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Effects of Acute Hyperoxia on Hyaluronic Acid and Histopathology of the Lung in Neonatal Rats†

Beyong Il Kim[1], Jung-Hwan Choi, Chong Ku Yun and Je G. Chi[2]

Department of Pediatrics and Pathology[2], Seoul National University College of Medicine, Seoul 110-744, Korea

= Abstract = **We studied the effects of hyperoxia(>95% oxygen for 10 days) on changes in body weight, changes in wet to dry lung weight ratio, changes in hyaluronic acid of the lung, and morphologic changes in lung tissue compared with those of normoxia (room air for 10 days) in Sprague-Dawley neonatal rat pups. In this acute hyperoxic neonatal rat model, we tried to analyze the relationship of relative water content of the wet lung and the amount of hyaluronic acid and morphologic change in lung tissue. The results were as follows: 1) Increase of postnatal body weight among survivors was stunted significantly under hyperoxia compared with normoxia during 10 days-experiment. 2) No appreciable difference of wet to dry lung weight ratio was noted on the beginning and the tenth day of experiment in neonatal rat pups between normoxia and hyperoxia, but considerable increased wet to dry lung weight ratio was noted significantly at 5, 7 days of hyperoxia suggesting that the relative water content of the wet lung was increased on the fifth, seventh day of experiment in hyperoxia compared with normoxia. 3) The amount of hyaluronic acid per wet lung decreased sequentially according to increase of postnatal age in normoxia, but a considerably increased amount of hyaluronic acid per wet lung was noted significantly on the fifth, the seventh day of experiment in hyperoxia. The difference in amount of hyaluronic acid per wet lung was not significant on the tenth day of experiment between normoxia and hyperoxia. These results suggested that the changing pattern of amount of hyaluronic acid per wet lung coincided with that of the relative water content of the wet lung in hyperoxia. 4) Pulmonary edema, interstitial inflammatory cell hypercellularity, and localization of hyaluronic acid in interstitial lung lesion were observed by light microscope at 7 days of exposure in hyperoxia compared with normoxia. These results suggest the possible role of hyaluronic acid on increase of water content and interstitial inflammatory cells in acute lung injury due to hyperoxia in an experimental neonatal rat model.**

Key Words: *Hyperoxia, Hyaluronic acid, Neonatal rat*

INTRODUCTION

Prematurely born infants with severe respiratory distress syndrome require protracted periods of assisted ventilation with high concentration oxygen in spite of exogenous pulmonary surfactant replacement(Jobe 1993). Lung damage resulting from hyperoxia which is

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[1] Author for correspondence: Tel. 760-2810
서울대학교 의과대학 소아과학교실 : 김병일, 최중환, 윤종구
서울대학교 의과대학 병리학교실 : 지제근

toxic to lung cells and degrades components of the extracellular matrix of the lung is well recognized(Andley and Chakrabarti 1983). One of fascinating aspects of in vivo research on pulmonary O_2 toxicity is a striking difference between responses of the neonatal and adult animals to hyperoxia. Whereas adult animals of many tested species die within 3 to 5 days of hyperoxia more than 95% O_2 with severe lung lesions of pulmonary edema, hemorrhage, and inflammatory cell influx, newborn animals ,however, survive with much less evidence of acute lung injury in a similar hyperoxic condition for >7 to 10 days(Frank 1991).

We tried to develop a simple, inexpensive model of hyperoxic lung injury in neonatal rats which mimic the functional and structural changes found in the lungs of prematurely delivered human newborn infants. We observed the effects of hyperoxia(>95% oxygen for 10 days) on body weight, wet to dry lung weight ratio, the content of hyaluronic acid, and morphologic changes in the lung tissue compared with those of normoxia(room air for 10 days) in Sprague-Dawley neonatal rat pups. In this acute hyperoxic neonatal rat model, we analyzed the relationship of the relative water content of the wet lung and the amount of hyaluronic acid and morphologic changes in the lung tissue.

MATERIALS AND METHODS

Animals and treatment

Newborn pathogen-free Sprague-Dawley rats were delivered from timed pregnant mothers. The rat pups were mixed half and half using double giving technique, so the mothers nursed both litters without problems. Beginning on the third day of life, each of two litters of newborn rats was continuously exposed to either room air(normoxia) or hyperoxia(〉95% O_2) for the limited period of the experiment. But normoxia and hyperoxia-exposed mothers were only switched every 24 hours to prevent oxygen toxicity induced pulmonary edema and death. Food and water were supplied ad libitum. Litters of pups were weighed daily to rule out poor nursing by any of the dams, but no dam replacements were needed throughout the course of these studies. Exposure to hyperoxia was conducted in a 40 L plexiglass isolation chamber with continuous monitoring of O_2(〉95%) and CO_2 concentration(〈0.4%), temperature(23 to 25℃), and humidity(40-60%). Hyperoxia was continuous except for a daily 10-20 minutes interval to allow for animal maintenance, dam switching, and pup weighing. Oxygen concentrations were measured continuously with a portable oxygen analyzer(model SMP-LA and T Co Ltd, Japan) and CO_2 concentrations were measured with a gas analyzer(Radiometric ABL 3000 gas analyzer, Copenhagen).

Analytical procedure

Daily body weights of normoxia and hyperoxia group of rat pups which survived until the 10 days experiment(n=16, respectively) were recorded chronologically during 10 days experiment. After this experiment, another normoxia and hyperoxia group of rat pups were sacrificed by an overdose of pentobarbital on the beginning day of experiment(n=5, respectively), the fifth(n=5, respectively), the seventh (n=5, respectively), and the tenth day of experiment(n=5, respectively). These removed fresh rat lungs(n=20, respectively) were used for calculation of wet to dry lung weight ratio and biochemical assay of hyaluronic acid of lung tissue. Normoxia and hyperoxia group of rat pups were also sacrificed for the histopathology on the seventh(n=5, respectively), and the tenth day of experiment(n=5, respectively).

Fresh rat lungs were weighed immediately (wet lung weight), and wet lungs were dryed for 4 days under 80℃ temperature until the lung weight was constant(dry lung weight) and the wet to dry lung weight ratio was calculated for evaluation of the relative water content of the lung tissue.

A radiometric assay was used to quantitate HA per g wet lung. Approximately 50 mg of lung was homogenized in 500 ml of 0.1 M Tris buffer,

and subjected to overnight pronase(Sigma, St. Louis, MO) digestion(25 μg per 500 μl sample) at 55℃. Samples were boiled for 2 minutes to stop the reaction. Samples were then centrifuged for 2 minutes at 14,000 r. p. m. , and the resulting supernatant was removed, weighed, and diluted 1:50. Duplicate aliquots were assayed for HA using a technique(Pharmacia Diagnostics, Uppsala, Sweden) based on the use of the specific binding region(HABr) from chondroitin sulfate proteoglycan core protein of bovine nasal cartilage. Hyaluronan from the sample was allowed to bind ^{125}I-labeled HABr. The unbound ^{125}I-HABr was then quantitated after interaction with HA covalently bound to AH Sepharose beads.

For the morphologic study lungs were fixed via intratracheal instillation of 10% buffered formalin using a constant inflation pressure of 13 cm H_2O. From all lungs sections were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. All sections were examined at low(10 X) and high(40 X) magnification by light microscopy to determine if two lesion patterns were present; interstitial cellularity, thickening, and alveolar edema.

For localization of HA in tissue sections, Biotinylated HA binding region(HABr) of cartilage aggrecan was prepared for use as a histochemical probe for HA. Lung sections were deparaffinized using xylene, and alcohol washes. They were then hydrated in phosphate buffered saline(PBS), and endogenous peroxide activity quenched with 0.3% H_2O_2 in 100% methanol. Slides were rinsed and incubated overnight at room temperature with biotinylated probe(4mg/ml) contained in 10% FBS. The probe was localized after exposure to streptavidine-conjugated peroxidase using DAB(Sigma, St. Louis, MO) as substrate. Sections were counterstained with methyl green for 2 minutes.

Statistical analysis

Statistical analysis was done by PC-SAS software program(SAS Institute, 1988). Analysis of variance testing followed by Duncan's multiple comparison test, and Repeated measures ANOVA were used for comparison of body weight, wet to dry lung weight ratio, and quantitation of HA per wet lung, using $p < 0.05$ as a level of significant difference between normoxia and hyperoxia group.

RESULTS

Changes in body weight

Increase of postnatal body weight among survivors was stunted significantly in the hyperoxia group compared with the normoxia group during the 10 days-experiment($p < 0.01$, Fig. 1).

Changes in wet to dry lung weight ratio

No appreciable difference in the wet to dry lung weight ratio was noted on the beginning day of experiment in newborn rat pups between the normoxia group and the hyperoxia group, but a considerably increased wet to dry lung weight ratio was noted significantly on the fifth, seventh day of experiment in the hyperoxia group compared with the normoxia group. The difference in the wet to dry lung weight ratio was not significant on the tenth day of experiment between the normoxia group and the

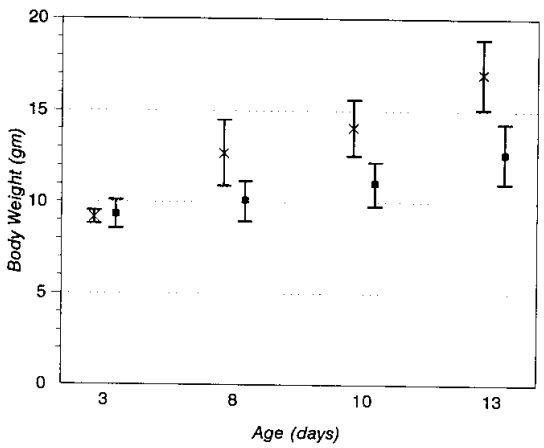

Fig. 1. Changes in body weight among survivors between normoxia(x ; n=16) and hyperoxia (■ ; n=16). Values are expressed as mean± SEM ; $p < 0.01$ by repeated measures ANOVA.

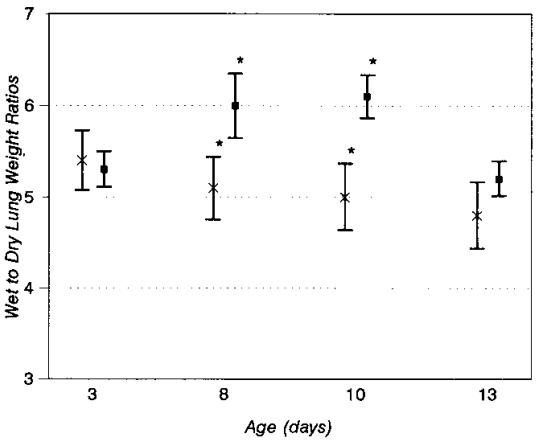

Fig. 2. Changes in wet to dry lung weight ratios between normoxia(x ; n=20) and hyperoxia(■; n=20). *denotes $p<0.05$. Values are expressed as mean±SEM.

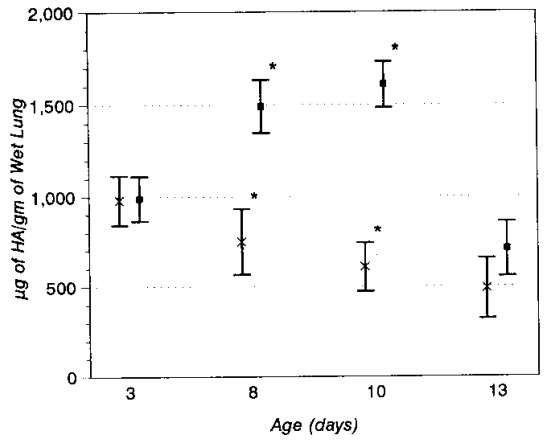

Fig. 3. Changes in amounts of hyaluronic acid per wet lung between normoxia(x ; n=20) and hyperoxia(■; n=20). *denotes $p<0.05$. Values are expressed as mean±SEM.

hyperoxia group. The relative water content of wet lung was at a peak on the seventh day of experiment in the hyperoxia group(Fig. 2).

Changes in amount of HA per wet lung

No appreciable difference in the amount of HA per wet lung was noted on the beginning day of experiment in rat pups between the normoxia group and the hyperoxia group, but a considerably increased amount of HA per wet lung was noted significantly on the fifth, seventh day of experiment in the hyperoxia group compared with the normoxia group. The amount of HA per wet lung was at a peak on the seventh day of experiment in the hyperoxia group. The difference in amount of HA per wet lung was not significant on the tenth day of experiment between the normoxia group and the hyperoxia group. The amount of HA per wet lung decreased sequentially according to increase of the postnatal age in the normoxia group(Fig. 3). The pattern of increase of amount of HA per wet lung coincided with that of increase of relative water content of wet lung in the hyperoxia group.

Morphologic changes in the lungs

Most lungs from control pups under room air for 7 days had many small alveoli and numerous septal buds(Fig. 4). After oxygen exposure()95% O_2) for 7 days, presence of pink staining material within the lumen of the air spaces(proteinaceous edema fluid) was observed(Fig. 5). This finding suggested acute exudative lung injury. In lung lesions with interstitial hypercellularity, there were multiple foci of thickening and hypercellularity of alveolar septa mainly due to infiltration by macrophages and neutrophils. Localization of HA in the interstitial lung region was not observed in 7 days of exposure to normoxia(Fig. 6), but localization of HA in the interstitial lung region was observed in 7 days of exposure to hyperoxia(Fig. 7).

Most lungs from control pups under room air for 10 days also had many small alveoli and numerous septal buds. But after oxygen exposure()95% O_2) for 10 days, absence of pink staining material within the lumen of the air spaces(proteinaceous edema fluid) was observed. This finding suggested resolved pulmonary edema. Localization of HA in the interstitial lung region was not observed in 10 days

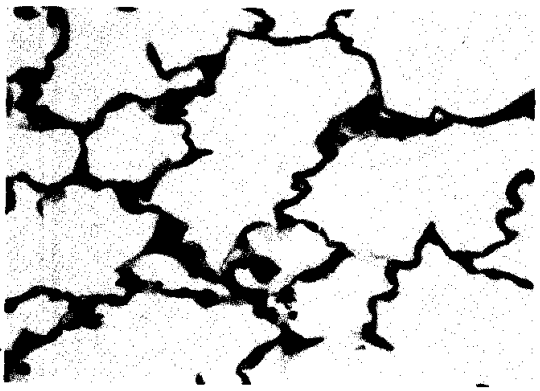

Fig. 4. Light micrograph of lung tissue from a developing rat pup exposed to room air for 7 days showing many alveoli and numerous septal buds(H & E X400).

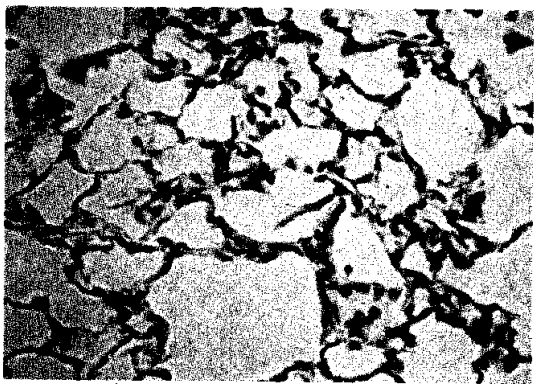

Fig. 6. Light micrograph of lung tissue from a developing rat pup exposed to room air for 7 days showing non localization of hyaluronic acid in the interstitial region(Immunohistochemical stain for HA X400).

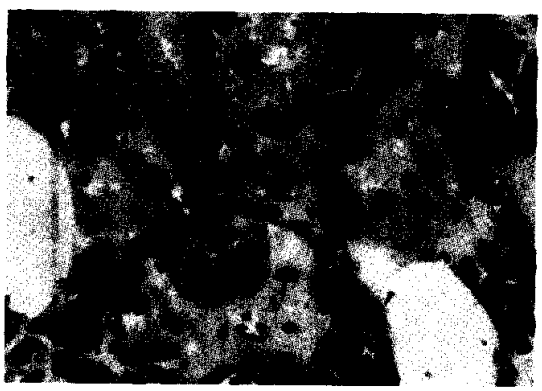

Fig. 5. Light micrograph of lung tissue from a developing rat pup exposed to hyperoxia for 7 days showing pulmonary edema and interstitial hypercellularity suggesting acute exudative lung injury (H & E X400).

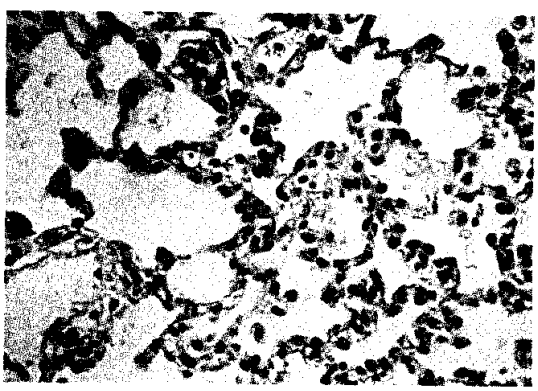

Fig. 7. Light micrograph of lung tissue from a developing rat pup exposed to hyperoxia for 7 days showing localization of hyaluronic acid in the interstitial region(Immunohistochemical stain for HA X 400).

of exposure to hyperoxia and normoxia. The pattern of pulmonary edema coincided with that of increase in amount of HA per wet lung and relative water content of wet lung in the hyperoxia group.

DISCUSSION

Since oxygen treatment of newborn babies was introduced, it has been known that oxygen exerts acute and chronic toxic effects on a number of organs particularly on the lung. Despite increased understanding on the pathology and mechanism of disease in the hyperoxic lung lesion, there are still no effective measures available for clinicians to help circumvent O_2 toxicity (Frank 1991). Extensive functional and morphologic changes in the lung and death

are also well-known in experimental animals under hyperoxia. The toxic effects varies according to the age of animals(Yam *et al.* 1978), concentration of oxygen, and duration of oxygen exposure(Rosenbaum *et al.* 1969; Crapo *et al.* 1980). In our neonatal rat model, we could find that many neonatal rat pups survive in spite of hyperoxia(>95% O_2) for 10 days, but most lung tissues of many survived neonatal rat pups had both initial exudative phase that lasted up to 7 days, which was characterized by pulmonary edema and inflammatory cell influx and inhibition of lung development. The increased wet to dry lung weight ratio also suggested that histopathologic findings of acute exudative lung injury such as pulmonary edema and interstitial hypercellularity of inflammatory cells in the hyperoxia group. The biochemical basis for the comparatively marked O_2 tolerance of neonatal animals is due to the ability of the newborn lung to respond to hyperoxic challenge with significantly increased pulmonary antioxidant enzyme levels. Adult animals, however, seem to be unable to manifest an adaptive biochemical response during hyperoxia(Bucher and Roberts 1981; Heffner and Roberts 1989; Frank 1991). Yet, despite this endogenous biochemical response, the newborn apparently seems to be unable to protect its growing lung from hyperoxia-associated inhibition of normal lung development and lung injury. Protection against hyperoxic lung injury with the antioxidant such as 21-aminosteroid drug was also suggested recently(Frank and Mclaughlin 1993). Decreased cell proliferation is a well-known concomitant of hyperoxic exposure in the newborn (Frank *et al* 1978). We could find that increase of postnatal body weight among survivors is stunted significantly under hyperoxia compared with normoxia during 10 days-experiment in this model.

Among many experimental animal models, the Sprague-Dawley rats have been frequently used for the study of effects of hyperoxia in newborn period because newborn is at a comparable level of structural immaturity of lung as prematurely delivered human infants and they have low cost and rapid development of alveolarization during postnatal 3-4 weeks(Burri 1974; Noguchi and Samaha 1991; Blanco and Frank 1993).

The extracellular matrix of the lung plays a major role in maintaining lung integrity. Collagen and elastic fibers contribute tensile strength and elastic recoil properties to the lung. Proteoglycans and hyaluronic acid fill the space between these fibrous proteins(Juul *et al.* 1991).

Hyaluronic acid is a component of extracellular matrix in lung, and it is a linear polysaccharide made up of repeating disaccharide units consisting of D-glucuronic acid and N-acetyl-D-glucosamine. It affects lung compliance and water balance in addition to contributing to the structural integrity of the lung by their ability to interact with and modulate other components of extracellular matrix. In addition, it influences a number of cellular events involved in lung development and cellular events including cellular adhesion, proliferation, and migration(Wiederhielm *et al.* 1976; Takahashi *et al.* 1983; Juul *et al.* 1991). It has been shown that hyaluronic acid and a receptor for hyaluronic acid are present in the lung and confined principally to the basolateral surfaces of bronchiolar epithelium, adventitia of blood vessels, and the space between large blood vessels and the respiratory pathways. The alveolar interstitium does not normally contain hyaluronic acid, however this may change under conditions of acute inflammation. hyaluronic acid and its receptor also appear on the surface of lung macrophages and may be important for macrophage attachment to specific regions within the lung(Green *et al.* 1988). Hyaluronic acid binding to its cell receptor has been shown to result in changes in protein phosphorylation, and to be associated with inflammatory cell migration(Turley 1989; Turley *et al.* 1989). Increased hyaluronic acid deposition has been associated with early injury in bleomycin treated animal lungs(Nettelbladt *et al.* 1989; Bray *et al.* 1991), adult respiratory distress syndrome(Hallgren *et al.* 1989), and hyperoxic lung injury in animal model(Domanico

et al. 1993). But it is not known which cells in the lung are responsible for the increase in hyaluronic acid accumulation in the lung injury. As suggested by Domanico *et al.* , we could also find that increased amount of hyaluronic acid of lung tissue was at a peak biochemically and morphologically at 7 days of acute hyperoxia in the neonatal rat model. Our study suggested that it was possible that the increased amounts of hyaluronic acid secreted by injured lung cells might participated in the chemotaxis and aggregation of inflammatory cells and increase of pulmonary fluid in the hyperoxic neonatal lung injury. As suggested by our study, hyaluronic acid is thought to have an important role in acute exudative lung injury in the hyperoxic neonatal rat model, because: 1) morphologic findings of pulmonary edema and interstitial inflammatory cell influx coincide with increased amounts of hyaluronic acid in lung tissue, 2) increased relative water content of lung tissue coincides with increased amounts of hyaluronic acid in lung tissue, 3) the increased amount of hyaluronic acid creates a favorable environment for inflammatory cells migration and increased water balance in lung tissue.

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REFERENCES

Andley UP, Chakrabarti. Role of singlet oxygen in the degradation of hyaluronic acid. Biochem Biophys Res Commun 1983; 115:894-901

Blanco LN, Frank L. The formation of alveoli in rat lung during the third and fourth postnatal weeks: Effect of hyperoxia, dexamethasone, and deferoxamine. Pediatr Res 1993; 34:334-40

Bray BA, Sampson PM, Osman M, Giandomenico A, Turino GM. Early changes in lung tissue hyaluronan (hyaluronic acid) and hyaluronidase in bleomycin induced alveolitis in hamsters. Am Rev Respir Dis 1991; 143:284-88

Bucher JR, Roberts RJ: The development of newborn rat lung in hyperoxia: Dose response study of lung growth, maturation, and changes in antioxidant enzyme activities. Pediatr Res 1981; 15:999-1008

Burri PH. The postnatal growth of the rat lung Ⅲ. Morphology. Anat Rec 1974; 180:77-98

Crapo JD, Barry BE, Foscue HA, Shelburne J: Structural and biochemical changes in rat lung occurring during exposures to lethal and adaptive doses of oxygen. Am Rev Respir Dis 1980; 122:123-43

Domanico RS, Juul SE, Hershenson MB. Exposure of neonatal rat pups to hyperoxia results in increased lung hyaluronan. Pediatr Res 1993; 33:323A (abst)

Frank L. Developmental aspects of experimental pulmonary oxygen toxicity. Free Radic Biol Med 1991; 11:463-94

Frank L, Bucher JR, Roberts RJ. Oxygen toxicity in neonatal and adult animals of various species. J Appl Physiol 1978; 45:463-94

Frank L, Mclaughlin GE; Protection against acute and chronic hyperoxic inhibition of neonatal rat lung development with 21-aminosteroid drug U74389F. Pediatr Res 1993; 33:632-38

Green SJ, Tarone G, Underhill CB; Aggregation of macrophages and fibroblasts is inhibited by a monoclonal antibody to the hyaluronate receptor. Exp Cell Res 1988;178:224-32

Hallgren R, Samuelsson T, Laurent TC, Modig J. Accumulation of hyaluronan (hyaluronic acid) in the lung in adult respiratory distress syndrome. Am Rev Respir Dis 1989; 139:682-87

Heffner JE, Repine JE. Pulmonary strategies of antioxidant defense. Am Rev Respir Dis 1989; 140:531-54

Jobe AH. Pulmonary surfactant therapy. N Engl J Med 1993; 328:861-8

Juul SE, Wight TN, Hascall VC. Proteoglycans. In Crystal RG(Ed) The lung:scientific foundation, Vol. Ⅰ, chapter 3. 3. 5. Raven Press Ltd. , New York, 1991:pp. 413-20

Nettelbladt O, Shenholm M, Tengbladt A, Hallgren R. Accumulation of hyaluronic acid in alveolar interstitial tissue in blemycin induced alveol-

itis. Am Rev Respir Dis 1989; 139:759-62

Noguchi A, Samaha H. Developmental changes in tropoelastin gene expression in the rat lung studied by in situ hybridization. Am J Respir Cell Mol Biol 1991; 5:571-8

Rosenbaum RM, Wittner M, Lenger M. Mitochondrial and other ultrastructural changes in great alveolar cells of oxygen-adapted and poisoned rats. Lab Invest 1969; 20:516-28

SAS Institute Inc. SAS/STAT user's guide, release 6. 03 edition. Cary, NC: SAS Institute Inc. , 1988

Takahashi K, Martin CJ, Young C. Saline immersion and the length-tension properties of alveolar wall. J Appl Physiol 1983; 54:1532-7

Turley EA. Hyaluronic acid stimulates protein kinase activity in intact cells and in an isolated protein complex. J Biol Chem 1989; 264:8951-55

Turley EA, Auersperg N. A hyaluronate binding protein transiently codistributes with p21k-ras in cultured cell lines. Exp Cell Res 1989;182: 340-8

Weiderhielm CA, Fox JR, Lee DR. Ground substance mucopolysaccharides and plasma proteins: Their role in capillary water balance. Am J Physiol 1976; 230:1121-5

Yam J, Frank L, Roberts RJ. Oxygen toxicity: Comparison of lung biochemical response in neonatal and adult rats. Pediatr Res 1978; 12:115-19