IMPACT OF TRANSITION METALS IN THE FREE–RADICAL DEGRADATION OF HYALURONAN BIOPOLYMERS

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

Received October 30, 2008

Due to their electronic configuration, transition metals readily participate in reductive-oxidative processes, thus contributing to the formation of free radicals, lipid peroxidation and oxidative DNA damage. In living (biogenic) organisms, there exist eight naturally-occurring transition metals, essential for the control of various metabolic and signaling pathways. In this overview, the role of these metals in physiological and pathophysiological processes is described, and their participation in the initiation and propagation of radical chain reactions – leading to oxidative stress and damage to the tissues – is critically assessed. Special attention is given to the involvement of biogenic transition metals in the catabolism of hyaluronan in the joint, where the oxidative degradation of this high-molar-mass glycosaminoglycan may lead to an impaired lubricating function of the synovial fluid, resulting in arthritic and inflammatory conditions.

Keywords: transition metals, biogenic elements, peroxidation, oxidative stress, hyaluronan, inflammation

Biogenic elements

Although, virtually, each chemical element plays some role in the Earth's living systems, it is only 24 elements that account for the vast majority of materials present in them. These elements are divided into: (**i**) 6 major biogenic elements, namely carbon, hydrogen, oxygen, nitrogen, sulfur and phosphorus; (**ii**) 5 minor biogenic elements: sodium, potassium, magnesium, calcium and chlorine; (**iii**) 13 biogenic trace elements comprising manganese, iron, cobalt, copper, zinc, boron, aluminum, vanadium, molybdenum, iodine, silicon, nickel and bromine.[1]

Transition metals

Thirty-eight chemical elements, with atomic numbers from 21 to 30, 39 to 48, 72 to 80, and 104 to 112, are termed transition metals (Table 1).

Eight of these – vanadium, manganese, iron, cobalt, nickel, copper, zinc and molybdenum – belong to biogenic trace elements. Thus V^{23}, Mn^{25}, Fe^{26}, Co^{27}, Ni^{28}, Cu^{29}, Zn^{30} and Mo^{42} could be classified as "biogenic transition metals".

In most cases, the valence electrons of the transition metal, *i.e*. the electrons used in the formation of bonds with other elements, are present in two outermost orbitals. That is why, transition metals may exist in several oxidation states (characterized by different oxidation numbers). The states/numbers for biogenic transition metals are listed in Table 2.

Table 1

Transition metals

Group	3 (III B)	4 (IV B)	5 (V B)	6 (VI B)	7 (VII B)	8 (VIII B)	9 (VIII B)	10 (VIII B)	11 (I B)	12 (II B)
Period 4	Sc^{21} 2, 8 9, 2	Ti^{22} 2, 8 10, 2	V^{23} 2, 8 11, 2	Cr^{24} 2, 8 13, 1	Mn^{25} 2, 8 13, 2	Fe^{26} 2, 8 14, 2	Co^{27} 2, 8 15, 2	Ni^{28} 2, 8 16, 2	Cu^{29} 2, 8 18, 1	Zn^{30} 2, 8 18, 2
Period 5	Y^{39} 2, 8, 18 9, 2	Zr^{40} 2, 8, 18 10, 2	Nb^{41} 2, 8, 18 12, 1	Mo^{42} 2, 8, 18 13, 1	Tc^{43} 2, 8, 18 14, 1	Ru^{44} 2, 8, 18 15, 1	Rh^{45} 2, 8, 18 16, 1	Pd^{46} 2, 8, 18 18, 0	Ag^{47} 2, 8, 18 18, 1	Cd^{48} 2, 8, 18 18, 2
Period 6	*	Hf^{72} 2, 8, 18, 32 10, 2	Ta^{73} 2, 8, 18, 32 11, 2	W^{74} 2, 8, 18, 32 12, 2	Re^{75} 2, 8, 18, 32 13, 2	Os^{76} 2, 8, 18, 32 14, 2	Ir^{77} 2, 8, 18, 32 15, 2	Pt^{78} 2, 8, 18, 32 17, 1	Au^{79} 2, 8, 18, 32 18, 1	Hg^{80} 2, 8, 18, 32 18, 2
Period 7	**	Rf^{104} 2, 8, 18, 32, 32 10, 2	Db^{105} 2, 8, 18, 32, 32 11, 2	Sg^{106} 2, 8, 18, 32, 32 12, 2	Bh^{107} 2, 8, 18, 32, 32 13, 2	Hs^{108} 2, 8, 18, 32, 32 14, 2	Mt^{109} 2, 8, 18, 32, 32 15, 2	Ds^{110} 2, 8, 18, 32, 32 17, 1	Rg^{111} 2, 8, 18, 32, 32 18, 1	Uub^{112} 2, 8, 18, 32, 32 18, 2

* La – lanthanide series; ** Ac – actinide series

The transition metals (in shaded boxes) – vanadium, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum – belong to biogenic trace elements. The numbers in the first line under the element symbol represent the number of electrons in the innermost (closest to the nucleus) orbitals, while those in the second line correspond to the electrons in the valence/binding orbitals.

Table 2
Oxidation states (oxidation numbers) of biogenic transition metals

Element	Oxidation states/numbers		Electronegativity (Pauling scale)	Ionization energies: 1st; 2nd; 3rd [kJ/mol]
Vanadium	V[a]	II, III, IV (I)[b]	1.63	650.9; 1414; 2830
Manganese	II	III, IV, VI, VII, (I, V)	1.55	717.3; 1509; 3248
Iron	III	II, V, (IV, VI)	1.83	762.5; 1561.9; 2957
Cobalt	II	III, (IV)	1.88	760.4; 1648; 3232
Nickel	II	III, (I, IV)	1.91	737.1; 1753; 3395
Copper	II	I, (III, IV)	1.90	745.5; 1957.9; 3555
Zinc	II		1.65	906.4; 1733.3; 3833
Molybdenum	VI	II, III, IV, V	2.16	684.3; 1560; 2618

[a,b] Most common (2nd column) and less common (3rd column) oxidation states/numbers
[b] Rare oxidation states/numbers are given in parentheses

Transition metals may form complexes with both charged and neutral ligands. A variety of different oxidation states, as well as the coordination by ligands provide redox and/or catalytic activity to transition metals, which serve as the catalytic centers of enzymes (oxidases, dehydrogenases).

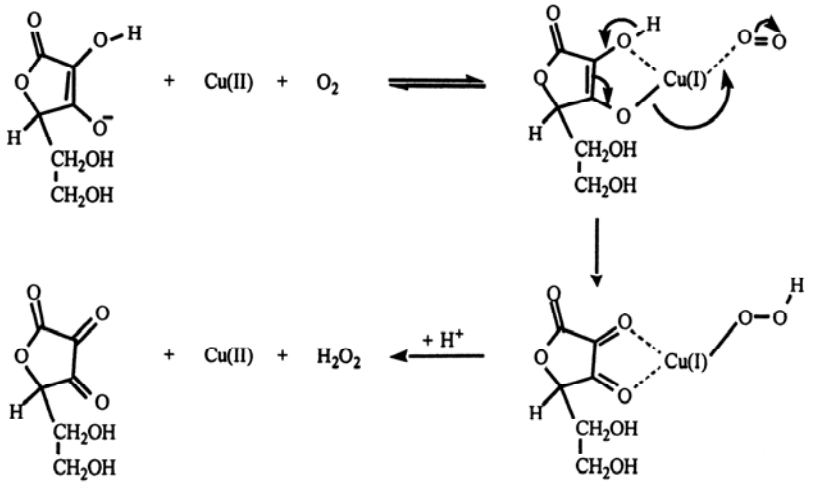
Most biogenic transition metals participate in the control of various metabolic and signaling pathways. However, their versatile coordination chemistry and redox properties allow them to escape the control mechanisms, such as homeostasis, transport, compartmentalization and binding to the designated tissue and cell constituents.[2]

When these metals are liberated from their catalytic sites within enzymes and sequestered abnormally by other ligands, the consequences can be deleterious for the environment. Under such circumstances, irreversible oxidative changes both in the metal-complexing ligand itself and in the neighboring molecules intercepting the reactive intermediates of the initial reaction may initiate a cascade of oxidative stress events.[3]

Moreover, transition metal ions have a specific feature, the so-called "isoelectronics". The manganeous ions are electronically equivalent to the ferric ones, both having five electrons (d^5 configuration) in the valence subshells, which predetermine their mutual substitutability, *e.g.* in octahedral coordination complexes. A similar isoelectronics, within tetrahedral coordination complexes, can be found for Zn(II) and Cu(I), *i.e.* both possess a d^{10} configuration, or for a less common pair – Fe(II) and Co(III) – the d^6 configuration of valence electrons. Such an isoelectronic effect may allow ions of two different elemental species, competing for the same ligands.

Iron coordination with biomolecules involves the participation of its *d* orbitals. Since molecular oxygen, using its electrons in antibonding π^* orbitals, can ligate to the iron by overlapping its *d* orbitals, iron may serve as a bridge between the biomolecule and oxygen. The "flexibility" of iron refers not only to its ability to vary the oxidation state, but also to its capacity of modifying the electronic spin properties and the relative redox potential in response to the interaction with different coordinating ligands. When ascorbate is the coordinating biomolecule/ ligand, it acts as both iron chelator and reductant. Hence, upon binding with Fe(III), ascorbate reduces the iron to the Fe(II) ion, which forms an ascorbate-Fe(II)-oxygen coordination complex.[2] This so-called Udenfriend's oxidative complex/system is a very efficient reagent, used by organic chemists to hydroxylate aromatic compounds, to saturate hydrocarbons to alcohols, olefins to epoxides, etc.[4] With copper, the so-called Weissberger's system – ascorbate-Cu(I)-oxygen[5,6] – may generate hydrogen peroxide (Scheme 1).[7-9] Due to H_2O_2 decomposition through the Fenton-like reaction, Weissberger's system represents one of the most efficient generators of hydroxyl radicals.[10]

If Udenfriend's or Weissberger's systems do not have additional oxidizable substrates, one may speak of "ascorbate auto-oxidation",[11] the resulting reaction product of which is dehydroascorbate (DHA). However, due to the simultaneous presence of oxidizable substrates, the reaction products contain both oxidized/decomposed substrates.[12]



Scheme 1: Generation of H_2O_2 by Weissberger's system from ascorbate and Cu(II), under aerobic conditions (adapted from Fisher and Naughton[9])

Vanadium

The oxidation states of vanadium include **+5**, +2, +3 and +4 (that of +1 being rarely observed – Table 2). Vanadium is an essential element of some enzymes (*e.g.*, nitrogenases in nitrogen-fixing microorganisms). Rats and chickens are known to require vanadium; deficiency in vanadium results in their reduced growth and impaired reproduction. The administration of oxovanadium compounds was shown to alleviate the *Diabetes mellitus* manifestations in certain animal models and humans.

Manganese

The common oxidation states/numbers of manganese are **+2**, +3, +4, +6 and +7, although oxidation states +1 and +5 have also been observed (Table 2). Compounds in which manganese occurs in higher oxidation states are powerful oxidizing agents. Potassium permanganate (oxidation number +7) is used in medicine as an efficient disinfectant. Mn(III)-acetate is a relatively powerful oxidizing agent; the Mn(III)/Mn(II) standard reduction potential equals +1.5 V.

Manganese ions act as cofactors for a number of enzymes, of which Mn-containing superoxide dismutase (Mn-SOD) is one of the essential substances required in almost all organisms living in the presence of oxygen, which use it against the toxic effects of the superoxide anion radicals ($O_2^{\bullet-}$), formed by one-electron reduction of dioxygen. Exceptions include *Lactobacilli* bacteria, which use for $O_2^{\bullet-}$ detoxification a different, non-enzymatic mechanism involving Mn(II) complexed directly with the polyphosphate. Further classes of enzymes, which may have manganese "cofactors", include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.

The human body contains about 300 ppm of manganese; the recommended daily intake of this essential element is of 3-9 mg.[13] The low manganese turnover observed in patients with rheumatoid arthritis may cause disturbances in the synthesis of mucopolysaccharides, as observed in experimental animals with manganese deficiency.[14] A heavy exposure to manganese can, however, cause chronic manganism – a form of Parkinson's disease.

Manganous ions are paramagnetic and hence they are detectable by EPR spectroscopy. A six-band EPR spectrum of Mn(II) has been detected in most natural products.

Iron

At pH 7, the Fe(III)/Fe(II) reduction potential equaling +0.48 V designates[15] iron as one of the major candidates for the production (and metabolism) of free radicals in biological systems. Contrary to ferric salts which, at neutral pH, precipitate, forming oxyhydroxide aggregates, the ferrous compounds are soluble, though unstable, since they tend to react with dioxygen, yielding $O_2^{\bullet-}$ and Fe(III).

In mammals, the iron distribution is heavily regulated, because the Fe(II) ions

represent a risk/toxic factor. Excessive iron intake may induce iron overload disorders, such as hemochromatosis, while a diminished absorption of this essential biogenic element from the duodenum or its deficit in food may lead to anemia.

The average-weight human body contains approximately 4-5 g of iron firmly bound/complexed in metallo-proteins, such as oxygen carrier haemoglobin, the enzymes containing heme prosthetic groups - catalases, cytochromes, as well as in iron transport/storage proteins – transferrin, ferritin and hemosiderin.

Transferrin, a glycoprotein, has two separate binding sites, to which Fe(III) attaches extremely tightly. Under physiological conditions, the transport protein – transferrin – present in the bloodstream is, however, loaded only to ca. 30% of its total iron-binding capacity, so that the amount of "free" iron salts available in plasma should be negligible.

Ferritin is a high-capacity and low-affinity storage protein with a capacity of about 4,500 atoms of iron per one protein macromolecule. The iron from ferritin can be removed by the action of some "bioreductants", such as ascorbate. However, in tissues, only trace amounts of iron are thought to remain "free" (non-chelated) or loosely chelated. Evidence was provided that the iron may be present in tissues over a micromolar range complexed/bound to ATP, AMP, GTP and possibly citrate.[15]

Cobalt

Cobalt is essential to humans. This metal ion is the central component of cobalamin (vitamin B_{12}).

Nickel

The most common oxidation state of nickel is **+2**, although, in Ni-complexes, oxidation states +1, +3 and +4 have also been observed. Solubilized Ni(II) ions in aqueous media at physiological pH are hydrated to a hexahydrate $[Ni(H_2O)_6]^{2+}$ cation.

The main Ni-transporting protein in blood is albumin, although nickeloplasmin also transports these metal ions. The role of nickel in enzymes, such as urease, glyoxalase and a class of superoxide dismutase, has been recently recognized.[16,17]

The daily intake of nickel ranges between 35 and 300 μg.

Copper

An average adult human body contains about 80 mg of copper, while the recommended copper intake for adults is of 0.9 mg/day. When copper is first absorbed in the gut, it is transported to the liver tightly bound to albumin.

Copper is stored in hepatocytes bound to metallothionein. In the bloodstream, copper is bound/carried within a plasma protein – ceruloplasmin. Each ceruloplasmin macromolecule, whose molar mass is around 134 kDa, complexes/binds up to eight copper ions, of which two can be relatively easily liberated.[18] Redox-active copper is the principal constituent in a variety of enzymes, including cytochrome *c* oxidase and CuZn-superoxide dismutase (CuZn-SOD).

Copper toxicity is derived from its ability to accept and donate single electrons. It has been claimed that cuprous/cupric ions catalyze the production of hydroxyl radicals, in a manner analogous to Fenton chemistry. This increase in unmediated reactive radicals, generally termed oxidative stress, is an active area of research in a variety of diseases, in which copper may play an insidious role.

In high amounts, copper can be poisonous and even fatal to organisms. This metal has been involved in the pathogenesis of neurodegenerative disorders, such as Alzheimer and Parkinson diseases, as well as amyotrophic lateral sclerosis. In Wilson disease, the body (mostly liver and brain) tissues retain too much copper. D-Penicillamine and oral Zn-supplements have been used for treating patients with Wilson disease.[19]

Zinc

Zinc is a component of many metalloproteins, such as metalloenzymes and metallothionein proteins. Chelatable redox-inert Zn(II) may serve as an antioxidant by preventing binding of pro-oxidant cuprous ions at the tissue sites.

The adult human body contains approximately 1.5-2.5 g of zinc. The recommended dietary allowance of zinc is of 11 mg for males and of 8 mg for females, higher amounts being recommended during pregnancy and lactation. Brain development is stunted by zinc insufficiency *in utero* and in youth.

Zinc is excreted mostly in feces (12-15 mg/day), lesser amounts (0.5 mg/day) being eliminated in urine.

Molybdenum

Molybdenum is present in several enzymes, such as aldehyde and sulfite oxidases. Purine catabolism, involving the oxidation of xanthine to uric acid, is catalyzed by xanthine oxidase – a Mo-containing enzyme.

A 70-kg human body contains about 10 mg of molybdenum, occurring in higher concentrations in liver and kidneys. The optimal daily intake of molybdenum is of 0.3 mg. Molybdenum deficiency may cause growth retardation and impaired reproduction. High amounts of molybdenum can interfere with the body's uptake of copper.

Non-enzymatic peroxidation – a radical chain reaction

As one may conclude from the above considerations, biogenic transition-metal ions are sequestered by proteins: iron in transferrin and ferritin, copper in ceruloplasmin, *etc*. However, a small amount of low-molar-mass metal chelates/complexes is likely to be present at all times, due to the transfer of metals from storage proteins to metalloproteins and to the turnover of these proteins.[15,20]

Metal ions – especially those of iron – are capable of redox cycling by either accepting [Fe(III) + $1e^-$ → Fe(II)] or donating [Fe(II) - $1e^-$ → Fe(III)] electron(s). These capabilities are closely related to the catalytic participation of iron in reactions producing oxygen-derived reactive species.

In a wide variety of *in vitro* systems, Fe(II) salts and/or non-enzyme chelated/complexed ferrous cations (*e.g*. Fe(II)-EDTA) were shown to enhance the radical damage by increasing the production of oxygen-derived reactive species.

Initiation reaction(s)

In a completely peroxide-free system, the initiation of a peroxidation sequence refers to the attack of any species with sufficient reactivity to abstract a hydrogen atom ($H^\bullet$ radical) from the (bio)substrate involved in a radical chain reaction. $O_2^{\bullet-}$ is insufficiently reactive to abstract the $H^\bullet$ radical. The protonated form of $O_2^{\bullet-}$, *i.e*. $HO_2^\bullet$, although present only as traces, is more reactive and appears as capable of abstracting the $H^\bullet$ radical.[20] 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.[21,22] However, as generally believed, the most efficient acceptor of an $H^\bullet$ radical should be the hydroxyl free radical – $^\bullet OH$, the redox potential ($^\bullet OH/H_2O$) of which equals +2.31 V at pH 7. For generating $^\bullet OH$ radical(s), the following sequence of reactions is usually suggested:

$2Fe(II) + 2O_2 \leftrightarrow 2Fe(III) + 2O_2^{\bullet-}$ (redox reaction; reversible) (***i***)

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ (dismutation reaction) (***ii***)

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

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$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^\bullet OH + HO^-$ (Fenton reaction) (**1**)

$O_2^{\bullet-} + H_2O_2 \leftrightarrow HO^\bullet + HO^- + O_2$ (Haber-Weiss reaction; reversible) (**2**)

It is, however, important that a high Fe(II) concentration can diminish the overall yield of the $^\bullet OH$ radicals, by scavenging them [$^\bullet OH + Fe(II) \rightarrow Fe(III) + HO^-$].

As evident, both reactions **1** and **2** yield $^\bullet OH$ radicals, which may initiate *e.g*. peroxidation of (polyunsaturated) fatty acids in lipids (LH) and thus generate peroxyl lipid radicals ($LOO^\bullet$), by the following sequence of reactions:

$LH + {}^\bullet OH \rightarrow L^\bullet + H_2O$ (**3**)

$L^\bullet + O_2 \rightarrow LOO^\bullet$ (**4**)

Propagation reaction(s)

The $LOO^\bullet$ radicals propagate the lipid peroxidation chain reactions as follows:

$LOO^\bullet + LH \rightarrow LOOH + L^\bullet$ (**5**)

reaction **5** being followed by reaction **4**.

It should be pointed out that, if a sufficiently high excess of ferrous cations is still present in the system, the produced lipid

hydroperoxides (may) undergo an iron(II)-driven "decomposition" reaction:

$$LOOH + Fe(II) \rightarrow LO^{\bullet} + HO^{-} + Fe(III) \quad (\mathbf{6})$$

yielding an alkoxyl lipid radical ($LO^{\bullet}$) which, due to its reactivity, qualitatively similar to that of the lipid peroxyl radical ($LOO^{\bullet}$), may propagate lipid peroxidation, as follows:

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet} \quad (\mathbf{7})$$

reaction **7** being also followed by reaction **4**.

It has to be admitted that, kinetically, the reaction of Fe(II) with lipid hydroperoxides (reaction **6**) is very plausibly to occur, since it is by one order of magnitude faster than the Fe(II)-mediated reaction of H_2O_2 decomposition (the rate constant for reaction **1** (and reaction **2** as well) is of about 76 M^{-1} s^{-1} while, for reaction **6**, the constant is equal to ca. 10^3 M^{-1} s^{-1}).[20]

To elucidate the frequently observed fact that the *in vitro* lipid peroxidation can be also initiated by ferric salts/complexes, it should be taken into account that, during storage and handling under an oxygen-containing atmosphere, all commercially available lipids are readily oxidized, yielding a "contaminant" – LOOH, which is exactly the substance acting as an initiator of (*in vitro*) lipid peroxidation, due to the following reaction:

$$LOOH + Fe(III) \rightarrow LOO^{\bullet} + Fe(II) + H^{+} \quad (\mathbf{8})$$

subsequently followed by reaction **5**.

An important fact should be noted: due to the high oxidation tendency of the ferrous ions (the Fe(III)/Fe(II) reduction potential is equal to +0.48 V at pH 7),[15] practically none of the Fe(II) salts/complexes is free from Fe(III) cations. Although Fe(III) should not trigger lipid peroxidation, due to an ubiquitous contamination of lipids with the LOOH hydroperoxides, the addition of Fe(III) initiates a radical chain reaction since its reaction with hydroperoxides generates $LOO^{\bullet}$ radicals, according to reaction **8**.

If the occurrence of the Fenton reaction is not unambiguously confirmed, one can reach the false conclusion that, when reacting with hydrogen peroxide, any (biogenic) transition metal in a lower oxidation state (M^{n+}) would generate hydroxyl radicals by the $M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + {}^{\bullet}OH + HO^{-}$ reaction. However, most researchers agree with the statement that, under normal/physiological *in vivo* conditions, it is only the iron(II)-dependent formation of $^{\bullet}OH$ radicals that may actually occur.[20]

The joint – role of hyaluronan

A joint is formed by the ends of two (or more) bones connected by connective tissues. The function of joints in the human organism is to ensure mutual motion of the adjacent bones in both plane (bending $x \leftrightarrow y$) and space (rotation $x \leftrightarrow y \leftrightarrow z$). The bone ends linked in a joint are covered by a thin soft layer – the cartilage, whose matrix is permanently restructured/rebuilt by the embedded chondrocytes.

Every joint is surrounded by a fibrous tissue capsule called synovium, the primary function of which is to produce a synovial fluid (SF), which reduces friction and wear-and-tear of the joint. The main components of this "lubricating" fluid are a filtrate of blood plasma and a high-molar-mass polysaccharide – hyaluronan (HA), extruded by type II synoviocytes, the cells localized within the synovial membrane.

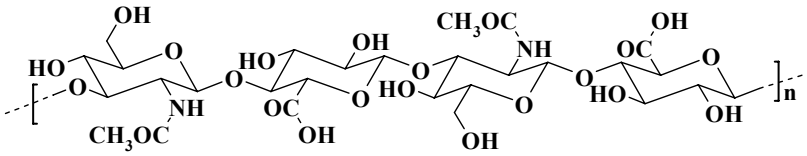


Figure 1: Hyaluronan – acid form

HA (Fig. 1) is a linear, high-molar-mass natural polysaccharide formed of disaccharide units containing *N*-acetyl-D-glucosamine and D-glucuronic acid. The chemical structure of HA is rather regular, the only deviation being a possible replacement of *N*-acetyl-D-glucosamine by deacetylated glucosamine residues. In an aqueous milieu, HA is represented by negatively charged hyaluronate macromolecules with extended conformations, which impart high viscosity/viscoelasticity to its solution.[23]

In human beings, HA is abundantly present in almost all body fluids and tissues. In the synovial fluid and vitreous humor, HA macromolecules reaching megadalton molar masses are present in a "free form", *i.e*. not

associated with proteins. In the articular cartilage matrix, however, HA is associated *via* a link protein with a proteoglycan – aggrecan – consisting of collagen and glycosaminoglycans, namely chondroitin sulfate and keratan sulfate.

In a healthy human being, the synovial fluid containing, besides blood plasma filtrate, entangled macromolecules of HA (1.4-3.6 mg/mL[24]), represents a gel-like colloidal medium. Upon crossing the synovial barrier, the nutrients, oxygen supply included, permeate the viscous colloid to the avascular articular cartilage where they are utilized by embedded chondrocytes. On the other hand, the chondrocyte catabolites (should) cross both the viscous fluid and the synovial membrane. It can thus be concluded that, within the articular gel-like fluid, the process of "mixing" at increased mobility of the joint may significantly affect joint homeostasis.

Joint hypoxia and re-oxygenation

As the cartilage contains no teleneurons, the regulation of chondrocytes should be of chemical nature. In a relaxed state – for example, at night – chondrocytes experience a decreased oxygen supply (a status termed "hypoxia"). However, when the status changes towards enhanced mobility in the morning, the SF joint receives an elevated oxygen supply (a situation termed "re-oxygenation"). Such an increased oxygen content can be, however, deleterious for the homeostasis of chondrocytes – the cells that, in adults, lack mitotic activity.

As the "normal" SF contains no hyaluronidase activity, it has been inferred that the oxygen-derived reactive species/metabolites are involved in a self-perpetuating process of hyaluronan catabolism within the joint,[25] considered responsible for the short – about twelve hours – half-life of the native HA macromolecules in SF.

To understand how to maintain a radical reaction active and self-perpetuating, its propagation stage should first be analyzed. Let us assume that a peroxyl-type macromolecular radical ($AOO^{\bullet}$) exists within SF. If considering the relatively high reactivity/affinity of the unpaired electron on the oxygen, the following particular reaction step can be assumed:

$$AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet} \qquad (\mathbf{9})$$

where HA stands for hyaluronan and H stands for a hydrogen atom – whose abstraction by the $AOO^{\bullet}$ radical leads to the formation of an $A^{\bullet}$ macroradical. When $A^{\bullet}$ is a carbon macroradical, it is especially this reactant that traps the excess of dioxygen molecule(s), according to the following reaction:

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \qquad (\mathbf{10})$$

Hence, by simply combining the two reactions, **9** and **10**, the resulting net reaction:

$$HA + O_2 \rightarrow AOOH$$

corroborates the idea that one particular function of (a high-molar-mass) HA will entrap the oxygen excess during joint re-oxygenation.

Participation of biogenic transition metals in the physiological catabolism of hyaluronan

Analogously to the decomposition of lipid hydroperoxides (reactions **6** and **8**) caused by a transition metal, similar reactions could be suggested for the decomposition of the cumulating AOOH hydroperoxides:

$$AOOH + Fe(II) \rightarrow AO^{\bullet} + HO^{-} + Fe(III) \qquad (\mathbf{11})$$

$$AOOH + Fe(III) \rightarrow AOO^{\bullet} + Fe(II) + H^{+} \qquad (\mathbf{12})$$

As evident, while the reactant/"propagator" entering reaction **9** is regenerated/recycled by reaction **12**, reaction **11** produces an alkoxyl macroradical. The ratio between the generated $AOO^{\bullet}$ to $AO^{\bullet}$ radicals is however governed by the present iron, or more precisely by the ratio of Fe(III) to Fe(II). However, in the case of a sufficiently high level of ascorbate, which is an efficient reductant of ferric cations, the actual concentration of Fe(II) ions exceeds that of Fe(III), so that the $AO^{\bullet}$ radicals should prevail. Similarly to the $AOO^{\bullet}$ ones, these radicals could propagate the radical chain reaction, as follows:

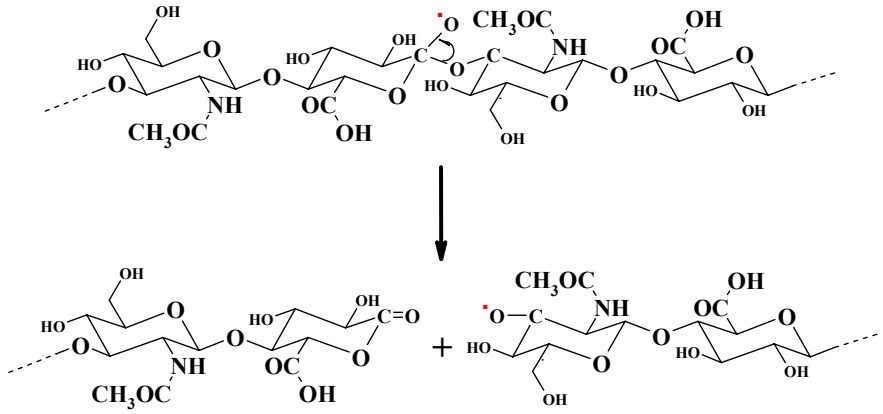
$$AO^{\bullet} + HA \rightarrow AOH + A^{\bullet} \qquad (\mathbf{13})$$

However, if assuming the quenching of the $AO^{\bullet}$ radicals by the ascorbate, the products would now contain "oxidized" hyaluronan macromolecules *plus* DHA and/or its hydrolyzates.

The higher reactivity of the $AO^{\bullet}$ radicals with ascorbate, compared to that of the $AOO^{\bullet}$ radicals, closely correlates with the about 1,000-times higher value of the rate-constant (k) in the case of reduction of alkoxyl radicals. For example, the k values estimated for the reactions between ascorbate

and a tocopheryl (T) radical $TO^{\bullet}$ or peroxyl-type radical $TOO^{\bullet}$, equal 1.6×10^{9} or $(1–2)\times10^{6}$ $M^{-1}s^{-1}$, respectively, and hence, the half-life of the alkoxyl radical is much shorter than that of peroxyl – microseconds *vs*. seconds. Analogously, the redox potential of the $RO^{\bullet}$, H^{+}/ROH pair = 1.6 V exceeds significantly that of $ROO^{\bullet}$, H^{+}/ROOH = 1.0 V.

Nevertheless, as to the radical reactions of carbohydrate polymers, the strand scission of $AO^{\bullet}$ and of $AOO^{\bullet}$ (intermediate) macroradicals represents a very plausible pathway, by which polymer fragments of smaller molecular size shall be generated (Scheme 2; see also refs. 26-28).



Scheme 2: The $AO^{\bullet}$ strand scission may be due to the *β*-cleavage of the radical formed at, *e.g*. C(1), on the ring of D-glucuronate/D-glucuronic acid

Along with the above-described hyaluronan fragmentation reactions, the radical attack on the D-glucuronate/D-glucuronic acid and *N*-acetyl-D-glucosamine moieties can also lead to the "opening" of rings, without breaking the polymer chain.[24,29-31]

Actors of physiological catabolism of hyaluronan in the synovial fluid

The concentration of ascorbate in the SF of healthy subjects reaches values close to those established in blood serum, *i.e*. 40-140 μM.[32] The contents of transition metals in both body fluids are listed in Table 3.

As evident from the data listed in Table 3, iron and copper are the two prevailing redox active transition metals in SF. It should be, however, pointed out that the respective levels, of ca. 5.2 and 4.3 μM, do not represent the ones (freely) disposable to catalyze the oxidative catabolism of hyaluronan within SF. As reported, the availability of iron to stimulate *in vivo* generation of $^{\bullet}OH$ radicals is very limited, since the concentrations of bleomycin-detectable, *i.e*. the "free" iron from the human samples, is rarely exceeding[20] 3 μM.

Table 3
Contents of transition metals in blood serum of healthy human volunteers and in SF collected *post mortem* from subjects with no evidence of any connective tissue disease

Element	Mean concentration in blood serum [μg/100 mL][a]	Mean concentration in the synovial fluid [μg/100 g][a]
Manganese	2.4 (0.44)[b]	2.4 (0.44)
Iron	131.7 (23.6)	29.0 (5.19)
Nickel	4.1 (0.70)	1.2 (0.20)
Copper	97.0 (15.3)	27.5 (4.33)
Zinc	115.4 (17.7)	17.6 (2.69)
Molybdenum	3.4 (0.35)	1.0 (0.10)

[a] Reported by Niedermeier and Griggs[14]
[b] Data in parentheses represent the values in μM calculated on considering that 100 g of SF has a volume of 100 mL

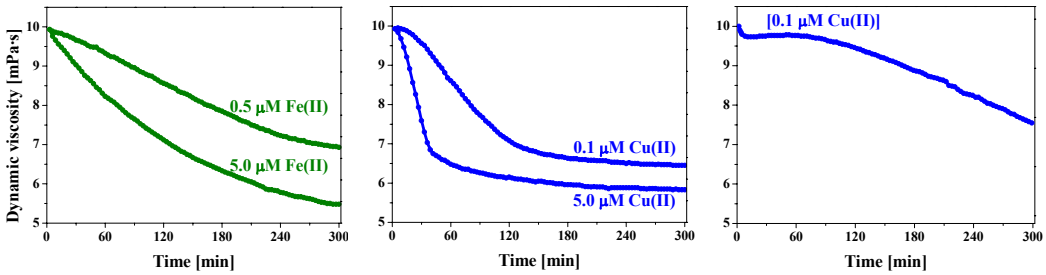


Figure 2: Time dependencies of dynamic viscosity in hyaluronan (P9710-2A) sample solutions (2.5 mg/mL), where left panel: Solutions of the HA sample with addition of 100 μM ascorbic acid immediately followed by admixing 0.5 or 5.0 μM of $FeCl_2$; middle panel: Solutions of the HA sample with addition of 100 μM ascorbic acid immediately followed by admixing 0.1 or 5.0 μM of $CuCl_2$; right panel: The curve represents an assay in which 0.1 μM of $CuCl_2$ was added to the HA sample solution, 9 minutes before admixing 100 μM ascorbic acid (for details, see ref. 33)

Figure 2 illustrates the viscosity–time profiles of a model situation, in which – along with 100 μM ascorbate – a single transition metal is involved.[34] As evident, a significant reduction in solution dynamic viscosity (η), corresponding to the degradation of the high-molar-mass HA sample, clearly indicates a concentration-dependent manner for each metal (see the left and central panels in Fig. 2). As one may see, the character of the time dependence of the η value upon the addition of $FeCl_2$ (5.0 μM) can be described as a gradual monotonous decline, while the addition of $CuCl_2$ (5.0 μM) results in a literally "dramatic" drop of the η value within a short time interval (30 min), after which the decrease in solution dynamic viscosity continued, yet at a much lower rate. A most probable explanation to this dissimilarity lies in the different reaction kinetics of the processes, leading to the generation of oxygen-derived reactive species in the ascorbate *plus* Cu(II) system, as well as in those containing ascorbate *plus* Fe(II), *i.e.* Weissberger's and Udenfriend's oxidative systems.

A similar drop in the η value and two-phase reaction kinetics may be identified upon the addition of even a minute (0.1 μM) amount of $CuCl_2$ (Fig. 2, central panel) although, when Cu(II) addition preceded that of ascorbate (right panel), the decline of solution viscosity was much less obvious. A plausible explanation is that a delay in ascorbate addition (9 min) allowed the Cu(II) ions to reach the "binding sites" within the HA macromolecule. The domain of the coordinate-bound copper thus formed seems to be "hindered" and, hence, less accessible to a direct complexation/interaction with the ascorbate anion, a condition required for the formation of Weissberger's oxidative complex. As postulated, the hyaluronate binding sites indicate a (weak) selectivity not only toward copper but also toward the (isoelectronic) zinc cations.[35] On the contrary, manganous and presumably, the (isoelectronic) Fe cations form simple hyaluronate salts with D-glucuronate moieties.[36] Thus, the four transition metal elements – iron, copper, manganese and zinc – may create, by their presence/absence, a variety of conditions, under which HA oxidative degradation proceeds.

There exists, however, a marked difference between the *in vitro*/*in situ* degradation of hyaluronan and its *in vivo* catabolism. Under physiological conditions, SF viscosity does not indicate any changes, since the content of "native" hyaluronan remains constant, as due to a permanent *de novo* production of megadalton HA macromolecules by (stimulated) synoviocytes. Thus, the self-perpetuating oxidative (non-enzymatic) HA catabolism in SF represents a rather delicate and properly balanced mechanism that presumably plays a significant role in regulating the physiological – normoxygen – homeostasis for chondrocytes. At the same time, the polymer fragments formed, which are removed by drainage pathways from the joint, serve most likely as chemical messengers/feedback molecules used for adjusting the optimum operation mode of the synovial membrane and of the HA-producing cells, synoviocytes, localized inside.

In other words, during physiologic joint functioning, the hyaluronan in SF plays the role of a "scavenger antioxidant", whereas the produced polymer fragments can subsequently serve as messengers mediating information on the changes occurring in the homeostasis of the joint.

The high "protective/scavenging efficiency" of hyaluronan against the action of, *e.g.* $^{\bullet}OH$ radicals, has been earlier pointed out by some authors.[37,38] Presti and Scott[38] described that high-molar-mass hyaluronan (1218 kDa) was much more effective than the lower-molar-mass (668 and 176 kDa) HAs in scavenging the $^{\bullet}OH$ radicals generated by a Fenton-type system comprising glucose and glucose oxidase *plus* Fe^{2+}-EDTA chelate.

Pathophysiological catabolism of hyaluronan in the synovial fluid

Despite their exceedingly simple primary structure, the HA macromolecules have extraordinarily wide-ranging and often opposing biological functions. As recently reported by Stern *et al.*,[39] the unadorned HA polymer functions size-specifically, its fragments constituting a highly information-rich system. While the "space-filling" megadalton hyaluronans synthesized by HA synthases (HAS1 and HAS2),[40,41] are immunosuppressive and anti-angiogenic,[42-44] the intermediate-sized HA-polymer fragments are inflammatory, immunostimulatory and highly angiogenic.[45-47]

There are two distinct modes to cleave *in vivo* the extended native HA chains: the enzymatic way, involving the participation of hyaluronidases (*e.g.* Hyal-2), and the chemical one, involving oxygen-derived reactive species.[28] Yet, due to the fact that SF is lacking any hyaluronidases, it is reasonable to assume that it is the latter mechanism which might be involved, too, during HA catabolism, in the inflamed joint. As illustrated in Figure 2, any transition metal, *i.e.* iron or copper, can play an active role in the oxidative HA catabolism. However, the increase in Cu(II) concentration within the joint (and particularly in SF) could lead to a really very rapid degradation of the native HA macromolecules.

Figure 3 exemplifies the impact of two different oxidative systems containing Cu(II) ions. As evident, a megadalton HA sample (M_w = 1.2 MDa) degrades *in situ* to intermediate-sized polymer fragments, which means that the molar masses are reduced by one or even two orders of magnitude. Interestingly, the unimodal and relatively narrow molar-mass distribution (MMD) of the megadalton HA sample (M_w/M_n = 1.8) remains retained even upon cleaving the polymer (compare the three MMD curves in Fig. 3).

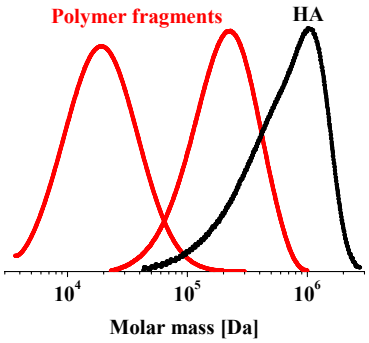


Figure 3: Comparison of MMD of the megadalton HA sample and of those degraded *in situ* by oxygen-derived reactive species (Fig. 2 and ref. 33)

Some differences should be, however, pointed out between the processes taking place in a joint (or locally, within the SF) and in a well-stirred reaction mixture under *in situ* conditions. In the bloodstream, the copper atoms are carried by ceruloplasmin, belonging to the so-called "acute-phase proteins", whose concentration increases markedly under certain circumstances.[18] A higher ceruloplasmin concentration in the blood plasma would mean a larger amount of copper crossing the synovial membrane. Yet, due to the SF gel-like consistency, the copper ions entering this specific environment should start their "dramatic" oxidative action in the vicinity of the synovial membrane. How efficiently the chemically-generated $^{\bullet}OH$ radicals are "scavenged" within this microenvironment by the locally disposable albumin, as well as by the HA polymer fragments of a lower molecular size, remains questionable. The oxidative process may escape the control mechanisms and damage/disrupt the synovial membrane. Moreover, the intermediate-sized HA-polymer fragments generated within this microenvironment could participate in the activation of "defender" cells. They may further increase the inflammation state of the injured tissue(s) because the HA-polymer

fragments can, in turn, augment the inflammatory responses. As reported by Jiang *et al.*, the HA fragments in the, *e.g.* 2×10^5 Da range, induce in macrophages the expression of a number of inflammatory mediators, including chemokines, cytokines, growth factors, proteases, and nitric oxide.[47] In this way, the oxidants generated by activated defender cells may enlarge the damage within the involved joint tissues, such as the synovial membrane.

Moreover, it appears that the reactive oxygen species disrupt copper binding to ceruloplasmin, thereby releasing the "free" copper ions, which in turn may promote oxidative pathology.[18] The damage can be manifested by visually localizable cardinal symptoms of inflammation – color, dolor, rubor, tumor and functiolaesa. Yet, although less distinct, repeated inflammatory injuries may lead to a disastrous outcome – *e.g.*, an autoimmune disease, such as rheumatoid arthritis.

The mutual substitution of (isoelectronic) transition metals, their versatile coordination chemistry and redox properties, along with changes in their compartmentalization and binding to the designed tissue/cell constituents, may reflect certain non-specific physiological responses of the organism to pathological stimuli.[2] Rheumatoid arthritis can be classified as one of the diseases of particular interest in this respect. Compared to the SF from normal/healthy subjects, in the synovial fluid from patients with rheumatoid arthritis, the mean concentration of copper is increased by a factor of three, while that of iron and zinc – by 2 and 3.5, respectively.[14]

There are several chronic (inflammatory) conditions under which "abnormalities" in the metabolism of trace/transition metal(s) are at present in the focus of biomedical research. These illnesses – such as autoimmune rheumatoid arthritis, neurodegenerative Alzheimer's and Parkinson's diseases – are classified as socially debilitating. Considering the permanently extending mean life-span of the human population, these diseases create serious ethical/societal issues, like that of euthanasia.

ACKNOWLEDGEMENTS: Grants VEGA 2/0003/8 and 2/7033/27 of the Slovak Academy of Sciences and Ministry of Education of Slovak Republic, and APVV-51-017905 from the Slovak Research and Development Agency are gratefully acknowledged.

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