Polymers **2014**, *6*, 1948-1957; doi:10.3390/polym6071948

OPEN ACCESS

polymers

ISSN 2073-4360

www.mdpi.com/journal/polymers

Article

Effect of Mannitol on Hyaluronic Acid Stability in Two *in Vitro* Models of Oxidative Stress

Marguerite Rinaudo [1], Bernard Lardy [2], Laurent Grange [3] and Thierry Conrozier [4,*]

[1] Biomaterials Applications, 6 rue Lesdiguières, 38000 Grenoble, France;
E-Mail: marguerite.rinaudo@sfr.fr

[2] Université Joseph Fourier, GREPI AGIM FRE 3405 CNRS, 38000 Grenoble, France;
E-Mail: blardy@chu-grenoble.fr

[3] Service de Rhumatologie, Centre hospitalier Universitaire A.Michallon, 38000 Grenoble, France;
E-Mail: lgrange@chu-grenoble.fr

[4] Service de Rhumatologie, Centre hospitalier de Belfort-Montbéliard, 90000 Belfort, France

* Author to whom correspondence should be addressed; E-Mail: tconrozier@chbm.fr;
Tel.: +33-384-986-051/+33-686-919-218; Fax: +33-384-985-908.

Received: 3 May 2014; in revised form: 20 June 2014 / Accepted: 23 June 2014 /
Published: 8 July 2014

Abstract: In this paper, we propose the evaluation of the mannitol's ability to reduce hyaluronic acid (HA) degradation using two different models of oxidative stress. Firstly, a solution of hyaluronan and a solution of the same HA including mannitol in PBS buffer were submitted to an oxidative stress generated by the addition of xanthine + xanthine oxidase generating oxygen free radicals. Different enzyme concentrations were used and the HA properties were studied after 24 h of contact at ambient temperature. Decreases of the viscosity of the solution were assessed by rheometry (viscous and elastic module) and that of HA molecular weight was determined by steric exclusion chromatography. Rheologic behavior was assessed on identical HA solutions subjected to another model of oxidative stress imposed by addition of hydrogen peroxide. The influence of mannitol concentration on HA degradation was also demonstrated. Whatever the stress applied, it appears very clearly that mannitol protects hyaluronic acid from mediated oxygen free radicals degradation. These *in vitro* results suggest that mannitol could be a simple way to significantly increase the intra-articular residence time of the injected hyaluronic acid and therefore might improve viscosupplementation effectiveness.

Keywords: hyaluronic acid; osteoarthritis; viscosupplementation; anti-oxidant; mannitol; xanthine oxidase; hydrogen peroxide; *in vitro*

1. Introduction

Intra-articular injections of hyaluronic acid (HA) of high molecular weight, is a symptomatic treatment of knee osteoarthritis [1,2] whose purpose is to replace [1–3] and or to induce the secretion [4] of endogenous HA in the osteoarthritic joint. This concept of viscosupplementation (VS) has been proposed by Balazs [1] noting that the elastic viscous behavior characterizing the healthy synovial fluid (SF) is altered in osteoarthritis and that alterations are directly correlated with the quantitative and qualitative decrease of the SF hyaluronate. The latter is indeed a key, but not the exclusive, element of the SF rheological properties [5,6], whose main roles are lubrication of the joint and shock absorption. In osteoarthritis, HA loss exposes the injured cartilage to increased mechanical stress and consequently to increase degradation.

After 20 years of use, viscosupplementation is widely recognized as an effective and well tolerated treatment for knee osteoarthritis [7–9], although the real level of its effectiveness and its specific indications remain controversial subjects [10,11]. Discrepancies between conclusions of some meta-analyzes [8–10] can come from methodological dissimilarities, but also from possible differences in efficacy between the studied products which widely vary in concentration, molecular weight and molecular organization [7,12]. Indeed, the HA injected into the joint is rapidly degraded, limiting the time of intra-articular residence ranging from few days for linear molecules [13] to up to several weeks [14,15] for solutions of cross-linked HA. Among the many pathogenic mechanisms contributing to HA degradation, reactive oxygen free radicals or reactive oxygen species (ROS) have a main role [16,17]. Osteoarthritis is a degenerative joint disease in the pathogenesis of which ROS play a major deleterious effect [18]. It has been shown that interleukin-1β (IL-1β) activates production of ROS, which are involved in the synthesis and or activation of metalloproteinases (MMPs) and in chondrocyte apoptosis [19]. On the opposite, inhibition of ROS production was shown to reduce the expression of the pro-collagenase MMPs [20].

In addition to their effect on degradation of the extracellular matrix, ROS are directly involved in the mechanisms of degradation of both endogenous and exogenous (*i.e.*, injected) HA in the synovial fluid [16]. Optimizing the efficiency and duration of action of VS by adding an antioxidant to HA in order to reduce its *in situ* degradation and to increase its time of contact with damaged tissue, is a challenging field of research. Mannitol and its isomer, sorbitol, which are known to be very well tolerated [21,22] are excellent candidates for this. In intra-articular injection into the knee, two studies with viscosupplements containing 0.5% mannitol [23] and 4% sorbitol [24] have been published. In both studies tolerance appeared similar to that of conventional viscosupplements and none of them revealed any serious or unexpected adverse effects. Addition of an antioxidant to HA has already been studied also in ophthalmology. In an animal model of oxidative stress using hydrogen peroxide (H_2O_2), Belda *et al.* [25] showed that HA associated with a low concentration of mannitol (0.5%) better protected the corneal endothelium, than HA alone.

Nevertheless the studied viscous products differed in molecular weight and concentration, which did not allow asserting with certainty that mannitol was responsible for this protection. Interestingly a number of hydroxyl rich polysaccharides, such as HA, have also antioxidant properties and are themselves inhibitors of oxygen free radical $^{-}O_2$* pointing the role of HA concentration [26,27].

The purpose of the present study was to investigate the ability of mannitol to reduce the degradation of HA by oxygen free radicals, using two different validated models of oxidative stress, firstly induced by xanthine (X) + xanthine oxidase (XO) (X/XO model), then by adding hydrogen peroxide (H_2O_2 model).

2. Experimental Section

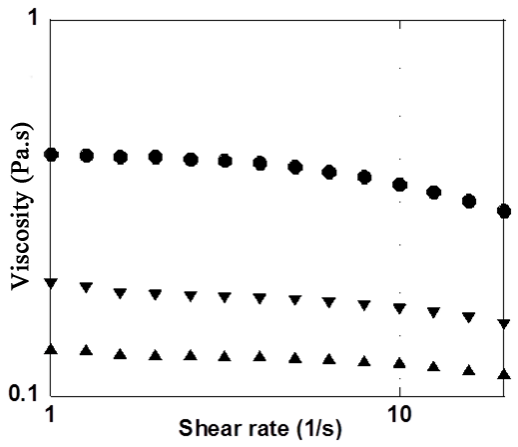
2.1. Induction of an Oxidative Stress

2.1.1. X/XO Model

A linear HA obtained by biofermentation (ARD Corporation, Pomacle, France), in purified sodium salt powder form with a weight-average molecular weight (MW) 800,000 g·mol^{-1}, was dissolved at a concentration of 8 g·L^{-1} of phosphate (PBS) buffer at pH = 7.4, and then a solution of the same HA supplemented with 35 g·L^{-1} of mannitol was made. Both were prepared in order to serve as control solutions. Before use, 3 mL of HA in PBS (or HA + 35 g·L^{-1} mannitol in PBS) were added of 300 µL of PBS. The rheological behavior of the control solutions was studied. They were the same and also compared with the same solutions added of 10 µL of XO at a concentration 30 mIU (International unite)·µL^{-1}. XO is a flavoprotein generating large amounts of superoxide anion O_2^- in the presence of xanthine catalyzing its oxidation in uric acid. The viscosity of these solutions was similar to that of the control thus showing that there was no XO/HA interaction and no degradation of HA in the presence of the enzyme alone (data not shown).

To generate oxidative stress, 300 µL of xanthine (at a concentration of 100 mM) was added as a substrate in place of the 300 µL PBS of the control solutions. The experiment was repeated with XO quantities of 10 (Figure 1), 16 µL and 32 µL at a concentration 30 mIU·µL^{-1}.

Figure 1. Measurement of the steady state viscosity η as a function of the shear rate for 3 solutions of hyaluronic acid (HA) at pH = 7.4: ● initial HA in phosphate (PBS); ▼ HA/PBS + xanthine; ▲ HA/PBS + xanthine + xanthine oxidase.



2.1.2. H_2O_2 Model

Hydrogen peroxide has been often used to degrade polysaccharides through a radical mechanism [28]. The same HA (MW = 800,000 g·mol^{-1}) used in the previous test, alone then added with mannitol, was subjected to oxidative stress generated by the addition of hydrogen peroxide (H_2O_2 30% supplied by Roth, South Watertown, NY, USA) at a final concentration of 2.7% and or 5.4% (v/v). The rheological behavior of the solutions was studied after exposure to oxidative stress of variable duration at room temperature [29]. The complex viscosity was measured using a cone-plate rheometer at 25 °C, at a frequency of 1 Hz as a function of the reaction rate. Two concentrations of mannitol, 10 g·L^{-1} and 35 g·L^{-1}, were studied to evaluate possible dose effect of the latter.

2.2. Rheology

The rheological properties were measured using a cone-plate rheometer (ARG2, Texas Instruments®, Dallas, TE, USA) at 25 °C on the different solutions tested before and 24 h after oxidative stress at room temperature. The steady state viscosity η (in Pa·s) was determined according to the shear rate ($\gamma \cdot s^{-1}$). The dynamic experiments were carried out in the region of linear viscoelasticity, where the G′ elastic and G″ viscous module are independent of the applied frequency. The dynamic moduli G′ and G″ (Pa) and the complex viscosity |η*| were determined according to the angular frequency (ω), expressed in Hertz.

2.3. Molecular Weight Measurement

Molecular weight of HA was measured by steric exclusion chromatography (SEC), before and after oxidative stress using a "Waters Alliance GPCV2000®" chromatograph (Milford, MA, USA) equipped with three detectors in line [30]. The injected concentration was 2 g·L^{-1}, with an injection volume of 100 µL using two columns in series (Shodex OH-Pack®, New York, NY, USA, 805 and 806). All samples were filtered through a membrane with pores of 0.2 µm (Sartorius® AG, Goettingen, Germany, cellulose acetate filter) prior to injection in order to retain any aggregates. The eluent used was 0.1 M $NaNO_3$ at an elution temperature of 30 °C and a flow rate of 0.5 mL·min^{-1}.

3. Results and Discussion

3.1. X/XO Stress Model

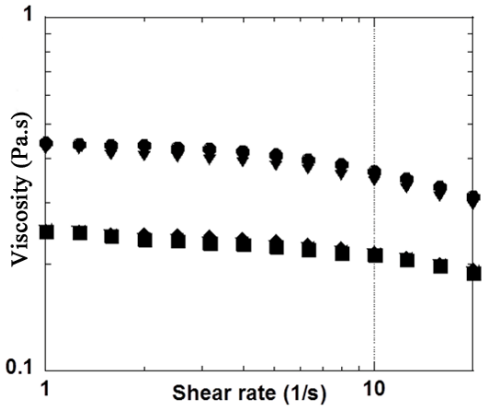
In the presence of substrate alone (X), the decrease of the HA solution viscosity was similar to that induced by the addition same amount of PBS (dilution effect). However the addition of xanthine to the HA solution containing XO, causing an X/XO reaction that leaded to ROS production, was responsible for HA degradation evidenced by a decrease of its viscosity (Figure 1), and a significant decrease (−36.6%) of its molecular weight (Table 1).

Table 1. Weight-average molecular weight (MW in $g \cdot mol^{-1}$) of hyaluronic acid alone and in presence of mannitol ($35 g \cdot L^{-1}$) before and after exposure to an oxidative stress induced by xanthine/xanthine oxidase (X/XO) at increasing doses added in 4mL HA solution.

Solution	Initial MW ($g \cdot mol^{-1}$)	Final MW ($g \cdot mol^{-1}$) +16 µL XO	Final Mw ($g \cdot mol^{-1}$) +32 µL XO	Difference (%)between initial and final MW with 32 µL XO
Hyaluronic acid (HA)	787,000	621,450	498,900	−36.6%
HA + Mannitol $35 g \cdot L^{-1}$	768,900	717,750	677,150	−11.9%

Similarly the addition of mannitol to the initial HA solution did not change significantly the rheological behavior of the latter (Figure 2). It is also shown that under the effect of oxidative stress, the viscosity of the solution of HA added with mannitol was not significantly changed compared with the HA in presence of xanthine alone. Concerning HA molecular weight, it is decreased very slightly (−11.9%) and only at the highest enzyme concentration (Table 1).

Figure 2. Measurement of the steady state viscosity η as a function of the shear rate of four solutions of hyaluronic acid (HA): ● HA/PBS; ▼ HA/mannitol/PBS; ◆ HA/mannitol + xanthine; ■ HA/mannitol + xanthine + xanthine oxidase.



3.2. H_2O_2 Model

Without mannitol, HA was rapidly degraded by hydrogen peroxide, as demonstrated by a rapid decrease of the complex viscosity |η*| (Figure 3).

However the rheological parameters of the HA solution containing 35 $g \cdot L^{-1}$ of mannitol were not modified in the presence of H_2O_2 over a period of 30 min. At a dose of 10 $g \cdot L^{-1}$, mannitol reduced the rate of HA degradation, but to a lesser extent and with a period of apparent induction (Figure 4 and Table 2).

Figure 3. Kinetic of hyaluronic acid degradation (HA 10 $g \cdot L^{-1}$) induced by hydrogen peroxide oxidative stress (0.3 mL of H_2O_2 in 3 mL HA solution) in presence (▼) and without mannitol (35 $g \cdot L^{-1}$) (●). The variation of the complex viscosity |η*| is measured according to the time of degradation.

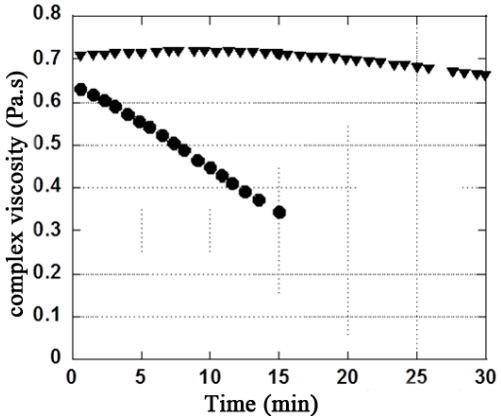


Figure 4. Kinetic of hyaluronic acid degradation (HA, 10 $g \cdot L^{-1}$) according to mannitol concentration (10 $g \cdot L^{-1}$ (▼) or 35 $g \cdot L^{-1}$ (●)) measured by the variation of the complex viscosity |η*| as a function of the time of hydrogen peroxide induced oxidative stress (0.6 mL of H_2O_2 in 3 mL HA solution).

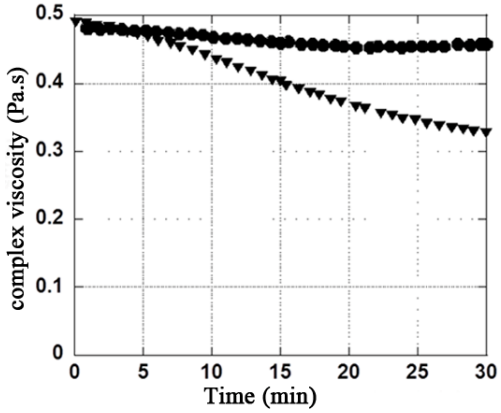


Table 2. Elastic modulus (G′), viscous modulus (G″) and complex viscosity (|η*|) of a solution of hyaluronic acid and mannitol (10 $g \cdot L^{-1}$ and 35 $g \cdot L^{-1}$), before and after 15 min exposure to an oxidative stress (0.6 mL H_2O_2 in 3 mL HA) at ambient temperature.

Mannitol Concentration ($g \cdot L^{-1}$)	**Rheological properties**	**Initial values**	**Final values in presence of H_2O_2 (2.7%)**	**Ratio (difference in %) Initial / Final**
10	G′ (Pa)	0.88	0.64	1.37 / (−27.2%)
10	G″ (Pa)	2.90	2.40	1.21 / (−17.2%)
10	|η*| (Pa·s)	0.49	0.40	1.22 / (−18.4%)
35	G′ (Pa)	0.87	0.82	1.06 / (−5.7%)
35	G″ (Pa)	2.90	2.77	1.05 / (−4.4 %)
35	|η*| (Pa·s)	0.48	0.46	1.04 / (−4.2%)

This *in vitro* study, using two different models of oxidative stress, shows clearly that the addition of mannitol to hyaluronic acid allows the reduction of HA-ROS-mediated degradation as related to its anti-oxidant power towards the rich reactive hydroxyl function previously shown in various diseases [31–35]. Then, the beneficial effect of mannitol ($C_6H_{14}O_6$) having potent free radical scavenger properties is confirmed from our data. Concerning the mechanism involved, when HA is administered intra articularly, HA macromolecules, containing many OH groups, react with ROS, resulting in the rupture of the macromolecular chains and accelerated degradation of the highly viscous solution (or partially crosslinked HA gel) [16]. The rapid depolymerisation of HA is a major reason for the short intra-articular half-life of viscosupplements made of non-cross-linked HA [13], cross-linking being another way to protect HA from degradation by ROS [14,15].

In addition, the chemical characteristics of mannitol make it as an antioxidant of choice in combination with HA, particularly because of its hydro-solubility compared with other antioxidants such as vitamin E which is lipid soluble, or β-caroten which is insoluble in water. Furthermore its resistance to heat allows sterilization by autoclaving, unlike other numbers thermolabile antioxidants (*i.e.*, polyphenols, vitamin C). Moreover mannitol does not increase the ionic strength of the medium and thus does not significantly alter the rheological performance of the HA as shown in Figure 2. As well as HA, mannitol has a perfect safety, many animal tests showing that it was non-cytotoxic, non-genotoxic, non-carcinogenic and non-mutagenic, even at high doses [21]. In humans it is widely used *per os* and through parenteral injections (intravenous, intra-ocular) at very high concentration including hyperosmolar one [22]. Almost non-metabolizable carbohydrate, mannitol is eliminated by the renal glomeruli and is not reabsorbed. That is the reason why it is also used as a substitute for glucose for diabetic patients; its sweetness is almost equivalent to the latter, while not being metabolized.

The benefit of combining HA and mannitol has previously been studied *in vitro*. In a model of oxidative stress using hydrogen peroxide and copper, Mendoza *et al.* [24] showed that 50% inhibition of degradation of HA by hydroxyl radicals was obtained from low concentrations of mannitol (26.5 mM or about 5 $g \cdot L^{-1}$). Our study suggests that the protective effect of mannitol on HA degradation is also dose-dependent and that, low concentrations (10 $g \cdot L^{-1}$) protected HA from ROS mediated depolymerisation to a lower degree than higher concentrations (35 $g \cdot L^{-1}$). It has to be noted that the concentrations of H_2O_2 used in our model are very high and clearly highlight the importance of mannitol content.

4. Conclusions

In summary, these *in vitro* results suggest that high concentrations of mannitol added into a viscosupplement, protect HA from oxidative degradation and could therefore increase its intra-articular residence time without modifying its rheological properties. Thereby, mannitol might improve the efficiency and or duration of action of viscosupplementation. These first *in vitro* results fully justify the *in vivo* studies already in progress, in order both to confirm or refute this hypothesis and to assess the safety of this new association.

Acknowledgments

The authors thank the Laboratory "Rhéologie et Procédés" of Grenoble University (France) for their technical contribution.

Author Contributions

Marguerite Rinaudo performed all the rheological measurements; Bernard Lardy performed the enzymatic degradation; Laurent Grange and Thierry Conrozier were involved in the data analysis in relation with the bibliographic references related to the medical applications.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Balazs, E.A.; Denlinger, J.L. Viscosupplementation: A new concept in the treatment of osteoarthritis. *J. Rheumatol. Suppl*. **1993**, *39*, 3–9.
2. Balazs, E.A. Viscosupplementation for treatment of osteoarthritis: From initial discovery to current status and results. *Surg. Technol. Int*. **2004**, *12*, 278–289.
3. Pelletier, J.P.; Martel-Pelletier, J. The pathophysiology of osteoarthritis and the implication of the use of hyaluronan and hylan as therapeutic agents in viscosupplementation. *J. Rheumatol.* **1993**, *20*, 19–23.
4. Bagga, H.; Burkhardt, D.; Sambrook, P.; March, L. Long term effects of intra-particular hyaluronan on synovial fluid in osteoarthritis of the knee. *J. Rheumatol.* **2006**, *33*, 946–950.
5. Mathieu, P.; Conrozier, T.; Vignon, E; Rozand, Y.; Rinaudo, M. Rheologic behavior of osteoarthritic synovial fluid after addition of hyaluronic acid: A pilot study. *Clin. Orthop. Relat. Res*. **2009**, *467*, 3002–3009.
6. Conrozier, T.; Mathieu, P.; Vignon, E.; Piperno, M.; Rinaudo, M. Differences in the osteoarthritic synovial fluid composition and rheology between patients with or without flare: A pilot study. *Clin. Exp. Rheumatol*. **2012**, *30*, 729–734.
7. Bellamy, N.; Campbell, J.; Robinson, V.; Gee, T.; Bourne, R.; Wells, G.A. Viscosupplementation for the treatment of osteoarthritis of the knee. *Cochrane Database Syst. Rev.* **2006**, *2* doi:10.1002/14651858.CD005321.pub2.
8. Miller, L.E.; Block, J.E. US-approved intra-articular hyaluronic acid injections are safe and effective in patients with knee osteoarthritis: Systematic review and meta-analysis of randomized, saline-controlled trials. *Clin. Med. Insight. Arthr. Musculoskelet. Disord*. **2013**, *6*, 57–63.
9. Bannuru, R.R.; Vaysbrot, E.E.; Sullivan, M.C.; McAlindon, T.E. Relative efficacy of hyaluronic acid in comparison with NSAIDs for knee osteoarthritis: A systematic review and meta-analysis. *Semin. Arthr. Rheum.* **2013**, *43*, 593–599.
10. Rutjes, A.W.; Jüni, P.; da Costa, B.R.; Trelle, S.; Nüesch, E.; Reichenbach, S. Viscosupplementation for osteoarthritis of the knee: A systematic review and meta-analysis. *Ann. Intern. Med.* **2012**, *157*, 180–191.

11. Hochberg, M.C.; Altman, R.D.; April, K.T.; Benkhalti, M.; Guyatt, G.; McGowan, J.; Towheed, T.; Welch, V.; Wells, G.; Tugwell, P. American college of rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. *Arthr. Care Res.* **2012**, *64*, 465–474.
12. Colen, S.; van den Bekerom, M.P.; Mulier, M.; Haverkamp, D. Hyaluronic acid in the treatment of knee osteoarthritis: A systematic review and meta-analysis with emphasis on the efficacy of different products. *Bio. Drugs* **2012**, *26*, 257–268.
13. Lindenhayn, K.; Heilmann, H.H.; Niederhausen, T.; Walther, H.U.; Pohlenz, K. Elimination of tritium-labelled hyaluronic acid from normal and osteoarthritic rabbit knee joints. *Eur. J. Clin. Chem. Clin. Biochem.* **1997**, *35*, 355–363.
14. Lindqvist, U.; Tolmachev, V.; Kairemo, K.; Aström, G.; Jonsson, E.; Lundqvist, H. Elimination of stabilised hyaluronan from the knee joint in healthy men. *Clin. Pharmacokinet*. **2002**, *4*, 603–613.
15. Larsen, N.E.; Dursema, H.D.; Pollak, C.T.; Skrabut, E.M. Clearance kinetics of a hylan-based viscosupplement after intra-articular and intravenous administration in animal models. *J. Biomed. Mater. Res. B Appl. Biomater.* **2011**, doi:10.1002/jbm.b.31971.
16. Stern, R.; Kogan, G.; Jedrzejas, M.J.; Soltés, L. The many ways to cleave hyaluronan. *Biotechnol. Adv.* **2007**, *25*, 537–557.
17. Hokputsa, S.; Jumel, K. A comparison of molecular mass determination of hyaluronic acid using SEC/MALLS and sedimentation equilibrium. *Eur. Biophys. J.* **2003**, *32*, 450–456.
18. Grange, L.; Nguyen, M.V.; Lardy, B.; Derouazi, M.; Campion, Y.; Trocme, C.; Paclet, M.H.; Gaudin, P.; Morel, F. NAD(P)H oxidase activity of Nox4 in chondrocytes is both inducible and involved in collagenase expression. *Antioxid. Redox. Signal.* **2006**, *8*, 1485–1496.
19. Rousset, F.; Nguyen, M.V.; Grange, L.; Morel, F.; Lardy, B. Heme oxygenase-1 regulates matrix metalloproteinase MMP-1 secretion and chondrocyte cell death via Nox4 NADPH oxidase activity in chondrocytes. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0066478.
20. Rousset, F.; Lardy, B.; Grange, L.; Morel, F. Differential impact of glucosamine sulfate and cuivramine on the il-1β stimulated c-20/a4 chondrocyte cell line *in vitro*. *Osteoarthr. Cartil.* **2012**, *20*, doi:10.1016/j.joca.2012.02.216.
21. Mannitol. INCHEM International program for Chemical safety (IPCS). Available online: http://www.inchem.org/documents/jecfa/jecmono/v21je10.htm (accessed on 6 June 2014).
22. VIDAL. Available online: http://www.vidal.fr/recherche/index/q:mannitol/sorbitol/classe_therapeutique (accessed on 6 June 2014).
23. Heisel, J.; Kipshoven, C. Safety and efficacy findings from a non-interventional study of a new hyaluronic acid/sorbitol formulation (GO-ON matrix) for intra-articular injection to relieve pain and disability in osteoarthritis patients. *Drug Res*. **2013**, *63*, 445–449.
24. Mendoza, G.; Alvarez, A.I.; Pulido, M.M.; Molina, A.J.; Merino, G.; Real, R.; Fernandes, P.; Prieto, J.G. Inhibitory effects of different antioxidants on hyaluronan depolymerization. *Carbohydr. Res.* **2007**, *342*, 96–102
25. Belda, J.I.; Artola, A.; García-Manzanares, M.D.; Ferrer, C.; Haroun, H.E.; Hassanein, A.; Baeyens, V.; Munoz, G.; Alió, J.L. Hyaluronic acid combined with mannitol to improve

protection against free-radical endothelial damage: Experimental model. *J. Cataract. Refract. Surg*. **2005**, *31*, 1213–1218.
26. Wang, C.; Chen, Y.; Hu, M.L.; Ding, J.; Xu, C.; Wang, R. *In vitro* antioxidant activities of the polysaccharides from Tricholoma lobayense. *Int. J. boil. Macromol.* **2012**, *50*, 534–539.
27. Raza, W.; Yang, W.; Jun, Y.; Shakoor, F.; Huang, Q.; Shen, Q. Optimization and characterization of a polysaccharide produced by Pseudomonas fluorescens WR-1 and its antioxidant activity. *Carbohydr. Polym.* **2012**, *90*, 921–929.
28. Yao, X.C.; Cao, Y.; Pan, S.K.; Wu, S.J. Preparation of peach gum polysaccharides using hydrogen peroxide. *Carbohydr. Polym.* **2013**, *94*, 88–90.
29. Tankovska, M.; Soltes, L.; Vikartovska, A.; Mendichi, R.; Lath, D.; Molnarova, M.; Gemeiner, P. Sudy of hyaluronan degradation by means of rotational viscometry: Contribution of the material of viscometer. *Chem. Pap.* **2004**, *58*, 348–352.
30. Milas, M.; Rinaudo, M. Characterization and Properties of Hyaluronic Acid (Hyaluronan). In *Polysaccharides: Structural Diversity and Functional Versatility*, Dimitriu, S. Ed.; Marcel Dekker Inc.: New York, NY, USA, 2004.
31. Magovern, G.J., Jr.; Bolling, S.F.; Casale, A.S.; Bulkley, B.H.; Gardner, T.J. The mechanism of mannitol in reducing ischemic injury: Hyperosmolarity or hydroxyl scavenger? *Circulation* **1984**, *70*, 91–95.
32. Suzuki, J.; Imaizumi, S.; Kayama, T.; Yoshimoto, T. Chemiluminescence in hypoxic brain—The second report: Cerebral protective effect of mannitol, vitamin E and glucocorticoid. *Stroke* **1985**, *16*, 695–700.
33. Fu, W.; Jiao, X. The effect of mannitol and anisodim on the prevention of free radical injury to post-ischemia flaps: an experimental study. *Br. J. Plast. Surg.* **1995**, *48*, 218–221.
34. Frati, E.; Khatib, A.M.; Front, P.; Panasyuk, A.; Aprile, F.; Mitrovic, D.R. Degradation of HA by photosensitized riboflavin *in vitro:* Modulation of the effect by transition metals, radical quenchers, and metal chelators. *Free Radic. Biol. Med.* **1997**, *22*, 1139–1144.
35. Borras-Verdera, A.; Calcedo-Bernal, V.; Ojeda-Levenfeld, J.; Clavel-Sainz, C. Efficacy and safety of a single intra-articular injection of 2% hyaluronic acid plus mannitol in knee osteoarthritis over a 6-month period. *Rev. Esp. Cir. Ortop. Traumatol.* **2012**, *56*, 274–280.