Aerosolized Hyaluronic Acid Decreases Alveolar Injury Induced by Human Neutrophil Elastase (44260)

JEROME O. CANTOR,[1] JOSEPH M. CERRETA, GERARD ARMAND, AND GERARD M. TURINO

St. Luke's-Roosevelt Institute for Health Sciences, New York, New York 10019, The Brooklyn Hospital Center, Brooklyn, New York 11201, and St. John's University School of Pharmacy, Jamaica, New York 11439

Abstract. **This laboratory has previously shown that an intratracheally instilled solution of hyaluronic acid (HA) protects the lung from elastase-induced airspace enlargement. In those studies, fluorescein-labeled HA was found to bind preferentially to lung elastic fibers, suggesting a mechanism for the protective effect. The current investigation extends these findings by examining the capacity of an aerosol preparation of HA to similarly inhibit elastase-induced lung injury. Syrian hamsters were exposed to aerosolized bovine tracheal HA (0.1% solution in water) for either 25 or 50 min, then immediately instilled intratracheally with 80 units of human neutrophil elastase. One week later the lungs were examined for airspace enlargement, using the mean linear intercept method. Animals exposed to HA for 50 min showed a significant decrease in airspace enlargement compared to controls exposed to aerosolized water alone (68.2 μm vs 85.9 μm; $P < 0.05$). The 25-min exposure to the HA aerosol also reduced the mean linear intercept compared to controls (73.7 μm vs 85.9 μm), but this decrease was not statistically significant. With regard to possible inflammatory effects of HA, there was no difference in the percentage of lavaged neutrophils between HA-treated and control lungs at 24 hr (1.4% vs 1.8%, respectively). As with earlier experiments using intratracheally instilled HA, aerosolized fluorescein-labeled HA was found to bind to lung elastic fibers. These results suggest that aerosolized HA may prevent elastase-mediated injury in pulmonary emphysema.** [P.S.E.B.M. 1998, Vol 217]

An imbalance between elastases and their inhibitors is believed to be responsible for the airspace enlargement that characterizes pulmonary emphysema (1–4). This protease-antiprotease concept has resulted in the design of therapeutic agents for pulmonary emphysema that focus on inhibiting the activity of elastases (5). However, such a treatment strategy assumes that the disease is caused by a single abnormality; namely, excess elastase activity. If emphysema represents a more general response of the lung to a variety of insults (with elastases playing a variable role), then enzyme inhibition may have only limited efficacy, and other potential forms of treatment may be required.

To determine if mechanisms other than elastase injury contribute to pulmonary emphysema, studies were performed by this laboratory using a variety of agents to induce lung elastic fiber breakdown and air space enlargement experimentally. In experiments using hyaluronidase and 60% oxygen, results showed that significant damage to elastic fibers occurs only when both agents are given concomitantly, suggesting the possibility that hyaluronidase may facilitate the breakdown of elastic fibers by making them more accessible to injury (6). This hypothesis was further tested by giving hamsters intratracheal instillments of hyaluronidase, followed by elastase, and then examining the lungs for airspace enlargement (7). The findings showed that pretreatment with hyaluronidase enhances elastase-induced emphysema. Furthermore, intratracheally instilled hyaluronic acid (HA) was found to have the opposite effect, significantly reducing elastase-induced airspace enlargement. Studies using fluorescein-labeled HA, indicated that

This work was supported by the Ned Doyle Foundation, the Charles A. Mastronardi Fund, and the Franklyn Bracken Fund for Pulmonary Research at St. Luke's-Roosevelt Hospital.

[1] To whom requests for reprints should be addressed at Antenucci Building (Room 101), St. Luke's-Roosevelt Hospital Center, 1000 Tenth Avenue, New York, NY 10019.

Received July 11, 1997. [P.S.E.B.M. 1998, Vol 217]
Accepted November 3, 1997.

0037-9727/98/2174-0471$10.50/0

this polysaccharide preferentially binds to and coats elastic fibers, possibly protecting them from injury (8).

In the current investigation, an aerosol solution of bovine tracheal hyaluronic acid was used to counteract the effects of intratracheally administered human neutrophil elastase. The results indicate that the inhaled HA rapidly coats pulmonary elastic fibers and significantly reduces the amount of airspace enlargement induced by elastase. These findings provide additional evidence that HA may be a useful alternative to elastase inhibitors in protecting the lung from injury.

Methods

Preparation of Fluorescein-Labeled HA. Fluorescein amine was coupled to bovine tracheal hyaluronic acid (Sigma Chemical Co., St. Louis, MO) according to previously published techniques (9). A solution of 100 mg of HA in 80 ml water was diluted with 40 ml dimethyl sulfoxide and combined with acetaldehyde (50 μl), cyclohexyl isocyanide (50 μl), and fluorescein amine (50 mg). The mixture was incubated at 22°C for 5 hr, and the resultant fluorescein-labeled HA was purified by alcohol precipitation and gel filtration on Sephacryl S-500, using a 1 × 135-cm column equilibrated with 0.2 *M* pyridine-acetate buffer at pH 6.2. The HA was eluted at room temperature at a rate of 8.0 ml/hr. Collected fractions were measured for HA with the carbazole reaction method (10). An unlabeled preparation of HA was subjected to the same purification procedure for comparison with the labeled material.

The intrinsic viscosity of both the labeled and unlabeled HA was then measured to determine average molecular weight. Viscosity measurements were made at 25°C in a Cannon semi-micro dilution viscometer (Cannon Instrument Company, State College, PA). Prior to measurement, the hyaluronic sample was dialyzed against 0.15 *M* NaCl. Intrinsic viscosity (η) was determined by extrapolating viscosity measurements to zero concentration (11). Average molecular weight was calculated by using intrinsic viscosity data in the Mark-Houwink equation, (i.e., $\eta = K(M)^a$ where a and K are constants for HA in saline solution) (11).

Aerosol Exposure to Fluorescein-Labeled HA. Syrian hamsters, weighing approximately 100 g, were placed inside a dual-port plexiglass chamber and exposed to aerosolized fluorescein-HA *via* a Whisper-Jet nebulizer (Marquest Medical Products, Englewood, CO) attached to a compressed air source. The animals were exposed to either 10 mg of HA in 10 ml water for 25 min or 20 mg HA in 20 ml water for 50 min.

Immediately following exposure to the aerosol, the animals were sacrificed, and their lungs were fixed *in situ* by inserting a catheter into the trachea and instilling 10% neutral-buffered formalin at a pressure of 20 cm H_2O. After 2 hr, both the lungs and the heart were removed from the chest as a single block and additionally fixed in 10% formalin for several days. The lungs were then dissected free of extraparenchymal structures, sectioned randomly, and histologically processed. Unstained slide sections were examined with a fluorescence microscope.

Exposure of Elastase-Treated Animals to Aerosolized HA. Hamsters were exposed to an aerosol solution of either 10 mg HA in 10 ml water for 25 min or 20 mg HA in 20 ml water for 50 min, as described above. Control animals were exposed to 20 ml water alone for 50 min. Approximately 30 min following aerosol exposure, the animals were anesthetized with ketamine and instilled intratracheally with 80 units of human neutrophil elastase (Elastin Products Company, Owensville, MO) dissolved in 0.2 ml normal saline solution. The elastase was delivered into the trachea *via* a 26-gauge needle mounted on a 1-ml syringe.

Determination of Mean Linear Intercept. One week following intratracheal instillment of HA and elastase, the animals were sacrificed by intraperitoneal injection of sodium pentobarbital. Their lungs were then fixed and histologically processed as described above. Slide sections stained with hematoxylin and eosin were coded, and mean linear intercept measurements were made by an experienced morphologist (JMC), according to published procedures (12).

Lung Lavage. Hamsters were exposed to either an aerosol preparation of 20 mg HA in 20 ml water for 50 min or, in the case of controls, 20 ml water alone for the same period. Twenty-four hr later, the animals were sacrificed, and their lungs were lavaged with a total of 12 ml of normal saline solution, administered in 3-ml aliquots. The recovered fluid was centrifuged, and the cell pellet was resuspended in Dulbecco's phosphate-buffered saline solution. Wright-stained smears were prepared, and the percentage of neutrophils was determined by counting 500 cells/specimen.

Data Analysis. Differences among treatment groups were analyzed for statistical significance ($P < 0.05$) with Fisher's LSD multiple comparison test.

Results

Characterization of Fluorescein-Labeled HA. As shown in Table I, the fluorescein-labeled HA had an average molecular weight of 87,214 compared to 86,137 for the unlabeled material. These values are relatively low compared to other preparations of HA, some of which may have molecular weights in excess of 3×10^6. The similarity in molecular weight between the two samples indicates that breakdown of HA did not occur during the labeling process. Furthermore, chromatography of both the labeled and unlabeled HA on Sephacryl S-500 produced very similar elution profiles (Fig. 1).

Table I. Physical Properties of HA Preparations

Sample	Intrinsic viscosity (cc/g)	MW
Fluorescein-HA	243	87,214
Control	240	86,137

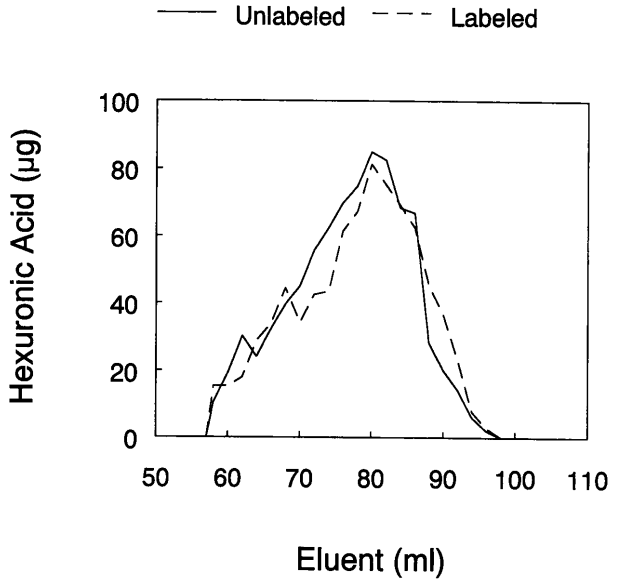

Figure 1. Chromatography of fluorescein-HA and unlabeled HA on Sephacryl S-500 resulted in similar elution profiles, indicating that no breakdown of HA occurs during the labeling process.

Aerosolization of Fluorescein-Labeled HA. The lungs were rapidly labeled by the aerosolized fluorescein-HA. Prominent fluorescence was observed after only a single 25-min exposure to a 0.1% solution of the labeled HA. Differences in the overall amount of lung fluorescence between 25- and 50-min exposures to the aerosol were not readily quantifiable. At both time points, there was preferential labeling of interstitial, vascular, and pleural elastic fibers (Fig. 2). The identity of these fibers was confirmed with the Verhoeff-Van Gieson elastic tissue stain. The pattern of fluorescence was similar to that observed in a previous study involving intratracheal instillation of fluorescein-HA (8). However, in the earlier study, there was additional labeling of alveolar macrophages. These cells presumably sequestered the relatively large amount of fluorescein-HA (2 mg) administered in that study. The aerosol preparation, in contrast, delivers a much smaller dose of the material to the lung and does not elicit a similar reaction.

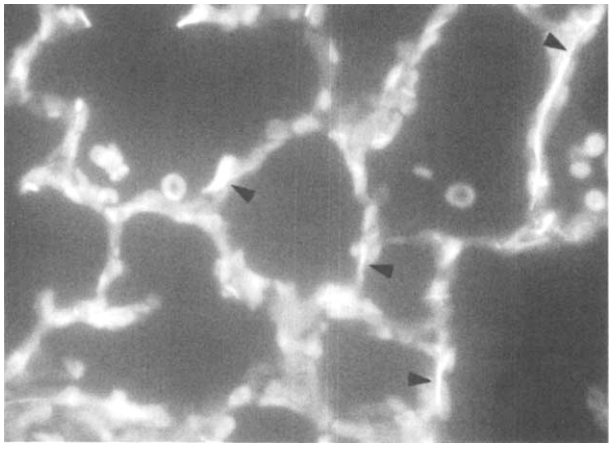

Figure 2. Fluorescence of elastic fibers in the lung interstitium (arrowheads) was seen immediately following a 50-min exposure to aerosolized fluorescein-HA. The visibility of other structures (e.g., alveolar septa, red cells) is most likely due to autofluorescence. Original magnification: 990×.

Effect of Aerosolized HA on Elastase-Induced Emphysema. Animals exposed to aerosolized HA (0.1% solution) for 50 min prior to intratracheal instillation of human neutrophil elastase had a significantly lower mean linear intercept at Week 1 compared to elastase-treated animals exposed to aerosolized water alone (68.2 µm vs 85.9 µm; $P < 0.05$; Fig. 3). Similarly, a 25-min exposure to the HA aerosol reduced the mean linear intercept (73.7 µm) compared to controls, but the difference was not statistically significant. Morphologically, both the HA-treated and control lungs showed minimal residual inflammation at Week 1 (Fig. 4).

The actual amount of HA delivered to the lungs is very much smaller than the 20 mg aerosolized over a 50-min period. The volume of air inspired per minute by an adult hamster is only approximately 30 ml whereas the airflow through the exposure chamber is approximately 8 l/min. Thus, as little as several milligrams of HA are available to protect the lung from elastase injury. This estimate does not include additional removal of HA by the upper respiratory system.

Effect of Aerosolized HA on Lavaged Neutrophils. Compared to animals receiving aerosolized water alone, those exposed to aerosolized HA for 50 min did not show an increase in the percentage of lavaged neutrophils at 24 hr (Fig. 5). This finding suggests that an aerosolized solution of 0.1% HA is not toxic to the lungs. In contrast, previous experiments involving intratracheal instillation of 2 mg of HA produced a significant increase in the percentage of lavaged neutrophils during the first 96 hr following treatment. The absence of inflammation associated with

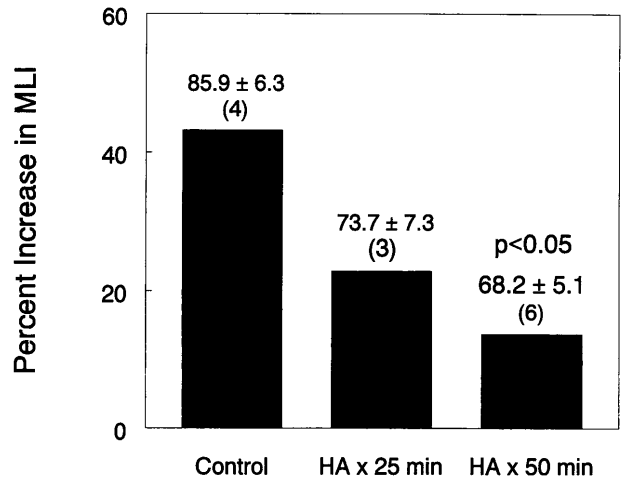

Figure 3. Animals exposed to aerosolized HA for 50 min showed a significant decrease in elastase-induced airspace enlargement compared to controls exposed to aerosolized water alone prior to elastase. Mean linear intercept (MLI) measurements were made 1 week following treatment. The percentage increase in MLI is based on a normal value of 60 µm (6). Values above bars represent mean linear intercept ± SEM and number of animals tested (figures in parentheses).

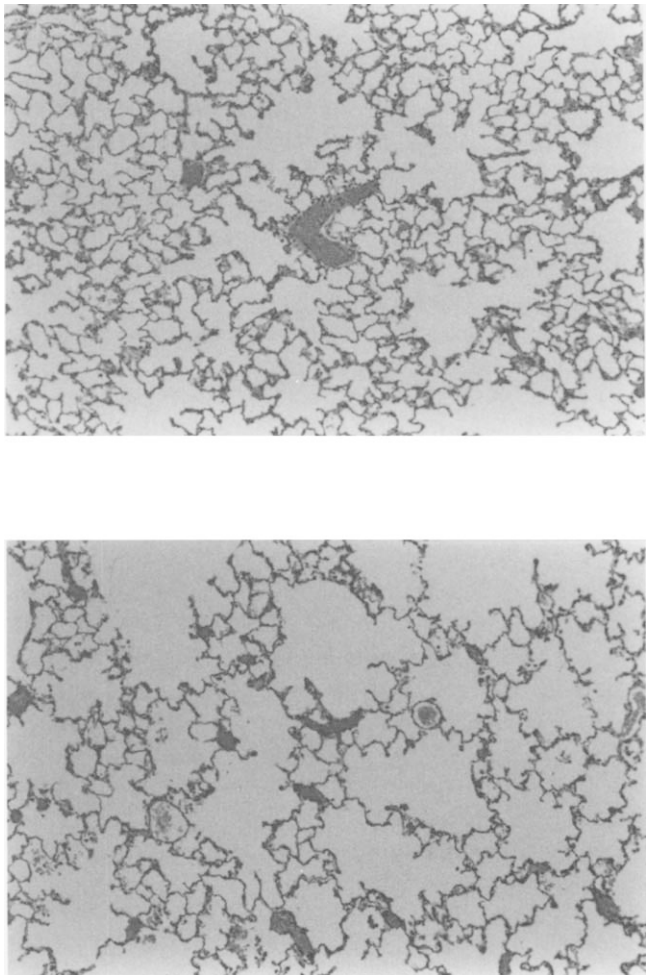

Figure 4. One week following elastase administration, there was no significant inflammation in either HA-treated (top) or control lungs (bottom). Original magnification of each photomicrograph: 200×.

aerosolized HA most likely reflects the much lower amount of material entering the lung. Nevertheless, long-term exposure studies are needed to confirm the lack of toxicity of the aerosol preparation.

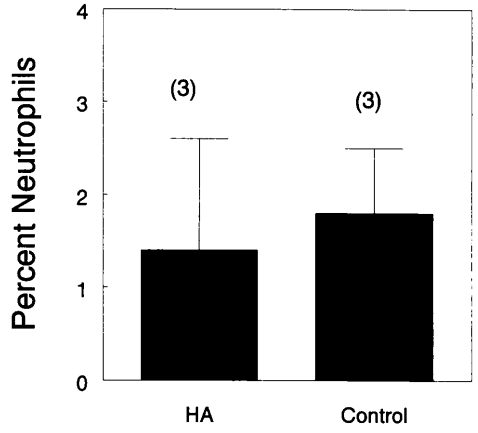

Figure 5. At 24 hrs, animals exposed to either HA or water alone (controls) had a similar percentage of lavaged neutrophils. T-bars indicate SEM; figures in parentheses represent number of animals tested.

Discussion

These results indicate that aerosol administration of HA can significantly decrease experimental pulmonary emphysema induced by neutrophil elastase. Direct inhibition of elastase does not appear to be responsible for this reduction in airspace enlargement since HA is unable to prevent the enzyme from degrading elastin *in vitro* (7). This finding distinguishes HA from other agents that ameliorate experimental emphysema by inactivating elastase (3–5) and suggests that intratracheally administered HA may provide an alternative approach to reducing lung parenchymal injury by elastases.

In a previous study from this laboratory, in which hyaluronidase was found to interact synergistically with 60% oxygen to produce airspace enlargement, it was hypothesized that HA and other glycosaminoglycans may protect elastic fibers (6). Several studies have supported this concept by providing evidence that HA is closely associated with elastic fibers (13, 14). Degradation of HA might be necessary for elastases and cells, such as monocytes or neutrophils, to gain access to these fibers (15). As shown by this laboratory and other investigators, pretreatment of the lung with hyaluronidase resulted in an additional significant increase in airspace enlargement over that induced by intratracheal instillment of elastase alone (7, 16).

The current studies provide additional evidence that HA forms a complex with elastic fibers. The strong association of the fluorescein-labeled HA with elastic fibers clearly indicates that the instilled HA coats these fibers, presumably protecting them from degradation by elastase. The mechanism responsible for this interaction may possibly involve formation of electrostatic or hydrogen bonds between carboxyl or hydroxyl groups of HA and elastic fibers.

HA may also influence airspace enlargement by virtue of its ability to retain water. It has been shown that a loss of HA can reduce extravascular water content in the lung interstitium (17). Negatively charged carboxyl groups attached to the saccharide moieties repel one another, enlarging the domain of HA and enhancing its ability to entrap water (18). The hydrated and expanded HA may protect alveolar elastic fibers from contact with elastase.

The fact that aerosolized HA is effective against neutrophil elastase increases the possibility that it may be useful in treating human emphysema. This enzyme has access to lung parenchyma through neutrophil migration and secretion, as well as by macrophage sequestration, and is thought to play an important role in the disease. The ubiquity of neutrophil elastase in various lung inflammatory reactions suggests the possibility that HA may be effective against other forms of pulmonary injury as well.

The current studies are difficult to compare to previous work using intratracheally instilled HA to protect the lung from neutrophil elastase (8). For one thing, HA was given 2 hr prior to intratracheal administration of neutrophil elastase

in the earlier studies, whereas there was no significant interval between the HA and elastase administration in the current work. Nevertheless, it is suspected that aerosolized HA would remain effective for several hours, since studies of the clearance of fluorescein-HA from the lung (8) show that it remains attached to elastic fibers for at least 4 hr.

As a possible treatment for protease-induced pulmonary injury, HA should be well-tolerated by the lung. It is a naturally occurring extracellular matrix constituent that has been administered to other tissues without adverse effects (19–23). In contrast to elastase inhibitors, which are now being considered as therapeutic agents for emphysema, HA might provide a more direct form of lung protection with fewer potential side effects.

The ability to slow the progression of emphysema, even a small amount, would be very beneficial to those who suffer from this disease. The disease generally results in a gradual loss of lung function and produces severe symptoms only late in its course. If a particular form of treatment altered development of the disease only modestly, then the devastating effects of emphysema might be postponed to extreme old age, effectively eliminating them from the life of most patients.

1. Gross P, Babyak MA, Tolker E, Kaschak M. Enzymatically produced pulmonary emphysema: A preliminary report. J Occup Med **6:**481, 1964.
2. Laurell C-B, Eriksson S. The electrophoretic alpha$_1$-globulin pattern of serum in alpha$_1$-antitrypsin deficiency. Scand J Clin Lab Invest **15:**132–140, 1963.
3. Janoff A. Elastases and emphysema: Current assessment of the protease-antiprotease hypothesis. Am Rev Respir Dis **132:**417–433, 1985.
4. Senior RM, Kuhn C III. The pathogenesis of emphysema. In: Fishman AP, Ed. Pulmonary Diseases and Disorders, 2d ed. New York: McGraw-Hill, pp 1209–1218, 1988.
5. Zimmerman M, Powers JC. Design and properties of synthetic elastase inhibitors. In: Robert L, Hornebeck W, Eds. Elastin and Elastases, Vol II. Boca Raton: CRC Press, pp 109–123, 1989.
6. Cantor JO, Cerreta JM, Armand G, Keller S, Turino GM. Pulmonary air-space enlargement induced by intratracheal instillment of hyaluronidase and concomitant exposure to 60% oxygen. Exp Lung Res **19:**177–192, 1993.
7. Cantor JO, Cerreta JM, Keller S, Turino GM. Modulation of air-space enlargement in elastase-induced emphysema by intratracheal instillment of hyaluronidase and hyaluronic acid. Exp Lung Res **21:**423–436, 1995.
8. Cantor JO, Cerreta JM, Armand G, Turino GM. Further investigation of the use of intratracheally administered hyaluronic acid to ameliorate elastase-induced emphysema. Exp Lung Res **23:**229–244, 1997.
9. Anthony N, Belder DE, Owe Wik K. Preparation and properties of fluorescein-labeled hyaluronate. Carbohyd Res **44:**251–257, 1975.
10. Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem **4:**330–334, 1962.
11. Cleland RL, Wang JL. Ionic polysaccharides. III. Dilute solution properties of hyaluronic acid fractions. Biopolymers **9:**799–810, 1970.
12. Dunnill MS. Quantitative methods in the study of pulmonary pathology. Thorax **17:**320–328, 1962.
13. Bray BA, Hsu W, Turino GM. Lung hyaluronan as assayed with a biotinylated hyaluronan-binding protein. Exp Lung Res **20:**317–330, 1994.
14. Baccarani-Contri M, Vincenzi D, Cicchetti F, Mori G, Pasquali-Ronchetti I. Immunocytochemical localization of proteoglycans within normal elastin fibers. Eur J Cell Biol **53:**305–312, 1990.
15. Campbell EJ, Senior RM, McDonald JA, Cox DL. Proteolysis by neutrophils: Relative importance of cell-substrate contact and oxidative inactivation of proteinase inhibitors *in vitro.* J Clin Invest **70:**845–852, 1982.
16. Ishibashi M, Nishida T, Murakami H, Shiraishi M, Aritomi T, Yoshida M. The role of interstitial hyaluronan in acute lung injury. Nippon Kyobu Shikkan Gakkai Zasshi **33**(Suppl):225–230, 1995.
17. Bhattacharya J, Cruz T, Bhattacharya S, Bray B. Hyaluronan affects extravascular water in lungs of unanesthetized rabbits. J Appl Physiol **66:**2595–2599, 1989.
18. Toole BP. Glycosaminoglycans and morphogenesis. In: Hay ED, Ed. Cell Biology of Extracellular Matrix. New York: Plenum, pp 259–294, 1981.
19. Leardini G, Mattara L, Franceschini M, Perbellini A. Intra-articular treatment of knee osteoarthritis: A comparative study between hyaluronic acid and 6-methyl prednisolone acetate. Clin Exp Rheumatol **9:**375–381, 1991.
20. Graf J, Neusel E, Schneider E, Niethard FU. Intra-articular treatment with HA in osteoarthritis of the knee joint: A controlled clinical trial versus mucopolysaccharide polysulfuric acid ester. Clin Exp Rheumatol **11:**367–372, 1993.
21. Aviad AD. The molecular weight of therapeutic hyaluronan (sodium hyaluronate): How significant is it? J Rheumatol **21:**297–301, 1994.
22. King SR, Hickerson WL, Proctor KG. Beneficial actions of exogeneous hyaluronic acid on wound healing. Surgery **109:**76–84, 1991.
23. Goldberg EP, Burns JW, Yaacobi Y. Prevention of postoperative adhesion by precoating tissues with dilute sodium hyaluronate solutions. Prog Clin Biol Res **381:**191–204, 1993.