HYALURONAN DEGRADATION BY ASCORBATE: PROTECTIVE EFFECTS OF MANGANESE(II) CHLORIDE

KATARÍNA VALACHOVÁ, GRIGORIJ KOGAN,*[1] PETER GEMEINER* and LADISLAV ŠOLTÉS

Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia
**Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Bratislava, Slovakia*
([1]Current address: Health Directorate, Directorate-General for Research, European Commission, B-1049, Brussels, Belgium)

Dedicated to Professor G. E. Zaikov,
on the occasion of his 75th anniversary

Received December 21, 2008

Six samples of high-molar-mass hyaluronan were subjected to radical-initiated degradation in an experimental system containing ascorbic acid, with the addition of transition metals – iron or copper – in different concentrations. Such a system closely resembles the environment occurring in the synovial fluid of the joints and thus can serve as a model for monitoring oxidative degradation of hyaluronan under physiological and pathophysiological conditions. Oxidative degradation of hyaluronan resulted in the decrease of its molar mass, which was monitored by rotational viscometry. The addition of manganese(II) chloride was found to retard/inhibit the oxidative damage of hyaluronan.

Keywords: hyaluronan degradation, rotational viscometry, ascorbic acid, $CuCl_2$, $FeCl_2$, $MnCl_2$

INTRODUCTION

Some biogenic transition metals, such as iron, copper, manganese, zinc and cobalt, participate at the control of various metabolic and signaling pathways. However, their versatile coordination chemistry and redox properties allow them to escape the control mechanisms, such as homeostasis, transport, compartmentalization and binding to the designated tissue and cell constituents.[1] In a wide variety of *in vitro* systems, Fe(II) salts and/or non-enzyme complexed ferrous cations (*e.g*. Fe(II)-EDTA) were shown to enhance oxygen radical damage by increasing the production of an oxidative species, generally believed to be the hydroxyl free radical. Iron ions are known to cause peroxidation of (polyunsaturated) fatty acids in lipids (LH) and to generate peroxyl lipid radicals ($LOO^\bullet$), by the following sequence of reactions:

$2Fe(II) + 2O_2 \leftrightarrow$
$2Fe(III) + 2O_2^{\bullet-}$ (redox reaction; reversible) (1)
$2O_2^{\bullet-} + 2H^+ \rightarrow$
$H_2O_2 + O_2$ (dismutation reaction) (2)

$2Fe(II) + O_2 + 2H^+ \rightarrow$
$2Fe(III) + H_2O_2$ net reaction

$Fe(II) + H_2O_2 \rightarrow$
$Fe(III) + {}^\bullet OH + HO^-$ (Fenton reaction) (3)
$O_2^{\bullet-} + H_2O_2 \leftrightarrow$
$HO^\bullet + HO^- + O_2$ (Haber-Weiss reaction) (4)
$LH + {}^\bullet OH \rightarrow$
$L^\bullet + H_2O$ (5)
$L^\bullet + O_2 \rightarrow LOO^\bullet$ (6)
peroxidation reaction.

The LOO• radicals propagate the lipid peroxidation chain reactions LOO• + LH → LOOH + L•. LOOH oxidizes the ferrous ions, yielding alkoxyl lipid radicals LOOH + Fe(II) → LO• + Fe(III) + HO–, while the generated LO• radicals participate in the propagation phase of the lipid peroxidation reaction LO• + LH → LOH + L•.

Ascorbate (Asc^-) is one of the most efficient (bio)reductants capable to keep the iron ions in a lower oxidation state and/or to recycle Fe(III) to Fe(II). The so-called iron-catalyzed ascorbate auto-oxidation yields an intermediate – the semidehydroascorbate radical ($Asc^{\bullet-}$) – a low-reactive radical that can undergo a dismutation/ disproportion-nation reaction to form Asc^- and dehydroascorbate (DHA):

$$Asc^- + Fe(III) \rightarrow Asc^{\bullet-} + Fe(II) \quad (7)$$

$$2Asc^{\bullet-} \rightarrow Asc^- + DHA \quad (8)$$

Alternatively, complexes of Fe(II) ions and dioxygen are also assumed to yield reactive species of unknown nature, which are subsequently able to oxidize the biological material.[2,3] A combination of ascorbate *plus* Cu(II) under aerobic conditions, the so-called Weissberger's system,[4,5] gives rise[6-8] directly to hydrogen peroxide (Scheme 1) and, taking into account the fact that ascorbate reduces Cu(II) to cuprous ions, it may be assumed that, during copper-catalyzed ascorbate auto-oxidation, $^{\bullet}OH$ radicals should be generated by a Fenton-type reaction $Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + HO^-$. This conclusion was recently supported by the unambiguous proof of the production of hydroxyl radicals in a system containing ascorbate *plus* $CuCl_2$ by the EPR spin-trap technique,[9] applying spin traps such as 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO).

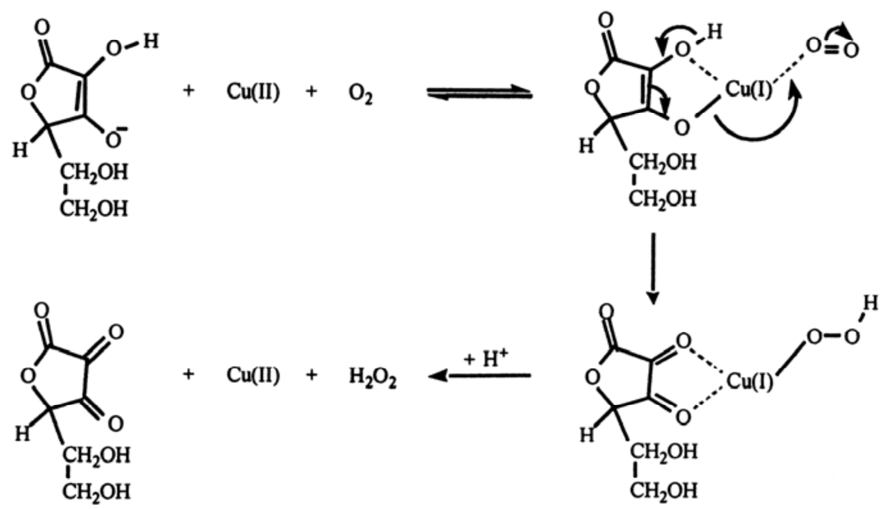
The oxidative damage of various biomolecules (lipids, enzymes, DNAs, etc.) with (catalytic) participation of inorganic Fe and/or Cu salts/complexes has been clearly demonstrated in many *in vitro* assays. Yet, under physiological conditions, and taking into account the negligible availability of the "free catalytic iron", the significance of, *e.g.* the Fenton reaction, cannot be fully clarified. The average-mass human body contains approximately 4-5 g iron bound to hemoglobin, myoglobin, cytochromes, iron-containing enzymes and also to the iron-storage proteins – ferritin, transferrin and hemosiderin. Similarly, about 95% of the copper circulating in the blood is bound to ceruloplasmin. Further, copper is bound/ligated to albumin, transcuprein and CuZn-superoxide dismutase.

Unlike Fe and Cu, inorganic salts/complexes of the biogenic transition metal – Mn – are known to occur at high concentrations in certain cells. As reported,[10] manganese concentrations in most adult human tissues range between 3 and 20 μM. The results of several *in vitro* studies suggest that Mn in various forms does indeed inhibit the damage mediated by $^{\bullet}OH$ radicals, but only if their production is dependent on the presence of $O_2^{\bullet-}$ or H_2O_2. Thus, Mn complexes appear to interact[11] with $^{\bullet}OH$, as well as with $O_2^{\bullet-}$ and H_2O_2, in a fundamentally different fashion than the Fe and Cu ones.

Hyaluronan (HA, Fig. 1) is a high-molar-mass glycosaminoglycan with important functions in the living organism.[12,13] HA macromolecules with molar mass equaling several MDa are extruded in the synovial fluid (SF) by synoviocytes/hyalocytes – the cells embedded in the synovial membrane. The free/non-associated HA present in SF determines its unique viscoelastic properties required for maintaining a proper functioning of joints in vertebrates.

It is noteworthy that the half-life of HA in SF is only of a few hours. Although, in most tissues, the relatively fast HA catabolism is controlled by hyaluronidases, in SF – due to the absence of these enzymes – different mechanisms are implemented in the rapid hyaluronan catabolism. One of the possible alternative mechanisms involved in the joints of healthy individuals is the oxidative/degradative action of the reactive oxygen species (ROS) generated during the ascorbate auto-oxidation catalyzed by transition metal (Fe and/or Cu) ions.[14] Evidence exists that ROS are responsible for HA degradation in inflammatory joint diseases, such as osteoarthritis and rheumatoid arthritis (RA). HA involvement in activation and modulation of the inflammatory response also includes[15] its scavenging action towards ROS, such as $^{\bullet}OH$ radicals.

Under aerobic conditions, a ternary system, comprising HA macromolecules *plus* ascorbate and traces of iron or copper ions, induces a gradual decrease in the viscosity of the HA solution, as a result of fragmentation/ degradation of the HA macromolecules. However, as to the effects of manganese on HA degradation invoked by ascorbate auto-oxidation, not a single study has been so far published. The impact of Mn(II) ions is evidenced[16,17] by a known catalytic participation of this transition metal essential for hyaluronan synthase(s). As reported, certain Mn(II) complexes, including biologically-relevant Mn(II) pyrophosphate and Mn(II) polyphosphate, can act[11] as very effective antioxidants by indirectly suppressing or blocking $^{\bullet}OH$ formation, due to Fenton-, Haber-Weiss-, xanthinoxidase-Fe-EDTA-, or Fe(III)-H_2O_2-type reactions, precisely as superoxide dismutase and catalase do.



Scheme 1: Generation of H_2O_2 *via* Weissberger's system from ascorbate and Cu(II), adapted from Fisher and Naughton[8]

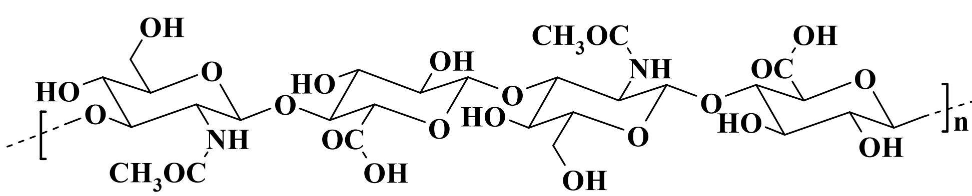


Figure 1: Hyaluronan – acid form

It has been established[18,19] that these two major antioxidatively acting enzymes are barely detectable in the rheumatoid synovial fluid. Their levels in SF, if any, do not exceed[20] 1 and 50 ng/mL, respectively.

The potential use of manganous salts/complexes for protecting lipids against oxidative stress has been demonstrated in several *in vitro* and *in vivo* studies.[11,21-34]

The results reported indicate that Mn(II) scavenges superoxide anion radicals already at nanomolar concentrations, whereas its micromolar concentrations are required to scavenge the hydroxyl radicals.

Increasing concentrations of manganese suppress lipid peroxidation even more strongly and complete inhibition is reached[21] at concentrations of 30 μM Mn(II). Manganese may act as a chain-breaker in inhibiting iron-induced lipid peroxidation chain reactions,[31,33] and, as proposed,[26,31] Mn(II) may scavenge peroxyl lipid radicals *via* the reaction below, quenching in this way the propagation reactions of lipid peroxidation caused by the hydroxyl radicals generated by pro-oxidative transition metals such as iron and copper:

$$LOO^{\bullet} + Mn(II) + H^{+} \rightarrow LOOH + Mn(III) \quad (9)$$

The present study investigates the function of trace concentrations of Fe(II), Cu(II), as well as of Mn(II) in ascorbate auto-oxidation, in which hyaluronans of various molar masses are involved as indicators of pro- or antioxidative properties of the system.

EXPERIMENTAL

Biopolymers

Six hyaluronan samples, covering by their molar-mass averages (M_w) the range of 0.43 to 1.3 MDa (Table 1), were kindly donated by or were purchased[35,36] from the following HA manufacturers: Genzyme Corporation, Cambridge, MA, USA; Sigma Chemicals Co., St. Louis, MO, USA; Lifecore Biomedical Inc., Chaska, MN, USA; and CPN Ltd., Ústí nad Orlicí, Czech Republic. In the HA samples P9706-6 and P9710-2, the contents of the following (transition) metals was stated by the manufacturer (in ppm): Fe = 27 and 13; Pb = 6 and 7; Cu = 3 and 4, respectively; Cr, Co, Ni < 3 and As, Cd, Hg < 1, in both samples – Certificate of Analysis (Lifecore Biomedical Inc., Chaska, MN, USA).

Table 1
Characteristic parameters of the six HA samples

Sample	Parameter[a] [unit]		
	M_w [kDa]	M_w/M_n	Rg [nm]
B22157	1340	1.50	129.8
53H0439	1017	1.82	130.7
P9710-2A[b]	808.7	1.63	110.0
P9706-6	803.9	1.64	107.9
CPN	659.4	1.88	97.4
1-9100-1	426.2	1.84	77.2

[a]M_w – weight-average of molar masses, M_w/M_n – polydispersity index and Rg – z-average of the gyration radius; [b]Aged HA sample[36]

Chemicals

Analytical purity grade NaCl and $CuCl_2{\cdot}2H_2O$ were obtained from Slavus Ltd., Bratislava, Slovakia; $FeCl_2{\cdot}4H_2O$ was purchased from Penta CZ Ltd., Chrudim, Czech Republic; $MnCl_2{\cdot}4H_2O$ was purchased from Lachema CZ Ltd., Brno, Czech Republic; and ascorbic acid (AA) – from Merck KGaA, Darmstadt, Germany. Redistilled de-ionized quality grade water with ≤ 0.055 μS/cm conductivity was prepared by the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

Hyaluronan degradation/Rotational viscometry

The HA sample (20 mg) was dissolved overnight in the dark in 0.15 M NaCl, in two steps: first, 4.0 mL of the solvent were added in the morning and 3.95 mL were added after 6 h. The following morning, 50.0 μL of 16.0 mM AA dissolved in 0.15 M NaCl were added to the HA solution and blended for 30 s. The resulting solution (8 mL) containing HA (2.5 mg/mL) and AA (100 μM) was transferred into a Teflon® cup reservoir of the Brookfield LVDV-II+PRO rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, USA). Recording of the viscometer output parameters started 2 min after the experimental onset. Solution dynamic viscosity (η) was measured at 25.0 ± 0.1 °C, over 3 min intervals, for up to 5 h. The viscometer Teflon® spindle rotated at 180 rpm, *i.e.* at a shear rate equal[36] to 237.6 s^{-1}.

When the effect of the addition of a single biogenic transition metal was investigated, the second portion of the aqueous NaCl solvent was only 3.90 mL. The following morning, the addition of 50.0 μL of 16.0 mM AA to the HA solution was followed by the admixing of 50.0

μL of appropriate $FeCl_2$, $CuCl_2$ or of $MnCl_2$ solutions in 0.15 M aqueous NaCl. The concentration of the biogenic transition metal salt in the system was of 0.5 or 5.0 μM, when using $FeCl_2$; 0.1, 1.0 or 5.0 μM with $CuCl_2$; and 0.5 μM with $MnCl_2$.

When assessing the (inhibitory) action of the Mn(II) ions on HA degradation by the system comprising AA (100 μM) and $CuCl_2$ (1.0 μM), the second portion of aqueous NaCl was of 3.85 mL. 50 μL of $MnCl_2$ solution in 0.15 M aqueous NaCl were added to adjust the final Mn(II) concentration to 30 μM.

Three different application schemes of AA and of the two metal ions were tested, namely: (**i**) Mn(II) followed by (AA) and Cu(II); (**ii**) AA followed by Mn(II) and Cu(II); and (**iii**) Mn(II) followed by Cu(II) and AA.

In each case, a homogenous solution was obtained after 30 s of moderate stirring of the mixture, upon addition of AA or transition metals. Under the above-specified experimental settings, the torque values ranged between 82 and 23%.

RESULTS

As shown in Figure 2, left panel, the dynamic viscosity *vs*. time relationship of the solutions containing HA (2.5 mg/mL) and AA (100 μM), with the exception of the two samples, CPN and 1-9100-1, indicates the presence of two distinct regions: (***i***) rheopectic and (***ii***) a region that should be assigned to the degradation of HA macromolecules. As calculated from the Certificate of Analysis of the HA samples P9706-6 and P9710-2, the solutions of these two samples contained the following concentrations of iron and copper ions: 1.209 and 0.118 μM (P9706-6) or 0.582 and 0.157 μM (P9710-2), respectively. Due to the presence of these biogenic transition metal ions, Fe(III)/Fe(II) and Cu(II)/Cu(I) catalyzed ascorbate auto-oxidation leads to the generation of $^{\bullet}OH$ radicals which, after a certain initiation period, promote the degradation of the HA macromolecules, manifested by a gradual decrease in solution dynamic viscosity.

The results presented in Figure 2, right panel, indicate that even a submicromolar addition of Mn(II) ions (0.5 μM) prolong the rheopectic region in the η *vs*. time plot up to 300 min, the total time of monitoring, which is especially recognizable for samples B22157, P9706-6 and P9710-2A. No changes at all or only small ones occurred when using 0.5 μM Mn(II) ions with HA samples with lower molar mass. It should be also noted that most curves suggested a slight decrease in viscosity after Mn(II) addition.

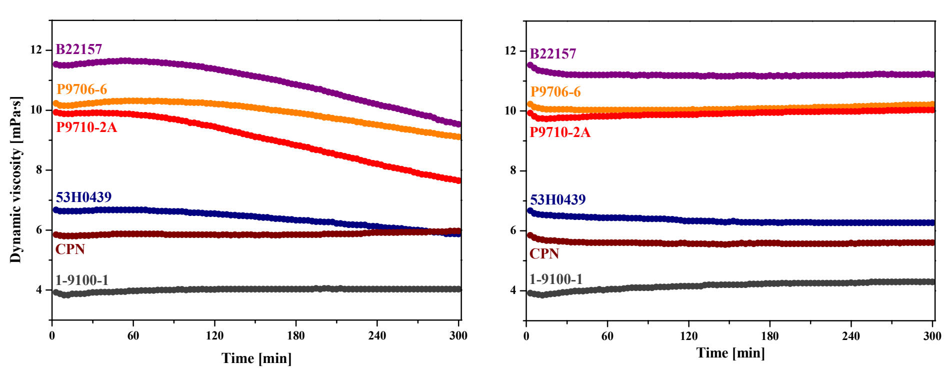


Figure 2: Time dependence of dynamic viscosity of the HA solutions (left panel: Solutions of hyaluronan samples with addition of 100 μM AA; right panel: Solutions of hyaluronan samples with addition of 100 μM AA and 0.5 μM $MnCl_2$)

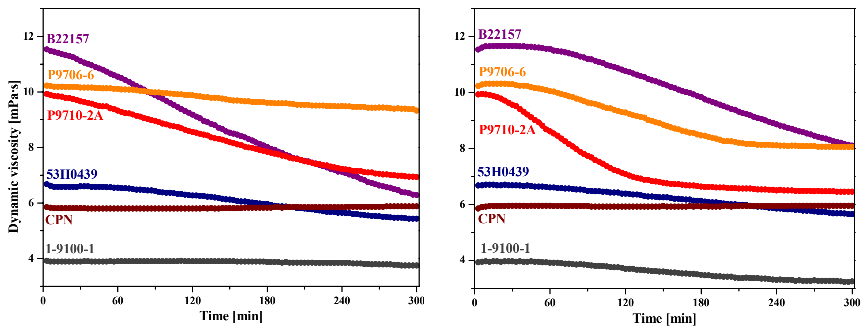


Figure 3: Time dependence of dynamic viscosity of the HA solutions (left panel: Solutions of hyaluronan samples with addition of 100 μM AA and 0.5 μM $FeCl_2$; right panel: Solutions of hyaluronan samples with addition of 100 μM AA and 0.1 μM $CuCl_2$)

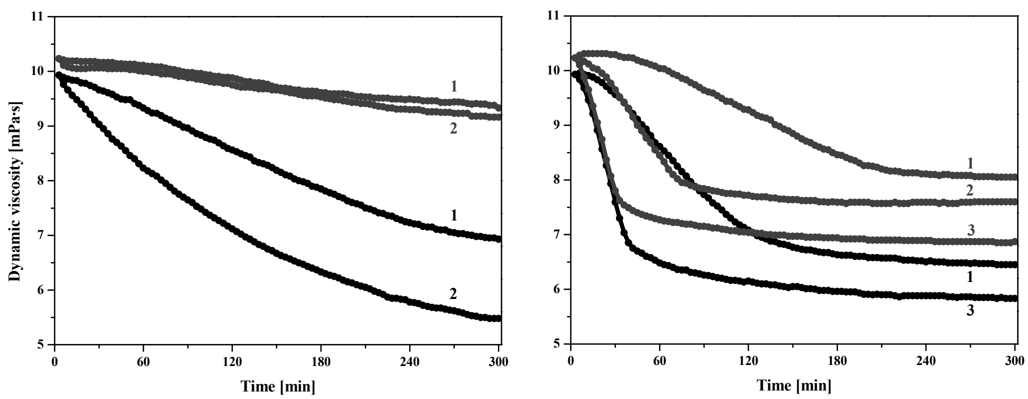


Figure 4: Time dependence of dynamic viscosity of HA P9710-2A (black lines) and P9706-6 (grey lines) sample solutions (left panel: Solutions of hyaluronan samples with addition of 100 μM AA and of 0.5 (1) or 5.0 μM (2) $FeCl_2$; right panel: Solutions of hyaluronan samples with addition of 100 μM AA and of 0.1 (1), 1.0 (2) or 5.0 μM (3) $CuCl_2$)

Contrary to the "antioxidative" action of $MnCl_2$, an identical 0.5 μM concentration of $FeCl_2$, or even a smaller (0.1 μM) concentration of $CuCl_2$ had a "pro-oxidative" effect on the degradation of HA macromolecules in most samples (compare the data presented in the left and right panels in Fig. 3 with those in Fig. 2, left panel). The only exception was observed for sample CPN, in which, however, a relatively high content of "a contaminant" – transition metal Mn ions – was detected (Prof. A. Staško, Slovak Technical University, Bratislava, Slovakia, personal communication).

The pro-oxidative effect of Fe or Cu ions addition is clearly indicated in a concentration-dependent manner (Fig. 4, left and right panels). A slight difference should be however pointed out as to the "nominal" $\eta_{2'}$ values, *i.e.* the values observed at the 2nd min after the addition of metal ions, in different concentrations (0.1-5.0 μM), to the solutions containing HA (2.5 mg/mL) and AA (100 μM). For a better visualization, the $\eta_{2'}$ value of the solutions containing metal salts was shifted to the value $\eta_{2'}$ valid for solutions comprising only HA and AA. By such "normalization", the changes in the values of dynamic viscosity caused by different amounts of added $FeCl_2$ and $CuCl_2$ became more visible (as presented in both panels of Fig. 4). As one can see, the character of the time dependence of η on the addition of $FeCl_2$ can be described as a gradual monotonous concentration-dependent decline, while the addition of $CuCl_2$ resulted in a literally "dramatic" drop of η over a very short time interval, after which its decrease continued, however at a much lower rate. A possible explanation of this dissimilarity may most probably lie in the different reaction kinetics of the processes leading to ROS generation in the ascorbate *plus* $FeCl_2$ system, as well as in the ascorbate *plus* $CuCl_2$ one.

The results presented in Figure 5 illustrate the effect of Mn(II) addition on η *vs*. time dependence of the solutions comprising high-molar-mass hyaluronan (2.5 mg/mL), ascorbate (100 μM) and $CuCl_2$ (1.0 μM). $MnCl_2$ addition in a relatively high concentration (30 μM) resulted in a significantly decreased degradation of the HA macromolecules, however, none of the application schemes used (**i**, **ii**, **iii**) inhibited totally biopolymer degradation. While, in the case of sample P9710-2A, by using the application scheme (**iii**), sample damage decreased to ca. 44% (Fig. 5, left panel), in the case of sample P9706-6, scheme (**i**) proved to be the most efficient one, *i.e.* the extent of degradation decreased to ca. 39% (Fig. 5, right panel).

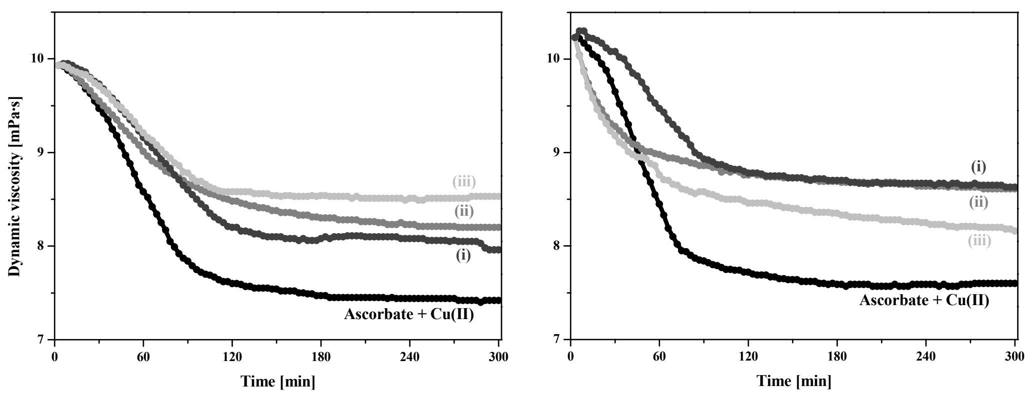


Figure 5: Time dependence of dynamic viscosity of HA sample solutions P9710-2A (left panel) and P9706-6 (right panel) after addition of AA and $CuCl_2$; (**i**) $MnCl_2$ followed by AA and $CuCl_2$; (**ii**) AA followed by $MnCl_2$ and $CuCl_2$; and (**iii**) $MnCl_2$ followed by $CuCl_2$ and AA. Concentrations used: 100 μM AA, 1.0 μM $CuCl_2$ and 30 μM $MnCl_2$

DISCUSSION

Under physiological conditions as well as in the early stages of acute-phase joint inflammation, the contribution of ascorbate auto-oxidation to the non-enzymatic catabolism of high-molar-mass hyaluronan in SF is plausible, since: (***a***) in a healthy human being, the content[12] of free HA macromolecules in SF is 1.4-3.6 mg/mL; (***b***) the concentration of ascorbate in the SF of healthy subjects reaches[18] values close to those established in blood serum, *i.e.* 40-140 μM; (***c***) the total concentrations of Fe and Cu ions in the SF of healthy human beings equal 5.2 and 4.3 μM, respectively, while rising[37] under pathological/inflammatory conditions, such as osteoarthritis (OA) and RA; (***d***) in the SF of individuals suffering from RA, total Cu concentration increases more than three times, compared to that of the healthy population,[37] while their Cu concentration in the SF ultrafiltrate equals[38] 0.125 ± 0.095 μM; (***e***) on ascorbate auto-oxidation with the catalytic contribution of Cu(II) traces, direct conversion/transformation of O_2 to H_2O_2 takes place;[4-8] (***f***) the average concentration of Mn ions in the SF of healthy persons and patients with RA is relatively low[37] (ca. 0.42 and 0.44 μM, respectively).

The investigation on the participation of biogenic transition metals in the oxidative damage of high-molar-mass HA appears to be very simple. However, at least two main technical/experimental obstacles should be pointed out. Firstly, due to the extremely high aggressiveness of the oxidative/radical processes, the materials contacting, *e.g*., the •OH radicals, should be non-metallic (preferably made of glass or Teflon®).[35,39] Secondly, at present, such studies are substantially limited due to the unavailability of the ultrapure HA reference preparations with sufficiently high molar masses completely devoid of contaminating metals.

The above limitations have been circumvented in our studies by using a Brookfield LVDV-II+PRO rotational viscometer equipped with a cup reservoir and spindle, both home-made of Teflon®, and HA samples with sufficiently high purity. The samples, coded P9706-6 and P9710-2, with a known content of the given (transition) metals, allowed to calculate the concentrations of iron and copper ions in the solutions of these samples. Despite their poor "identity" as to the content of metal impurities, the other four samples were used since their mean molar masses covered a relatively broad range, from 0.43 to 1.3 MDa (Table 1).

Under the conditions applied in the study (neutral pH), the HA macromolecules are present in a highly ionized state:[40] the pK_a value for the D-glucuronic acid residues is 3.12. The D-glucuronic structural units of the HA polyanion form salts with transition metal counter-cations. As formerly reported,[41] HA is able to weakly bind cupric ions (the binding constant[42] being 3.0×10^3 L/mol). Generally, the transition metal counter-cations form coordinate complex compounds, in which the metal cation can be fixed[41,43] either intra- or inter-molecularly, simply *via* the carboxyl groups of HA. However, especially for the copper-hyaluronate coordinates, it has been suggested that, in the metal ion, "binding" site electrons of the nearest *N*-acetyl group from the same HA molecule might be involved,[44] along with the COO^- group.

The •OH radical, whose redox potential (•OH/H_2O) equals +2.31 V at pH 7, is classified as the most efficient initiator of radical oxidation/degradation accepting an H• radical from the hyaluronan macromolecule. The formation of •OH radicals may occur in several ways while by far the most important *in vivo* mechanism is mediated by hydrogen peroxide. Under aerobic conditions, its generation proceeds according to reactions **1** and **2**, as well as *via* those depicted in Scheme 1. Thus, on addition of a high excess of AA (100 μM), trace amounts (submicro/micro) of Fe and/or Cu ions present in the HA samples undergo multiple redox cycles, generating a flux of •OH radicals. A lag-phase, registered during the experiments (Fig. 2, left panel), means that a given amount of initiating •OH radicals should be generated right in the beginning. An excess amount of either Fe or Cu ions added to the system comprising hyaluronan and ascorbate yields a much higher flux of •OH radicals (compare the results in Figs. 3 and 4 with those from Fig. 2, left panel). The higher the metal content, the shorter or even absent is the lag-phase. Moreover, when assessing the effect of $CuCl_2$ added to the system (Fig. 4, right panel), a really dramatic decline in the η *vs*. time dependence can be observed, for all HA sample solutions investigated.

The pro-oxidative effect of both biogenic transition metals, *i.e*. Fe and Cu, evidenced in the present study, corroborates the observations already published by several authors[5,18,45-53] on HA degradation caused by ascorbate alone or in combination with Fe and/or Cu ions. However, the study protocol including assays on the effect of another biogenic transition metal, manganous ions, may to our knowledge be classified as pioneering. When added into the system comprising hyaluronan macromolecules, the reductant – the ascorbate – and the pro-oxidatively acting Fe and/or Cu ions, Mn(II) ions demonstrate a significant antioxidative effect. The high-molar-mass HA samples were protected against degradation by the addition of a relatively low amount of $MnCl_2$ (0.5 μM; Fig. 2, right panel), *i.e*. comparable[37] to the Mn contents in the SF of healthy persons (0.42 μM on average). On the other hand, in the case of a "massive" load of Cu(II) ions (1.0 μM), the added $MnCl_2$ was also effective (Fig. 5, both panels), yet it should be pointed out that not even the highest concentration applied (30 μM) could prevent the oxidative damage of the HA macromolecules.

Changes in the chemical structure of the HA chain, occurring during the metal ion-catalyzed ascorbate auto-oxidation with the participation of manganous salts, leading to the formation of an increased fraction of hyaluronan hydroperoxides (and/or of AOH-type derivatives), according to reactions **10** and **11**:

$$AOO^{\bullet} + Mn(II) + H^{+} \rightarrow AOOH + Mn(III) \quad (10)$$

$$AO^{\bullet} + Mn(II) + H^{+} \rightarrow AOH + Mn(III) \quad (11)$$

analogously to reaction **9** suggested by Coassin *et al*. and Sziraki *et al*.,[26,31,33] are still to be demonstrated. The hyaluronan hydroperoxides thus produced should, however, be decomposed under the action of the two transition metal cations occurring in lower oxidation states – Cu(I) and Fe(II).

For detecting such AOOH and/or AOH type derivatives, non-isothermal chemi-luminometry and MALDI-TOF mass spectrometry are proposed[9,54,55] as relevant analytical methods.

CONCLUSIONS

The structure[56] of the electronic orbitals of iron – $1s^22s^22p^63s^23p^63d^64s^2$ – and its high redox potential value – Fe(III)/Fe(II) equaling +0.48 V at pH 7 – predetermine iron as one of the major participants in the production (and metabolism) of free radicals in biological systems. While, at physiological pH values, Fe(III) precipitates as oxyhydroxide aggregates, compounds containing Fe(II) are soluble, though unstable, and tend to react with oxygen to form the superoxide anion radical ($O_2^{\bullet-}$) and Fe(III). However, the biological reductant present in the system, *i.e.* ascorbate (Asc^-), restores iron's lower oxidation state, according to reaction **7**. Thus, the so-called "auto-oxidation" of ascorbate is actually mediated by trace amounts of transition metals, such as iron.[57] In any case, it should be pointed out that the biological consequences of the interaction of ascorbate (vitamin C) with iron are not yet fully understood.

Over the past decades, the pro-oxidant properties of ascorbate have been also investigated, in addition to its better explored antioxidant role.

The copper electron configuration is $1s^22s^22p^63s^23p^63d^{10}4s^1$ and the value of its redox potential Cu(II)/Cu(I) is +0.16 V at pH 7. The bivalent Cu(II) is paramagnetic ($3d^9$) and represents the most stable oxidation state of copper. Ceruloplasmin carries copper atoms in both Cu(II) and Cu(I) states. However, it has been demonstrated that a fraction of loosely bound copper may be "liberated" under certain circumstances. Moreover, reactive oxygen species appear to disrupt copper binding to ceruloplasmin, thereby impairing its normal protective function while releasing free copper, which, in turn, may promote oxidative pathology.[58] Since ascorbate acts as a powerful reducing agent with a redox potential of +0.282 V for the redox couple $Asc^{\bullet-}/Asc^-$ at pH 7, it should reduce Cu(II) to Cu(I) and hence cuprous ions should be able to reduce the dioxygen molecules directly to H_2O_2.

The human body contains about 300 ppm of manganese; the recommended daily intake of this essential element is 3–9 mg.

The Mn electron configuration is $1s^22s^22p^63s^23p^63d^54s^2$ and the Mn(III)/Mn(II) redox potential equals +1.5 V at pH 7. Manganese exists[10] in several different oxidation states; within biological systems, however, the +2 valence prevails. Manganous ions are paramagnetic and thus detectable by EPR spectroscopy. The six-band EPR spectrum of Mn(II) has been detected in most natural (bio)products. A sub-ppm/ppm concentration of Mn in the HA samples is an established fact, which should be taken into account when performing experiments (Prof. V. Brezová, Slovak Technical University, Bratislava, Slovakia, personal communication).

Various carbohydrate-based preparations with specifically designed precise structures and molecular parameters are currently viewed as future effective tools/remedies applicable in the treatment of various diseases.[59] The so-called visco-supplementing injections of high-molar-mass hyaluronan directly into the osteoarthritic joints could be one such example. A further step forward could be the elimination or minimalization of the Fe and Cu content and a potentially advantageous addition of an appropriate Mn(II) salt/complex to the injection mixtures to be administered during visco-supplementary treatment of OA.

ACKNOWLEDGEMENTS: Grants VEGA 2/0003/08 and 2/7033/27 from the Slovak Academy of Sciences and from the Ministry of Education of the Slovak Republic, and APVV-51-017905 from the Slovak Research and Development Agency are gratefully acknowledged. The kind gift of the hyaluronan samples P9710-2 and P9706-6 from the part of Dr. K. Thacker – Lifecore Biomedical Inc., Chaska, MN, USA, is also appreciated.

REFERENCES

[1] M. Valko, H. Morris and M. T. D. Cronin, *Curr. Med. Chem.*, **12**, 1161 (2005).
[2] S. Y. Qian and G. R. Buettner, *Free Radical. Bio. Med.*, **26**, 1447 (1999).
[3] J. Flemmig and J. Arnhold, *Eur. Biophys. J.*, **36**, 377 (2007).
[4] A. Weissberger, J. E. LuValle and D. S. Jr. Thomas, *J. Am. Chem. Soc.*, **65**, 1934 (1943).
[5] M. M. Khan and A. E. Martell, *J. Am. Chem. Soc.*, **89**, 4176 (1967).
[6] A. E. O. Fisher and D. P. Naughton, *Med. Hypotheses*, **61**, 657 (2003).
[7] A. E. O. Fisher and D. P. Naughton, *Nutrition J.*, **3**, 1 (2004).
[8] A. E. O. Fisher and D. P. Naughton, *Curr. Drug Del.*, **2**, 261 (2005).
[9] L. Šoltés, M. Stankovská, V. Brezová, J. Schiller, J. Arnhold, G. Kogan and P. Gemeiner, *Carbohyd. Res.*, **341**, 2826 (2006).
[10] J. A. Roth, *Biol. Res.*, **39**, 45 (2006).
[11] P. L.-B. Cheton and F. S. Archibald, *Free Radical. Bio. Med.*, **5**, 325 (1988).
[12] G. Kogan, L. Šoltés, R. Stern and R. Mendichi, in "Handbook of Polymer Research: Monomers, Oligomers, Polymers and Composites", edited by R. A. Pethrick, A. Ballada and G. E. Zaikov, Nova Science Publishers, New York, 2007, Chapter 31, pp. 393-439.
[13] G. Kogan, L. Šoltés, R. Stern, J. Schiller and R. Mendichi, in "Studies in Natural Products Chemistry", Vol. 34, Bioactive Natural Products, edited by Atta-ur-Rahman, Elsevier, Amsterdam, 2008, Part D, pp. 789-882.
[14] L. Šoltés, M. Stankovská, G. Kogan, P. Gemeiner and R. Stern, *Chem. Biodivers.*, **2**, 1242 (2005).
[15] G. Mendoza, A. I. Álvarez, M. M. Pulido, A. J. Molina, G. Merino, R. Real, P. Fernandes and J. G. Prieto, *Carbohyd. Res.*, **342**, 96 (2007).
[16] K. A. Johnson, D. A. Hulse, R. C. Hart, D. Kochevar and Q. Chut, *Osteoarthr. Cartilage*, **9**, 14 (2001).
[17] P. H. Weigel and P. L. DeAngelis, *J. Biol. Chem.*, **282**, 36777 (2007).
[18] S. F. Wong, B. Halliwell, R. Richmond and W. R. Skowroneck, *J. Inorg. Biochem.*, **14**, 127 (1981).
[19] B. J. Parsons, S. Al-Assaf, S. Navaratnam and G. O. Phillips, in "Hyaluronan: Chemical, Biochemical and Biological Aspects", Vol. 1, edited by J. F. Kennedy, G. O. Phillips, P. A. Williams and V. C. Hascall, Woodhead Publishing Ltd, Cambridge, MA, 2002, pp. 141-150.
[20] J. M. McCord, *Science*, **185**, 529 (1974).
[21] G. S. Shukla and S. V. Chandra, *Acta Pharmacol. Toxicol.* (Copenhagen), **48**, 95 (1981).
[22] F. S. Archibald and I. Fridovich, *Arch. Biochem. Biophys.*, **214**, 452 (1982).
[23] L. Cavallini, M. Valente and A. Bindoli, *Inorg. Chim. Acta*, **91**, 117 (1984).
[24] E. C. Chang and D. J. Kosman, *J. Biol. Chem.*, **264**, 12172 (1989).
[25] J. Varani, I. Ginsburg, D. F. Gibbs, P. S. Mukhopadhyay, C. Sulavik, K. J. Johnson, J. M. Weinberg, U. S. Ryan and P. A. Ward, *Inflammation*, **15**, 291 (1991).
[26] M. Coassin, F. Ursini and A. Bindoli, *Arch. Biochem. Biophys.*, **299**, 330 (1992).
[27] B. Gray and A. J. Carmichael, *Biochem. J.*, **281**, 795 (1992).
[28] R. K. Singh, K. M. Kooreman, C. F. Babbs, J. F. Fessler, S. C. Salaris and J. Pham, *Am. J. Vet. Res.*, **53**, 1822 (1992).
[29] Y. Tampo and M. Yonaha, *Free Radical. Bio. Med.*, **13**, 115 (1992).
[30] K. Sakurai, M. Andoh, M. Yamada, Y. Kodera, H. Nishimura, M. Hiroto, A. Matsushima, M. Aoyama, H. Yamamoto and Y. Inadal. *Jpn. J. Pharmacol.*, **74**, 117 (1997).
[31] I. Sziraki, K. P. Mohanakumar, P. Rauhala, H. G. Kim, K. J. Yeh and C. C. Chiueh, *Neuroscience*, **85**, 1101 (1998).
[32] S. Hussain and S. F. Ali, *Neurosci. Lett.*, **261**, 21 (1999).
[33] I. Sziraki, P. Rauhala, K. K. Koh, P. V. Bergen and C. C. Chiueh, *Neurotoxicology*, **20,** 455 (1999).
[34] C. G. Worley, D. Bombick, J. W. Allen, R. L. Suber and M. Aschner, *Neurotoxicology*, **23**, 159 (2002).
[35] M. Stankovská, L. Šoltés, A. Vikartovská, R. Mendichi, D. Lath, M. Molnárová and P. Gemeiner, *Chem. Pap.*, **58**, 348 (2004).
[36] L. Šoltés, K. Valachová, R. Mendichi, G. Kogan, J. Arnhold and P. Gemeiner, *Carbohyd. Res.*, **342**, 1071 (2007).
[37] W. Niedermeier and J. H. Griggs, *J. Chron. Dis.*, **23**, 527 (1971).

[38] D. P. Naughton, J. Knappitt, K. Fairburn, K. Gaffnev, D. R. Blake and M. Grootveld, *FEBS Lett*., **361**, 167 (1995).
[39] M. Stankovská, L. Šoltés, A. Vikartovská, P. Gemeiner, G. Kogan and D. Bakoš, *Biologia*, **60**/Suppl. 17, 149 (2005).
[40] J. W. Park and B. Chakrabarti, *Biopolymers*, **17**, 1323 (1978).
[41] L. Nagy, S. Yamashita, T. Yamaguchi, P. Sipos, H. Wakita and M. Nomura, *J. Inorg. Biochem*., **72**, 49 (1998).
[42] N. Figueroa, B. Nagy and B. Charkrabarti, *Biochem. Biophys. Res. Commun*., **74**, 460 (1977).
[43] E. T. Pirc, I. Arčon, A. Kodre and P. Bukovec, *Carbohyd. Res*., **339**, 2549 (2004).
[44] A. Magnani, V. Silvestri and R. Barbucci, *Macromol. Chem. Phys*., **200**, 2003 (1999).
[45] G. Matsumura and W. Pigman, *Arch. Biochem. Biophys*., **110**, 526 (1965).
[46] M. M. T. Khan and A. E. Martell, *J. Am. Chem. Soc*., **89**, 7104 (1967).
[47] W. Niedermeier, C. Dobson and R. P. Laney, *Biochim. Biophys. Acta*, **141**, 366 (1967).
[48] W. Niedermeier, R. P. Laney and C. Dobson, *Biochim. Biophys. Acta*, **148**, 400 (1967).
[49] D. A. Swann, *Biochem. J*., **102**, 42 (1967).
[50] B. Halliwell and C. H. Foyer, *Biochem. J*., **155**, 697 (1976).
[51] M. J. Harris and W. Pigman, *J. Am. Chem. Soc*., **94**, 7570 (1976).
[52] A. Samuni, J. Aronovitch., D. Godinger, M. Chevion and G. Czapski, *Eur. J. Biochem*., **137**, 119 (1983).
[53] G. B. Buettner and B. A. Jurkiewicz, *Radiat. Res*., **145**, 532 (1996).
[54] J. Rychlý, L. Šoltés, M. Stankovská, I. Janigová, K. Csomorová, V. Sasinková, G. Kogan and P. Gemeiner, *Polym. Degrad. Stabil*., **91**, 3174 (2006).
[55] L. Šoltés, G. Kogan, M. Stankovská, R. Mendichi, J. Rychlý, J. Schiller and P. Gemeiner, *Biomacromolecules*, **8**, 2697 (2007).
[56] W. H. Koppenol, in "Free Radical Damage and Its Control", edited by C. A. Rice-Evans and R. H. Burdon, Elsevier Science B. V., Amsterdam, 1994, pp. 3-24.
[57] G. R. Buettner and B. A. Jurkiewicz, *Free Radical. Bio. Med*., **14**, 49 (1993).
[58] N. Shukla, J. Maher, J. Masters, G. D Angelini and J. Y. Jeremy, *Atherosclerosis*, **187**, 238 (2006).
[59] N. Volpi, *Curr. Med. Chem*., **13**, 1799 (2006).