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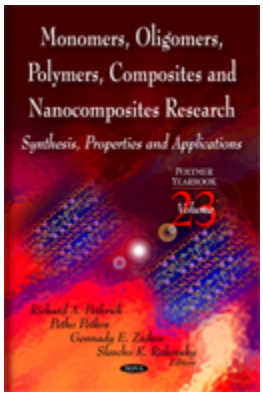

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Chapter 8

PEROXYNITRITE: A POTENT ENDOGENOUS PRO-OXIDANT AGENT PLAYING AN IMPORTANT ROLE IN DEGRADATION OF HYALURONAN BIOPOLYMER

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ABSTRACT

This mini review chapter is aimed at giving some important facts on the present state of peroxynitrite biosynthesis *in vivo* and to its damaging actions studied in vitro *via* monitoring the kinetics of hyaluronan degradation. The complex biochemical behavior of peroxynitrite is ruled by its chemistry, conformational structures differing in energy content, reactive intermediates released, contribution of carbon dioxide, trace transition metal ions, the presence of trace organics, concentration of this species in solution, and, also, by the reaction kinetics.

The *in vitro* study of hyaluronan degradation has been, recently, the subject of an enormously growing interest. Up-to-date understanding of the role of peroxynitrite in ethiopathogenetic mechanisms of selected human diseases such as cardio-vascular diseases, stroke, cancer, inflammation, neurodegenerative disorders, diabetes mellitus, and diabetic complications has still been challenging.

Keywords: Reactive free radicals, nitric oxide, peroxynitrite, hyaluronan degradation, and pathophysiological consequences

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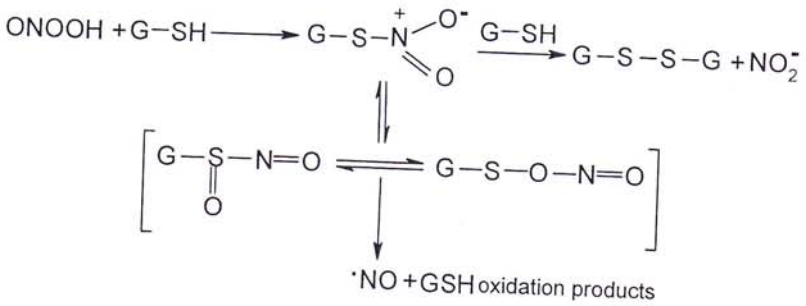
1. INTRODUCTION

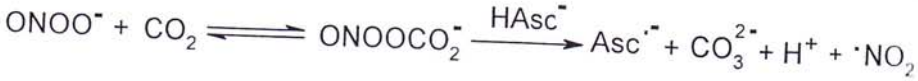
A great many of review papers have been published on peroxynitrite, one of the most important endogenous reactive oxidizing and nitrating species and a mediator of cellular and tissue injury in various pathological situations. Biomolecular pathways of this metabolite, producing free radicals, are responsible for the oxidative stress in biological systems [1-14].

Free radicals, possessing one or more unpaired electrons (atoms, molecules, or their fragments), are usually able to exist for a short time period. They, in general, function as pro-oxidants in biological environment. These species are either oxygen-derived such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), hydroperoxyl radical ($HO_2^{\bullet}$), and trioxocarbonate anion radical ($CO_3^{\bullet-}$) or nitrogen-derived such as nitric oxide radical ($^{\bullet}NO$) and nitrogen dioxide radical ($^{\bullet}NO_2$). The non-radical reactive metabolites are possible radical precursors, such as hydrogen peroxide (H_2O_2), hydrogen disulphide (H_2S), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), ozone (O_3), and peroxynitrite ($ONOO^-$). Besides these two groups, organic compound-derived free radicals, *e.g.* thiyl ($RS^{\bullet}$), alkoxyl ($RO^{\bullet}$), and peroxyl ($ROO^{\bullet}$) radical species have been the subject of interest.

Anti-oxidants in human body, particularly thiol-based species such as glutathione and thioredoxin functioning together with ascorbate and α-tocopherol, at low concentrations, play a crucial role in *in vivo* preventing biological systems from oxidative damage evoked by pro-oxidants (Scheme 1 and 2). According to their variable molecular size and structure, these potential scavengers can be classified as high-molar-mass (superoxide dismutase, SOD; catalase; glutathione peroxidase; transferrin; albumin; polyphenols), and low-molar-mass compounds (ascorbate; glutathione; uric acid; vitamin E; coenzyme Q) [15].



Scheme 1. Proposed mechanism of nitric oxide radical formation *via* reaction of peroxynitrous acid and glutathione [16].



Scheme 2. Formation of ascorbyl anion radical and nitric dioxide radical *via* reaction of peroxynitrite anion and carbon dioxide in the presence of ascorbate anion [17].

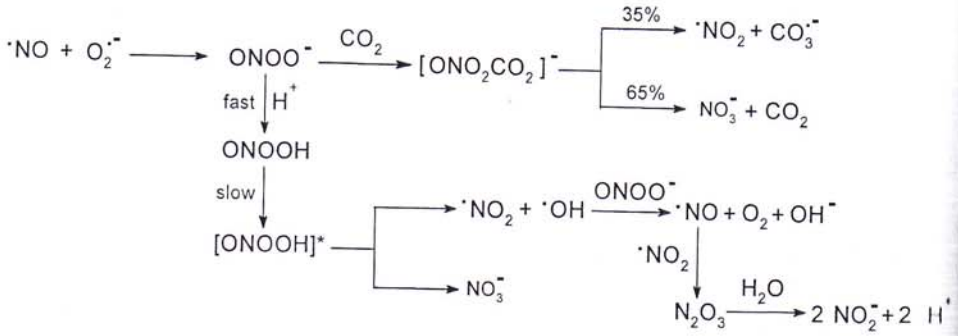
2. Are Free Radicals Reactive Predators in Biomolecular Systems?

Though traditionally understood as deleterious cellular by-products potentially able to damage cell components, reactive free radicals, due to their various redox actions, play a key role in preventing biomolecular systems (cells, tissues, and organs) from great many of stress factors. They are formed and released by cells possessing the privilege to function as the protectors for their homeostasis. *In vivo* redox pathways mediated by reactive oxygen, as well as their nitrogen and thiol analogues play pivotal roles in many physiological and pathological events, regarding intra- and inter-cellular signaling. Several free radicals, *e.g.* thiyl- and nitrogen dioxide radicals are known to promote *cis–trans* isomerization of double bonds of poly-unsaturated fatty acids. Nitrogen dioxide radical recombines *in vivo* with superoxide anion radical giving peroxynitrate anion (O_2NOO^-) whose conjugative acid is a strong and toxic oxidant. The reaction is limited *via* nitrogen dioxide radical scavenging by ascorbate and glutathione. A hydro-sulphide radical ($HS^\bullet$) is formed *via* oxidation by Cu-Zn superoxide dismutase enzyme (Cu-Zn-SOD) from an ionized form of hydrogen disulphide which is a signaling molecule *in vivo* in cardiovascular functions. A trioxocarbonate radical anion is a potential oxidant of nucleic acids, e.g guanine bases of DNA. Thiyl radicals, the products of the reduction of transition metals by thiol compounds, abstract hydrogen atom from lipids leading to a lipid peroxidation, from organic compounds forming C-centered organic radicals, and, from ascorbate-forming ascorbyl anion radical [18].

3. Nitric Oxide, a Peroxynitrite Precursor

Nitric oxide ($^\bullet NO$) is a biologically-ubiquitous radical. It was discovered as a signaling, reactive free radical species, acting as a specific "messenger" in numerous biological processes [19]. Since it has been found out that nitric oxide serves as one of key modulators of vascular tone in a role of endothelium derived relaxing factor (EDRF), many studies were performed covering often unlike functions of $^\bullet NO$. Activity of nitric oxide synthases (NOS), a family of enzymes catalyzing production of $^\bullet NO$, has been found in most cell types. In addition to the direct interactions of $^\bullet NO$ with signal transduction pathways of the cell, the specific reactions of $^\bullet NO$-derived products, such as peroxynitrite, with intra- and extracellular components may be responsible for damaging effects of $^\bullet NO$. Direct and indirect actions of $^\bullet NO$, and its derivatives are quite diverse and depend primarily on concentration and cell type. This results also in seemingly controversial findings on involvement of $^\bullet NO$ in various pathological processes.

The most significant *in vivo* reaction of $^\bullet NO$ is its combination with $O_2^{\bullet-}$ (Scheme 3), released from several cellular sources, to form peroxynitrite. This species is relatively stable; however, peroxynitrous acid ONOOH undergoes rapid decay forming harmful $^\bullet OH$ and $^\bullet NO_2$ radicals.



Scheme 3. Hypothetical mechanism of various biomolecular pathways of peroxynitrite [20].

•NO is synthesized *in vivo* by a wide variety of cell types, including macrophages, vascular endothelial cells, neutrophils, hepatocytes, phagocytes, and neurons. Neutrophils and macrophages generate •NO radical *via* an L-arginine-dependent pathway. Large quantities of •NO are produced by the endothelial-constitutive (ecNOS) and inducible (iNOS) forms of nitric oxide synthase enzyme in neutrophils and monocytes upon inflammatory stimulation [21].

Balazy *et al* [16] report evidence for the reaction of peroxynitrite with glutathione, provided by mass spectrometric data, giving an important biologically-active metabolite, *S*-nitroglutathione ($GSNO_2$), promoting a time-dependent production and oxidation of nitric oxide (Scheme 1). On the other hand, Hofstetter *et al* [22] claim that *S*-nitrosoglutathione (GSNO), generated *in vivo*, undergoes homolysis forming nitric oxide and glutathione thiyl radical (GS•). However, no GSNO is produced reacting •NO with GS• for a very rapid interconversion of GS• to •GS, and, since the reaction of GS• with •NO is very slow.

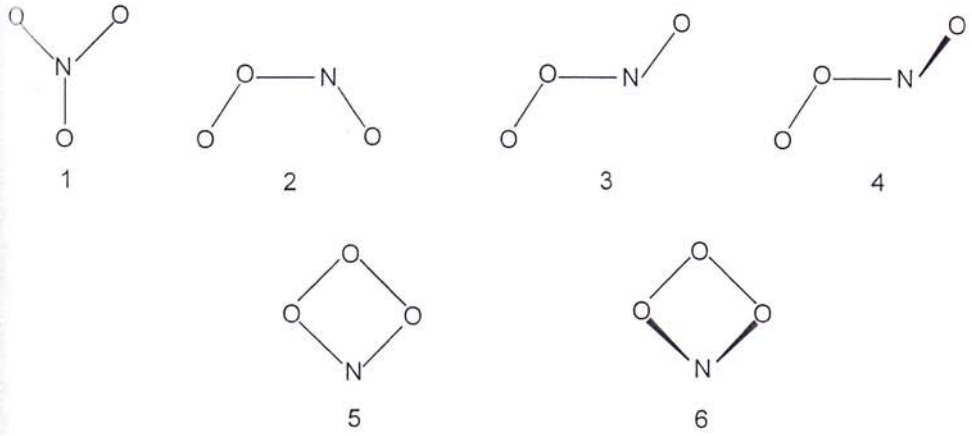
Diffusion conditions of cellular oxidants (superoxide anion radical, nitric oxide radical, peroxynitrite, and hydroxyl radical) are demonstrated in detail by Pacher *et al* [2].

4. Peroxynitrite Anion – Product of Nitric Oxide and Superoxide Anion Radical Interaction

4.1. Biosynthesis vs. Fate and Tissue Injury-related Pathophysiology of Peroxynitrite Anion

Peroxynitrite is generated *in vivo* by a fast radical-radical recombination reaction of $O_2^{\bullet-}$ and a free nitric oxide radical (•NO). Peroxynitrous acid is generated by the protonation of peroxynitrite (Scheme 3).

Various thioperoxynitrite ($SSNS^-$) [23] – like conformational forms of peroxynitrite molecule, might be expected (Scheme 4). On the other hand, Symons [19] reports the only two possible planar conformations of peroxynitrite, *'cyclic' cis- and trans*-form, both almost equaling in energy.



Scheme 4. Various conformational alternatives for the peroxynitrite anion: 1-branched chain; 2-cis (planar open-chain); 3-*trans* (planar open-chain); 4-helical (non-planar open-chain); 5-cyclic (planar chain); 6-bent (nonplanar chain) [23].

As to either direct cytotoxic oxidative or indirect radical-mediated interactions of peroxynitrite with cellular components and important bio-macromolecules (lipids, DNA, proteins), protein oxidation and nitration are important features of peroxynitrite-induced bio-molecular injury. Peroxynitrite can cause chain scission in DNA promoting oxidation and nitration of DNA fragments resulting in *e.g.* 8-nitroguanine [21]. Peroxynitrite is believed to play a cytotoxic role in the development of post-bypass systemic inflammatory response.

A natural flavonoid, quercetin has been proven as the most efficient peroxynitrite scavenger mediating the reduction of nitrotyrosine formation. Under biological conditions, peroxynitrite can convert to nitrosothiols *via* the detoxification reaction *in vivo* regenerating thus nitric oxide [24]. Under mild physiological conditions, peroxynitrite is mostly trapped by the substrates, such as thiols or metaloproteins through a bimolecular pathway, and, only its minor fraction is quickly converted into "radical-like" reactive species [25]. Bio-molecular pathways of peroxynitrite-mediated cell death and the role of nitric oxide radical and peroxynitrite in cardiovascular pathophysiology is outlined by Pacher *et al* [2].

Peroxynitrite is involved in pathogenesis of many diseases such as acute and chronic inflammatory processes, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, adult respiratory distress syndrome, sepsis, ischemia-reperfusion, vascular injuries, or neurodegenerative disorders. A short overview on peroxynitrite mediated pathophysiological processes is given by Hrabárová et al [21].

4.2. Scission of Glycosaminoglycans Mediated by Peroxynitrite

Complex polysaccharides, glycosaminoglycans (GAGs) are widely distributed in the endothelial cell extracellular matrix (ECM) of tissues among which heparin, hyaluronan as well as heparan/keratan/dermatan/chondroitin sulfates have been the subject of an intensive study. They differ in their sugar composition and in the position of the sulfate group as well as the degree of sulfation. They are typically heterogeneous in chain length and negatively

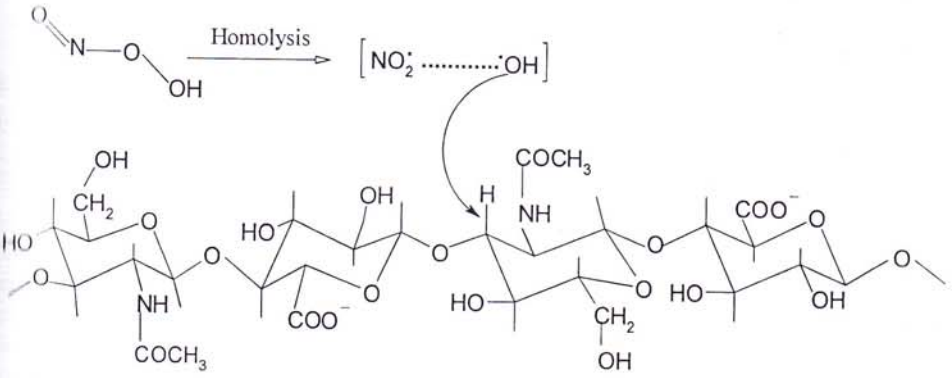
charged. They also differ in the type of uronic acid, *e.g.* glucuronic or iduronic acid. A relatively anaerobic cartilage matrix is mainly composed of the two major protein groups, collagens and proteoglycans. Regulation of breakdown *versus* synthesis of these complex bio-macromolecules consisting of a core protein with one or more covalently bound GAG chains specifies the cartilage matrix integral function. They contain large quantities of water *via* multiple hydrogen bonds and thus expanding their three-dimensional space.

Rheumatoid arthritis and osteoarthritis exist both also as a consequence of the degradation of glycosaminoglycans mostly *via* hydroxyl radical, the product of peroxynitrite fate. The $^{\bullet}$NO-mediated degradation of GAGs has two pathways. The first one is initiated by the conversion of nitric oxide to nitrous acid, while the second one includes peroxynitrite. Heparin and heparan sulfate are susceptible to degradation *via* nitrous acid, hyaluronan *via* peroxynitrite, and chondroitin sulfates partially *via* both reagents. Decomposition of heparan sulfate and other GAGs of the extracellular matrix by $^{\bullet}$NO may be important in pathophysiological states (bone development, apoptosis, atherosclerotic plaque release, metastatic, inflammatory conditions). Endothelial-cell-derived $^{\bullet}$NO is capable of degrading heparin and heparan sulfate *via* HNO_2 rather than peroxynitrite. Along with cleavage of the glycosidic bond, the amino and sulfate groups are both eliminated. Inflammatory processes may be responsible for release of excess $^{\bullet}$NO and $O_2^{\bullet-}$, forming peroxynitrite capable to degrade hyaluronan but not heparan sulfate. The balance between $^{\bullet}$NO and $O_2^{\bullet-}$ determines which glycosaminoglycan component of the extracellular matrix is destroyed and it may be important in regulating the disease processes [21].

4.3. High-molar-mass Hyaluronan Susceptibility against Peroxynitrite Pro-oxidative Action

Hyaluronan is a non-branched glycosaminoglycan bearing no sulphate groups, composed of alternating disaccharide units: D-glucuronic acid (GlcU) and *N*-acetyl-d-glucosamine (GlcNAc). It is a naturally occurring biopolymer widely distributed in vertebrate tissues. Aqueous solutions of hyaluronan (pK_a 3.2) are represented by negatively-charged macromolecules exhibiting a three-dimensional structure with extensive intramolecular hydrogen bonding. Highly viscous hyaluronan solutions – with average molar mass (10^5 – 10^7 Da) – have gel-like properties at the concentration range of 2–4 mg mL^{-1} in the synovial fluid, in which it serves also as the joint-lubricating agent.

At neutral pH, decomposition products of the probably more reactive *trans*-peroxynitrite, released from a complex radical pair [$^{\bullet}$OH - ONO$^{\bullet}$], nitric dioxide radical and hydroxyl radical, degrade hyaluronan, as depicted in Scheme 5. The polymeric chains are predominantly cleaved by hydroxyl radicals. The concentration of hyaluronan fragments has a progressive tendency linearly with peroxynitrite concentration up to 0.15 mmol L^{-1}. It reaches 4 μmol L^{-1} at high peroxynitrite concentration. Each peroxynitrite molecule generates 1.12×10^{-2} hyaluronan chain breaks resulting in reduced solution viscosity. Protective scavengers against deleterious action of reactive oxidant species in the attack of this biopolymer, are *e.g.* thiourea (extremely effective), dimethyl sulfoxide (moderately effective), sodium benzoate and mannitol (slightly effective).



Scheme 5. Hyaluronan attack mediated by peroxynitrite.

Peroxynitrite exhibits hydroxyl radical-like reactivity derived from the vibrationally-exciting state of probably less stable *trans*-peroxynitrous acid. Hyaluronan does not undergo the degradation by $^{\bullet}NO$ and HNO_2 due to its *N*-acetyl groups. At acidic pH, peroxynitrite-dependent C-centered carbon radicals are formed in monomers, in the tetrasaccharide as well as in the hyaluronan polymer, as revealed by spin-trapping electron paramagnetic resonance (EPR) spectroscopic experiments. This fact supports the hypothesis of oxidative pathway involved in the degradation of hyaluronan playing a key role in the development and progression of rheumatoid arthritis [21]. Trioxocarbonate anion radical ($CO_3^{\bullet-}$), the product of the biologically-relevant reaction of peroxynitrite with carbon dioxide, causes about 20 % yields of hyaluronan chain scission *via* a not well-known mechanism [26].

Monitoring the kinetics of hyaluronan degradation by peroxynitrite containing H_2O_2 or H_2O_2- free peroxynitrite using a Brookfield rotational viscometer was properly introduced by *Stankovská et al.* [27]. Various investigations on peroxynitrite pro-oxidative action resulting in hyaluronan fragmentation are summarized in the Appendix.

4.4. Analytical Approach-based Investigation of Hyaluronan Degradation by Reactive free Radicals

Šoltés et al. [34] give overview on currently used analytical methods to evaluate the impact of different pathways of oxygen- and nitrogen-derived free radical species on high-molar-mass hyaluronan degradation resulting in certain changes in its structure (chain size, molar mass, and solution viscosity reduction). Hyaluronans are well-characterized by their molar mass distribution *via* viscometry, osmometry, and light scattering.

Rheological parameters - markers of hyaluronan degradation can be detectable *via* capillary or rotational viscometry. Capillary viscometry – based Mark-Houwink equation was used by Gura *et al.* [35] to characterize hyaluronan fragments undergone ultra-sonicatious degradation.

Chemical modification of hyaluronan monosaccharide units after degradation due to the action of free radicals is best characterized by EPR method. Identification of the generation of free radicals as a consequence of anti- and pro-oxidative action of D-penicillamine in the degradative system {hyaluronan *plus* ascorbate *plus* Cu(II)} was monitored *via* EPR method

[36]. Al-Assaf *et al.* [37] studied, by the EPR method, the formation of various radicals on the reaction of a Ti(III) *plus* H_2O_2 redox couple generating hydroxyl radicals, reacting subsequently with hyaluronan, supporting the thesis where each hydroxyl radical resulted in a single hyaluronan chain scission. An $^{\bullet}OH$ attack-derived radical of hyaluronan was identified at the C_5 (GlcU) and C_6 (GlcNAc) moieties.

Pro- and anti-oxidative effects of potential low-molar-mass thiol-based scavengers of hydroxyl radicals, D-penicillamine and reduced L-glutathione, were studied monitoring the kinetics of hyaluronan degradation *via Weissberger's* system using rotational viscometry. Among others, fragmented hyaluronan samples were also tested by non-isothermal chemiluminescence method confirming the effect of these thiol compounds on the decomposition of hydroperoxides and the removal of oxygenated structures. Fourier-transformed infrared spectroscopy revealed a possible thiol incorporation into a hyaluronan network [38].

5. Concluding Remarks

The research on ubiquitous endogenous biomolecules, playing important role in biological systems, such as nitric oxide radical and peroxynitrite, has recently led to a numerous publication activity meeting all aspects of physiological and pathological areas of interest. Enormous challenge still remains to reduce a disease derived-human suffering if a biological specificity against deleterious actions of oxidants in living systems is better understood. A special interest must be focused on experimental investigations elucidating specific cellular mechanisms such as the effects of oxidative stress in the respiratory systems, reactive radical species- signaling pathways as well as the role of anti-oxidant systems. The management of the pathogenesis of various diseases may eventually lead to discovery of novel therapeutic and clinical strategies.

Acknowledgments

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Appendix

Hyaluronan degradation by peroxynitrite-mediated free radical species

The aim of the work	Na-HA average-molar weight (M_w), [MDa]	Na-HA concentration [mg mL^{-1}] or * [mmol L^{-1}]	$ONOO^-$ concentration [mmol L^{-1}]	$ONOO^-$-mediated free radical species	Effects of TMIs on HA degradation	Methodology used on experimental degradative study	Scavenging conditions for ROS	Pathophysiological consequences and therapeutic aspects	Refe-rence
Comparison of the kinetics of HA degradation by H_2O_2- $ONOO^-$ /H_2O_2-free- $ONOO^-$ system	1.378	2.50 (0.15 M NaCl)	10^{-3}; 10^{-4}	$^{\bullet}OH$; $RO^{\bullet}$; $ROO^{\bullet}$	$ONOO^-$ and HA-radicals decomposition accelerated by Mn(II)	Brookfield rotational viscometer	Not mentioned	Lowered HA- viscosity leading to a joint inflammation	[27]
Study of HA chain breaks in context with potential $ONOO^-$ pathways to $^{\bullet}OH$ formation	0.44	* 5.30 (0.01 M PB/DTPA)	3.06	$^{\bullet}OH$; $CO_3^{\bullet-}$; $RO^{\bullet}$; $ROO^{\bullet}$	Fenton reaction due to trace TMI ($^{\bullet}OH$ formation)	Stopped-flow system (Applied Photophysics Reactor)	$^{\bullet}OH$ scavenging by NO_2^- ions at high $ONOO^-$ concentrations	$ONOO^-$-mediated chronic inflammation (HA fragmentation induced by NOS in murine macrophages)	[28]
Study of the degradation and antioxidative effect of Na-, Zn-, Co-, Cu-, and Mn-HA associates	1.20	* 6.30 (Mops; glycine)	0.0011-0.0085 mmol $ONOO^-$ /mmol T	$^{\bullet}OH$; $RO_2^{\bullet}$;$O_2^{\bullet-}$	TMI-HA associates resistance against $ONOO^-$-induced HA degradation	Spectro-photometry	The $^{\bullet}OH$ and $O_2^{\bullet-}$ scavenging capacity and TRAP of HA associates assay; $ONOO^-$-scavenging effects: $Co^{2+}>Cu^{2+}/Mn^{2+}>Zn^{2+}$	The correlation between ROS-mediated TMI-HA-degradation and its scavenging capacity ⇒ TMI-HA associates effective in therapy (SF protection)	[29]

Appendix (Continued)

Study of antioxidative effects and degradation of Zn-HA complex against ROS in comparison with Na-HA	1.43-3.30×10^{-4}	* 4.50 (1.0 mM $ZnSO_4$)	Not mentioned	$O_2^{\bullet-}$; $ONOO^-$-derived radicals	$ONOO^-$ inhibition by Zn-HA	Pyrogallol red bleaching assay; SEC	$ONOO^-$-induced HA degradation limited by scavenging effect of Zn-HA complex	Enhancement of therapeutic benefits of Na-HA by Zn antioxidative effects	[30]
Comparison of the effects of $ONOO^-$ on HA (P, T, M) degradation	Not mentioned	10.0 P (PB/DTPA); 2.0 M (150 mM PB); 4.0 T (25 mM AF)	5.0; 20	$^{\bullet}OH$; C-centered carbon radicals; $RO^{\bullet}$;$ROO^{\bullet}$	Metal-catalyzed nitration by $ONOO^-$ avoided	^{1}H-NMR; ESI-MS; EPR	Not discussed	Involvement of $NO^{\bullet}$ pathway in development and progression of RA ⇒ $^{\bullet}OH$ progression at low pH in inflamed SF	[31]
Comparison of the effect of TMs on HA degradation induced by $ONOO^-$ and TMI/H_2O_2 system	2.0-3.0 (apparent)	3.60 (0.15 M NaCl/ 0.10 M PB)	0.04-0.67	$^{\bullet}OH$; $^{\bullet}NO_2$	H_2O_2-Cu^{2+}/Fe^{3+} /EDTA-mediated $^{\bullet}OH$ formation	AGE; CV	HA protection from $ONOO^-$ $^{\bullet}OH$ attack by TM addition (TH>DMSO>SB/ML)	Biological impact on HA cleavage may differ *via* either free $^{\bullet}OH$ or $ONOO^-$ /ONOOH degradative action	[32]
Comparison of HA and other GAGs degradation by $ONOO^-$/ONOOH or SIN-1 system	0.12	2.0 (Chelex-treated 0.10 M PB)	10	$^{\bullet}OH$; $^{\bullet}C(O)R$; C-centered carbon radicals; $ROO^{\bullet}$	Contaminating TMIs removal *via* Chelex resin	PAGE; EPR	Not discussed	Time-, concentration-, and pH-dependent fragmentation of extracellular matrix GAGs into disaccharides at sites of inflammation	[33]

Abbre*via*tions: Na-HA: Hyaluronan, sodium salt; R: alkyl; M_w: Average-molar-weight; TMIs : Transition metal ions; TRAP: Total peroxyl radical-trapping antioxidant parameter; SEC: Size-exclusion chromatography;P, T, M: Polymer, tetrasaccharide, monomer of HA (monomeric units: GlcNAc and GlcU); ESI-MS: ElectroSpray Ionisation Mass Spectrometry; RA: Rheumatoid arthritis;

AF: Ammonium formiate; PB: Phosphate buffer; DTPA: Diethylenetriaminepentaacetate; SF: Syno*vial* fluid; TM: Target molecule;

TH: Thiourea; DMSO: Dimethyl sulfoxide; SB: Sodium benzoate; ML: Mannitol; AGE: Agarose gel electrophoresis; CV: Capillary viscometry; PAGE: Polyacrylamide gel electrophoresis; EDTA: Ethylenediaminetetraacetate; SIN-1: 3-Morpholinosydnonimine (oxygen-dependent peroxynitrite generator); $^{\bullet}C(O)R$: Acyl radical of HA.

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