

Aerosolised hyaluronic acid prevents exercise-induced bronchoconstriction, suggesting novel hypotheses on the correction of matrix defects in asthma

G. Petrigli, L. Allegra*

Institute of Respiratory Disease of the University, IRCCS Policlinico Hospital, via F. Sforza 35, 20122 Milano, Italy

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Abstract

Hyaluronic acid (HA), a biopolymer, member of the family of the glycosaminoglycans (GAGs) is one of the major natural components of the connective amorphous matrix. The lungs, together with skin and intestine, contain >50% of HA of the body: it provides to several biologic functions and presents the unique capacity to link and retain a particularly relevant number of water molecules. Since other GAGs have been proven to be provided with anti-asthmatic properties and HA has been employed with positive results by intra-tracheal instillation in experimental models of lung emphysema and COPD, we have explored the efficacy of the pre-administered aerosol of HA (compared to placebo) in preventing in asthmatic patients the bronchoconstriction induced by a challenge test such as that obtained with muscular exercise. In a randomised, cross-over, single-blind study design, saline as placebo (P) or HA have been administered by aerosol, in two non-consecutive days, 30 min prior to the beginning of the challenge (10 min free running), to 14 patients (13–36 years old; 7 teenagers, 7 young adults; 11 males, 3 females; 12 allergic, 2 non-allergic), all suffering from mild bronchial asthma. The bronchoconstrictive effect induced by the muscular exercise has been relevant and statistically significant. With the P pre-treatment, the average FEV1 measured 5 min after the end of exercise was reduced by 36.14% from the baseline FEV1. Pre-treatment with HA determined a partial but clear-cut protection of the FEV1 impairment due to the challenge: the average post-challenge FEV1 resulted to be 12.43% less than the pre-challenge baseline value. No significant difference was observed in the level of HA protection in the subgroup of teenagers when compared to that of young adults. The protection induced by HA, when compared with P, resulted particularly significant by the statistical point of view ($p < 0.0001$). We conclude that aerosol HA administration significantly reduces the bronchial hyper-reactivity to muscular exercise in asthmatics. Such effect could be attributed to the correction of the pathological remodelling, one of the main features of asthma: a correction which could be attributed to the unique physicochemical properties of this major component of the loose connective amorphous matrix of the airways, which is undoubtedly involved in the remodelling process.

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1. Introduction

Hyaluronic acid (HA) is a naturally occurring biopolymer which serves several important biological functions in bacteria and superior organisms, including human. In human tissues, naturally occurring HA may be found ubiquitously, in particular as intercellular space filler of the extra-cellular matrix, with higher concentration in

the vitreous humour and synovial liquid, but also in the loose connective tissue [1].

HA is a member of the family of the glycosaminoglycans (GAGs): the molecule is constituted by a linear chain without ramifications, containing a repeated series of disaccharide units of glucuronic acid and *N*-acetyl-glucosamine linked by glucoside chains (Fig. 1). One single molecule contains up to 10,000 disaccharide units and it may have a molecular weight of several million Daltons. Different from other GAGs, HA does not contain sulphur groups, is free from covalent links to proteins and presents the simplest chemical structure.

HA presents the unique capacity to link and retain a relevant number of water molecules in the inter-fibrillar

* Corresponding author. Tel.: +39 0255190306; fax: +39 0250320628.
E-mail address: luigi.allegra@unimi.it (L. Allegra).

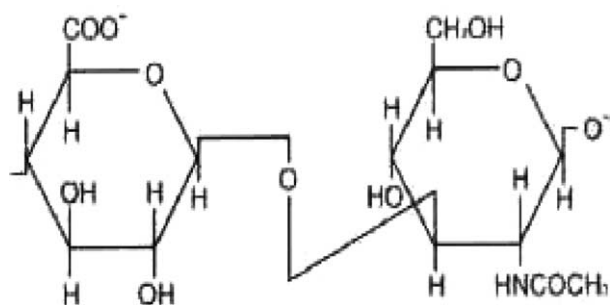


Fig. 1. Disaccharide repeating unit of HA comprising glucuronic acid and *N*-acetyl-glucosamine linked by glucoside chain.

spaces contributing to constitute the fundamental part of the amorphous colloidal matrix which glues cells and connective fibres, and to determine relevant effects on tissue morphogenesis [2], cells growth, differentiation and migration, hydration, lubrication, solute transport, as well as on microcirculatory exchanges, due to its influence on interstitial volume, hydraulic conductivity and macromolecule diffusibility [3].

In serum, HA levels are 10–100 µg/l. Skin, intestine and lung contain more than 50% of HA of the body [4]. In the lungs, HA content is 15–150 µg/g dry weight (different species), mainly localised in the peri-bronchial and inter-alveolar/peri-alveolar tissue. It is drained by the lymphatic vessels and catabolised in the local lymph nodes [5] and liver [6].

Up to some years ago, HA was extracted from natural sources such as cockscomb or bovine connective tissues and the procedure consisted in enzymatic digestion and specific separation from the proteins in order to purify the extract. This procedure presented several disadvantages: bad molecular weight standardisation, risk of viral infection, high costs.

More recently [7], such disadvantages have been largely avoided and good results have been obtained thanks to a biotechnological procedure consisting in the bio-fermentation of G+ bacteria such as *Streptococcus zooepidermicus* (Fig. 2), a micro-organism which is coated with a mucoid

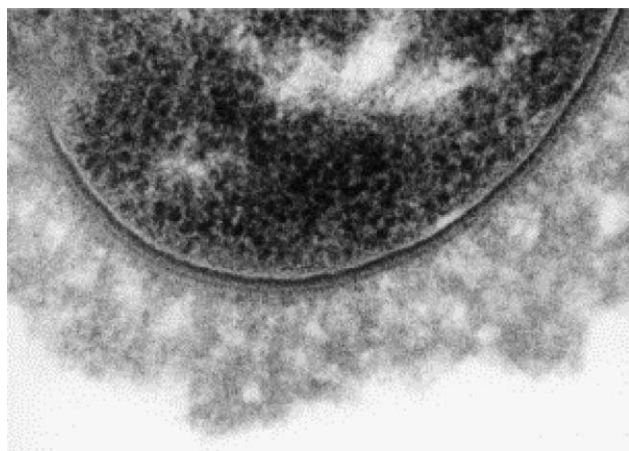


Fig. 2. Cross-sectioned *Streptococcus zooepidermicus* cell with its HA capsule from aerated culture (particular): ~3000×.

HA capsule and can so theoretically provide unlimited HA biopolymers with a molecular weight going from 10,000 up to 4 million Daltons (depending on temperature, initial glucose concentrations, speed of bacterial growth): this is presently the favourite production procedure worldwide.

HA is synthesised into the fibroblast and other cellular plasmatic membranes through the joining of glycidic residuals to the reducing chain extremity [8]. HA receptors have been localised on fibroblasts and 3T3 cells surface [9] and recently on endothelial surface [10] probably influencing angiogenesis. Furthermore, HA reduces erythrocyte viscosity [11].

1.1. Aim and objectives of the investigation

Although less investigated than heparin, HA, due to its peculiar physico-chemical characteristics, has been proven to be provided with several biologic functions in the tissues of animals and humans, such as stabilisation of proteoglycans in the extra-cellular matrix [12], high hydrating properties [13], contribution to tissue repair [14], inhibition of migration, chemotaxis and aggregation of polymorphonuclear leucocytes and monocytes [15–17], regulation of cellular growth and solute transfer.

Furthermore, its concentration is high in several tissues and parenchymas, large quantity of HA being especially present in the lung. Therapeutic applications of HA in diseases interesting domains such as the orthopaedic, ophthalmic and dermatologic ones have already been extensively studied and reproduced in animal models [18–22].

Together with its molecular similarities with other GAGs, especially heparin [23] which according to the literature is provided with anti-asthmatic properties [24–28], the above mentioned HA biological activities and characteristics give evidence in favour of its possible therapeutic interventions in such respiratory disease. Some studies have been performed on the activity of HA in models of other obstructive diseases such as emphysema and exacerbation of COPD [29,30]; our preliminary data have shown the possibility of preventing induced bronchoconstriction in asthma thanks to the pre-treatment with aerosolised HA [31,32]: such aerosol had been proven to protect the asthmatics from different specific and non-specific challenges [33]. In this study we present data on the effect of inhaled HA in adult asthmatics, namely on the bronchial response to a non-specific challenge such as physical exercise.

2. Subjects and methods

2.1. Production plan for the preparation of the HA used in the present study

Preliminarily, solutions of HA with a molecular weight variable from 400 to 4000 kilodaltons (KD) were prepared [7].

A condensation reaction between glucuronic acid of vegetal origin and hydrolysed chitin solution (acetylglucosamine 85–90% the dry weight) in presence of a biological catalysator, at a temperature of 18–20 °C, is successively obtained. A 10% water mixture of about 90% disaccharide (provided with a structure which is identical to the HA basic monomer) and HA polymers for the resting 10%, is so obtained, under the form of a translucent colloid. The above mentioned proportions have been selected with the intention of optimising (reducing) the viscosity of the preparation so facilitating its aerosolisation.

2.2. Patients

Fourteen patients (11 males), mean age 21.36 (range: 13–36 years old; 7 teenagers, 7 young adults; 11 males, 3 females), all suffering from mild persistent asthma ($n=12$ allergic, 2 non-allergic) have been selected for the present study: all patients were asymptomatic at the enrolment, with a FEV1 > 80% (predicted). All asthmatic patients participated to the study out of the season of their individual allergy.

The study design was randomised, cross-over, single-blind.

All patients gave their informed consent.

At 9 a.m. each patient has been submitted to a non-specific bronchial challenge consisting in physical exercise. The respiratory function has been studied before and after such challenge by the mean of a body plethysmograph VMax Autobox, V6-20 provided with a pneumotachograph Mass Flow Meter (Sensor Medics, Yorba Linda, CA, USA):

FEV1 was the functional variable taken into consideration for the purpose of the present study.

2.3. Exercise test

Four millilitres of saline (placebo=P) or isoosmolar solution containing 0.3% of the above mentioned colloidal preparation of HA in saline were administered at random by aerosol to each patient in two non-consecutive days via a nebuliser (Turbo Boy, Pari, Starnberg, Germany) 30 min prior to the beginning of the challenge, which consisted of a free run of 10 min. All subjects were invited to run on a 100 m track on a flat paved oval surrounding our Hospital pavilion. Heart rate was measured every 200 m. Each subject was encouraged to run at an intensity such as to reach and maintain a heart rate of about 85% of his own predicted value. All tests were performed in good weather days and adequate clothes.

Clinical symptoms and spirometric data have been evaluated before the physical exercise and 5 min following its stop.

2.4. Statistics

The results have been statistically analysed on the basis of paired *T*-test.

3. Results

The bronchoconstrictive effect induced by physical exercise has been relevant (Fig. 3) and statistically

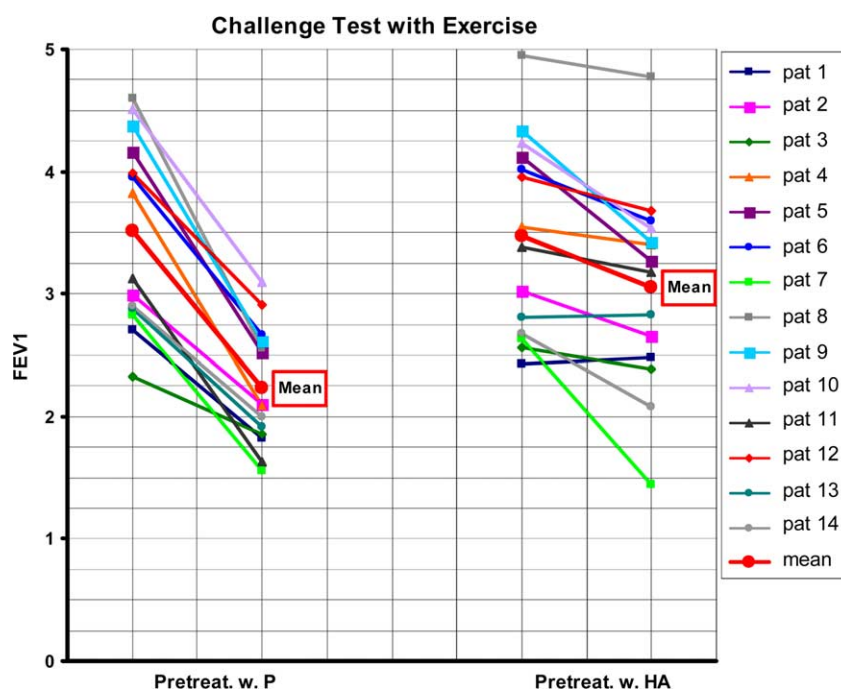


Fig. 3. Protective effect of the pre-treatment with aerosolised hyaluronic acid on bronchoconstriction induced by muscular exercise in asthmatics, compared with the effect of the pre-treatment with aerosolised placebo. Vertical axis: FEV1 (l/s).

Table 1
Exercise challenge test

Patient's initials	Sex	Age	Placebo base line FEV1	(%Pred)	Placebo post-chall FEV1	%Diff. post-challenge Baseline	HA base line FEV1	(%Pred)	HA post-challenge FEV1	%Diff. post-challenge Baseline
BBD	M	13	2.71	(107)	1.82	−33	2.43	(96)	2.48	+2
SB	M	14	2.99	(91)	2.10	−30	3.02	(94)	2.65	−12
SG	M	14	2.33	(88)	1.85	−21	2.56	(97)	2.39	−7
FR	M	15	3.82	(103)	2.09	−45	3.54	(96)	3.40	−4
TAR	M	16	4.16	(101)	2.52	−39	4.12	(100)	3.27	−21
BI	M	17	3.95	(99)	2.66	−33	4.02	(101)	3.60	−10
PG	M	17	2.83	(86)	1.56	−45	2.63	(82)	1.44	−45
NB	F	20	3.13	(88)	1.63	−52	3.38	(95)	3.18	−6
AA	M	23	4.60	(94)	2.55	−46	4.95	(98)	4.77	−4
FE	M	24	4.37	(85)	2.61	−40	4.33	(85)	3.42	−21
ICH	M	26	4.51	(108)	3.09	−31	4.23	(101)	3.53	−17
FM	M	30	3.99	(100)	2.91	−27	3.96	(99)	3.68	−8
SP	F	34	2.88	(105)	1.92	−33	2.81	(102)	2.83	+1
TRR	F	36	2.90	(99)	2.00	−31	2.67	(91)	2.08	−22
Mean		21.36	3.51	96.71	2.24	−36.14	3.48	95.36	3.05	−12.43
SEM		2.05	0.20	2.50	0.13	2.30	0.23	2.34	0.23	3.27
%Diff.					−36.3	$p < 0.0001$		$p < 0.001$	−12.3	$p < 0.001$

significant (Table 1): the average FEV1 measured 5 min post-exercise was reduced by 36.14% from the baseline values ($p < 0.0001$) which have been obtained in the subjects pre-treated with aerosolised P.

Pre-treatment with aerosolised HA determined partial but clear protection on the FEV1 reduction due to the bronchoconstriction induced by the challenge: the average post-challenge FEV1 was proven to be 12.43% less than the pre-challenge baseline ($p < 0.001$).

In the asthmatic patients, the protection induced by HA (when compared to P) on the bronchoconstriction due to muscular exercise resulted particularly significant by the statistical point of view ($p < 0.0001$) when evaluated on the basis of the respective FEV1 individual values. Incidentally, once the patients are divided into two subgroups (A: teenagers and B: young adults), the results obtained in the subgroup A (7 individuals: 6 allergic, 1 non-allergic; mean age 15.14, range 13–17 years) vs B (7 individuals: 6 allergic, 1 non-allergic; mean age 25.57, range 20.36 years) were not proven statistically different (NS).

4. Discussion

The physiological and physico-chemical HA properties derive from its molecular structure which in solution is a casual winding. This occupies a volume which is 1000 times larger than that occupied by organic materials [34], so that the neighbouring molecules are trapped by HA in concentrations > 1 mg/ml [35].

Since two molecules cannot occupy the same space in the same time and since the volume occupied by HA is very large, HA solutions of 5 or 15 mg/ml exclude albumin, respectively, from 25 to 75% of the total fluid volume [36].

Due to such 'exclusion', the volume available for other macromolecules (inflammatory ones included) is consequently limited, so that their total number per volume unit decreases and/or concentration/colloidal osmotic pressure in such limited volume increases remarkably, facts which can deeply influence fluid lymphatic transcapillary drainage [37] and reduce interstitial protein content [38]. These can be the main reasons for the higher 'restriction' of macromolecular traffic in HA solutions than in water [39]. Then, the importance of HA in microcirculatory exchanges is mainly due to its action on the interstitial connective tissue, so that, once HA concentration increases, diffusion of macromolecules decreases.

The multiple functions of HA and especially the limitation of neighbouring molecules and cells (including the inflammatory ones) and its high concentration in the lung matrix let conclude that a substantial role can be attributed to this molecule as a major component of the extra-cellular matrix in different connective tissues, contributing to their local homeostasis. Extensive orthopaedic studies in arthritic patients have demonstrated clear clinical success when high molecular weight hyaluronic acid solutions have been locally injected: an effect which has been attributed to the modulation of the inflammation [40], persisting for several weeks [41], due to specific interaction with ICAM-1 receptors exposed on the surface of inflammatory cells such as macrophages and T-lymphocytes.

Due to its high concentration in the lung, the hypothesis of a possible analogous anti-inflammatory, or at least protective, activity on the respiratory apparatus could be legitimated. In fact, it has been demonstrated that in the airways wall HA positively influences bio-mechanical forces, hydric balance, cellular functions, activity of growth factors and cytokine behaviour [35].

4.1. Conclusions

On such hypothetical basis, attention has been put on the possible activity of HA on pulmonary matrix (notoriously altered in the remodelled bronchial wall as well in the emphysematous lung tissue), so that the initial models were intended to study its activity in animal experimental lung emphysema [35], also on the basis of the observed low concentration of the molecule in emphysematous patients [42].

Furthermore, according to more or less recent observations, airways remodelling is presently considered one of the main features to be investigated in asthma: a concept which legitimates the hypothesis that the correction of the asthmatics' bronchial wall alterations which obviously involves its major amorphous matrix component (HA), up-to-date nearly neglected, could be claimed as one of the main needs to prevent abnormal reactivity or to treat structural and/or functional bronchial deficiencies in asthma as well as in other obstructive diseases.

Of course, it is not easy to imagine that a single inhalation of HA before exercise could induce alone, as a possible outcome, the minimisation of the complex phenomenon of asthma remodelling. To explain our observations we have, first of all, to take in consideration the increase in osmolarity of airways surface fluid, which results from water loss by evaporation during exercise, being believed to significantly contribute to the bronchoconstriction induced by such a stimulus. Therefore, the results of the present study are explained, at least in part, by the capacity of HA of preventing airway dehydration during exercise. A phenomenon, the latter, which can directly or indirectly contribute to the partial correction of the pathological remodelling of bronchial wall composition and consequent asset. Only long-lasting therapeutical plans will furnish an answer to the question whether and to which extent a treatment with HA and/or other GAGs can contribute to the correction of bronchial remodelling.

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References

- [1] O'Regan M, Martini L, Crescenzi F, De Luca C, Lansing M. Molecular mechanisms and genetics of hyaluronan biosynthesis. *Intern J Biol Macromol* 1994;16:283–6.
- [2] Laurent UBG, Reed RK. Turnover of hyaluronan in the tissues. *Adv Drug Deliv Rev* 1991;7:237–56.
- [3] Reed RK, Laurent UBG. Turnover of hyaluronan in the microcirculation. *Am Rev Respir Dis* 1992;146:S37–S9.
- [4] Reed RK, Lilja K, Laurent TC. Hyaluronan in the rat with special references to the skin. *Acta Physiol Scand* 1988;134:405–11.
- [5] Fraser JRE, Kimpton WG, Laurent TC, Cahill RN, Vakakis N. Uptake and degradation of hyaluronan in lymphatic tissue. *Biochem J* 1988;256:153–8.
- [6] Smedsrön B, Perthof H, Gustavson S, Laurent TC. Scavenger function of the liver endothelial cell. *Biochem J* 1990;266:313–27.
- [7] Goh LT. Effect of culture conditions on rates of intrinsic hyaluronic acid production by *Streptococcus equi* spp. *Zoepidermicus*. *Biotechnol Lett* 1998;16:507–12.
- [8] Prehm P. Hyaluronate is synthesized at plasma membranes. *Biochem J* 1984;220:597–600.
- [9] Turley EA. Hyaluronan-binding proteins and receptors. *Adv Drug Deliv Rev* 1991;7:257–64.
- [10] Antonelli A, D'Amore PA. Density-dependent expression of hyaluronic acid binding to vascular cells in vitro. *Microvasc Res* 1991;41:239–51.
- [11] Clarke DN, Sirs JA. Hyaluronic acid and erythrocyte flexibility. *Intern J Microcirc Clin Exp* 1988;S153 [Special Issue].
- [12] Rosenberg RC, Varma R. An overview of proteoglycans in physiology and pathology. In: Varma RS, Varma R, editors. *Glycosaminoglycans and proteoglycans in physiological and pathological processes of body systems*. Basel: Karger; 1982. p. 1–4.
- [13] Bhattacharya J, Cruz T, Bhattacharya S, Bray BA. Hyaluronan affects extravascular water in lung of anesthetized rabbits. *J Appl Physiol* 1989;66:2595–9.
- [14] Toole BP. Glycosaminoglycans and morphogenesis. In: Hay ED, editor. *Cell biology and extracellular matrix*. New York: Plenum Press; 1991. p. 259–94.
- [15] Forrester JV, Wilkinson PC. Inhibition of leukocyte locomotion by hyaluronic acid. *J Cell Sci* 1981;48:315–31.
- [16] Partsch G, Schwartz C, Neumüller J, Dunky A, Petera P, Broll H, et al. Modulation of chemotaxis and migration of PMN cells by hyaluronic acid. *Z Rheumatol* 1989;48:123–8.
- [17] Forrester JV, Lackie JM. Effect of hyaluronic acid on neutrophil adhesion. *J Cell Sci* 1981;50:329–44.
- [18] Balasz EA, Denlinger JL. The role of hyaluronic acid in arthritis and its therapeutic use. In: Peyron JC, editor. *Osteoarthritis current clinical and fundamental problems*. Basel: Geigy; 1984. p. 165–74.
- [19] Brandt K. Modification of chemiotaxis by synovial fluid hyaluronate. *Arthritis Rheum* 1970;13:308–9.
- [20] Knudson JB, Knudson W. Hyaluronic-binding proteins in development tissue homeostasis and disease. *FASEB J* 1993;7:1233–41.
- [21] Balasz EA, Lechner A. Cross-linked gels of hyaluronic acid used as drug delivery systems and in cosmetic formulations. *Biomatrix Inc*. 1997.
- [22] McDonald CC, Kaye SB, Figueiredo FC, Macintosh G, Lockett C. A randomised, crossover, multicentre study to compare the performance of 0.01% (w/v) sodium hyaluronate with 1.4% (w/v) polyvinyl alcohol in the alleviation of symptoms associated with dry eye syndrome. *Eye* 2002;16:601–7.
- [23] Lever R, Page C. Glycosaminoglycans, airways inflammation and bronchial hyperresponsiveness. *Pulm Pharmacol Ther* 2001;14:249–54.
- [24] Ahmed T, Abraham WM, D'Brot J. Effects of inhaled heparin on immunologic and non-immunologic bronchoconstrictor responses in sheep. *Am Rev Respir Dis* 1992;145:566–70.
- [25] Ahmed T, Garrigo J, Danta I. Preventing bronchoconstriction in exercise-induced asthma with inhaled heparin. *N Engl J Med* 1993;329:90–5.
- [26] Garrigo J, Danta I, Ahmed T. Time course of the protective effect of inhaled heparin on exercise-induced asthma. *Am J Respir Crit Care Med* 1996;153:1702–7.
- [27] Ahmed T, Gonzales BJ, Danta I. Prevention of exercise-induced bronchoconstriction by inhaled low-molecular-weight heparin. *Am J Respir Crit Care Med* 1999;160:576–81.

- [28] Tranfa CM, Vatrella A, Parrella R, Pelaia G, Bariffi F, Marsico S. Effect of inhaled heparin on water-induced bronchoconstriction in allergic asthmatics. *Eur J Clin Pharmacol* 2001;57:59.
- [29] Cantor JO, Cerreta JM, Keller S, Turino GM. Modulation of airspace enlargement in elastase-induced emphysema by intratracheal instillation of hyaluronidase and hyaluronic acid. *Exp Lung Res* 1995;21: 423–6.
- [30] Venge P, Pedersen B, Hakansson L, Hallgren R, Lindblad G, Dahl R. Subcutaneous administration of hyaluronan reduce the number of infectious exacerbations in patients with chronic bronchitis. *Am J Respir Crit Care Med* 1996;153:312–6.
- [31] Petrigni G, Comi AL, Guerra S, Longo L, Misuraca C, Capone P, et al. Hyaluronic acid in pediatric patients with exercise-induced asthma. *Eur Respir J* 2000;16(Suppl. 31):307s.
- [32] Petrigni G, Comi AL, Guerra S, Capone P, Cerotti L, Allegra L. Hyaluronic acid protects asthmatics from exercise-induced bronchoconstriction. *Eur Respir J* 2000;16(Suppl. 31):458s.
- [33] Petrigni G, Guerra S, Comi AL, Misuraca C, Allegra L. Hyaluronic acid, a novel way of protecting asthmatics from non-specific bronchoprovocation. *Eur Respir J* 2000;16(Suppl. 31):458s.
- [34] Granger HJ. Physicochemical properties of the extracellular matrix. In: Hargens AR, editor. *Tissues fluid pressure and composition*. Baltimore: Williams & Wilkins; 1981. p. 43–61.
- [35] Bothner H, Wik O. Rheology of hyaluronate. *Acta Otolaryngol* (Suppl.) 1987;442:25–30.
- [36] Bert JL, Pierce RH. The interstitium and microvascular exchange. In: Renkin EM, editor. *Handbook of physiology. Section 2: the cardiovascular system. Pt 1: Microcirculation, vol. IV*. Bethesda: American Physiological Society; 1984. p. 521–47.
- [37] Granger HJ, Laine GA, Barnes JE, Lewis RE. Dynamics and control of transmicrovascular fluid exchange. In: Staub NC, Taylor AE, editors. *Edema*. New York: Raven Press; 1984. p. 189–228.
- [38] Reed RK, Bowen BD, Bert JL. Microvascular exchange and interstitial volume regulation in the rat: implication of the model. *Am J Physiol* 1989;257:2081–91.
- [39] Laurent TC, Persson H. On the interaction between polysaccharides and other macromolecules. *Biochem Biophys Acta* 1963; 78:351–9.
- [40] Haynes BF, Liao HX, Patton KL. The transmembrane hyaluronate receptors (CD44): multiple functions, multiple forms. *Cancer Cells* 1991;3:347–50.
- [41] Ialenti A, DiRosa M. Hyaluronic acid modulates acute and chronic inflammation. *Agents Actions* 1994;43:44–7.
- [42] Cantor JO, Cerreta JM, Armand G, Osman M, Turino GM. The pulmonary matrix, glycosaminoglycans and pulmonary emphysema. *Connect Tissue Res* 1999;40:97–104.