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*Chapter 10*

# EFFECTS OF BIOGENIC TRANSITION METAL IONS ZN(II) AND MN(II) ON HYALURONAN DEGRADATION BY ACTION OF ASCORBATE *PLUS* CU(II) IONS

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## ABSTRACT

High-molar-mass hyaluronan (HA) degradation was initiated by two oxidative systems, i.e. ascorbate *plus* Fe(II) ions and ascorbate *plus* Cu(II) ions. By using the method of rotational viscometry, we observed dose-dependent degradation of HA by the above mentioned oxidative systems, which however differed in the reaction kinetics. The effects of two biogenic transition metal ions, i.e. Zn(II) and Mn(II) ions, on degradation of the high-molar-mass HA sample by the oxidative system ascorbate *plus* Cu(II) ions (Weissberger's oxidative system) were investigated. Two experimental settings were tested: Zn(II) or Mn(II) ions were added to the reaction system (i) before the reaction onset or (ii) 1 h after initiating the HA degradation. It was found that under the conditions specified in (i), Mn(II) and Zn(II) ions caused an inhibitory effect on HA degradation in a dose-independent manner up to a certain period of time (appr. 30 min). On the contrary, when the Mn(II) or Zn(II) ions were applied 1 h after the reaction onset (ii), a pro-oxidative effect of each element on degradation of the high-molar-mass HA chains was evidenced.

**Keywords:** Hyaluronan, zinc, manganese, rotational viscometry, Weissberger's oxidative system.

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## 1. INTRODUCTION

Hyaluronan (HA, Figure 1) is a high-molar-mass glycosaminoglycan composed of repeating disaccharide units consisting of D-glucuronate and *N*-acetyl-D-glucosamine [1]. This linear polysaccharide can reach a size of 6-8 MDa [2]. It is an important component of most connective tissues, including vitreous body, skin, synovial fluid, and umbilical cord [3]. It affects many cell functions, such as proliferation, differentiation and migration in a concentration and molar-mass dependent manner [1]. The turnover of HA is extremely rapid. It is estimated that from 15 g of HA in the vertebrate body, 5 g turns over daily. The half-life of HA in the blood circulation is between 2-5 min. In the epidermis of the skin, where one half of HA of the body is found, it is up to 2 days, and in an apparently inert tissue as cartilage, it is appr. 1-3 weeks [2]. HA is susceptible to degradation by reactive oxygen species (ROS), especially by highly reactive $^{\bullet}OH$ radicals [4].

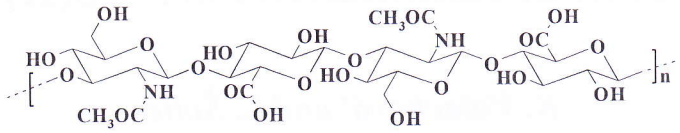
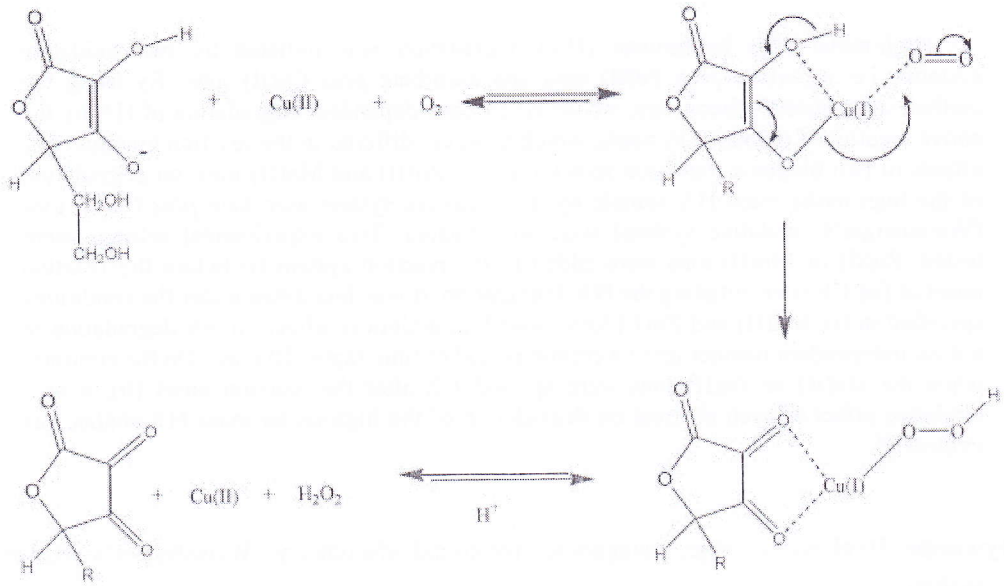


Figure 1. Hyaluronan – the acid form.

The system containing ascorbate and Cu(II) ions is known as Weissberger's oxidative system (Scheme 1) [5] and it is an efficient $^{\bullet}OH$ radical generating system [6].



Scheme 1. Weissberger's oxidative system.

Superoxide dismutase (SOD), the first ROS-metabolizing enzyme discovered, is a unique metal enzyme containing copper and zinc or manganese at its catalytic site [7,8]. Mn-SOD

and Cu,Zn-SOD, present in mitochondria and cytoplasmatic membrane, respectively, dismutate $O_2^{\bullet-}$ to generate $H_2O_2$ [8,9].

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Zinc is the second most prevalent trace element in the human body [10]. Total human body zinc content has been estimated to be 2 g [11]. It participates in the regulation of gene expression, in the structural maintenance of chromatin and biomembranes, in immunity, and in protection against free radicals [10,12]. Zn is a component of more than 300 different enzymes that function in many aspects of cellular metabolism of proteins, lipids, and carbohydrates. Zinc ions are bound to a low-molar-mass protein – metallothionein, whereby they maintain its adequate level. This protein binds except zinc also copper and various heavy metals, such as cadmium and mercury [13,14,15]. Zinc in plasma is bound to the protein with high affinity. The free metal concentration in plasma is estimated to be much less than 0.015 μM [15]. In numerous studies, zinc was found to antagonize catalytic properties of the redox-active transition metals (Fe and Cu) with respect to their abilities to promote formation of superoxide anion radicals and $^{\bullet}OH$ radicals from $H_2O_2$ [10,12,16]. Zn ions do not participate in redox reactions since they are stable. The recommended dietary allowance for adults is 8 mg/day for women and 11 mg/day for men. Zinc ions protect sulfhydryl groups of most proteins against oxidation, thus they prevent intramolecular disulfide formation, further they prevent lipid peroxidation in mitochondria and microsome membranes [10,15,17]. It has been shown that the extent and severity of the immune inflammatory rheumatoid process is associated with low serum zinc concentration. Zinc and copper are present in very small quantities in both mud packs and sulphur healing baths [18].

Manganese is one of the essential trace elements required for all living organisms in certain physiological functions such as brain development and metabolism. This element participates in enzymatic activity as a cofactor in various enzymes such as hydrolases, kinases, carboxylases, and transferases [19].

Manganese is also the cofactor of SOD. It has been shown that Mn ions inhibit lipid peroxidation induced *in vitro*. On the other hand, an excessive intake of manganese may lead to toxic effects on the central nervous system [20, 21] causing e.g. Parkinson-like symptoms, however its mechanism of action is still unclear [22].

Some organisms, such as lactic acid bacteria, are deficient in SOD, yet Mn(II) ions can substitute for SOD [23]. The total body burden of Mn for the standard 70 kg-weighing man is estimated to be approximately 10 to 20 mg of which 25 to 40% is present in bone. The average daily Mn intake is estimated to be between 3 and 9 mg/day.

Concentrations in most adult tissues range between 3 and 20 μM. Manganese scavenges $O_2^{\bullet-}$ at nanomolar concentrations, while $^{\bullet}OH$ radicals are scavenged at micromolar concentrations [24].

The aim of this work was to study the effects of two biogenic transition metal ions, namely zinc and manganese, in the form of $ZnCl_2$ and $MnCl_2$, on hyaluronan degradation initiated by Weissberger's oxidative system.

# EXPERIMENTAL

## Material and Methods

Hyaluronan sample P9710-2A ($M_w$ = 808.7 kDa) was purchased from HA manufacturer Lifecore Biomedical Inc., Chaska, MN, U.S.A. The content of some trace transition metals in the original HA sample was stated as follows Fe = 13, and Cu = 4 ppm. ["Certificate of Analysis" (Lifecore Biomedical Inc., Chaska, MN, U.S.A.)].

## Chemicals

The analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were from Slavus Ltd., Bratislava, Slovakia, $ZnCl_2$ was purchased from Aldrich-Chemie GmbH, Steinheim, Germany, $MnCl_2 \cdot 4H_2O$ and $FeCl_2 \cdot 4H_2O$ were from Lachema s.p., Brno, Czech Republic. L-Ascorbic acid was purchased from Merck KGaA, Darmstadt, Germany.

Redistilled deionized high quality grade water, with conductivity of ≤0.055 μS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

## Study of Hyaluronan Degradation

The HA sample (20 mg) was dissolved overnight in the dark in 0.15 M aqueous NaCl in two steps: first, 4.0 mL of the solvent was added in the morning. Then, the next portion of the solvent in the volume of 3.90 mL was added after 6 h.

On the following morning, the addition of 50 μL of 16 mM ascorbate to the HA solution was followed by admixing 50 μL of 160 μM $CuCl_2$. The solutions of $CuCl_2$ and ascorbic acid were also prepared in 0.15 M aqueous NaCl and their concentrations in the system tested were 1.0 μM and 100 μM, respectively.

## Study of Effects of $ZnCl_2$ or $MnCl_2$ on Hyaluronan Degradation

Antioxidant and pro-oxidative effects of two biogenic transition metal ions, i.e. Zn(II) and Mn(II), on the kinetics of HA degradation were studied by using the oxidative system comprising 100 μM ascorbate *plus* 1.0 μM Cu(II) ions. Yet, when preparing the HA solution, the second portion of aqueous NaCl was 3.85 mL. Fifty μL of $MnCl_2$ or $ZnCl_2$ solution in 0.15 M aqueous NaCl was added to adjust the final concentration of these metal ions to 30 or 300 μM, respectively.

The order of components added to the solution of HA were: a) Mn(II), Cu(II) followed by ascorbic acid, or b) Zn(II), Cu(II) followed by ascorbic acid. The solution mixture was stirred for 30 s prior to each processing.

The second experimental set involved the same concentrations of the components, each stirred for 30 s, however, Mn(II) or Zn(II) ions were added 1 h after the addition of ascorbate to the HA solution.

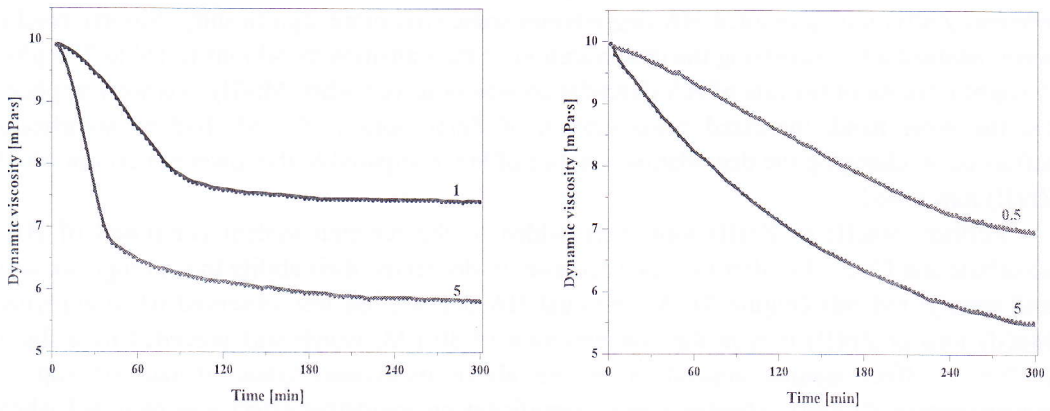
## Rotational Viscometry

The resulting solution mixtures (8 mL) were 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. Dynamic viscosity of the system 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 $s^{-1}$ [6].

## Results and Discussion

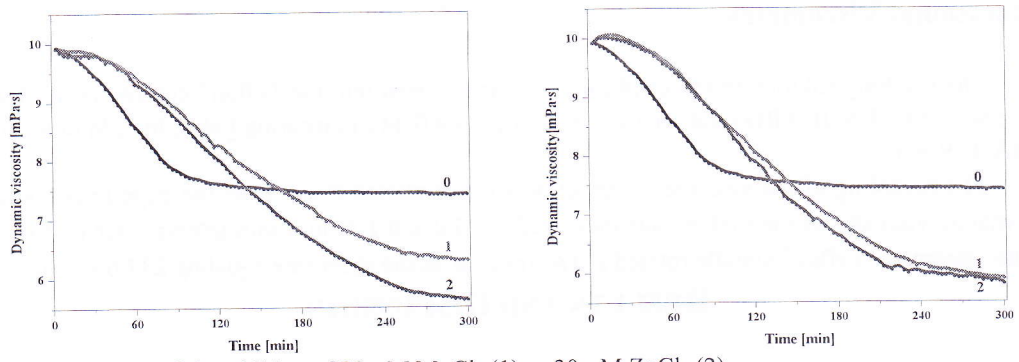
Figure 1 illustrates the comparison of dynamic viscosity *vs.* time profiles of the P9710-2A sample solution (2.5 mg/mL) with the addition of 100 μM ascorbate *plus* $CuCl_2$ (1.0 or 5.0 μM) or $FeCl_2$ (0.5 or 5.0 μM). The addition of Fe(II) or Cu(II) ions was shown to have a dose-dependent pro-oxidative effect, however differences are seen in the degradation kinetics of the two reaction systems tested. The degradation of HA in the presence of the two Cu(II) ions concentrations tested was rapid for a certain time period (30 or 90 min, respectively), followed by a slight HA degradation. On the contrary, HA degradation in the presence of Fe(II) ions was shown to be continual during the whole period. For a better visualization, the initial values of dynamic viscosity of all solutions containing metal salts were shifted to the initial value valid for the solutions comprising only HA and ascorbate.




Left panel: Solutions of HA with the addition of 100 μM ascorbic acid and 1.0 μM $CuCl_2$ (black curve) or 5.0 μM $CuCl_2$ (blue curve).

Right panel: Solutions of HA with the addition of 100 μM ascorbic acid and 0.5 μM $FeCl_2$ (green curve) or 5.0 μM $FeCl_2$ (blue curve).

Figure 1. Kinetics of HA degradation.




Left panel: effect of the addition of 30 μM $MnCl_2$ (1) or 30 μM $ZnCl_2$ (2).
Right panel: effect of the addition of 300 μM $MnCl_2$ (1) or 300 μM $ZnCl_2$ (2).

Figure 2. Kinetics of HA degradation in the presence of 100 μM ascorbate *plus* 1.0 μM $CuCl_2$ (0).

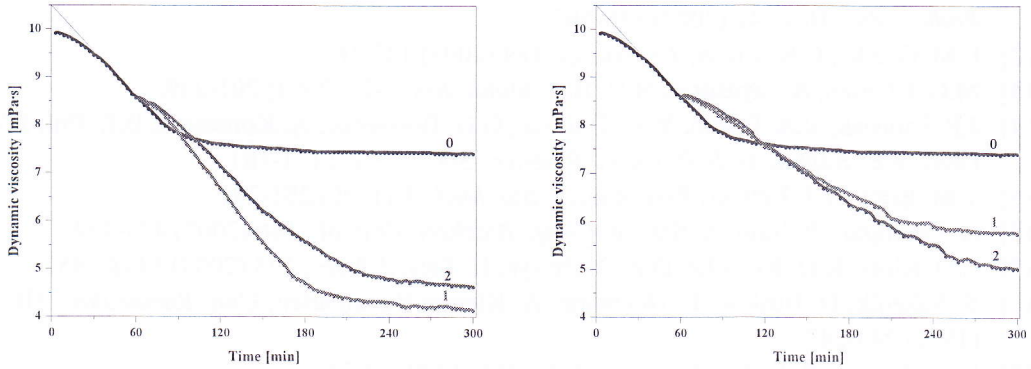
Results in Figure 2 display the effect of Mn(II) or Zn(II) ions in concentrations of 30 or 300 μM, respectively, on scavenging $^{\bullet}OH$ radicals in HA solution degraded by the oxidative system ascorbate *plus* Cu(II) ions. Our study is the first one referring to the action of Mn(II) or Zn(II) ions on oxidatively damaged saccharides. Addition of either Mn(II) or Zn(II) ions in the concentration of 30 μM was shown to have a moderate inhibitory effect, the curves (marked 1 and 2) were almost parallel with the reference curve (up to approximately 120 min). Further a continual decrease of the dynamic viscosity values of HA solution is evident, whereas Zn(II) ions promoted HA degradation somewhat more significantly. Similar results were obtained after increasing the concentration of the transition metal ions tested to 300 μM. A slight increase of the rate of HA degradation was observed when Mn(II) ions were applied. On the other hand, increased concentration of Zn(II) ions (300 μM) had no significant influence on changing the degradation kinetics of HA compared to the lower concentration of Zn(II) ions tested.

Further, Mn(II) or Zn(II) ions were added to the reaction system composed of HA, ascorbate and $CuCl_2$ 1 h after the reaction onset to determine their ability to scavenge alkoxyl and peroxyl radicals (Figure 3). A continual HA degradation was observed when applying Mn(II) ions or Zn(II) ions in the concentration of 30 μM, which was preceded by a slight inhibitory effect against generation of the above mentioned types of radicals (up to approximately 30 min), whereas a more significant pro-oxidative effect was recorded when working with Mn(II) ions. Yet, an increase of the concentration of Mn(II) ions or Zn(II) ions to 300 μM led to a somewhat greater protection of HA for a certain period of time, compared to the former concentration tested. In the following 5-h period, the pro-oxidant effect recorded was in this case more evident in the system composed of Zn(II) ions.

On concluding, our experimental data indicate that Mn(II) or Zn(II) ions promote the oxidative degradation of the probe – high-molar-mass HA. As evident, the generated $^{\bullet}OH$ radicals initiate degradation of the biopolymer, as documented by a decrease of the dynamic viscosity value from 9.93 to 7.42 mPa·s (cf. Figures 2 and 3, curves marked 0). Although addition of Mn(II) or Zn(II) ions at the reaction onset indicate a slight inhibitory zone – appr. 30 min – in both doses tested (cf. Figure 2, both panels, curves marked 1 and 2), yet then the degradation of HA macromolecules is really very extensive, as documented by the following courses of all $\eta$ *vs.* time dependencies showing a decline in the dynamic viscosity values

equaling up to 5.63 mPa·s. However, when Mn(II) or Zn(II) ions were applied 1 h after the reaction onset, a pro-oxidative effect of each element applied on the degradation of the high-molar-mass hyaluronan chains was evidenced (cf. Figure 3, both panels, curves marked 1 and 2).

Under our experimental conditions, both transition metal cations, namely Mn(II) and Zn(II), demonstrated properties which could be classified as typical ones for "prevention antioxidants", i.e. partly hindering the formation of hydroxyl radicals (cf. Figure 2, both panels, curves marked 1 and 2).




Left panel: effect of the addition of 30 μM $MnCl_2$ (1) or 30 μM $ZnCl_2$ (2) 1 h after the reaction onset.
Right panel: effect of the addition of 300 μM $MnCl_2$ (1) or 300 μM $ZnCl_2$ (2) 1 h after the reaction onset.

Figure 3. Kinetics of HA degradation in the presence of 100 μM ascorbate *plus* 1 μM $CuCl_2$ (0).

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