Free-Radical Degradation of High-Molar-Mass Hyaluronan Induced by Ascorbate *plus* Cupric Ions: Evaluation of Antioxidative Effect of Cysteine-Derived Compounds

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Dedicated to Prof. *Radomír Nosál'*, MD, DSc on the occasion of his 70th birthday

Based on our previous findings, the present study has focused on free-radical-mediated degradation of the synovial biopolymer hyaluronan. The degradation was induced *in vitro* by the *Weissberger*'s system comprising ascorbate *plus* cupric ions in the presence of oxygen, representing a model of the early phase of acute synovial joint inflammation. The study presents a novel strategy for hyaluronan protection against oxidative degradation with the use of cysteine-derived compounds. In particular, the work objectives were to evaluate potential protective effects of reduced form of L-glutathione, L-cysteine, *N*-acetyl-L-cysteine, and cysteamine, against free-oxygen-radical-mediated degradation of high-molar-mass hyaluronan *in vitro*. The hyaluronan degradation was influenced by variable activity of the tested thiol compounds, also in dependence of their concentration applied. It was found that L-glutathione exhibited the most significant protective and chain-breaking antioxidative effect against the hyaluronan degradation. Thiol antioxidative activity, in general, can be influenced by many factors such as various molecule geometry, type of functional groups, radical attack accessibility, redox potential, thiol concentration and pK_a, pH, ionic strength of solution, as well as different ability to interact with transition metals. Antioxidative activity was found to decrease in the following order: L-glutathione, cysteamine, *N*-acetyl-L-cysteine, and L-cysteine. These findings might be beneficial in future development of potential drugs in the treatment of synovial hyaluronan depletion-derived diseases.

Introduction. – Although in the past exclusively considered as deleterious agents, reactive oxygen species (ROS) are now recognized as important mediators in cell signaling and maintenance of homeostasis [1]. Redox status of the cell is controlled *via* a variety of regulatory mechanisms. It is kept in equilibrium unless the cells are exposed to some overwhelmingly oxidizing (or reducing) agents. When the antioxidative capacity of the cell is exceeded due to the ROS over-production during oxidative stress, the ROS, however, may exert their toxic effect which may eventually result in developing various pathologies. The redox homeostasis is crucial for numerous biological processes such as enzyme activation, DNA synthesis, cell metabolism, and many others. Moreover, it controls basal level of permanently formed ROS, the side products of electron transfer from reduced compounds to molecular O_2, a universal electron acceptor in biological systems [2–4].

There are 38 chemical elements termed transition metals, and eight of them are present in biological systems. Those are vanadium, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum. A variety of different oxidation states, as well as the complexation by either charged or neutral ligands determine redox and/or catalytic activity of transition metal ions (TMIs) serving mostly as catalytic centers of enzymes like oxidases and dehydrogenases [5]. Due to the presence of unpaired electrons in the outer/valence electronic orbital of TMIs and the capacity to change their oxidation state, they may be promoters of free-radical reactions analogously to that of the *Fenton* reaction [6]. Iron and copper ions are the two most abundant redox active TMIs in the joint synovial fluid. These two TMIs can play an active role in the oxidative hyaluronan degradation, which may be accompanied by the joint inflammation [5].

Biothiols, occurring under physiological conditions in well-balanced redox pairs (thiol/disulfide), are very efficient endogenous antioxidants protecting the cells from deleterious ROS action. The protective and repairing action of these endobiotics depends not only on their capacity to detoxify ROS, but also on chemical character and reactivity of deleterious thiyl radicals, which may be formed in the presence of trace TMIs and O_2 [6]. Besides ROS-detoxifying enzymes and low-molar-mass antioxidant vitamines C and E, thiols are responsible for maintaining the cell homeostasis; they scavenge and/or detoxify ROS, block their production, or sequester TMIs as the source of free electrons [2]. Amphoteric behavior of thiols, ruled by various factors such as molecule geometry, type of functional groups, radical attack accessibility, redox potential, thiol pK_a, pH, as well as thiol and solution concentration, has already been reviewed [6–9]. Miscellaneous effects of thiols to react preferentially with TMIs or block TMI catalytic action *via* their chelating [10], determine the ability of thiols to scavenge HO$^{\bullet}$ radicals or promote their production. Depending upon the molar ratio of thiol/TMI, the action of thiols may lead to the formation either of $(TMI)^{I}$ redox-inactive chelates to oxygen or $(TMI)^{II}$ ascorbate-reducible chelates with free-radical-scavenging activities [9].

Reduced L-glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is a thiol compound present in the cell in millimolar concentrations [9]. GSH is the major regulator of cell redox status acting through the reversible redox reactions of its reduced (GSH) and oxidized (GSSG) forms [2].

L-Cysteine (Cys), a naturally occurring amino acid, contributes to the intracellular thiol pool, and is a precursor of GSH synthesis [9].

N-Acetyl-L-cysteine (NAC), another significant precursor of the GSH biosynthesis, has broadly been used as effective antioxidant in a form of nutritional supplement [10][11]. At low concentrations, it is a powerful protector of α-1-antiproteinase against the enzyme inactivation by HOCl. NAC reacts with HO$^{\bullet}$ radicals and slowly with H_2O_2; however, no reaction of this endobiotics with superoxide anion radical was detected [12].

An endogenous amine, cysteamine (CAM) is a cystine-depleting compound with antioxidative and anti-inflammatory properties; it is used for treatment of cystinosis – a metabolic disorder caused by deficiency of the lysosomal cystine carrier. CAM is widely distributed in organisms and considered to be a key regulator of essential metabolic pathways [13].

Synovial joint enables free movement of connected bones and is composed of the articular cartilage, synovial membrane, synovium, and synovial fluid. Hyaluronic acid,

mostly present as sodium salt, hyaluronan (HA), is the main component of synovial fluid in the concentration range of 2–4 mg/ml. Oxygen-dependent synovial fluid HA degradation has recently drawn attention of biochemists, in particular, for the messenger-like activity of various HA fragments [14][15]. In this context, oxidative pathway-driven formation of ROS, ruled by the catalytic action of TMIs, may play an important role.

Native HA is a high-molar-mass (4–7 MDa) unsulfated glycosaminoglycan, composed of strictly alternating sequences of 3-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 4-linked β-D-glucuronic acid residues. Due to the presence of free COOH groups on glucuronic units, HA has a polyanionic character predicting it to bind cations and/or forming chelate structures. Such modified HA, especially, when applied with other antioxidants, has revealed the protective role of these antioxidants against HA degradation by ROS [16]. During physiological joint functioning, HA in synovial fluid plays also a role of scavenging antioxidant, and the produced HA fragments can mediate information on the changes in the joint [5].

The aim of the present study was to investigate potential protective effects of various thiol compounds, in comparison to a standard thiol antioxidant, GSH, towards *Weissberger*'s oxidative system-induced degradation of high-molar-mass HA *in vitro* (*Scheme*) in the presence of O_2, *i.e.*, condition simulating the early phase of acute synovial joint inflammation.

Results and Discussion. – Time-dependent rheological changes of HA macromolecules in solution can directly be assessed by rotational viscometry [18].

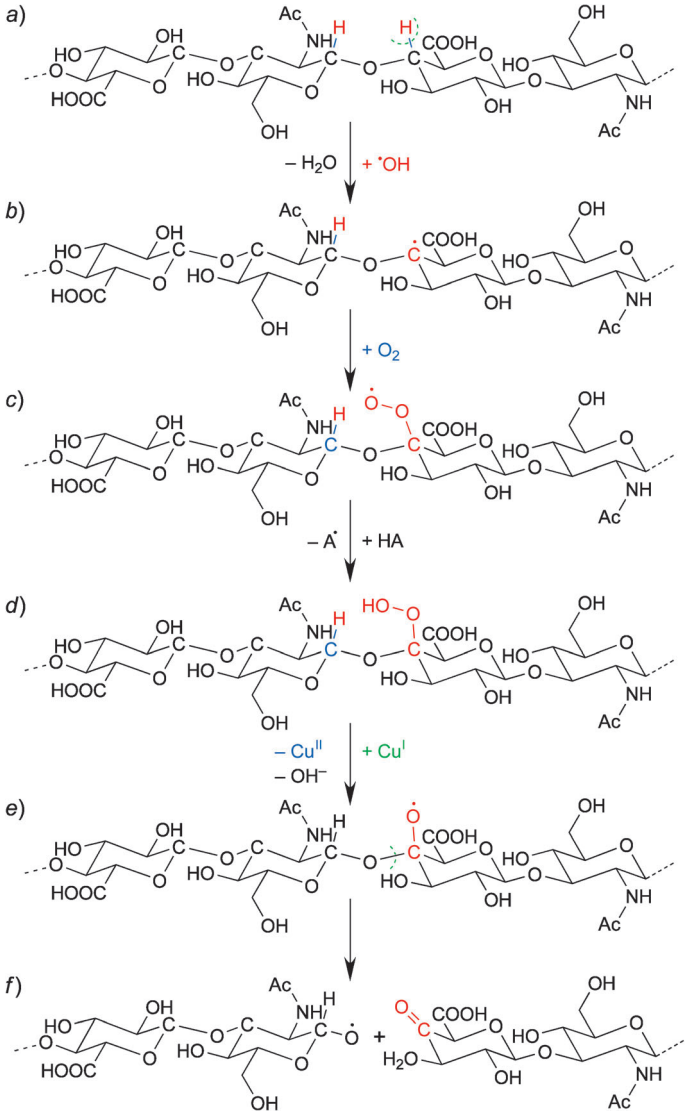
The well-known biogenic *Weissberger*'s H_2O_2-generating system – *via* the reaction of ascorbate *plus* Cu^{II} ions, under aerobic conditions [19] – was, for the first time, circumstantiated on the investigation of the HA polymer chain degradation *in vitro* [18].

Ferrous- and ascorbate-dependent lipid peroxidation studied *in vitro* revealed different $HO^{\bullet}$ radical-scavenging properties of various endogenous (L-glutathione, *S*-methyl-L-glutathione, cysteamine, *N*-acetyl-L-cysteine, L-cysteine) and exogenous thiols (*dl*-dithiothreitol, *d*-penicillamine), among them, CAM was found to be the most effective antioxidant [20]. Efficient antioxidative action of an exogenous compound, dithioerythritol, containing the two SH groups, was demonstrated against the HA *in vitro* degradation induced by the *Weissberger*'s oxidative system, exhibiting comparable protective effect as that of GSH [21].

The dynamic viscosity (η) *vs.* time profiles of the P9710–2A sample solution (2.5 mg/ml) with the addition of Cu^{II} (1.0 μM) *plus* L-ascorbic acid (100 μM), to which either 25, 50, or 100 μM of L-glutathione, L-cysteine, *N*-acetyl-L-cysteine, and cysteamine were added, at the beginning of the reaction or after 1 h, are illustrated in *Figs. 1–4*. As evident, within the time interval examined (5 h), application of the GSH concentrations (50 and 100 μM) resulted in a marked protection of the HA macromolecules against degradation, leading to the total inhibition of the viscosity decay. The higher the GSH concentration used, the longer was the observed stationary interval in the sample η values. However, the inhibitory effect of the other thiols demonstrated another kinetic course.

The application of the 1 h-delayed addition of the SH compound has been designed supposing that the depletion of formed $HO^{\bullet}$ radicals, initiating the HA degradation

Scheme. a) *Initiation Phase: the Intact Hyaluronan Macromolecule Entering the Reaction with the HO• Radical Formed* via *the* Fenton-*Like Reaction:* $Cu^{I} + H_2O_2 \rightarrow Cu^{II} + HO^{\bullet} + OH^{-}$. H_2O_2 has its origin due to the oxidative action of the *Weissberger*'s system: $Asc + Cu^{II} + 2\,O_2 + 4\,H^{+} \rightarrow Asc^{\bullet} + Cu^{I} + 2\,H_2O_2$. b) *Formation of an Alkyl Radical* (*C*-centered hyaluronan macroradical) *Initiated by the HO• Radical Attack*. c) *Propagation Phase: Formation of a Peroxy-Type* C*-Macroradical of Hyaluronan in a Process of Oxygenation after Entrapping a Molecule of* O_2. d) *Formation of a Hyaluronan-Derived Hydroperoxide* via *the Reaction with Another Hyaluronan Macromolecule.* e) *Formation of Highly Unstable Alkoxy-Type* C*-Macroradical of Hyaluronan on Undergoing a Redox Reaction with a Transition Metal Ion in a Reduced State.* f) *Termination Phase: Quick Formation of Alkoxy-Type* C*-Fragments and the Fragments with a Terminal C=O Group Due to the Glycosidic Bond Scission of Hyaluronan.* Alkoxy-type C-fragments may continue the propagation phase of the free-radical hyaluronan degradation reaction. Both fragments are represented by reduced molar masses [14][17].



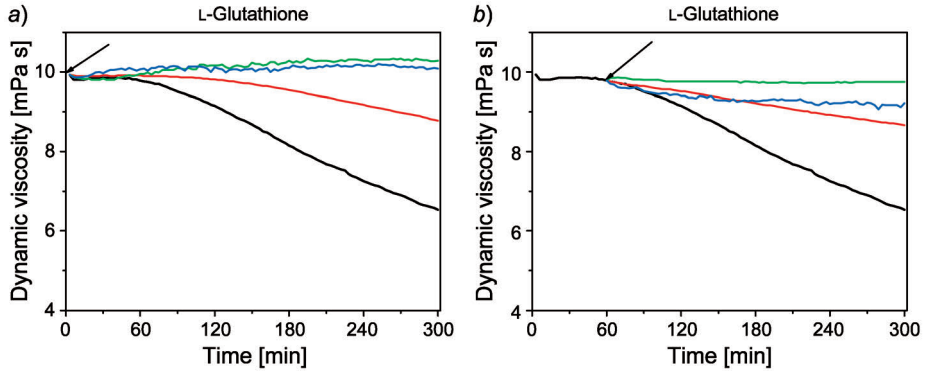


Fig. 1. *Evaluation of antioxidative effects of* L-*glutathione against high-molar-mass hyaluronan degradation* in vitro *induced by* Weissberger*'s oxidative system*. Reference sample (black): 1 μM Cu^{II} ions *plus* 100 μM ascorbic acid; nil thiol concentration. L-Glutathione addition at the onset of the reaction (*a*) and after 1 h (*b*) (red: 25 μM; green: 50 μM; blue: 100 μM).

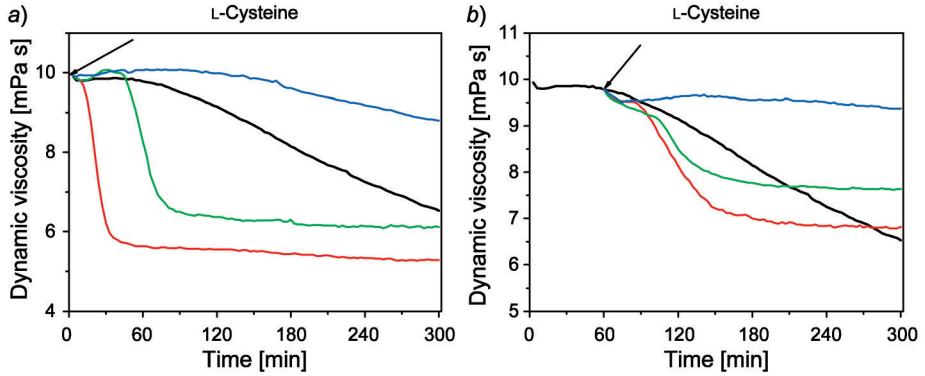


Fig. 2. *Evaluation of antioxidative effects of* L-*cysteine against high-molar-mass hyaluronan degradation* in vitro *induced by* Weissberger*'s oxidative system*. Reference sample (black): 1 μM Cu^{II} ions *plus* 100 μM ascorbic acid; nil thiol concentration. L-Cysteine addition at the onset of the reaction (*a*) and after 1 h (*b*) (red: 25 μM; green: 50 μM; blue: 100 μM).

processes, was almost accomplished within this time period as confirmed by the EPR study [22].

Investigation of the Antioxidative Effect of L-*Glutathione.* Application of L-glutathione (50 and 100 μM) evidenced that, when applied before the onset of the reaction (*Fig. 1*,*a*), it behaved as a preventive antioxidant, scavenging HO• radicals, whereas, when applied after 1 h (*Fig. 1*,*b*), it exerted a chain-breaking antioxidative effect, scavenging peroxy-type radicals [23]. This action of GSH could be ascribed to its ability to form glutathionyl radical (GS•), which is known to recombine to form glutathione disulfide (GSSG). Due to its bulky molecule, GSH has a high potential to inhibit HO• production *via* chelating Cu^{I} ions and thus blocking their reaction with H_2O_2, *i.e.*, by disabling the *Fenton*-derived HO• formation.

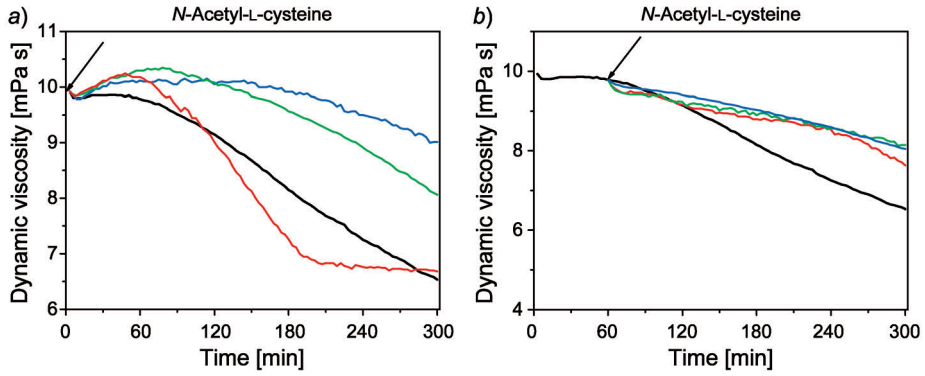


Fig. 3. *Evaluation of antioxidative effects of* N-*acetyl*-L-*cysteine against high-molar-mass hyaluronan degradation* in vitro *induced by* Weissberger*'s oxidative system*. Reference sample (black): 1 μM Cu^{II} ions *plus* 100 μM ascorbic acid; nil thiol concentration. *N*-Acetyl-L-cysteine addition at the onset of the reaction (*a*) and after 1 h (*b*) (red: 25 μM; green: 50 μM; blue: 100 μM).

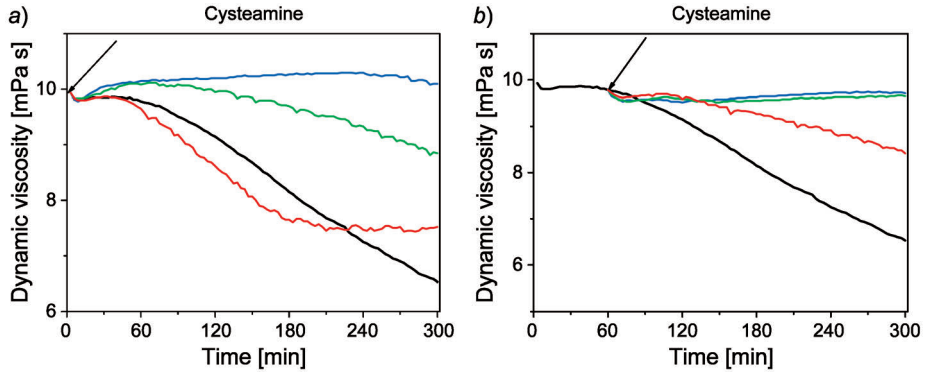


Fig. 4. *Evaluation of antioxidative effects of cysteamine against high-molar-mass hyaluronan degradation* in vitro *induced by* Weissberger*'s oxidative system*. Reference sample (black): 1 μM Cu^{II} ions *plus* 100 μM ascorbic acid; nil thiol concentration. Cysteamine addition at the onset of the reaction (*a*) and after 1 h (*b*) (red: 25 μM; green: 50 μM; blue: 100 μM).

Investigation of the Antioxidative Effect of L-*Cysteine.* L-Cysteine (100 μM) exhibited similar effect as *N*-acetyl-L-cysteine (100 μM) when added before the onset of the reaction (*Figs. 2,a*, and *3,a*). However, Cys (25 and 50 μM) caused a fast drop of the HA η value during the first hour of the recording, indicating a rapid HA degradation likely due to the Cys pro-oxidative action (*Fig. 2,a*). On thiol addition after 1 h of the onset of the reaction (*Fig. 2,b*), Cys (25 and 50 μM) behaved, during pro-oxidative phase, as an inhibitor of peroxy-type radical production, and, on the other hand, under aerobic conditions, it may function as a generator of $O^{\bullet}_{2}^{-}$ anion radicals that, in turn, formed H_2O_2. The latter, due to the catalytic interaction with TMIs, such as Cu^{I} ions, promoted peroxidative degradation of HA by *in situ* formation

of HO• radicals. Surprisingly, Cys (100 μM) exhibited comparable antioxidative effect to that of GSH (*Fig. 1,b*, and *Fig. 2,b*).

Investigation of the Antioxidative Effect of N-*Acetyl*-L-*cysteine.* Unlike L-glutathione, *N*-acetyl-L-cysteine was found to have preferential tendency to reduce Cu^{II} ions to Cu^{I}, forming *N*-acetyl-L-cysteinyl radical (NAC•) that may subsequently react with molecular O_2 to give $O^{\bullet}_2^-$ [10][11].

On the contrary to Cys, NAC (25 and 50 μM), when added at the beginning of the reaction, exhibited a clear antioxidative effect within *ca.* 60 and 80 min, respectively (*Fig. 3,a*). Subsequently, NAC exerted a modest pro-oxidative effect, more profound at 25-μM than at 100-μM concentration (*Fig. 3,a*). Application of NAC 1 h after the onset of the reaction (*Fig. 3,b*) revealed its partial inhibitory effect against formation of the peroxy-type radicals, independently from the concentration applied.

Investigation of the Antioxidative Effect of Cysteamine. Cysteamine (100 μM), when added before the onset of the reaction, exhibited an antioxidative effect very similar to that of GSH (*Fig. 1,a*, and *Fig. 4,a*). Moreover, the same may be concluded, when applied 1 h after the onset of the reaction (*Fig. 1,b*, and *Fig. 4,b*), at the two concentrations (50 and 100 μM), suggesting that CAM may be an excellent scavenger of peroxy radicals generated during the peroxidative degradation of HA.

Conclusions. – Processes involving free-oxygen-radical-mediated degradation of HA, due to the reduced thiol-derived antioxidative capacity of synovial joints under pathophysiological conditions, have been recently studied [5][16][23–25].

Inhibitory Effect of Thiols against the HA Degradation. Our results provide evidence that L-glutathione (50 and 100 μM) and cysteamine (100 μM) may be effective antioxidants inhibiting the HA free-oxygen-radical-mediated *in vitro* degradation preferentially caused by a detrimental HO• radical action during initiation phase. Both GSH and CAM (50 and 100 μM) also acted as effective inhibitors of peroxy-type radical species generated from the *C*-centered macroradicals of the HA chain during propagation phase. In this context, remarkable antioxidative effect of L-cysteine, comparable with both GSH and CAM, was observed at the highest concentration applied (100 μM).

Retarding Effect of Thiols against the HA Degradation. N-Acetyl-L-cysteine (100 μM) exhibited a significantly retarding effect against the HA degradation during propagation phase after *ca.* 1 h (thiol addition at the beginning). Initiation phase can be classified as a period of the HA rheopectic behavior. A less retarding effect was observed at a lower concentration applied (50 μM). NAC (50 and 100 μM) was found to have similar retarding effect against the HA degradation as that of CAM (50 μM). During propagation phase (thiol addition after 1 h), retarding effect of NAC against the HA degradation was shown to be concentration independent. GSH (25 μM) and CAM (50 μM) exhibited similar retardation of the HA degradation during propagation phase (thiol addition at the beginning), however, it was less effective in the case of CAM. The same can be concluded for GSH and CAM (25 μM) regarding propagation phase (thiol addition after 1 h).

Pro-Oxidative Effect of Thiols Accelerating the HA Degradation. CAM and NAC (25 μM) exhibited a pro-oxidative effect accelerating the HA degradation during propagation phase (thiol addition at the beginning) when compared to that of a

reference experiment. The most significant pro-oxidative effect causing extensive HA polymeric chain scission, within *ca.* 15–20 min since the reaction beginning, was, however, observed in the case of Cys (25 μM), whereas, in the case of Cys (50 μM), it was within *ca.* 45 min. Taking into account propagation phase, Cys (25 and 50 μM) exhibited a different mechanism of the HA degradation when compared with that of a reference experiment.

In conclusion, the paradoxical behavior of the tested thiols, in the context of the HA free-oxygen-radical-mediated *in vitro* degradation, can be ascribed to their various molecule geometry, type of functional groups, radical attack accessibility, redox potential, thiol pK_a, pH, thiol and solution concentration, and to their different ability to interact with transition metals. It is worth to emphasize that, independently on thiol used, except for the *N*-acetyl-L-cysteine added after 1 h, the higher thiol concentration used the stronger was the antioxidative effect.

It has been well-established that SH groups of cysteine residues of proteins are readily susceptible to oxidation by the action of ROS [26]. When low-molar-mass thiols are present, the oxidation of cysteine residues may lead to the reversible formation of mixed disulfides between protein SH groups and the low-molar-mass thiols. In this case, we can speak of *S*-thiolation [26][27].

Short-sized HA molecular fragments are usually formed as a result of our investigations. One can assume that, on processing, a thiol-related residue could be, in some way, chemically incorporated into the modified HA chain. The proof/disproof of the HA *S*-thiolation, leading to the formation of polyanionic HA-derived thiomers, calls for further investigative work. This could have a really high impact in the given research scope.

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Experimental Part

Biopolymer and Chemicals. High-molar-mass hyaluronan (HA) sample *Lifecore P9710-2A* (M_w 808.7 kDa; M_w/M_n 1.63) was kindly donated by the manufacturer *Lifecore Biomedical Inc.*, Chaska, MN, USA. The anal. purity-grade L-glutathione, cysteamine, L-cysteine, and *N*-acetyl-L-cysteine were purchased from *Sigma–Aldrich Chemie GmbH*, D-Steinheim; NaCl and $CuCl_2 \cdot 2\,H_2O$ were purchased from *Slavus Ltd.*, SK-Bratislava. L-Ascorbic acid was the product of *Merck*, D-Darmstadt. Deionized high-purity-grade H_2O, with conductivity of ≤ 0.055 μ*S*/cm, was produced by using the *TKA* water purification system (*Water Purification Systems* GmbH, D-Niederelbert).

Procedures. Preparation of HA soln. (2.5 mg/ml), stock solns. of ascorbic acid, $CuCl_2$, and corresponding thiol solns. (16 mM) in an aq. NaCl soln. (0.15 M), the experiment onsets, as well as monitoring the kinetics of the HA degradation *via* rotational viscometry were already described in [23]. Data of dynamic viscosity were obtained using *Brookfield LVDV-II+PRO* digital rotational viscometer (*Brookfield Engineering Labs., Inc.*, Middleboro, MA, USA) within 2 min after the experiment onset. 3-Min intervals were taken monitoring the changes of dynamic viscosity of the system, as a function of time and thiol concentration applied, measured at $25.0 \pm 0.1^\circ$ within 5 h. The viscometer *Teflon*® spindle rotation rate was as optimal as 180 rpm, *i.e.*, at the shear rate equaling 237.6 s^{-1} [18][28].

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