Protective effects of manganese(II) chloride on hyaluronan degradation by oxidative system ascorbate plus cupric chloride

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ABSTRACT
The degradation of several high-molar-mass hyaluronan samples was investigated in the presence of ascorbic acid itself and further by an oxidative system composed of ascorbic acid plus transition metal ions, i.e. Fe(II) or Cu(II) ions. The latter oxidative system imitates conditions in a joint synovial fluid during early phase of acute joint inflammation and can be used as a model for monitoring oxidative degradation of hyaluronan under pathophysiological conditions. The system Cu(II) plus ascorbate (the Weissberger oxidative system) resulted in a more significant decrease of hyaluronan molar mass compared to the oxidative system Fe(II) plus ascorbate. Addition of manganese(II) chloride was found to decrease the rate of the oxidative damage of hyaluronan initiated by ascorbate itself and by the Weissberger system.

KEY WORDS: hyaluronan; Weissberger oxidative system; reactive oxygen species; dynamic viscosity; ascorbic acid; Fenton reaction; manganese(II) chloride

Introduction

Some biogenic transition metals, such as iron, copper, manganese, zinc, and cobalt, participate in 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 (Valko et al., 2005; Šoltés and Kogan, 2009).

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 hydroxyfree radical. Iron ions are known to cause peroxidation of (polyunsaturated) fatty acids in lipids (LH) and generate peroxyl lipid radicals (LOO•) by a sequence of the following reactions

\[
2\text{Fe(II)} + 2\text{O}_2 \leftrightarrow 2\text{Fe(III)} + 2\text{O}_2^{•-} \quad \text{(redox reaction; reversible)} \quad (1)
\]

\[
2\text{O}_2^{•-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad \text{(dismutation reaction)} \quad (2)
\]

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{O}_2^{•-} + \text{HO}^- \quad \text{(Fenton reaction)} \quad (3)
\]

\[
\text{O}_2^{•-} + \text{H}_2\text{O}_2 \leftrightarrow \text{HO}^- + \text{HO}^- + \text{O}_2 \quad \text{(Haber-Weiss reaction; reversible)} \quad (4)
\]

\[
\text{LH} + \text{OH} \rightarrow \text{LOO}^{•-} + \text{H}_2\text{O} \quad (5)
\]

\[
\text{LOO}^{•-} + \text{O}_2 \rightarrow \text{LOOH} + \text{L}^{•} \quad (6)
\]

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

Ascorbate (Asc•−) belongs to the most efficient (bio)reductants capable to keep iron ions in the 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•−) – a low-reactive radical that can undergo dismutation/disproportionation reaction to form Asc•− and dehydroascorbate (DHA)
Alternatively, complexes of Fe(II) ions and dioxygen are also assumed to yield reactive species of unknown nature, which are subsequently able to oxidize biological material (Qian and Buettner, 1999; Flemmig and Arnhold, 2007).

A combination of ascorbate plus Cu(II) under aerobic conditions, the so-called Weissberger’s system (Weissberger et al., 1943; Khan and Martell, 1967), gives rise directly to hydrogen peroxide (cf. Scheme 1, Fisher and Naughton, 2003; 2004; 2005) and, taking into account the fact that ascorbate reduces Cu(II) to cuprous ions, it is feasible to assume that at copper-catalyzed ascorbate auto-oxidation, •OH radicals should be generated by a Fenton-type reaction Cu(I) + H2O2 → Cu(II) + •OH + HO–. This conclusion was recently supported by an unambiguous proof of production of hydroxyl radicals in a system comprising ascorbate plus CuCl2 by using EPR spin-trap technique, applying such spin traps as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DIPPMPO) (Šoltés et al., 2006).

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, taking into account the negligible availability of “free catalytic iron”, the significance of e.g. the Fenton reaction can not be fully clarified. The average-weight human body contains approximately 4–5 g iron bound to hemoglobin, myoglobin, cytochromes, iron-containing enzymes, and to the iron storage proteins – ferritin, transferrin, and hemosiderin. Similarly, about 95% of 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, manganese concentrations in most adult human tissues range between 3 and 20 μM (Roth, 2006). The results of several in vitro studies imply that Mn in various forms does indeed inhibit damage mediated by •OH radicals, however only if their production is dependent on the presence of O2•– or H2O2. Thus, Mn complexes appear to interact with •OH, as well as with O2•– and H2O2, in a fundamentally different fashion than those of Fe and Cu (Cheton and Archibald, 1988).

Hyaluronan (HA; Figure 1) is a high-molar-mass glycosaminoglycan that has important functions in living organisms (Kogan et al., 2007; 2008). HA macromolecules with molar mass equaling several MDa are extruded into synovial fluid (SF) by synoviocytes/hyalocytes – the cells embedded in the synovial membrane. Free/non-associated HA present in SF determines its unique viscoelastic properties required for maintaining the proper functioning of the joints in vertebrates.

It is noteworthy that the half-life of HA in SF is only a few hours. Although the relatively fast HA catabolism is controlled by hyaluronidases in most tissues, 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 reactive oxygen species (ROS) generated during the ascorbate auto-oxidation catalyzed by the transition metal (Fe and/or Cu) ions (Šoltés et al., 2005). Evidence exists that ROS are responsible for HA degradation in inflammatory joint diseases, such as osteoarthritis and rheumatoid arthritis (RA). HA involvement in activation of hyaluronidases by ROS may contribute to the pathophysiology of inflammatory joint diseases.

\[
\text{Asc}^- + \text{Fe(II)} \rightarrow \text{Asc}^{2-} + \text{Fe(II)} \quad (7)
\]
\[
2\text{Asc}^{2-} \rightarrow \text{Asc}^- + \text{DHA} \quad (8)
\]
and modulation of the inflammatory response includes also its scavenging action toward ROS, such as •OH radicals (Mendoza et al., 2007).

As demonstrated in many studies, under aerobic conditions a ternary system, comprising HA macromolecules plus ascorbate and traces of iron or copper ions, induces gradual decrease of the viscosity of the HA solution, as a result of fragmentation/degradation of 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 stipulated by a known catalytic participation of this essential transition metal in the function of Mn(II) ions is stipulated by a known catalytic participation of this essential transition metal in the function of Mn(II) ions is stipulated by a known catalytic participation of this essential transition metal in the function of Mn(II) ions in ascorbate auto-oxidation, in which hyaluronans of various molar masses are involved as indicators of pro- or antioxidative properties of the system.

Material and methods

Biopolymers

Six hyaluronan samples, covering by their molar-mass averages (Mw) the range of 0.43 to 1.3 MDa (cf. Table 1), were kindly donated by or purchased from the following HA manufacturers: Genzyme Corporation, Cambridge, MA, U.S.A.; Sigma Chemicals Co., St. Louis, MO, U.S.A.; Lifecore Biomedical Inc., Chaska, MN, U.S.A.; and CPN Ltd., Ústí nad Orlicí, Czech Republic (Stankovská et al., 2004, Šoltés et al., 2007). In the HA samples P9706-6 and P9710-2, the contents of the following (transition) metals were stated by 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, U.S.A.)].

Chemicals

Analytical purity grade NaCl and CuCl2·2H2O were from Slavus Ltd., Bratislava, Slovakia; FeCl2·4H2O was purchased from Penta CZ Ltd., Chrudim, Czech Republic; MnCl2·4H2O was from Lachema CZ Ltd., Brno, Czech Republic; ascorbic acid (AA) was from Merck KGaA, Darmstadt, Germany. Redistilled de-ionized quality grade water with ≤ 0.055 μS/cm conductivity was prepared by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

Table 1. Summary of the characteristic parameters of the six HA samples

| Sample designation | Parametera [unit] | Parameterb [unit] | Parameterc [unit] |
|--------------------|------------------|------------------|------------------|
|                    | Mw [kDa]         | Mw/Mn [-/-]      | Rg [nm]          |
| B22157             | 1340             | 1.50             | 129.8            |
| 53H0439            | 1017             | 1.82             | 130.7            |
| P9710-2A           | 808.7            | 1.63             | 110.0            |
| P9706-6            | 803.9            | 1.64             | 1079             |
| CPN                | 659.4            | 1.88             | 974              |
| 1-9100-1           | 426.2            | 1.84             | 77.2             |

aMw – weight-average of molar masses, Mw/Mn – polydispersity index, and Rg – z-average of the radius of gyration.
bAged HA sample (Šoltés et al., 2007).
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 was added in the morning. The next 3.95 mL of the solvent was added after 6 h. On the following morning, 50.0 μL of 16.0 mM AA dissolved in 0.15 M NaCl was 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 the Teflon® cup reservoir of the Brookfield LVDV-II+PRO rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). The recording of the viscometer output parameters started 2 min after the experiment onset. The solution dynamic viscosity (η) was measured at 25.0 ± 0.1 °C in 3 min intervals for up to 5 h. The viscometer Teflon® spindle rotated at 180 rpm, i.e. at the shear rate equaling 237.6 s–1 (Šoltés et al., 2007).

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. On 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 FeCl₂, CuCl₂, or MnCl₂ solutions in 0.15 M aqueous NaCl. The concentration of the biogenic transition metal salt in the system was 0.5 or 5.0 μM when using FeCl₂; 0.1, 1.0, or 5.0 μM with CuCl₂; and 0.5 μM with MnCl₂.

When the (inhibitory) action of the Mn(II) ions on HA degradation by the system comprising AA (100 μM) and CuCl₂ (1.0 μM) was assessed, the second portion of aqueous NaCl was 3.85 mL. Fifty μL of the MnCl₂ solution in 0.15 M aqueous NaCl was 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: (i) Mn(II) followed by (AA) and Cu(II); (ii) Mn(II) followed by AA 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 were in the interval 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, namely CPN and 1-9100-1, indicates the presence of two distinct regions: (i) rheopectic and (ii) the 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, 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, the Fe(III)/Fe(II) and Cu(II)/Cu(I) catalyzed ascorbate auto-oxidation leads to the generation of •OH radicals that, in the presence of these biogenic transition metal ions, the catalyzed ascorbate auto-oxidation leads to the generation of •OH radicals that, after a certain initiation period, promote degradation of HA macromolecules, which is manifested by a gradual decrease of the 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 in the case of the samples B22157, P9706-6, and P9710-2A. No or only small changes occurred using 0.5 μM Mn(II) ions with HA samples having lower molar mass. It should be noted that most curves implied that a slight decrease in viscosity took place after addition of Mn(II).

Figure 2. Time dependence of dynamic viscosity of 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 MnCl₂.
Contrary to an “antioxidative” action of MnCl₂, an identical 0.5 μM concentration of FeCl₂ or even smaller 0.1 μM concentration of CuCl₂ had a “pro-oxidative” effect on the degradation of HA macromolecules in most samples (compare the data presented in left and right panels in Figure 3 with those in Figure 2, left panel). The only exception was observed with the 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 the addition of Fe or Cu ions is clearly indicated in a concentration-dependent manner (cf. Figure 4, left and right panels). A slight difference should be however pointed out concerning the “nominal” η₂’ values, i.e. the values observed at the 2nd min after 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, we shifted the η₂’ value of the solutions containing metal salts to the value η₂’ valid for the solutions comprising only HA and AA. By such “normalization”, the changes in the values of dynamic viscosity caused by different amounts of added FeCl₂ and CuCl₂ became more visible (as presented in both panels of Figure 4). As can be seen, the character of the time dependence of η value upon the addition of FeCl₂ can be described as a gradual monotonous concentration-dependent decline, while the addition of CuCl₂ resulted in a literally “dramatic” drop of η value in a very short time interval, after which its decrease continued, however at a much lower rate. A possible explanation of this dissimilarity may lie most probably in different reaction kinetics of the processes leading to generation of ROS in a system ascorbate plus FeCl₂ and in that comprising ascorbate plus CuCl₂.

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 CuCl₂ (1.0 μM). The addition of MnCl₂ in a relatively high concentration (30 μM) resulted in a significant decrease of the degradation of HA macromolecules, however, none of the used application schemes (i, ii, iii) resulted in total inhibition of the biopolymer degradation. While in the case of sample P9710-2A using the application scheme (iii), the sample damage was decreased to ca. 44% (cf. Figure 5, left panel), in the case of sample P9706-6, scheme (i) proved to be the most efficient, i.e. extent of degradation decreased to ca. 39% (cf. Figure 5, right panel).

Discussion

Under physiological conditions as well as at the early stage of acute-phase joint inflammation, 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 of free HA macromolecules in SF is 1.4–3.6 mg/mL (Kogan et al., 2007); (b) the concentration of ascorbate in SF of healthy subjects reaches the values closely to those established in blood serum, i.e. 40–140 μM (Wong et al., 1981); (c) the total concentrations of Fe and Cu ions in SF of healthy human equal 5.2 and 4.3 μM, respectively, and they rise under pathological/inflammatory conditions such as osteoarthritis (OA) and RA; (d) in SF of the individuals suffering from RA, the total Cu concentration is increased more than three-fold compared to that of the healthy population (Niedermeier and Griggs, 1971) and their Cu concentration in SF ultrafiltrate equals 0.125 ± 0.095 μM.
(Naughton et al., 1995); (e) at ascorbate auto-oxidation with the catalytic contribution of Cu(II) traces, direct conversion/transformation of O₂ to H₂O₂ takes place (Weissberger et al., 1943; Khan and Martell, 1967; Fisher and Naughton 2003; 2004; 2005); (f) the average concentration of Mn ions in SF of healthy persons and patients with RA is relatively low (ca. 0.42 and 0.44 μM, respectively) (Niedermeier and Griggs, 1971).

Investigation of the participation of biogenic transition metals in oxidative damage of high-molar-mass HA appears to be very simple. However, at least two main technical/experimental obstacles should be pointed out. First: due to an extremely high aggressiveness of the oxidative/radical processes, the materials, which come into contact with e.g. the "OH radicals, should be non-metallic (preferably made of glass or Teflon) (Stankovská et al., 2004; 2005). Second: 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 the Brookfield LVDV-II+PRO rotational viscometer equipped with a cup-reservoir and spindle both home-made of Teflon, and by using HA samples which purity was sufficiently high. The samples coded P9706-6 and P9710-2 with the known content of the given (transition) metals allowed us to calculate the concentrations of iron and copper ions in the solutions of these samples.

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 0.3 (1) or 3.0 μM (2) FeCl₂. 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) CuCl₂.

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 CuCl₂: (i) MnCl₂ followed by AA and CuCl₂; (ii) AA followed by MnCl₂ and CuCl₂; and (iii) MnCl₂ followed by CuCl₂ and AA. The concentrations used were 100 μM AA, 1.0 μM CuCl₂, and 30 μM MnCl₂.
The other four samples, despite their poor “identity” as concerns the content of metal impurities, were used since their mean molar masses covered a relatively broad range of 0.43 to 1.3 M Da (cf. Table 1).

Under the conditions used in our studies (neutral pH), the HA macromolecules are present in a highly ionized state: the pKₐ value for the d-glucuronic acid residues is 3.12 (Park and Chakrabarti, 1978). The d-glucuronic structural units of the HA polyanion form salts with transition metal counter-cations. As formerly reported (Nagy et al., 1998), HA is able to weakly bind cupric ions (the binding constant being 3.0 × 10³ L/mol (Figueroa et al., 1977)). Generally, the transition metal counter-cations form coordinate complex compounds, in which the metal cation can be fixed intra- or intermolecularly simply via the carboxyl groups of HA (Nagy et al., 1998; Pirc et al., 2004). 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 along with the COO⁻ group (Magnani et al., 1999).

The •OH radical, which redox potential (•OH/H₂O) equals +2.31 V at pH 7, is classified as the most efficient initiator of the radical oxidation/degradation accepting an H⁺ radical from the macromolecule of hyaluronan. The formation of •OH radicals can occur in several manners, 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 represented in Figure 2 (left panel) means that a given amount of initiating •OH radicals must be generated right at 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 shown in Figure 2, left panel). The greater the metal content, the shorter or even absent is the lag-phase. Moreover, in the case of assessing the effect of CuCl₂ added to the system (cf. Figure 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 presented study corroborates the observations already published by several authors studying HA degradation caused by ascorbate alone or in combination with Fe and/or Cu ions (Khan and Martell, 1967; Wong et al., 1981; Matsumura and Pigman, 1965; Niedermeier et al., 1967a; 1967b; Swann, 1967; Halliwell and Foyer, 1976; Harris and Pigman, 1976; Samuni et al., 1983; Buettner and Jurkiewicz, 1996). However, the study protocol including the assays of 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 – 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 addition of a relatively low amount of MnCl₂ (0.5 μM; cf. Figure 2, right panel), i.e. one comparable to Mn contents in SF of healthy persons (0.42 μM on average) (Niedermeier and Griggs, 1971). On the other hand, in the case of a “massive” load of Cu(II) ions (1.0 μM), the added MnCl₂ was also effective (cf. Figure 5, both panels), yet it should be pointed out that not even the highest concentration applied (30 μM) was able to prevent the oxidative damage of HA macromolecules.

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

\[
\text{AOO}^+ + \text{Mn}(II) + \text{H}^+ \rightarrow \text{AOOH} + \text{Mn}(III)
\]  
\[
\text{AO}^+ + \text{Mn}(II) + \text{H}^+ \rightarrow \text{AOH} + \text{Mn}(III)
\]

analogously to reaction 9 suggested by Coaslin et al. (1992) and Sziraki et al. (1998; 1999) have yet to be proven. The hyaluronan hydroperoxides produced in this way should, however, be decomposed by the action of the two transition metal cations in lower oxidation states – Cu(I) and Fe(II).

For detection of such AOOH and/or AOH type derivatives, the non-isothermal chemilumimetry and MALDI-TOF mass spectrometry could be proposed as relevant analytical methods (Šoltés et al., 2006; 2007; Rychlý et al., 2006).

Concluding Remarks

The structure of the electronic orbitals of iron – 1s²2s²2p⁶3s²3p⁶3d⁴4s² – and its high redox potential value – Fe(II)/Fe(II) equaling +0.48 V at pH 7 (Koppenol, 1994) – predetermine iron as one of the major participants in the production (and metabolism) of free radicals in biological systems. While Fe(III) at physiological pH values precipitates as oxyhydroxide aggregates, compounds containing Fe(II) are soluble, though unstable, and tend to react with oxygen to form superoxide anion radical (O₂⁻) and Fe(III). Yet 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 (Buettner and Jurkiewicz, 1993). 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 also been investigated, in addition to its better explored antioxidant role.

The copper electron configuration is 1s²2s²2p⁶3s²3p⁶3d¹⁰4s¹ and the value of its redox potential...
Cu(II)/Cu(I) is +0.16 V at pH 7. The bivalent Cu(II) is paramagnetic (3d⁹) 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 (Shukla et al., 2006). Since ascorbate acts as a powerful reducing agent with a redox potential of +0.282 V for the redox couple Asc⁻/Asc at pH 7, it should reduce Cu(II) to Cu(I) and hence cuprous ions should be able to reduce molecules of dioxygen directly to H₂O₂.

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 1s²2s²2p⁶3s²3p⁶3d⁵4s² and the Mn(III)/Mn(II) redox potential equals +1.5 V at pH 7. Manganese exists in several different oxidation states; within biological systems, however, the +2 valence prevails (Roth, 2006). Manganese 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 HA samples is an established fact, which should be taken into account at 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 believed to be the future effective tools/remedies applicable at treating a number of diseases (Volpi, 2006). So-called viscosupplementing injections of high-molar-mass hyaluronan directly into the osteoarthritic joints could be classified as one of such examples. A further step forward could be elucidation or minimalization of the content of Fe and Cu and a potentially advantageous addition of an appropriate Mn(II) salt/complex to the injection mixtures to be administered at viscosupplementary treatment of OA.

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REFERENCES

Archibald FS, Fridovich I. (1982). The scavenging of superoxide radical by manganese complexes: in vitro. Arch Biochem Biophys. 214: 452–463.

Buettnner GR, Jurkiewicz BA. (1993). Ascorbate free radical as a marker of oxidative stress: an EPR study. Free Radic Biol Med 14: 49–55.

Buettnner GR, Jurkiewicz BA. (1996). Catalytic metals, ascorbate and free radicals: combinations to avoid. Radiat Res 145: 532–541.

Cavallini L, Valente M, Bindoli A. (1984). On the mechanism of inhibition of lipid peroxidation by manganese. Inorg Chem Acta 91: 317–120.

Chang EC, Kromann DJ. (1989). Intracellular Mn (II)-associated superoxide scavenging activity protects Cu,Zn superoxide dismutase-deficient Saccharomyces cerevisiae against dioxygen stress. J Biol Chem 264: 12172–12178.

Cheton PL-B, Archibald FS. (1988). Manganese complex(es) and the generation and scavenging of hydroxyl radicals. Free Radic Biol Med 5: 325–333.

Coassin M, Urzini F, Bindoli A. (1992). Antioxidant effect of manganese. Arch Biochem Biophys 299: 330–333.

Figueroa N, Nagy B, Charkrabarti B. (1977). CuZn - Hyaluronic acid complex: spectrophoto- metric detection. Biochem. Biophys Res Commun 74: 460–465.

Fisher AEO, Naughton DP. (2004). Iron supplements: The quick fix with long-term consequences. Nutr J 3: 1–5.

Fisher AEO, Naughton DP. (2005). Therapeutic chelators for the twenty first century: new treatments for iron and copper mediated inflammatory and neuro- logical disorders. Curr Drug Deliv 2: 261–268.

Fisher AEO, Naughton DP. (2005). Vitamin C contributes to inflammation via radical generating mechanisms: a cautionary note. Med Hypotheses 61: 657–660.

Flennig J, Arnhold J. (2007). Ferrous iron-induced strand breaks in the DNA plasmid pBR322 are not mediated by hydrogen peroxide. Eur J Biochem 367: 377–384.

Gray B, Carmichael AJ. (1992). Kinetics of superoxide scavenging by dismutase enzymes and manganese mimics determined by electron spin resonance. Biochem J 281: 795–802.

Hallwell B, Foyer CH. (1976). Ascorbic acid, metal ions and the superoxide radical. Biochem J 155: 697–700.

Harris MJ, Hesp A, Pigman W. (1976). Metal catalysis in the depolymerization of hyaluronic acid by autoxidants. J Am Chem Soc 94: 7570–7572.

Hussain S, Ali SF. (1999). Manganese scavenges superoxide and hydroxyl radicals: an in vitro study in rats. Neurosci Lett 261: 21–24.

Johnson KA, Hulse DA, Hart RC, Kochevar D, Chut Q. (2001). Effects of an orally viscosupplementary treatment of OA.

Kogan G, Šoltés L, Stern R, Schiller J, Mendichi R. (2007) Hyaluronic acid: A Bio- polymer with Versatile Physico-Chemical and Biological Properties, in Handbook of Polymer Research: Monomers, Oligomers, Polymers and Composites (Pethrick RA, Ballada A, Zaikov GE eds) pp. 393–439, New York, Nova Science Publishers.

Kogan G, Šoltés L, Stern R, Schiller J, Mendichi R. (2008) Hyaluronic Acid: Its Function and Degradation in In Vivo Systems, in Studies in Natural Products Chemis- try, Vol. 35 Bioactive Natural Products, Part D (Attar-ur-Rahman ed) pp. 789–882, Amsterdam, Elsevier.

Koppensol WH. (1994). Chemistry of Iron and Copper in Radical Reactions, in Free Radical Damage and Its Control (Rice-Evans CA, Burdon RH eds) pp. 3–24, Amster- dam, Elsevier Science B. V.
Magnani A, Silvestri V, Barbucci R. (1999). Hyaluronic acid and sulphated hyaluronic acid in aqueous solution: effect of the sulphation on the protonation and complex formation with Cu²⁺ and Zn²⁺ ions. Macromol Chem Phys. 200:2003–2014.

Matsumura G, Pignan W. (1965). Catalytic role of iron ion in the depolymerization of hyaluronic acid by ascorbic acid. Arch Biochem Biophys. 110:526–533.

McCord JM. (1974). Free radicals and inflammation. protection of synovial fluid by superoxide dismutase. Science 185:529–531.

Mendoza G, Álvarez AI, Pulido MM, Molina AJ, Merino G, Real R, Fernandes P, Prieto JG. (2007). Antioxidant profile of hyaluronan: physico-chemical features and its role in pathologies. Carbohydr Res 342:96–102.

Nagy L, Yamashita S, Yamaguchi T, Sipos P, Wakita H, Nomura M. (1998). The local structures of Cu(I) and Zn(I) complexes of hyaluronate. J Inorg Biochem 72:49–55.

Naughton DP, Knappitt J, Fairburn K, Gaffney K, Blake DR, Grooteveld M. (1995). Detection and investigation of the molecular nature of low-molecular-mass copper ions in isolated rheumatoid knee-joint synovial fluid. FEMS Lett 167:1–10.

Niedermeier W, Dobson C, Laney RP. (1967a). Studies on the ascorbic acid-induced depolymerization of hyaluronic acid. Biochim Biophys Acta 141:366–373.

Niedermeier W, Laney RP, Dobson C. (1967b). The mechanism of action of ascorbic acid–induced depolymerization of hyaluronic acid. Biochim Biophys Acta 148:400–405.

Niedermeier W, Griggs, J.H. (1971). Trace metal composition of synovial fluid and blood serum of patients with rheumatoid arthritis. J Chronic Dis 23:527–538.

Park JW, Chakrabarti B. (1978). Optical properties and viscosity of hyaluronic acid in mixed solvent: evidence of conformational transition biopolymers. Biopolymers 17:1323–1333.

Parsons BJ, Al-Assaf S, Navaratnam S, Phillips GO. (2002). Comparison of the Reactivity of Different Oxidative Species (ROS) Towards Hyaluronan, in Hyaluronan: Chemical, Biochemical and Biological Aspects, Vol. 1 (Kennedy JF, Phillips GO, Williams PA, Hascal VC edis.) pp. 141–150, Cambridge, MA, Woodhead Publishing Ltd.

Pic ET, Ačton I, Kodre A, Bukovec P. (2004). Metal-ion environment in solid Mn(II), Co(II) and Ni(II) hyaluronates. Carbohydr Res 339:2549–2552.

Roth JA. (2006). Homeostatic and toxic mechanisms regulating manganese uptake, retention, and elimination. Biol Res 39:45–57.

Rychlý J, Šoltés L, Stankovská M, Janůvá I, Csomorová K, Sasisová V, Kogan G, Gmeiner P. (2006). Unexplored capabilities of chemiluminescence and thermoanalytical methods in characterization of intact and degraded hyaluronans. Polym Degrad Stab 91:3174–3184.

Qian SY, Buettern Gr. (1999). Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: an electron paramagnetic resonance spin trapping study. Free Radic Biol Med 26:1447–1456.

Sakuragi K, Andoh M, Yamada M, Koderer, Nishimura H, Hiroto M, Matsushima A, Aoyama M, Yamamoto H, Inada Y. (1997). Anti-inflammatory activity of superoxide dismutase conjugated with sodium hyaluronate. Biochem Pharmacol 74:117–120.

Samuni A, Aroivotich J, Godinger D, Chevion M, Crapski G. (1983). On the cytotoxicity of vitamin C and metal ions. A site-specific Fenton mechanism. Eur J Biochem 137:119–124.

Shukla GS, Chandra SV. (1981). Manganese toxicity: Lipid peroxidation in rat brain. Acta Pharmacol Toxicol 48:95–100.

Shukla N, Maher J, Masters J, Angelini GD, Jeremy JY. (2006). Does oxidative stress change ceruloplasmin from a protective to a vasculopathogenic risk factor? Atherosclerosis 187:238–250.

Singh RK, Kooreman KM, Babbs CF, Fessler JF, Salaris SC. (1992). Potential use of simple manganese salts as antioxidant drugs in horses. Am J Vet Res 53:1822–1829.

Stankovská M, Šoltés L, Vikartovská A, Gmeiner P, Kogan G, Bakol D. (2005). Degradation of high-molecular-weight hyaluronan: rotational viscometry study. Biochim Biophys Acta 167:198–202.

Stankovská M, Šoltés L, Vikartovská A, Méndez R, Lath O, Molnárová M, Gmeiner P. (2004). Study of hyaluronan degradation by means of rotational viscometry: contribution of the material of viscometer. Chem Pap 58:348–352.

Swann DA. (1967). The degradation of hyaluronic acid by ascorbic acid. Biochem J 102:42–44.

Sziráki I, Mohanakumar KP, Rauhala P, Kim HG, Yeh KJ, Chiuvec CE. (1999). Manganese: a transition metal protects nigrostriatal neurons from oxidative stress in the iron-induced animal model of Parkinsonism. Neuroscience 85:1101–1111.

Sziráki I, Rauhala P, Koh KH, Bergen PV, Chiuvec CE. (1999). Implications for asysis of antioxidative properties of manganese in iron-induced brain lipid peroxidation and copper-dependent low density lipoprotein conjugation. Neurotoxicology 20:455–466.

Šoltés L, Kogan G, Stankovská M, Méndez R, Rychlý J, Schiller J, Gmeiner P. (2007). Degradation of high-molar-mass hyaluronan and characterization of fragments. Biomacromolecules 8:2677–2705.

Šoltés L, Kogan G. (2009). Catabolism of hyaluronan: involvement of transition metals. Interdisciplinary Toxicology 2:229–238.

Šoltés L, Stankovská M, Brezová V, Schiller J, Arnhold J, Kogan G, Gmeiner P. (2006). Hyaluronan degradation by copper(II) chloride and ascorbate: rotational viscometric, EPR spin-trapping, and MALDI–TOF mass spectrometric investigations. Carbohydr Res 341:2826–2834.

Šoltés L, Stankovská M, Kogan G, Gmeiner P, Stern R. (2005). Contribution of oxidant-reductive reactions to high-molecular-weight hyaluronan catabolism. Chem Biodivers 2:1242–1245.

Šoltés L, Valachová K, Méndez R, Kogan G, Arnhold J, Gmeiner P. (2007). Solution properties of high-molar-mass hyaluronans: the biopolymer degradation by ascorbate. Carbohydr Res 342:1071–1077.

Tampo Y, Yonaha M. (1992). Antioxidant mechanism of Mn(II) in phospholipid peroxidation. Free Radic Biol Med 13:115–20.

Vallo M, Morris H, Cronin MTO. (2005). Metals, toxicity and oxidative stress. Curr Med Chem 12:1161–1208.

Varani J, Ginsburg I, Gibbs DF, Mukhopadhyay PS, Sulavik C, Johnson KJ, Weinberg JM, Ryan US, Ward PA. (1991). Hydrogen peroxide-induced cell and tissue injury: protective effects of Mn++. Inflammation 15:291–301.

Volpi N. (2006). Therapeutic applications of glycosaminoglycans. Curr Med Chem 13:1799–810.

Wiegell PH, DeAngelis PL. (2007). Hyaluronan synthases: a decade-plus of novel glycosyltransferases. J Biol Chem 282:36777–36781.

Weissberger A, LuValle JE, Jr. Thomas DS. (1943). Oxidation processes. XVI. The autoxidation of ascorbic acid. J Am Chem Soc 65:1934–1939.

Wong SF, Halliwell B, Skowroneck WR. (1981). The role of superoxide and hydroxyl radicals in the degradation of hyaluronic acid induced by metal ions and ascorbic acid. J Inorg Biochem 14:127–134.

Worley CG, Bombick D, Allen JW, Suber RL, Aschner M. (2002). Effects of manganese on oxidative stress in CATH.a cells. Neuro toxicology 23:159–164.