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Chapter 8

HYALURONAN – A HIGH-MOLAR-MASS MESSENGER REPORTING ON THE STATUS OF SYNOVIAL JOINTS: PART I. PHYSIOLOGICAL STATUS

Grigorij Kogan
Directorate Health, Directorate General Research, European Commission,
B-1049, Brussels, Belgium

ABSTRACT

Since the content of hyaluronan-degrading enzymes in synovial fluid, if any, is extremely low, the high rate of this glycosaminoglycan turnover in synovial fluid – $t_{1/2} \approx$ 12 hours – is to result from a cause different from enzymatic catabolism. An alternative and plausible mechanism is that of oxidative-reductive degradation of the biopolymer chains by a combined action of oxygen, transition metal cations, and ascorbate. This oxidative-reductive hyaluronan cleavage, under physiological homeostasis, has recently drawn much attention of biochemists for an excellent messenger-like capability of hyaluronan fragments generated *in vivo*.

Keywords: Glycosaminoglycans, Hyaluronan catabolism, Reactive oxygen species, Synovial fluid, Transition metals.

INTRODUCTORY REMARKS

On average, a healthy person living in the developed countries currently reaches lifespan of ca. 80–85 years. Women often live longer than men. This fact could be associated with their enhanced redox load during the reproductive phase of their life. Physiological bleeding (with a periodicity of ca. 4 weeks) is accompanied by changes in the concentration of iron ions. Pre-menopausal women are believed to have a lower risk of common diseases because amounts of iron in their body are unlikely to be excessive at this time [1].

Fe ions are regarded as one of the most important catalytical agents that contribute to the augmented generation of the reactive oxygen species (e.g. $^{\bullet}OH$ radicals). However, such "radical training" of female organism lasting on average 40 years (i.e. in a period between ca. 15 to 55 years) can have a positive effect on females in the sense that their organism is better adjusted to the oxidative stress. In the "free radical theory of ageing" oxidative stress is considered to be a risk factor that is usually associated with such negative consequences as serious diseases or even premature death [2,3].

Life can be in a simplified way divided into three periods: childhood, maturity, and senescence. Maturity is the longest lasting part of human life. It lasts from the end of development and growth of a skeleton (around ca. 20 years) till the old age, which start can be marked as at ca. 70–75 years. Thus, maturity lasts about half a century. During this period, human skeleton can be considered invariable regarding the number of bones (206), their size, and mass.

The human skeleton consists of both fused and individual bones supported and supplemented by ligaments, tendons, and skeletal muscles. Articular ligaments and tendons are the main parts holding together the joint(s). In respect to the movement, there are freely moveable, partially moveable, and immovable joints. Synovial joints, the freely moveable ones, allow for a large range of motion and encompass wrists, knees, ankles, shoulders, and hips.

THE STRUCTURE OF A SYNOVIAL JOINT

Figure 1 illustrates a normal healthy synovial joint indicating its major parts.

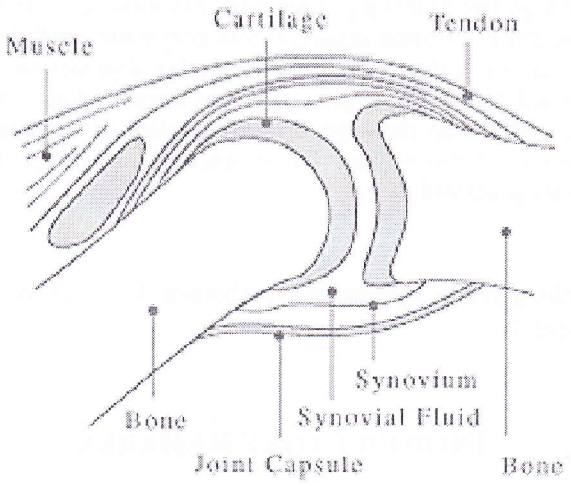


Figure 1. Normal, healthy synovial joint [4].

Cartilage

In a healthy synovial joint, heads of the bones are encased in a smooth (hyaline) cartilage layer. These tough slippery layers – e.g. those covering the bone ends in the knee joint – belong to mechanically highly stressed tissues in the human body. At walking, running, or sprinting the strokes frequency attain approximately 0.5, 2.5 or up to 10 Hz.

Cartilage functions also as a shock absorber. This property is derived from its high water-entrapping capacity, as well as from the structure and intermolecular interactions among polymeric components that constitute the cartilage tissue [5]. Figure 2 sketches a section of the cartilage – a chondrocyte cell that permanently restructures/rebuilds its extracellular matrix.

Three classes of proteins exist in articular cartilage: collagens (mostly type II collagen); proteoglycans (primarily aggrecan); and other noncollagenous proteins (including link protein, fibronectin, COMP – cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin, and fibromodulin). The interaction between highly negatively charged cartilage proteoglycans and type II collagen fibrils is responsible for the compressive and tensile strength of the tissue, which resists applied load *in vivo*.

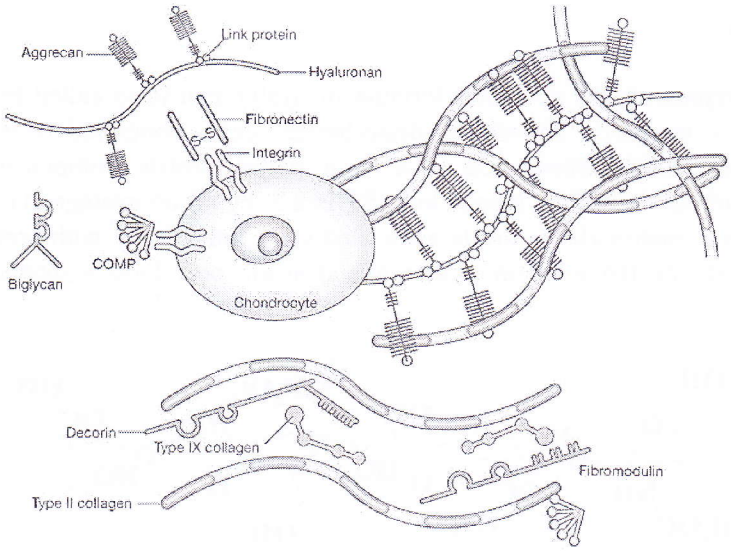


Figure 2. Articular cartilage main components and structure [6].

Synovium/Synovial Membrane

Each synovial joint is surrounded by a fibrous, highly vascular capsule/envelope called synovium, which internal surface layer is lined with a synovial membrane. Inside this membrane, type B synoviocytes (fibroblast-like cell lines) are localized/embedded. Their primary function is to continuously extrude high-molar-mass hyaluronans (HAs) into synovial fluid (SF).

Synovial Fluid

The synovial fluid, which consists of an ultrafiltrate of blood plasma and glycoproteins, in normal/healthy joint contains HA macromolecules of molar mass ranging between 6–10 megaDaltons [7]. SF serves also as a lubricating and shock absorbing boundary layer between moving parts of synovial joints. SF reduces friction and wear and tear of the synovial joint playing thus a vital role in the lubrication and protection of the joint tissues from damage during the motion [8].

The nutrients, including oxygen supply, upon crossing the synovial barrier, permeate through the viscous colloidal SF to the avascular articular cartilage, where they are utilized by the embedded chondrocytes. On the other hand, the chondrocyte catabolites (should) cross the viscous SF prior to being eliminated from the synovial joint [9]. It can thus be concluded that within SF, the process of "mixing" at the joint motion, significantly affects the equilibrium of influx and efflux of all low- and high-molar-mass solutes. It appears that the traffic of solutes is determined by molecular size, with small polar molecules being cleared by venular reabsorption, while high-molecular-sized solutes are removed by lymphatic drainage [10].

Hyaluronan

Figure 3 represents the structural formula of hyaluronan (also called hyaluronic acid, hyaluronate) – regularly alternating disaccharide units composed from *N*-acetyl-D-glucosamine and D-glucuronic acid. HA is a polyelectrolyte component of SF; the concentration of HA in healthy human knee SF is 2.5 mg/ml on average [11]. While in the articular cartilage matrix HA is firmly associated via a link protein with proteoglycans (cf. Figure 2), in SF the HA macromolecules are, if at all, only loosely interacting/bound to proteins.

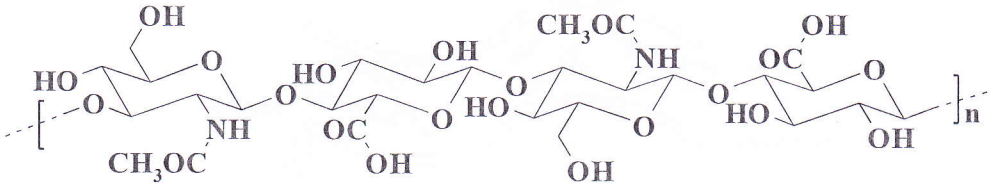


Figure 3. Hyaluronan – the acid form.

HA is a linear non-branched non-sulfated glycosaminoglycan (bio)polymer. In aqueous solutions, HA is represented by negatively charged hyaluronate macromolecules (p*K*a = 3.21 [12]) with extended conformations, which impart high viscosity/viscoelasticity, accompanied also by low compressibility – the characteristic property of SF [13].

Reactive Oxygen Species in Articular Cartilage

Articular cartilage is an avascular, acidic (pH 6.6–6.9) and hyperosmotic tissue dependent on diffusion of nutrients supplied mainly from SF (and perhaps partly from subchondral bone

[14]) to provide for the metabolic requirements of chondrocytes. The oxygen levels in this tissue are low, ranging between 1 and 6% (cf. Figure 4). While reduction in O_2 tension to 6% in all other tissues is already hypoxic, for chondrocytes such oxygen level is normoxic.

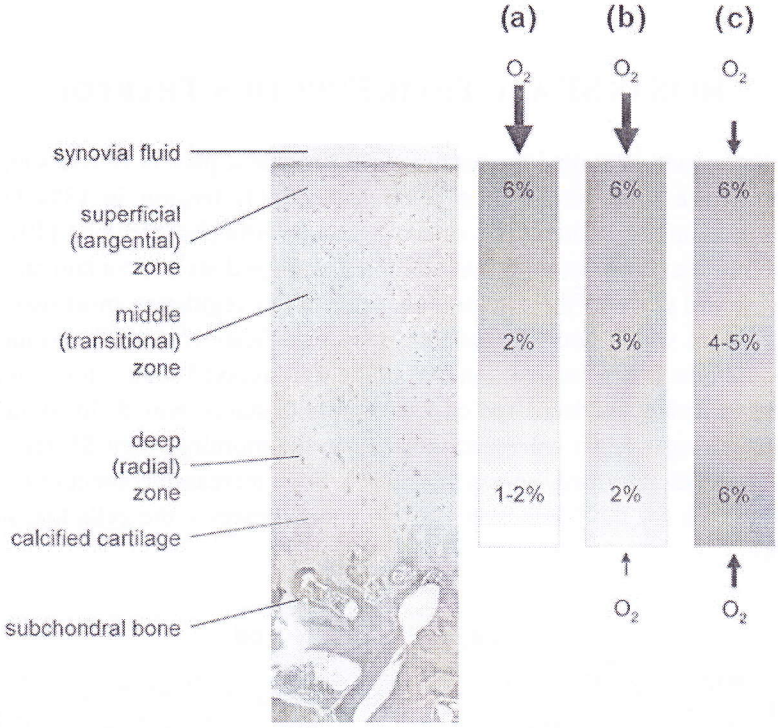


Figure 4. The structure of articular cartilage and its oxygen supply (adapted from [14]). Estimated levels of O_2 within the cartilage tissue are shown for three scenarios: (a) penetration of O_2 exclusively from SF; (b) O_2 supply mostly from SF with a small contribution from subchondral bone; (c) supply of O_2 in equivalent amounts from SF and subchondral bone.

In the mitochondria of the eukaryotic cells, not all O_2 is fully reduced to water. A small fraction of oxygen is reduced incompletely yielding reactive oxygen species (ROS), which are assigned to the defense of the organism against viral/bacterial invaders [15]. It has been established that while ROS content within the articular cartilage tissue remains normal at 6% O_2, it decreased at 1% O_2 [14].

Since hydrogen peroxide generated within the mitochondria of chondrocytes can freely permeate through the chondrocyte cell wall, one should admit the presence of H_2O_2 in all (deep, middle, and superficial) zones of the articular cartilage (cf. Figure 4). The higher the O_2 tension, the greater is the content of H_2O_2 and vice-versa.

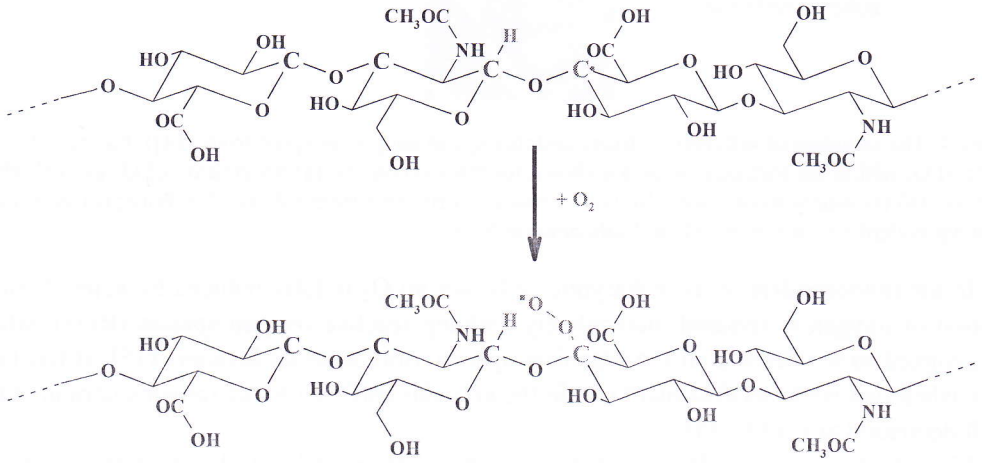
The ROS within the cartilage tissue could serve both as intra- and inter-cellular signaling devices and a reactant participating in the so-called Fenton reaction

$$H_2O_2 + Me^{n+} \rightarrow {}^{\bullet}OH + Me^{(n+1)+} + OH^- \qquad (1)$$

where Me^{n+} and $Me^{(n+1)+}$ represent a (biogenic) transition metal ion in reduced and oxidized state. Among these metals, primarily iron and copper are usually ranked, however, several further trace/biogenic metals can be taken into account as well [1,16].

ROS IN SF AND THEIR FUNCTION THEREOF

The capillaries within synovium continuously provide a plasma filtrate supplying in this way nutrients to the joint tissues (the arterial blood O_2 tension is 13% [17]). This is particularly important for homeostasis of the avascular articular cartilage [10]. As recently stated [16], taking into consideration that articular cartilage does not contain any teleneurons, chondrocytes should perform their autonomic (metabolic) regulation most plausibly using a chemical process, in which both O_2 and ROS play significant roles [17]. To understand this tenet, one should take into consideration that in the joint relaxed state – for example, at night – chondrocytes experience a decreased oxygen supply (a status termed "hypoxia"). However, when the status changes to an enhanced mobility in the morning, joint SF receives elevated supply of O_2 (a situation termed "re-oxygenation"). Such increased content of oxygen can be, however, deleterious for the homeostasis of the chondrocytes – the cells that in adults lack mitotic activity.



Scheme 1. Entrapment of oxygen by the hyaluronan C-(macro)radical (A•) yielding a peroxyl (macro)radical (A–O-O•).

Let us assume that Me^{n+} ions in a given concentration are "entrapped" by (highly) negatively charged cartilage glycosaminoglycans (GAGs) within the superficial (tangential) zone of the articular cartilage (cf. Figure 4). During the utilization of O_2 – respiration – by chondrocytes, a limited amount of H_2O_2 liberated from their mitochondria can react with the entrapped transition metal ions generating hydroxyl (•OH) radicals. Due to extremely short half-life of these species (picoseconds), they react *in situ nascendi* with GAGs – chondroitin sulfate (CS) and/or keratan sulfate (KS). The C-type radicals of CS or KS can, however, instantly undergo a reaction of hydrogen radical transfer onto the neighboring HA

macromolecules within the SF. In such a way, free C-(macro)radicals of hyaluronan appear nearby the superficial zone of the articular cartilage. And it is this very C-(macro)radical (denoted later as $A^{\bullet}$), which reacts and in this way reduces the (free "hyperoxic") O_2 tension within and nearby the superficial zone of the articular cartilage – according to the reaction presented in the following scheme:

or briefly

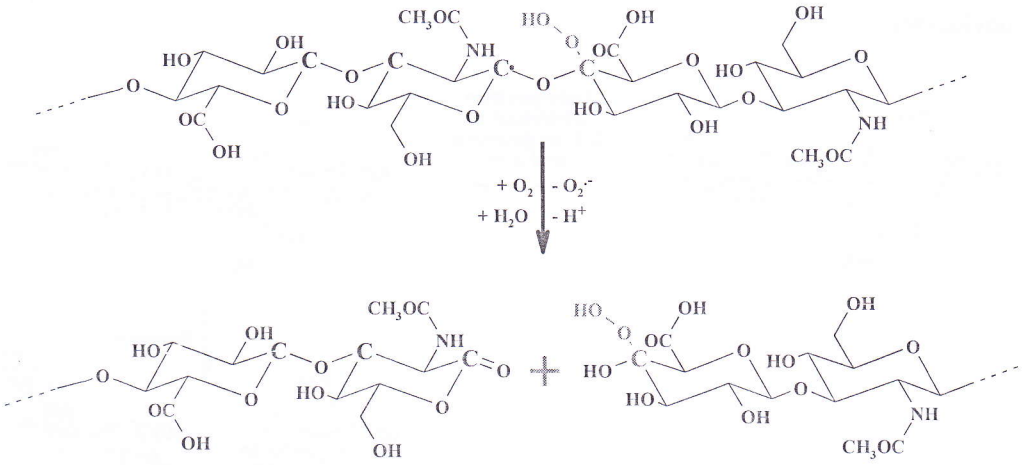
$$A\text{–}H + {}^{\bullet}OH \rightarrow A^{\bullet} + H_2O \qquad (2)$$

$$A^{\bullet} + O_2 \rightarrow A\text{–}O\text{-}O^{\bullet} \qquad (3)$$

where A–H represents the intact hyaluronan macromolecule (cf. Figure 3 and Scheme 1).

Subsequently, this $A\text{–}O\text{-}O^{\bullet}$ peroxyl (macro)radical can transform simply by an intramolecular 1,5-hydrogen shift to another C-(macro)radical – $A^{\bullet}$ (cf. Scheme 2). By participation of another O_2 molecule, this $A^{\bullet}$ radical can yield two fragments of the HA biopolymer: (i) the fragment, which possesses an aldehyde terminus, and (ii) the fragment bearing a hydroperoxide functional group. It is naturally evident that both fragments differ in their chemical structure from the initial HA macromolecule, not only due to the included novel substituents (–C=O; –O-OH) but above all by a reduced molar mass of both polymer fragments compared to that of the parent biopolymer.

Since the intermolecular reaction between the CS and KS radicals and the native HA macromolecule could yield various $A^{\bullet}$ radicals – formed for example at C(4) of the D-glucuronate/D-glucuronic acid (GlcA) unit (cf. Scheme 1) or at C(1) of GlcA unit, as well as at C(1) or C(3) of *N*-acetyl-D-glucosamine (GlcNAc) [18] – various biopolymer fragments are produced.

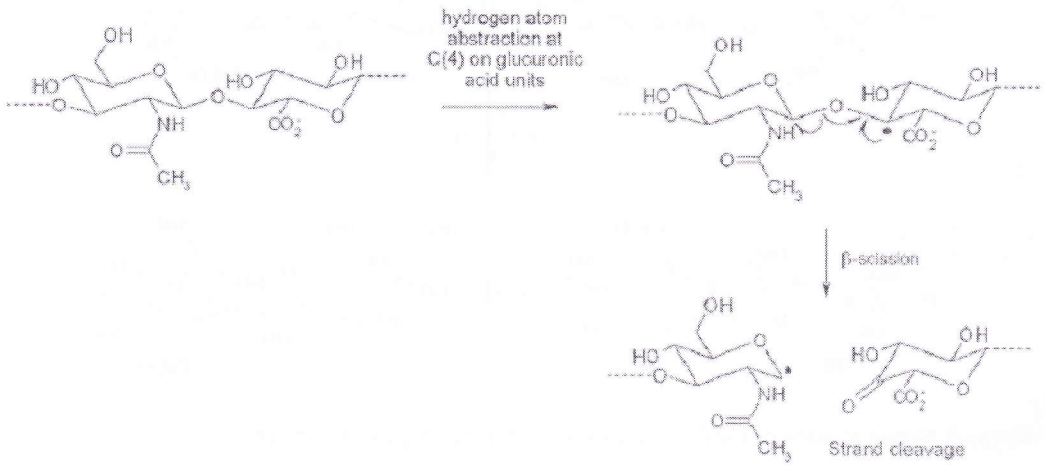


Scheme 2. Strand scission of the C-(macro)radical ($A^{\bullet}$) yielding two fragments.

Very recently Kennett and Davies [19] reported the data obtained with both the C(1)- and the C(2)- ^{13}C-labeled *N*-acetyl-D-glucosamine, and the apparent highly selective generation of radicals at the C(2) position of the isopropyl group of the β-isopropyl glycoside, which allow the authors to rationalize the specific banding pattern observed on oxidation of hyaluronan: The lack of reactivity at C(1)/C(2) of the *N*-acetyl-D-glucosamine monomers and the specific formation of radicals on the isopropyl group, which models the C(4) glycosidic linkage site of the glucuronic acid, implicate attack at C(4) of the glucuronic acid subunits and subsequent β-scission of this radical as a major route to cleavage of the hyaluronan backbone (Scheme 3). A contribution from reaction at C(1) of the glucuronic acid and subsequent cleavage of the alternative glycosidic linkage cannot be discounted; however, it is clear that an alternative route involving C(3) on the *N*-acetyl-D-glucosamine monomer is less favored, as only low levels of initial hydrogen atom abstraction seem to occur at this position as judged by the low yield of radicals that did not have additional ^{13}C couplings observed with the two labeled *N*-acetyl-D-glucosamine species. It should be pointed, however, that the products of the hyaluronan strand cleavage depicted in Scheme 3 do not take into account that the ubiquitous oxygen participate within the strand scission reaction and thus, analogously to Scheme 2, the involved O_2 molecule with the $A^{\bullet}$ radical yields two fragments of the HA biopolymer: (i) the fragment bearing a hydroperoxide functional group, and (ii) the fragment, which possesses an aldehyde terminus. As stated above, both fragments naturally differ in their chemical structure due to the included –C=O or –O-OH substituent and, above all, by the reduced molar mass of both polymer fragments compared to that of the parent HA biopolymer.

Along with the fragmentation reactions shown in Schemes 2 and 3, the radical attack on the GlcA and GlcNAc moieties can also lead to the ring opening without breaking the polymer chain [11,18,20,21].

There exists, however, a remarkable phenomenon of *in vivo* free-radical oxidative degradation of hyaluronan: Under physiological conditions, the SF viscosity does not undergo any changes since the content of "native" hyaluronan remains constant due to permanent *de novo* production of megaDalton HA macromolecules by (stimulated) type B synoviocytes.



Scheme 3. Potential mechanism of hyaluronan strand cleavage as a result of hydrogen abstraction and radical formation on C(4) of the glucuronic acid unit (adapted from [19]).

Thus, the self-perpetuating oxidative (non-enzymatic) HA "catabolism" in SF represents a rather delicate and properly balanced mechanism that presumably plays significant role in regulating the physiological – normoxygen – homeostasis for chondrocytes. At the same time, the produced polymer fragments, which are probably cleared from the joint by drainage pathways, serve most likely as chemical messengers/feedback molecules. These play role in the adjustment of the optimum mode of functioning of the synovial membrane and of the HA-producing cells, B synoviocytes, localized within. 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 [16].

High "protective/scavenging efficiency" of hyaluronan against the *in vitro* action of $^{\bullet}OH$ radicals has been earlier pointed out by some authors [22,23]. Presti and Scott [23] described that high-molar-mass hyaluronan (megaDalton HA) was much more effective than the lower-molar-mass HAs (hundreds of kiloDaltons HAs) in scavenging $^{\bullet}OH$ radicals generated by a Fenton-type system comprising glucose and glucose oxidase *plus* Fe^{2+}-EDTA chelate.

HYPOXIA AND RE-OXYGENATION OF THE JOINT

As SF of healthy human exhibits no activity of the hyaluronidase enzyme, it has been inferred that oxygen-derived free radicals are involved in a self-perpetuating process of HA catabolism within the joint [24]. This radical-mediated process is considered to account for ca. twelve-hour half-life of native HA macromolecules in SF.

To understand how to maintain a radical reaction active/self-perpetuating, its propagation stage should first be analyzed. If a peroxyl-type (macro)radical ($A–O-O^{\bullet}$) exists within SF, due to the relatively high reactivity of the unpaired electron on oxygen, the following intermolecular reaction can be assumed

$$A–O-O^{\bullet} + A–H \rightarrow A–O-OH + A^{\bullet} \qquad (4)$$

In the case when $A^{\bullet}$ is a C-type (macro)radical, it is this very reactant that traps the dioxygen molecule, dissolved in SF, according to the reaction

$$A^{\bullet} + O_2 \rightarrow A–O-O^{\bullet} \qquad (5)$$

Hence, by combining the reactions 4 and 5, the net reaction

$$A–H + O_2 \rightarrow A–O-OH \qquad \text{(net reaction)}$$

corroborates the statement that one particular function of (a high-molar-mass) HA is to trap the oxygen excess during the phase of joint re-oxygenation [16].

PHYSIOLOGIC OXIDATIVE CATABOLISM OF HYALURONAN: PARTICIPATION OF BIOGENIC TRANSITION METAL IONS

As stated in Scheme 2 and reaction 4, A–O-OH hydroperoxides are generated during the self-perpetuating – propagation – stage of the hyaluronan oxidative catabolism. The fate of A–O-OH type hydroperoxides, however, is significantly dependent on the presence or absence of the transition metal ions within SF. In the former case, the following reactions could be suggested for decomposition of the generated A–O-OH hydroperoxides

$$A\text{–O-OH} + Me^{n+} \rightarrow A\text{–O}^{\bullet} + HO^{-} + Me^{(n+1)+} \quad (6)$$

$$A\text{–O-OH} + Me^{(n+1)+} \rightarrow A\text{–O-O}^{\bullet} + Me^{n+} + H^{+} \quad (7)$$

As can be seen, while the "propagator" that participates in reaction 4 is (re)generated by reaction 7, reaction 6 produces an alkoxyl type (macro)radical A–O$^{\bullet}$. The ratio of the A–O-O$^{\bullet}$ to A–O$^{\bullet}$ radicals is, however, governed by the present transition metal ions, or, more precisely, by the ratio of $Me^{(n+1)+}$ to Me^{n+}. To answer the question, which transition metals may be present in SF and cells or tissues of healthy human beings, one should take into account the data presented in Tables 1 and 2.

Table 1. Contents of transition metals in blood serum of healthy human volunteers and in *post mortem* collected SF from subjects without evidence of connective tissue disease

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

[a]Reported by Niedermeier and Griggs [25].

[b]Data in parentheses are the values in µM calculated in assumption that 100 g of SF has a volume of 100 mL.

Based on the data listed in Table 1, iron and copper are the two prevailing redox active transition metals in SF. It should be, however, pointed out that the respective concentrations of ca. 5.2 µM of iron ions and 4.3 µM of copper ones do not represent those, which are (freely) disposable to catalyze the oxidative catabolism of hyaluronan within SF. As has been reported, the availability of iron to stimulate *in vivo* generation of $^{\bullet}$OH radicals is very limited, since concentrations of "free" iron, are seldom larger than 3 µM in human samples [27].

Table 2. Average relative abundance of some biogenic transition metals in the mammalian blood plasma and cells/tissues

Element	Blood plasma [μM][a]	Cell/Tissue [μM][a]
Iron	22	≈ 68
Copper	8-24	0.001-10
Zinc	17	180
Manganese	0.1	180
Nickel	0.04	2
Molybdenum	-	0.005

[a]Adapted from [26].

Let us now deal with the oxidation states of iron within SF of a healthy human. By accepting that the concentration of ascorbate in SF of healthy subjects reaches the values close to those established in blood serum, i.e. 40–140 μM [28], it must be admitted that the transition metal ions in SF of a healthy human being are in the reduced oxidation state, i.e. Me^{n+}. Thus, in the case of the ascorbate level, which many times exceeds the concentration of transition metal ions, the actual concentration of ferrous ions should exceed that of ferric ones, and thus A–O$^{\bullet}$ radicals should prevail. These radicals could, similarly to the A–O-O$^{\bullet}$ ones, propagate the radical chain reaction as follows

$$A\text{–}O^{\bullet} + A\text{–}H \rightarrow A\text{–}OH + A^{\bullet} \quad (8)$$

Yet, due to the redox potential of the pair $RO^{\bullet},H^{+}/ROH = +1.6$ V, which surpasses significantly that of $ROO^{\bullet},H^{+}/ROOH = +1.0$ V, the actual content of A–O$^{\bullet}$ in SF is practically nil; the half-life of the A–O$^{\bullet}$ radicals is much shorter than that of A–O-O$^{\bullet}$ ones – microseconds *vs*. seconds.

CONCLUSIONS

It has been inferred that ROS are involved in a self-perpetuating process of HA catabolism within the joint. The radical mediated process is considered to account for about 12-h half-life of native high-molar-mass HAs in SF.

During physiological joint functioning, the HA 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.

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REFERENCES

[1] M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, *Chem.-Biol. Interact.*, 160 (2006) 1-40.

[2] D. Harman, *J. Gerontol.*, 11 (1956) 298–300.

[3] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, *Int. J. Biochem.* Cell Biol., 39 (2007) 44-84.

[4] http://www.niams.nih.gov/Health_Info/Rheumatic_Disease/graphics/joint_vert.gif (valid: December 4, 2009).

[5] R. Servaty, J. Schiller, H. Binder, K. Arnold, *Int. J. Biol. Macromol.*, 28 (2001) 121-127.

[6] http://www.nature.com/ncprheum/journal/v2/n7/fig_tab/ncprheum0216_F1.html#figure-title (valid: December 4, 2009) by F.H. Chen, K.T. Rousche, R.S. Tuan, *Nat. Clin. Prac. Rheumatol.*, 2 (2006) 373-382.

[7] B.M. Praest, H. Greiling, R. Kock, *Carbohydr. Res.*, 303 (1997) 153-157.

[8] K.M.N. Oates, W.E. Krause, R.H. Colby, *Mat. Res. Soc. Symp. Proc.*, 711 (2002) 53-58.

[9] E. Rovenská, *Rheumatologia*, 15 (2001) 57-64.

[10] R.O. Day, A.J. McLachlan, G.G. Graham, K.M. Williams, *Clin. Pharmacokin.*, 36 (1999) 191-210.

[11] G. Kogan, L. Šoltés, R. Stern, R. Mendichi, "Hyaluronic acid: A biopolymer with versatile physico-chemical and biological properties" in the book "Handbook of Polymer Research: Monomers, Oligomers, Polymers and Composites, Chapter 31", pp. 393-439, Eds. R.A. Pethrick, A. Ballada, G.E. Zaikov, Nova Science Publishers, New York 2007.

[12] V.R. Ryabina, S.E. Vasyukov, V.P. Panov, S.G. Starodubtsev, *Khim.-Farm. Zh.* 21 (1987) 142-149.

[13] T. Hardingham, "Solution properties of hyaluronan" in the book "Chemistry and Biology of Hyaluronan", pp 1-19, Eds. H.G. Garg, C.A. Hales, Elsevier Press, Amsterdam 2004.

[14] J.S. Gibson, P.I. Milner, R. White, T.P. Fairfax, R.J. Wilkins, *Pflugers Arch.* 455 (2008) 563-573.

[15] Y.-Z. Fang, S. Yang, G. Wu, *Nutrition,* 18 (2002) 872-879.

[16] L. Šoltés, G. Kogan, "Impact of transition metals in the free-radical degradation of hyaluronan biopolymer" in the book "Kinetics and Thermodynamics for Chemistry and Biochemistry, Vol. 2", pp. 181-199, Eds. E.M. Pearce, G.E. Zaikov, G. Kirshenbaum, Nova Science Publishers, New York 2009; ibid *Polym. Res. J.*, 2 (2008) 269-288.

[17] P.I. Milner, R.J. Wilkins, J.S. Gibson, Osteoarthr. *Cartilage*, 15 (2007) 735-742.

[18] C.L. Hawkins, M.J. Davies, *Free Radic. Biol. Med.*, 21 (1996) 275-290.

[19] E.C. Kennett, M.J. Davies, *Free Radic. Biol. Med.*, 47 (2009) 389–400.

[20] C.L. Hawkins, M.J. Davies, *Biochem. Soc. Trans.*, 23 (1995) S248.

[21] G. Kogan, L. Šoltés, R. Stern, J. Schiller, R. Mendichi, "Hyaluronic Acid: Its Function and Degradation in In Vivo Systems", in the book "Studies in Natural Products Chemistry, Vol. 35 Bioactive Natural Products, Part D", pp. 789-882, Ed. by Atta-ur-Rahman, Elsevier, Amsterdam 2008.

[22] P. Myint, D.H. Deeble, P.C. Beaumont, S.M. Blake, G.O. Phyllips, *Biochim. Biophys. Acta*, 925 (1987) 194-202.
[23] D. Presti, J.E. Scott, *Cell Biochem. Func.*, 12 (1994) 281-288.
[24] M. Grootveld, E.B. Henderson, A. Farrell, D.R. Blake, H.G. Parkes, P. Haycock, *Biochem. J.*, 273 (1991) 459-467.
[25] W. Niedermeier, J.H. Griggs, *J. Chronic Dis.*, 23 (1971) 527-536.
[26] "Transport and Storage of Metal Ions in Biology", in the book "Biological Inorganic Chemistry: Structure and Reactivity" pp. 57-77, Eds. I. Bertini, H.B. Gray, E.I. Stiefel, J.S. Valentine, University Science Books, *Sausalito California* 2007.
[27] B. Halliwell, J.M.C. Gutteridge, *Methods Enzymol.*, 186 (1990) 1-85.
[28] S.F. Wong, B. Halliwell, R. Richmond, W.R. Skowroneck, *J. Inorg. Biochem.*, 14 (1981) 127-134.