |
|---|---|---|---|---|---|
| 03.38 | 0.1% | 4 | 433.9 ± 17.4 | 7.2 | 67.1 |
| 06.75 | 0.1% | 4 | 382.6 ± 36.2 | 6.4 | 59.1 |
| 13.50 | 0.1% | 4 | 320.3 ± 22.5 | 5.3 | 49.5 |
| 27.00 | 0.1% | 4 | 295.8 ± 14.2 | 4.9 | 45.7 |
| 0 | 0.1% | 4 | 647.0 ± 21.8 | 10.8 | 100.0 |
| 0 | 10% | 4 | 1444.9 ± 45.3 | 24.1 | 223.3 |

In general, the results varied considerably between different experiments, with a mean coefficient of variation of 0.17 (Exosurf 0.18, Curosurf 0.14, N-acetylcysteine 0.19).

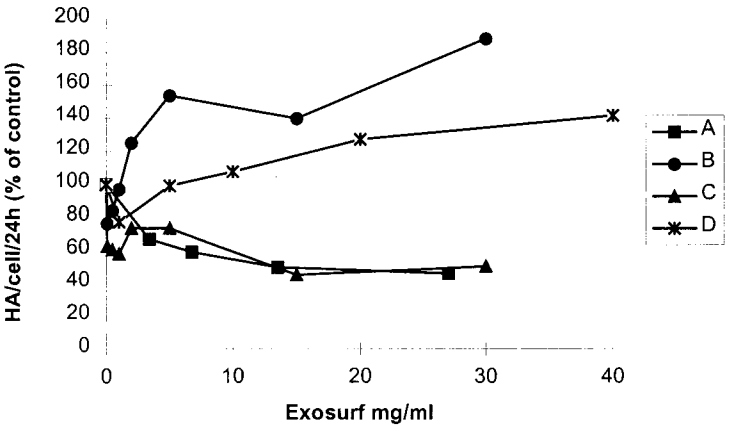


**Figure 1 A.** Hyaluronan (HA) in culture medium in relation to Exosurf exposure. Summary of 4 experiments (A-D) with human fetal fibroblasts.

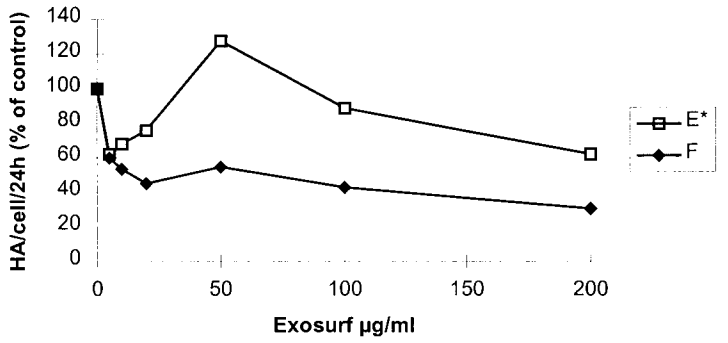


**Figure 1 B.** Hyaluronan (HA) in culture medium in relation to Exosurf exposure. Summary of 2 experiments, E* with feline fetal epithelial cells, and F with human fetal fibroblasts.

After exposure to Exosurf, no increase in the hyaluronan concentration in the culture medium, compared to the control (starvation medium alone), was found in three experiments with fibroblasts (A,C,F) and one with epithelial cells (E). In two experiments with fibroblasts (B,D) a progressive increase to 188% and 141%, respectively, was noted after exposure to Exosurf 2-40 mg/mL (*Fig. 1*).

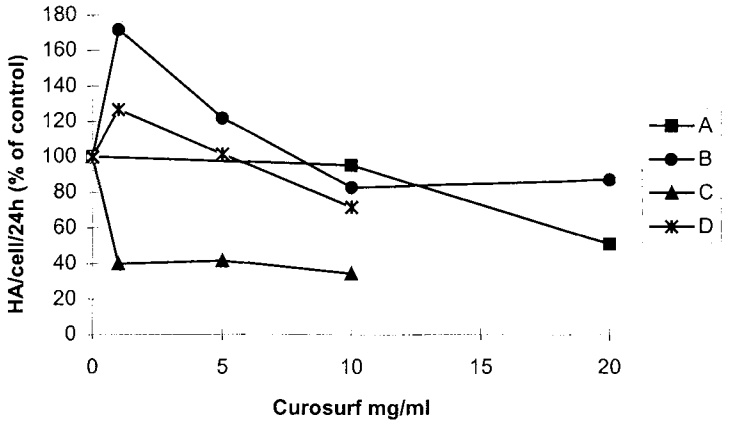


**Figure 2 A.** Hyaluronan (HA) in culture medium in relation to Curosurf exposure. Summary of 4 experiments (A-D) with human fetal fibroblasts.

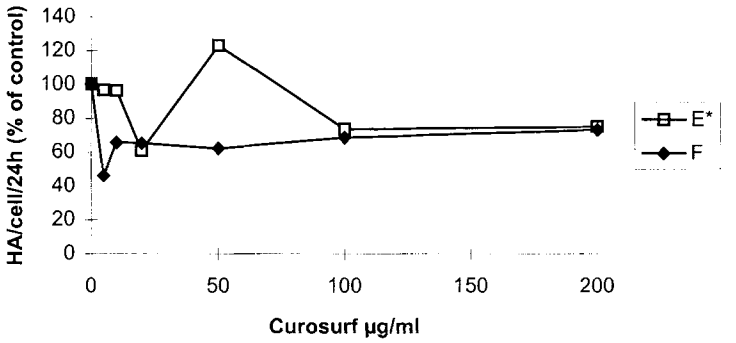


**Figure 2 B.** Hyaluronan (HA) in culture medium in relation to Curosurf exposure. Summary of 2 experiments, E* with feline fetal epithelial cells, and F with human fetal fibroblasts.

Following Curosurf exposure, three experiments with fibroblasts (A,C,F) and one with epithelial cells (E) showed no increase in the hyaluronan concentration compared to the control (starvation medium alone). In two experiments with fibroblasts (B,D) maximal increases to 172 and 122%, respectively, were observed with Curosurf at 1 and 5 mg/mL. Higher concentrations yielded hyaluronan values below the control level (*Fig. 2*).

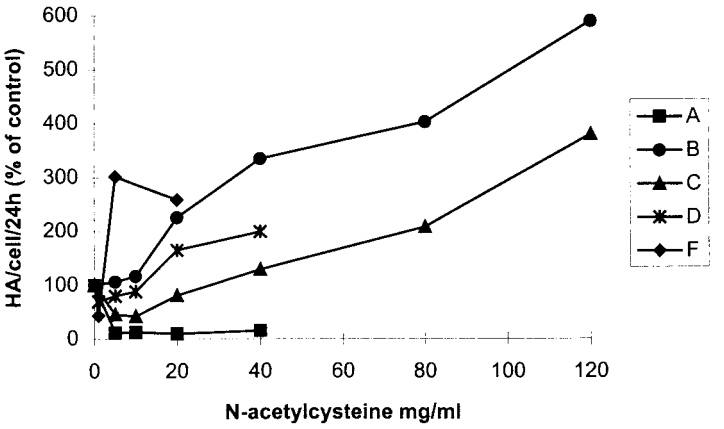


**Figure 3 A.** Hyaluronan (HA) in culture medium in relation to N-acetylcysteine exposure. Summary of 5 experiments (A-D, F) with human fetal fibroblasts.

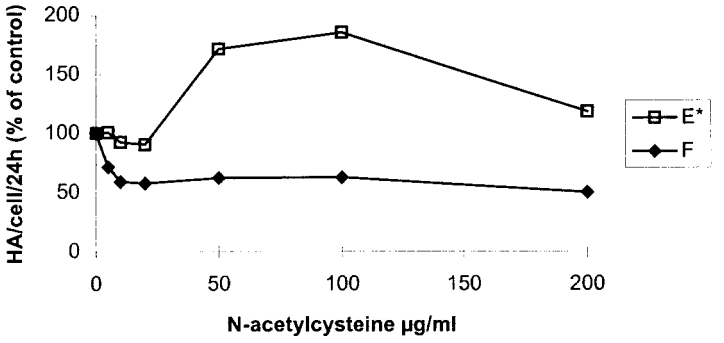


**Figure 3 B.** Hyaluronan (HA) in culture medium in relation to N-acetylcysteine exposure. Summary of 2 experiments, E* with feline fetal epithelial cells, and F with human fetal fibroblasts.

After exposure to N-acetylcysteine, one experiment with fibroblasts (A) showed no increase in the hyaluronan concentration compared to the control (starvation medium alone), while in four experiments with fibroblasts (B,C,D,F) a mainly progressive increase to 185-589 % was noted with N-acetylcysteine 5-100 mg/mL. In the experiment with epithelial cells (E*), there was an increase to 171-185% after exposure to N-acetylcysteine 50-100 µg/mL, but only to 118% after 200 µg/mL (*Fig. 3*).

## DISCUSSION

Our findings do not indicate that hyaluronan synthesis by fetal lung epithelial cells or fetal lung fibroblasts is stimulated by either artificial or natural surfactant in the concentration ranges 5 μg/mL - 40 mg/mL, and 5 μg/mL - 20 mg/mL, respectively. This is in accordance with the finding by Juul *et al* (2) that *in vivo* surfactant treatment of IRDS reduces the hyaluronan concentration in the lung, and with the report by Thomassen *et al* (23) that surfactants inhibit *in vitro* synthesis of inflammatory mediators in normal human lung fibroblasts. Oxidants are involved in the signal transduction pathways for cytokine secretion, and the inhibitory effect of surfactant supports a proposed antioxidant effect (14).

Progressive stimulation of hyaluronan synthesis by fetal human lung fibroblasts was induced by N-acetylcysteine in the doserange 5-100 mg/mL, and fetal feline lung epithelial cells responded with increased hyaluronan synthesis after addition of N-acetylcysteine in the doserange 50-100 μg/mL. This could have some clinical implications, since N-acetylcysteine is commonly used in concentrations of 20 - 200 mg/mL and administered directly as a fluid into the airway or by nebulizer. Thus, the treatment may result in *in vivo* concentrations comparable to those used in our *in vitro* experiments.

In a preliminary communication (32) we reported a moderate increase in the hyaluronan concentration after *in vitro* stimulation of fibroblasts with Exosurf or Curosurf . The present results, based on several additional experiments, show a more varied picture, with no stimulatory effect of surfactant preparations in most of the experiments. The results of addition of Curosurf in concentrations above 20 mg/mL have now been omitted, as the volume of the drug did not allow for sufficient culture medium. Our preliminary finding of increased hyaluronan concentrations after exposure to N-acetylcysteine has been confirmed in the present study.

We conclude that surfactants used for treatment of IRDS have only minor effects on the hyaluronan production by fetal pulmonary epithelial cells and fibroblasts *in vitro*, and that exposure to N-acetylcysteine results in increased synthesis of hyaluronan by fetal pulmonary fibroblasts, but only in a moderate increase in fetal epithelial cells.

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## REFERENCES


1. Allen SJ, Sedin G, Jonzon A, Wells AF, Laurent TC. Lung hyaluronan during development: A quantitative and morphological study. Am J Physiol 260 (Heart Circ Physiol 29): H 1449-54, 1991.
2. Juul SE, Kinsella MG, Jackson JG, Troug WE, Standaert TA, Hodson WA. Changes in hyaluronan deposition during early respiratory distress syndrome in premature monkeys. Pediatr Res 35: 238-43, 1994.
3. Nettelbladt O, Tengblad A, Hällgren R. Lung accumulation of hyaluronan parallels pulmonary edema in experimental alveolitis. Am J Physiol 257(Lung Cell Mol Physiol 1): L379-84, 1989.
4. Nettelbladt O, Bergh J, Schenholm M, Tengblad A, Hällgren R. Accumulation of hyaluronic acid in the alveolar interstitial tissue in Bleomycin-induced alveolitis. Am Rev Respir Dis 139: 759-62, 1989.
5. Johnsson H, Sedin G, Jonzon A, Laurent TC. Hyaluronan and water content in lungs from preterm rabbit pups kept in air or oxygen (abstract). Biol Neon 62: 180, 1992.
6. Johnsson H, Sedin G, Jonzon A, Laurent TC. Hyaluronan and water content in the lungs of term rabbit pups kept in air or oxygen (abstract). Ped Res 35: 280, 1994.
7. Elias JA, Krol RC, Freundlich B, Sampson PM. Regulation of human lung fibroblast glycosaminoglycan production by recombinant interferons, tumor necrosis factor, and lymphotoxin. J Clin Invest 81: 325-33, 1988.
8. Sahu SC. Hyaluronic acid. An indicator of pathological conditions of human lungs? Inflammation 4: 107-12, 1980.
9. Robertson B. New targets for surfactant replacement therapy: experimental and clinical aspects. Arch Dis Child 75: F1-F3, 1996.
10. Modig J, Hällgren R. Increased hyaluronan production in lung - a possible important factor in interstitial and alveolar edema during general anesthesia and in adult respiratory distress syndrome. Resuscitation 17: 223-31, 1989.
11. Hällgren R, Samuelsson T, Laurent TC, Modig J. Accumulation of hyaluronan (hyaluronic acid) in the lung in adult respiratory distress syndrome. Am Rev Respir Dis 139: 682-87, 1989.
12. Gregory TJ, Longmore WJ, Moxley MA, Whitsett JA, Reed CR, Fowler AA, Hudson LD, Maunder RJ, Crim C, Hyers TM. Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. J Clin Invest 88: 1976-81, 1991.
13. Jackson JG, Mackenzie AP, Chi EY, Standaert TA, Troug WE, Hodson WA. Mechanisms for reduced total lung capacity at birth and during hyaline membrane disease in premature monkeys. Am Rev Respir Dis 142: 413-19, 1990.
14. Ghio AJ, Fracia PJ, Young SL, Piantadosi CA. Synthetic surfactant scavenges oxidants and protects against hyperoxic lung injury. J Appl Physiol 77: 1217-23, 1994.
15. Arnon S, Griggs J, Silverman M. Pulmonary inflammatory cells in ventilated preterm infants: effect of surfactant treatment. Arch Dis Child 69: 44-48, 1993.
16. Gerdes J, Whisett J, Long W. Elastase activity and surfactant protein concentration in tracheal aspirates from neonates receiving synthetic surfactant. J Pediatr 120: S34-S39, 1992.
17. Geertsma MF, Teeuw WL, Nibbering PH, van Furth R. Pulmonary surfactant inhibits activation of human monocytes by recombinant interferon-$\gamma$. Immunology 82: 450-56, 1994.
18. Les Brown D, Pattishall EN. Other uses of surfactant. Clin Perinat 20: 761-89, 1993.

19. Herting E, Jarstrand C, Rasool O, Curstedt T, Håkansson S, Robertson B. Effect of surfactant on nitroblue tetrazolium reduction of polymorphonuclear leukocytes stimulated with type Ia group B streptococci. Acta Paediatr 84: 922-6, 1995.
20. Speer CP, Götze B, Curstedt T, Robertson B. Phagocytic functions and tumor necrosis factor secretion of human monocytes exposed to natural porcine surfactant (Curosurf). Ped Res 1991; 30: 69-74, 1991.
21. Benoit J, Cormier M, Wepierre J. Comparative effects of four surfactants on growth, contraction and adhesion of cultured human fibroblasts. Cell Biol Toxicol 4: 111-22, 1988.
22. Rice WR, Sarin VK, Fox JL, Baatz J, Wert S, Whitsett JA. Surfactant peptides stimulate uptake of phosphatidylcholine by isolated cells. Biochem Biophys Acta 1006: 237-45, 1989.
23. Thomassen MJ, Antal JM, Barna BP, Divis LT, Meeker DP, Wiedemann HP. Surfactant downregulates synthesis of DNA and inflammatory mediators in normal lung fibroblasts. Am J Physiol 270 (Lung Cell Mol Physiol 14): L159-L163, 1996.
24. Phibbs RH, Ballard RA, Clements JA et al. Initial clinical trial of Exosurf, a protein-free synthetic surfactant, for the prophylaxis and early treatment of hyaline membrane disease. Pediatrics 88: 1-9, 1991.
25. Moya FR, Hoffman DR, Zhao B, Johnston JM. Platelet-activating factor in surfactant preparations. Lancet 341: 858-60, 1993.
26. Ziment I. Acetylcysteine: A drug with an interesting past and a fascinating future. Respiration 50 (suppl 1): 26-30, 1986.
27. Bernard GR. N-acetylcysteine in experimental and clinical acute lung injury. Am J Med 91 (suppl 3C): 54S-59S, 1991.
28. Raes M, Renard P, Bosmans E, Delaive E, Remacle J. Effects of antioxidants on IL-6 secretion induced by IL-1 in human cultured lung fibroblasts. Involvement of NFκB. In: Oxidative stress, cell activation and viral infection (eds. C Pasquier et al.), pp. 77-90. Birkhäuser Verlag, Basel, Switzerland 1994.
29. Honda S, Matsuo M. Relationship between the cellular glutathione level and in vitro life span of human diploid fibroblasts. Exp Gerontol 23: 81-86, 1988.
30. Smith BT, Post M. Fibroblast-pneumonocyte factor. Am J Physiol 257 (Lung Cell Mol Physiol 1): L174-L178, 1989.
31. Chen YJ, Grant ME, Schor AM, Schor SL. Differeces between adult and foetal fibroblasts in the regulation of hyaluronan synthesis: correlation with migratory activity. J Cell Sci 94: 577-84, 1989.
32. Johnsson H, Heldin P, Sedin G, Laurent TC. Surfactant and N-acetylcysteine stimulate the hyaluronan synthesis by human fetal lung fibroblasts (Abstract). Ped Res 36: 19A, 1994.
33. Johnsson H, Heldin P, Sedin G, Laurent TC. Hyaluronan production in vitro by fetal lung fibroblasts and epithelial cells exposed to surfactants (Abstract). Biol Neonate 71 (suppl 1): 65, 1997.
34. Brandt R, Hedlöf E, Asman I, Bucht A, Tengblad A. A convenient radiometric assay for hyaluronan. Acta Otolaryngol Suppl 442; 31-35, 1987.
35. Bland M. An introduction to medical statistics, 2nd ed, p 267. Oxford University Press, Oxford, UK, 1995.

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