Engineering of Polymers and Chemical Complexity

Volume 2 New Approaches, Limitations, and Control

Editors
Walter W. Focke, PhD
Hans-Joachim Radusch, PhD

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Volume II: New Approaches, Limitations, and Control

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Walter W. Focke, PhD and Hans-Joachim Radusch, PhD

Gennady E. Zaikov, DSc, and A. K. Haghi, PhD

Reviewers and Advisory Board Members

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CHAPTER 6

HYALURONAN DEGRADATION UNDER FREE-RADICAL OXIDATION STRESS: ACTION AND HEALING

T. M. TAMER

CONTENTS

6.1 INTRODUCTION

Oxidation stress is unbalanced between prooxidants and natural antioxidants in body that lead to several diseases such as rheumatoid. Hyaluronic acid (HA), is a high molecular weight biopolysacharide, is found in the extracellular matrix of soft connective tissues and is particularly concentrated in synovial fluid (SF). Half-live time of Hyaluronan in SF is approximately 12 hrs in normal conditions. This process is accelerated under normal oxidation stress that generates troubles in human joints.

This chapter describe oxidation stress-source and effects-Hyaluronan origin, properties and functions, and finally thiol compounds as antioxidants preventing HA degradations under conditions of oxidation stress.

The ability to utilize oxygen has provided humans with the benefit of metabolizing lipids, proteins, and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called "free radicals." Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. The cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction. [1]. Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases. [2,3]

Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is when the availability of antioxidants is limited that this damage can become cumulative and debilitating.

Oxidative stress is the phenomenon that occurs when the steady-state balance of pro-oxidants to antioxidants is shifted in the direction of the former, creating the potential for organic damage. Pro-oxidants are by definition free radicals, atoms or clusters of atoms with a single unpaired electron. Physiologic concentrations of pro-oxidants are determined both by internal and external factors. Pro-oxidant reactive oxygen species (ROS), for example, are normal products of aerobic metabolism. However, under pathological conditions ROS production can increase, surpassing the body's detoxification capacity and thus contribute to molecular-level organic pathology. External sources of free radicals include exposures to environmental toxins such as ionizing radiation, ozone and nitrous oxide, cigarette smoke (including passive inhalation) and heavy metals, as well as dietary intake of excess alcohol, unsaturated lipid, and other chemicals and compounds present in food and water.

Antioxidants are chemical compounds that can bind to free radicals and thus prevent them from damaging healthy cells. Antioxidants can be divided into enzymatic and non-enzymatic subtypes. Several antioxidant enzymes are produced by the body, with the three major classes being catalase, the glutathione (GSH) peroxidases, and the superoxide dismutases (SODs). Non-enzymatic antioxidants include the innate compound glutathione as well as antioxidant vitamins obtained through the diet, such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), and β-carotene.

6.2 FREE RADICALS DEFINITION AND FORMATION

Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves.

Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction.

6.2.1 SOURCE OF FREE RADICAL

In cells, there are two main sources of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Hydroxyl radical (HO) is generated from superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2).

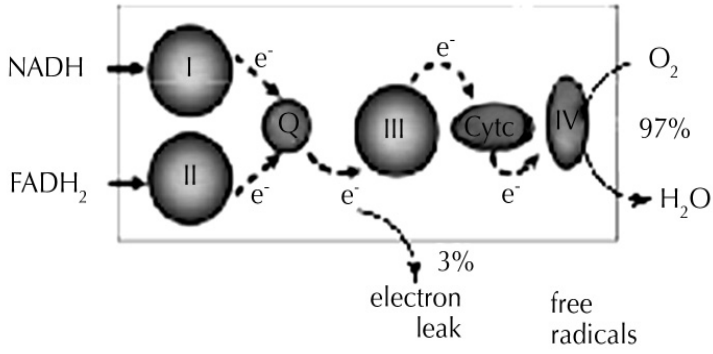
6.2.2 SOURCES OF SUPEROXIDE RADICAL (O_2^-)

The following Table (1) illustrates the most important reactions within the cell that generate superoxide anion (O_2^-).

TABLE 1 Reaction sources of superoxide anion radical (O_2^-)

Source	*Pathophysiological significance*
• ***Enzymic reactions***	
- xanthine oxidase	Intestinal ischemia/reperfusion
- NADH oxidase	Present in leukocytes: bactericidal activity
- NADPH-cytochrome P450 reductase	
•Cellular sources	
- leukocytes and macrophages	Bactericidal activity
- mitochondrial electron transfer	
- microsomal monooxygenase	
• ***Environmental factors***	
- ultraviolet light	
- X rays	
- toxic chemicals	
- aromatic hydroxylamines	

- aromatic nitro compounds
- insecticides, such as paraquat
- chemotherapeutic agents, such as quinines



Mitochondria are major cellular sources of reactive oxygen species. Mitochondria consume oxygen associated with the process of oxidative phosphorylation. Under normal conditions, approximately 95–97% of the oxygen is reduced to water; electron leakage, accounting for about 3–5% of the total oxygen consumed by mitochondria, is associated with the generation of oxygen radicals.

6.2.3 SOURCES OF HYDROGEN PEROXIDE

Hydrogen peroxide (H_2O_2) is generated within the cells by two distinct processes: 1) nonradical or enzymic generation and 2) radical or from superoxide anion disproportionation.

NONRADICAL OR ENZYMIC GENERATION

The following enzymes do generate (H_2O_2) upon reduction of their cosubstrate, molecular oxygen:

Glycolate oxidase, d-amino acid oxidase, urate oxidase, acetyl-CoA oxidase, NADH oxidase and monoamine oxidase.

The latter enzyme, monoamine oxidase (MAO) occurs in two forms A and B and it catalyzes the oxidative deamination of biogenic amines. It is present in the outer mitochondrial membrane.

RADICAL GENERATION FROM SUPEROXIDE ANION DISPROPORTIONATION

This is achieved upon dismutation or disproportionation of superoxide anion ($O_2^{\cdot -}$), according to the reaction mentioned before:

$$O_2^{\cdot -} + O_2^{\cdot -} + H^+ \rightarrow H_2O_2 + O_2$$

As mentioned above, *mitochondria are major cellular sources of oxyradicals*. Superoxide anion radical ($O_2^{\cdot -}$), generated upon autoxidation of ubisemiquinone, is vectorially released into the inter membrane space and the mitochondrial matrix. In the latter compartment, $O_2^{\cdot -}$ dismutates to H_2O_2.

The H_2O_2 is a freely diffusible species that can cross membranes. Hence, mitochondria have two major sources of H_2O_2: On the one hand, H_2O_2 generated by disproportionation of superoxide anion in the mitochondrial membrane and, on the other hand, the oxidative deamination of biogenic amines by the outer mitochondrial membrane-bound monoamine oxidase activity. Mitochondrion-generated H_2O_2 is involved in the redox regulation of cell signaling pathways. The steady-state levels of H_2O_2 ($[H_2O_2]ss$) determine the cellular redox status and the transition from proliferation to apoptosis and necrosis.

6.2.4 SOURCES OF HYDROXYL RADICAL

The most of the hydroxyl radical ($HO^{\cdot}$) generated *in vivo*, except for that during excessive exposure to ionizing radiation, originates from the breakdown of hydrogen peroxide (H_2O_2) *via* a *Fenton reaction*.

The Fenton reaction entails a metal-dependent reduction of hydrogen peroxide (H_2O_2) to hydroxyl radical (HO). *Transition metals*, such as copper (Cu), iron (Fe), and cobalt (Co), in their reduced form catalyze this reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\cdot}$$

As indicated above, the Fenton reaction requires the transition metal in its reduced state. Reduction of the transition metal may be accomplished by superoxide anion ($O_2^{\cdot -}$), as in the following example with Fe^{+++}:

$$Fe^{+++} + O_2^{\cdot -} \rightarrow Fe^{++} + O_2$$

The overall reaction, involving iron reduction by superoxide anion ($O_2^{\cdot -}$) and iron oxidation by hydrogen peroxide (H_2O_2), is as follows:

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$
$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + HO^- + HO$$

$$O_2^- + H_2O_2 \rightarrow O_2 + HO^- + HO^{\cdot}$$

The latter reaction ($O_2^- + H_2O_2 \rightarrow O_2 + HO^- + HO^{\cdot}$), is known as the *Haber-Weiss reaction*. This reaction, as such, proceeds at very slow rates. The *Fenton reaction*, that is, metal catalyzed reduction of hydrogen peroxide (H_2O_2), prevails in a biological environment. It is worth noting that, at variance with superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), there is no direct generation of hydroxyl radical (HO) in the cell. Both, superoxide anion and hydrogen peroxide are required to form the highly reactive hydroxyl radical (HO).

6.2.5 SINGLET OXYGEN

Singlet oxygen is a reactive oxygen species that can be formed not only by energy transfer (as mentioned above), but also by electron-transfer reactions.

Electron transfer reactions: Of biological interest, the enzyme *myeloperoxidase*, present in neutrophiles, can catalyze the formation of hypochlorite from Cl^- and $H_2O_2^{\cdot}$ The further reaction of hydrogen peroxide (H_2O_2) with formed HOCl yields singlet oxygen ($^1O_2$):

$$Cl^- + H_2O_2 \xrightarrow{myeloperoxidase} HOCl + H_2O$$

$$HOCl + H_2O_2 \rightarrow Cl^- + H_2O + H^+ + \mathbf{^1O_2}$$

Energy transfer reactions: This is another way to generate singlet oxygen as it is comprised in the photosensitization of different chemotherapeutic agents. The chemotherapeutic agent (or sensitizer = S) absorbs energy upon irradiation and transfers this energy to molecular oxygen with formation of singlet oxygen ($^1O_2$).

$$S + h\upsilon \rightarrow S^*$$

$$S^* + O_2 \rightarrow S + {}^1O_2$$

As mentioned before, singlet oxygen ($^1O_2$) is a reactive species that reacts with molecules, such as vitamin E, vitamin C, DNA, cholesterol, carotenoids, polyunsaturated fatty acids in membranes, and certain amino acids.

HARMFUL OF FREE RADICAL

All the biological molecules present in our body are at risk of being attacked by free radicals. Such damaged molecules can impair cell functions and even lead to cell death eventually resulting in diseased states.

It can be considered that superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are less reactive than hydroxyl radical ($HO^{\cdot}$) and singlet oxygen. However, in a suitable biological setting the two first species may display considerable chemical reactivity leading to damage of various biomolecules

LIPIDS AND LIPID PEROXIDATION

Membrane lipids present in subcellular organelles are highly susceptible to free radical damage. Lipids when reacted with free radicals can undergo the highly damaging chain reaction of lipid peroxidation (LP) leading to both direct and indirect effects. During LP a large number of toxic byproducts are also formed that can have effects at a site away from the area of generation, behaving as 'second messengers'. The damage caused by LP is highly detrimental to the functioning of the cell (Ramsarma T et al., 2003).

Lipid peroxidation (LP) is a free radical mediated process. Initiation of a peroxidative sequence is due to the attack by any species, which can abstract a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon atom (CH). The resultant carbon radical is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to give a lipid peroxyl radical (LOO) These radicals can further abstract hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate LP further. The peroxidation reaction can be terminated by a number of reactions. The major one involves the reaction of $LOO^{\cdot}$ or lipid radical ($L^{\cdot}$) with a molecule of antioxidant such as vitamin E or α-tocopherol (α-TOH) forming more stable phenoxyl radical that is not involved in further chain reactions. This can be 'recycled' by other cellular antioxidants such as vitamin C or GSH.

$$LH^{\cdot} + OH \rightarrow L^{\cdot} + H_2O$$

$$L^{\cdot} + O_2 \rightarrow LOO$$

$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH$$

$$LOO^{\cdot} + \alpha\text{-}TOH \rightarrow LOOH + \alpha\text{-}TO\bullet$$

The process of LP, gives rise to many products of toxicological interest like malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and various 2-alkenals. Isoprostanes are unique products of lipid peroxidation of arachidonic acid and recently tests

such as mass spectrometry and ELISA-assay kits are available to detect isoprostanes [4].

6.2.6 CARBOHYDRATES

Free radicals such as ˙OH react with carbohydrates by randomly abstracting a hydrogen atom from one of the carbon atoms, producing a carbon-centered radical. This leads to chain breaks in important molecules like hyaluronic acid. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals that is also being implicated in rheumatoid arthritis.

6.2.7 DNA OXIDATION

Hydroxyl radical is endowed with unique properties: due to a combination of high electrophilicity, high thermochemical reactivity, and a mode of production that can occur in the vicinity of DNA (site specific mechanism), it can both abstract H atoms from the sugar in the DNA helix and add to DNA bases, leading to single strand breaks and nucleobase (8- hydroxydesoxyguanosine) oxidation, respectively.

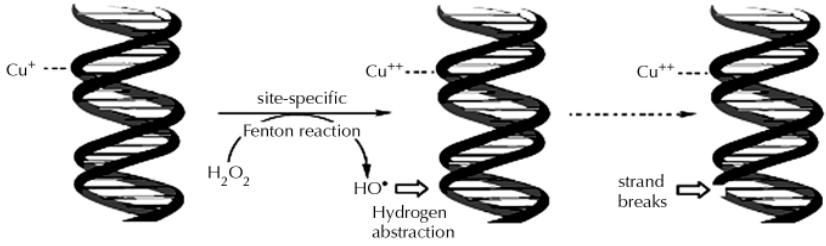
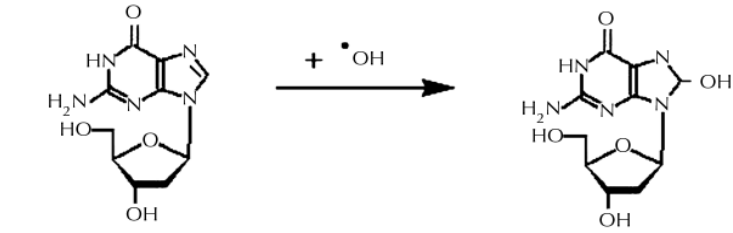


FIGURE 1 Hydrogen abstraction–DNA strand breaks.

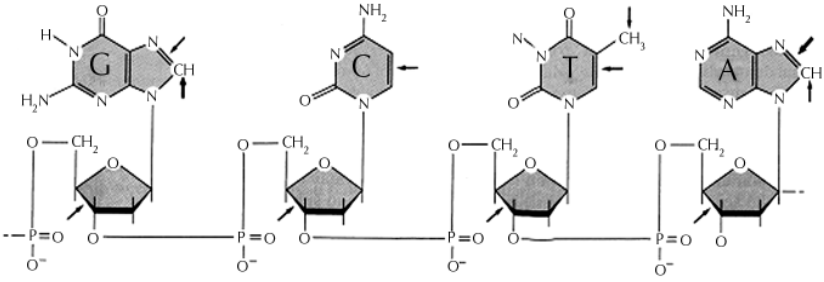
ADDITION - NUCLEOBASE OXIDATION

Hydroxyl radical addition to bases such as guanine, proceeds very rapidly and leads to the formation of 8-hydroxydesoxyguanosine, which is used as a fingerprint of nucleobase oxidative damage.



The DNA is susceptible to changes that would lead to mutations. For example, DNA bases are damaged by an encounter with free radicals or environmental chemicals. Hydroxyl radical mediated damage on sugars (deoxyribose) is a part of the known C'4 mechanism and leads to strand breaks. Oxidative damage of bases, usually leads to adduct formation, as exemplified above with 8-hydroxydesoxyguanosine.

Reactive oxygen species can damage DNA at different levels: hydroxyl radical through addition reactions can cause single-strand base damage (e.g., formation of 8-hydroxyldeoxyguanosine) and through H abstraction single strand DNA nick (ssDNA nick) or double strand DNA break (dsDNA break).



Oxidative damage to DNA is a result of interaction of DNA with ROS or RNS. Free radicals such as OH and $H^{\cdot}$ react with DNA by addition to bases or abstractions of hydrogen atoms from the sugar moiety. The C4–C5 double bond of pyrimidine is particularly sensitive to attack by OH, generating a spectrum of oxidative pyrimidine damage products, including thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin and others. Similarly, interaction of $^{\cdot}OH$ with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products. Several repair pathways repair DNA damage (Halliwell B and Aruoma OI., 1993). 8-OHdG has been implicated in carcinogenesis and is considered a reliable marker for oxidative DNA damage.

6.2.8 PROTEINS

Oxidation of proteins by ROS/RNS can generate a range of stable as well as reactive products such as protein hydroperoxides that can generate additional radicals particularly upon interaction with transition metal ions. Although most oxidized proteins that are functionally inactive are rapidly removed, some can gradually accumulate with time and thereby contribute to the damage associated with ageing as well as various diseases. Lipofuscin, an aggregate of peroxidized lipids and proteins accumulates in lysosomes of aged cells and brain cells of patients with Alzheimer's disease [5].

6.2.9 METHODS TO OVERCOME FREE RADICAL RISKS

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals[6].

In their definition of the term antioxidant, Halliwell and Gutteridge (1989) state, 'any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate'. This definition would comprise compounds of nonenzymic as well as enzymic nature. Table 2 overviews some of the antioxidants of biological interest [7].

TABLE 2 Antioxidant defense in biological systems. Condensed list of antioxidant compounds and enzymes. Modified from (Sies, H., 1985)

System	Remarks
Non-enzymic	
α-Tocophero1 (vitamin E)	radical chain-breaking
ß-Carotene	singlet oxygen quencher
Lycopene	singlet oxygen quencher
Ubiquinol-10	radical scavenger
Ascorbate (vitamin C)	diverse antioxidant functions
Glutathione (GSH)	diverse antioxidant functions
Urate	radical scavenger
Bilirubin	plasma antioxidant
Flavonoids	plant antioxidants (rutin, etc.)
Plasma proteins	metal binding, e.g. coeruloplasmin

TABLE 2 *(Continued)*

chemical	food additives, drugs
Enzymic (direct)	
superoxide dismutases	CuZn enzyme, Mn enzyme, Fe enzyme
glutathione peroxidases	enzymes (GPx, PHGPx)
	ebselen as enzyme mimic
catalase	heme protein, peroxisomes
Enzymatic (ancillary enzymes)	
conjugation enzymes	glutathione-S-transferases
	UDP-glucuronosyl-trans ferases
NADPH-quinone oxidoreductase	two-electron reduction
GSSG reductase	maintaining GSH levels
NADPH supply	NADPH for GSSG reductase
transport systems	GSSG export thioether (S-conjugate) export
repair systems	DNA repair systems oxidized protein turnover

6.3 HYALURONAN

In 1934, Karl Meyer and his colleague John Palmer isolated a previously unknown chemical substance from the vitreous body of cows' eyes. They found that the substance contained two sugar molecules, one of which was uronic acid. For convenience, therefore, they proposed the name "hyaluronic acid". The popular name is derived from "hyalos", which is the Greek word for glass + uronic acid [8]. At the time, they did not know that the substance which they had discovered would prove to be one of the most interesting and useful natural macromolecules. HA was first used commercially in 1942 when Endre Balazs applied for a patent to use it as a substitute for egg white in bakery products[9].

The term "hyaluronan" was introduced in 1986 to conform to the international nomenclature of polysaccharides and is attributed to Endre Balazs (Balazs E.A, et al., 1986), who coined it to encompass the different forms of the molecule can take, e.g., the acid form, hyaluronic acid, and the salts, such as sodium hyaluronate, which forms at physiological pH [10]. HA was subsequently isolated from many other sources and the physicochemical structure properties, and biological role of this polysaccharide were studied in numerous laboratories [11]. This work has been summarized in a Ciba Foundation Symposium [12] and a recent review [13].

Hyaluronan (HA) (Figure 2) is a unique biopolymer composed of repeating disaccharide units formed by *N*-acetyl-d-glucosamine and d-glucuronic acid. Both sugars are spatially related to glucose which in the beta configuration allows all of its bulky groups (the hydroxyls, the carboxylate moiety and the anomeric carbon on the adjacent sugar) to be in sterically favorable equatorial positions while all of the small hydrogen atoms occupy the less sterically favorable axial positions. Thus, the structure of the disaccharide is energetically very stable. HA is also unique in its size, reaching up to several million Daltons, and is synthesized at the plasma membrane rather than in the Golgi, where sulfated glycosaminoglycans are added to protein cores [14, 15].

In a physiological solution, the backbone of a HA molecule is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds, and interactions with the solvent. The axial hydrogen atoms form a non-polar, relatively hydrophobic face while the equatorial side chains form a more polar, hydrophilic face, thereby creating a twisting ribbon structure. Solutions of hyaluronan manifest very unusual rheological properties and are exceedingly lubricious and very hydrophilic. In solution, the hyaluronan polymer chain takes on the form of an expanded, random coil. These chains entangle with each other at very low concentrations, which may contribute to the unusual rheological properties. At higher concentrations, solutions have an extremely high but shear-dependent viscosity. A 1% solution is like jelly, but when it is put under pressure it moves easily and can be administered through a small-bore needle. It has therefore been called a "pseudoplastic" material. The extraordinary rheological properties of hyaluronan solutions make them ideal as lubricants. There is evidence that hyaluronan separates most tissue surfaces that slide along each other. The extremely lubricious properties of hyaluronan, meanwhile, have been shown to reduce postoperative adhesion formation following abdominal and orthopedic surgery. As mentioned, the polymer in solution assumes a stiffened helical configuration, which can be attributed to hydrogen bonding between the hydroxyl groups along the chain. As a result, a coil structure is formed that traps approximately 1000 times its weight in water [16].

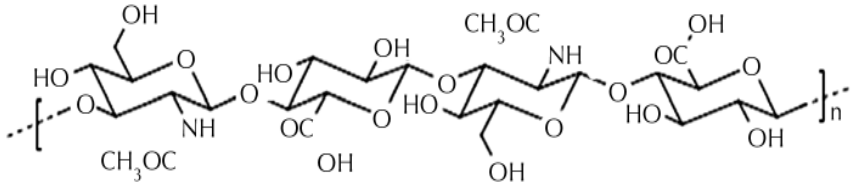


FIGURE 2 Structural formula of hyaluronan – the acid form.

6.3.1 HYALURONAN SOURCE

The HA is an important component of most connective tissues, including vitreous body, skin, synovial fluid, and umbilical cord. Comparative data for a variety of species, tissues and organs are shown in Table 3 [17]. It affects many cell functions, such

as proliferation, differentiation and migration in a concentration and molar-mass dependent manner. The turnover of HA is extremely rapid. It is estimated that from 15 g of HA in the vertebrate body, 5 g turns over daily. The half-life of HA in the blood circulation is between 2-5 min. In the epidermis of the skin, where one half of HA of the body is found, it is up to 2 days, and in an apparently inert tissue as cartilage, it is approximate 1-3 weeks[18].

TABLE 3 Normal concentrations (μg/g) of hyaluronan (HA) in various organs of different species. (Laurent T C and Fraser J R E., 1996, Reed R K et al., 1988, Laurent UBC and Laurent T C., 1981, Laurent UBG., 1981)

Organ or fluid	Man	Sheep	Rabbit	Rat
Umbilical cord	4100			
Synovial fluid	1400-3600	540	3890	
Dermis	200			
Vitreous body	140-338	260	29	
Lung		98-243		34
Kidneys			93-113	30
Renal Papillae			250	
Renal cortex			4	
Brain	35-115		54-76	74
Muscles			27	
intestine				44
Thoracic lymph	8.5-18	1-34		5.4
Liver			1.5	4
Aqueous humour	0.3-2.2	1.6-5.4	0.6-2.5	0.2
Urine	0.1-0.3			
Lumbar CSF	0.02-0.32			
Plasma (serum)	0.01-0.1	0.12-0.31	0.019-0.086	0.048-0.26

The cellular synthesis of HA is a unique and highly controlled process. HA is naturally synthesized by a class of integral membrane proteins called hyaluronan synthases, of which vertebrates have three types: HAS1, HAS2, and HAS3 [19, 20]. Secondary structure predictions and homology modeling indicate an integral membrane

protein (IMP). The IMP is a protein molecule (or assembly of proteins) that in most cases spans the biological membrane with which it is associated (especially the plasma membrane) or which, is sufficiently embedded in the membrane to remain with it during the initial steps of biochemical purification (in contrast to peripheral membrane proteins). Hyaluronan synthase enzymes synthesize large, linear polymers of the repeating disaccharide structure of hyaluronan by alternate addition of glucuronic acid and N-acetylglucosamine to the growing chain using their activated nucleotide sugars (UDP = glucuronic acid and UDP-N-acetyl glucosamine) as substrates.

6.3.1 HYALURONAN PRODUCTION

Currently there are two competing methods for industrial HA production that are extraction from animal sources, such as bovine eyes and rooster combs, and microbial production through the use of large scale fermentors. Both will be discussed in the following sections, in addition the opportunity of using novel genetically engineered microbial factories and a chemo-enzymatic synthesis approach will be reported from the very recent literature.

TRADITIONAL EXTRACTION PROCESSES

The traditional method for HA production is based on solvent extraction from animal tissue extracts, eventually using cetylpiridinium chloride (CPC) precipitation. One of the first paper presented by Swann (1968), reported the following procedure: (1) mechanical slicing of the rooster combs to obtain small pieces, (2) washing with ethanol (4 L ethanol to 1 Kg comb), this operation could be repeated until the solvent would not appear cloudy; (3) extracting the minced combs with a water/chloroform mixture (2.5 Kg combs: 10 L water: 0.5 L chloroform), while stirring to allow combs to swell; (4) filtering the solids from the broth and adding NaCl, successively carrying an additional chloroform extractions; (5) accomplishing protease (pronase) digestion, followed by chloroform extraction and centrifugation (Swann DA., 1968).

In alternative methods (29) the crude extracts were purified by epichlorohydrin triethanolamine- (ECTEOLA-) chromatography and by fractionation with CPC. In addition, repeated ethanol precipitation (1:3 water/ethanol ratio), before and after CPC (1%) HA precipitation, were reported [21]. In all the cases the product is then filtered through sterilizing filters, followed by solvent precipitation, finally HA is formulated into medical devices and pharmaceutical products. HA purified by these procedures was recovered with a yield greater than 90% with respect to the uronic acid evaluated in the starting material.

However, the collection of rooster combs and the extraction and purification procedures of HA from these tissues are time-consuming and labour intensive, making hyaluronan production very costly [22]. In fact in animal tissues hyaluronan is complexed with proteoglycans and often contaminated with HA degrading enzymes, making the isolation of high purity and high molecular sized polysaccharide very difficult. Moreover the use of animal-derived biomolecules for biopharmaceutical applications is facing growing opposition because of the risk of cross-species viral and other ad-

ventitious agent contaminations. Hence, since '80 microbial production is gradually replacing extraction from animal tissues in HA industrial manufacturing.

6.3.2 BIOTECHNOLOGICAL PRODUCTION OF HA

Bacteria known to be capable of the synthesis of HA are Streptococci of groups A and C, gram-positive bacteria such as *Streptococcus equi*, an equine pathogen, *Streptococcus equisimilis*, that is infective for different animals, *Streptococcus pyogenes*, a human pathogen and *Streptococcus uberis*, a bovine pathogen. These β-hemolytic bacteria, able to digest blood based agar medium, also present a slimy translucent layer surrounding bacterial colonies that can be attributed to HA synthesis. Figure (3) describes schematically steps of production of HA from Strepococcus species.

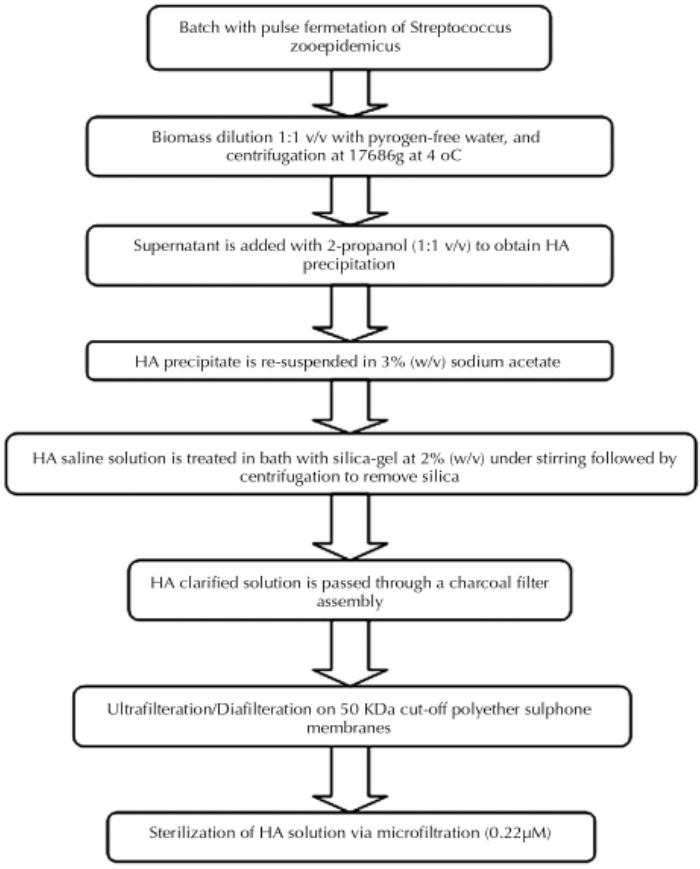


FIGURE 3 Overview of hyaluronic acid biotechnological production process from *Streptococcus zooepidemicus* fermentation to recently proposed downstream procedure as described by Rangaswamy and Jain (2008).

6.3.3 PROPERTIES OF HYALURONAN

HYALURONAN NETWORKS

The physico-chemical properties of hyaluronan were studied in detail from 1950 onwards (Comper WD and Laurent TC., 1978).

The molecules behave in solution as highly hydrated randomly kinked coils, which start to entangle at concentrations of less than 1 mg/mL. The entanglement point can be seen both by sedimentation analysis [23] and viscosity. (Morris ER et al., 1980) More recently Scott *et al. (1991)* have given evidence that the chains when entangling also interact with each other and form stretches of double helices so that the network becomes mechanically more firm.

RHEOLOGICAL PROPERTIES

Solutions of hyaluronan are viscoelastic and the viscosity is markedly shearing dependent. [24, 25] Above the entanglement point the viscosity increases rapidly and exponentially with concentration ($\sim c^{3.3}$) [26] and a solution of 10 g/L may have a viscosity at low shear of $\sim 10^6$ times the viscosity of the solvent. At high shear the viscosity may drop as much as $\sim 10^3$ times. [27] The elasticity of the system increases with increasing molecular weight and concentration of hyaluronan as expected for a molecular network. The rheological properties of hyaluronan have been connected with lubrication of joints and tissues and hyaluronan is commonly found in the body between surfaces that move along each other, for example, cartilage surfaces and muscle bundles. [28]

WATER HOMEOSTASIS

A fixed polysaccharide network offers a high resistance to bulk flow of solvent. [29, 30]This was demonstrated by Day, (1950) who showed that hyaluronidase treatment removes a strong hindrance to water flow through a fascia. Thus HA and other polysaccharides prevent excessive fluid fluxes through tissue compartments. Furthermore, the osmotic pressure of a hyaluronan solution is non-ideal and increases exponentially with the concentration. In spite of the high molecular weight of the polymer the osmotic pressure of a 10 g/L hyaluronan solution is of the same order as a l0g/L albumin solution. The exponential relationship makes hyaluronan and other polysaccharides excellent osmotic buffering substances — moderate changes in concentration lead to marked changes in osmotic pressure. Flow resistance together with osmotic buffering makes hyaluronan an ideal regulator of the water homeostasis in the body.

NETWORK INTERACTIONS WITH OTHER MACROMOLECULES

The hyaluronan network retards the diffusion of other molecules. [31]. It can be shown that it is the steric hindrance which restricts the movements and not the viscosity of the solution. The larger the molecule the more it will be hindered. In *vivo* hyaluronan will therefore act as a diffusion barrier and regulate the transport of other substances through the intercellular spaces. Furthermore, the network will exclude a certain volume of solvent for other molecules; the larger the molecule the less space will be available to it [32]. A solution of 10 g/L of hyaluronan will exclude about half of the solvent to serum albumin. Hyaluronan and other polysaccharides therefore take part in the partition of plasma proteins between the vascular and extravascular spaces. The excluded volume phenomenon will also affect the solubility of other macromolecules in the interstitium, change chemical equilibria and stabilize the structure of, for example, collagen fibers.

6.3.4 MEDICAL APPLICATIONS OF HYALURONIC ACID

The HA's viscoelastic matrix can act as a strong biocompatible support material and therefore is commonly used as growth scaffold in surgery, wound healing and embryology. In addition, administration of purified high molecular weight HA into orthopaedic joints can restore the desirable rheological properties and alleviate some of the symptoms of osteoarthritis. [33-36] The success of the medical applications of HA has led to the production of several successful commercial products, which have been extensively reviewed previously.

Table 4 summarizes both the medical applications and the commonly used commercial preparations containing HA used within this field. HA has also been extensively studied in ophthalmic, nasal and parenteral drug delivery. In addition, more novel applications including, pulmonary, implantation and gene delivery have also been suggested. Generally, HA is thought to act as either a mucoadhesive and retain the drug at its site of action/absorption or to modify the in vivo release/absorption rate of the therapeutic agent. A summary of the drug delivery applications of HA is shown in Table 5.

6.3.5 COSMETIC USES OF HYALURONIC ACID

The HA has been extensively utilized in cosmetic products because of its viscoelastic properties and excellent biocompatibility. Application of HA containing cosmetic products to the skin is reported to moisturize and restore elasticity thereby achieving an anti wrinkle effect, albeit no rigorous scientific proof exists to substantiate this claim. The HA-based cosmetic formulations or sunscreens may also be capable of protecting the skin against ultraviolet irradiation due to the free radical scavenging properties of HA. [37] HA, either in a stabilized form or in combination with other polymers, is used as a component of commercial dermal fillers (e.g. Hylaform®, Restylane® and Dermalive®) in cosmetic surgery. It is reported that injection of such products into the dermis, can reduce facial lines and wrinkles in the long term with

fewer side-effects and better tolerability compared with the use of collagen. [38-42]. The main side-effect may be an allergic reaction, possibly due to impurities present in HA. [43]. Lin et al ((2000 have also investigated the feasibility of using HA as an alternative implant filler material to silicone gel in plastic surgery. These workers found that when using HA, the implanted organ structure was visually better than that obtained using silicone gel and saline implants, Moreover there were no reported in vivo side-effects 1 year after the implantation.

TABLE 4 Summary of the medical applications of hyaluronic acid (Brown MB and Jones SA., 2005)

Disease state	Applications	Commercial products	Publications
Osteoarthritis	Lubrication and mechanical support for the joints	Hyalgan® (Fidia, Italy), Artz® (Seikagaku, Japan) ORTHOVISC® (Anika, USA) Healon®, Opegan® and Opelead®	Hochburg, 2000, Altman, 2000, Dougados, 2000, Guidolin et al., 2001, Maheu et al., 2002, Barrett and Siviero, 2002, Miltner et al., 2002, Tascioglu and Oner, 2003, Uthman et al., 2003, Kelly et al., 2003, Hamburger et al., 2003, Kirwan, 2001, Ghosh and Guidolin, 2002, Mabuchi et al., 1999, Balazs, 2003, Fraser et al., 1993, Zhu and Granick, 2003.
Surgery and wound healing	Implantation of artificial intraocular lens Viscoelastic gel	Bionect®, Connettivina® and Jossalind®	Ghosh and Jassal, 2002, Risbert, 1997, Inoue and Katakami, 1993, Miyazaki et al., 1996, Stiebel-Kalish et al., 1998, Tani et al., 2002, Vazquez et al., 2003, Soldati et al., 1999, Ortonne, 1996, Cantor et al., 1998, Turino and Cantor, 2003.

TABLE 4 *(Continued)*

Embryo implantation	Culture media for the use of invitro fertilization	EmbryoGlue® (Vitrolife, USA)	Simon et al., 2003, Gardner et al., 1999, Vanos et al., 1991, Kemmann, 1998, Suchanek et al., 1994, Joly et al., 1992, Gardner, 2003, Lane et al., 2003, Figueiredo et al., 2002, Miyano et al., 1994, Kano et al., 1998, Abeydeera, 2002, Jaakma et al., 1997, Furnus et al., 1998, Jang et al., 2003.

TABLE 5 Summary of the drug delivery applications of hyaluronic acid

Route	Justification	Therapeutic agents	Publications
Ophthalmic	Increased ocular residence of drug, which can lead to increased bioavailability	Pilocarpine, tropicamide, timolol, gentimycin, tobramycin, arecaidine polyester, (S) aceclidine	Jarvinen et al., 1995, Sasaki et al., 1996, Gurny et al., 1987, Camber et al., 1987, Camber and Edman, 1989, Saettone et al., 1994, Saettone et al., 1991, Bucolo et al., 1998, Bucolo and Mangiafico, 1999, Herrero-Vanrell et al., 2000, Moreira et al., 1991, Bernatchez et al., 1993, Gandolfi et al., 1992, Langer et al., 1997.
Nasal	Bioadhesion resulting in increased bioavailability	Xylometazoline, vasopressin, gentamycin	Morimoto et al., 1991, Lim et al., 2002.
Pulmonary	Absorption enhancer and dissolution rate Modification	Insulin	Morimoto et al., 2001, Surendrakumar et al., 2003.
Parenteral	Drug carrier and facilitator of liposomal entrapment	Taxol, superoxide dismutase, human recombinant insulin-like growth factor, doxorubicin	Drobnik, 1991, Sakurai et al., 1997, Luo and Prestwich, 1999, Luo et al., 2000 Prisell et al., 1992, Yerushalmi et al., 1994, Yerushalmi and Margalit, 1998, Peer and Margalit, 2000, Eliaz and Szoka, 2001, Peer et al., 2003.

TABLE 5 *(Continued)*

Implant	Dissolution rate modification	Insulin	Surini et al., 2003, Takayama et al., 1990.
Gene	Dissolution rate modification and Protection	Plasmid DNA/monoclonal antibodies	Yun et al., 2004, Kim et al., 2003.

6.3.6 BIOLOGICAL FUNCTION OF HYALURONAN

Naturally, hyaluronan has essential roles in body functions according to organ type that it distributes in it [44].

SPACE FILLER

The specific functions of hyaluronan in joints are still essentially unknown. The simplest explanation for its presence would be that a flow of hyaluronan through the joint is needed to keep the joint cavity open and thereby allow extended movements of the joint. Hyaluronan is constantly secreted into the joint and removed by the synovium. The total amount of hyaluronan in the joint cavity is determined by these two processes. The half-life of the polysaccharide at steady-state is in the order of 0.5–1 days in rabbit and sheep [44-46]. The volume of the cavity is determined by the pressure conditions (hydrostatic and osmotic) in the cavity and its surroundings. Hyaluronan could, by its osmotic contributions and its formation of flow barriers in the limiting layers, be a regulator of the pressure and flow rate. [47] It is interesting that in fetal development the formation of joint cavities is parallel with a local increase in hyaluronan. [48]

LUBRICATION

Hyaluronan has been regarded as an ideal lubricant in the joints due to its shear-dependent viscosity [49] but its role in lubrication has been refuted by others [50]. However, there are now reasons to believe that the function of hyaluronan is to form a film between the cartilage surfaces. The load on the joints may press out water and low-molecular solutes from the hyaluronan layer into the cartilage matrix. As a result the concentration of hyaluronan increases and a gel structure of micrometric thickness is formed which protects the cartilage surfaces from frictional damage [52]. This mechanism to form a protective layer is much less effective in arthritis when the synovial hyaluronan has both a lower concentration and a lower molecular weight than normal. Another change in the arthritic joint is the protein composition of the synovial fluid.

Fraser *et al* (1972) showed 25 years ago that addition of various serum proteins to hyaluronan substantially increased the viscosity and this has received a renewed interest in view of recently discovered hyaladherins (see above). The TSG-6 and inter-α-trypsin inhibitor and other acute phase reactants such as haptoglobin are concentrated to arthritic synovial fluid [52]. It is not known to what extent these are affecting the rheology and lubricating properties.

SCAVENGER FUNCTIONS

Hyaluronan has also been assigned scavenger functions in the joints. It has been known since the 1940s that hyaluronan is degraded by various oxidizing systems and ionizing irradiation and we know today that the common denominator is a chain cleavage induced by free radicals, essentially hydroxy radicals [53]. Through this reaction hyaluronan acts as a very efficient scavenger of free radicals. Whether this has any biological importance in protecting the joint against free radicals is unknown. The rapid turnover of hyaluronan in the joints has led to the suggestion that it also acts as a scavenger for cellular debris. [54] Cellular material could be caught in the hyaluronan network and removed at the same rate as the polysaccharide.

REGULATION OF CELLULAR ACTIVITIES

As discussed above, more recently proposed functions of hyaluronan are based on its specific interactions with hyaladherins. One interesting aspect is the fact that hyaluronan influences angiogenesis but the effect is different depending on its concentration and molecular weight [55]. High molecular weight and high concentrations of the polymer inhibit the formation of capillaries, while oligosaccharides can induce angiogenesis. There are also reports of hyaluronan receptors on vascular endothelial cells by which hyaluronan could act on the cells [56]. The avascularity of the joint cavity could be a result of hyaluronan inhibition of angiogenesis.

Another interaction of some interest in the joint is the binding of hyaluronan to cell surface proteins. Lymphocytes and other cells may find their way to joints through this interaction. Injection of high doses of hyaluronan intra-articularly could attract cells expressing these proteins. Cells can also change their expression of hyaluronan-binding proteins in states of disease whereby hyaluronan may influence immunological reactions and cellular traffic in the path physiological processes cells [57].The observation often reported that intra-articular injections of hyaluronan alleviates pain in joint disease [58] may indicate a direct or indirect interaction with pain receptors.

6.4 HYALURONAN AND 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 mega Daltons [59]. 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 [60].

As SF of healthy human exhibits no activity of the hyaluronidase, it has been inferred that oxygen-derived free radicals are involved in a self-perpetuating process of HA catabolism within the joint [61]. This radical-mediated process is considered to account for ca. twelve-hour half-life of native HA macromolecules in SF.

Acceleration degradation of high-molecular-weight HA occurring under inflammation and/or oxidative stress is accompanied by impairment and loss of its viscoelastic properties [62]. Low-molecular weight HA was found to exert different biological activities compared to the native high-molecular-weight biopolymer. The HA chains of 25–50 disaccharide units are inflammatory, immune-stimulatory, and highly angiogenic. HA fragments of this size appear to function as endogenous danger signals, reflecting tissues under stress [63-65]. Figure 4 describe the fragmentation mechanism of HA under free radical stress.

a) Initiation Phase: the Intact Hyaluronan Macromolecule Entering the Reaction with the $HO^{\cdot}$ Radical Formed via the Fenton-Like Reaction:

$$Cu^{+} + H_2O_2 \rightarrow Cu\ (II) + HO^{\cdot} + OH^{-}$$

H_2O_2 has its origin due to the oxidative action of the Weissberger_s system:

$$Asc + Cu^{2+} + 2\ O_2 + 4\ H^{+} \rightarrow Asc^{\cdot} + Cu^{+} + 2\ H_2O_2.$$

b) Formation of an alkyl radical (C-centered hyaluronan macroradical) initiated by the $HO^{\cdot}$ 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 [65-70].

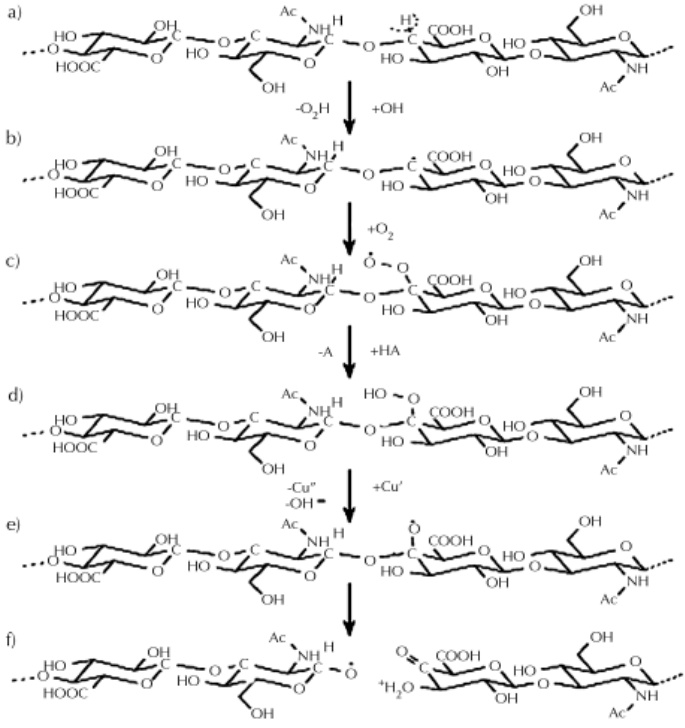


FIGURE 4 Schematic degradation of HA under free radical stress (E. Hrabarova et. al., 2012).

Several thiol compounds have attracted much attention from pharmacologists because of their reactivity toward endobiotics such as hydroxyl radical-derived species. Thiols play an important role as biological reductants (antioxidants) preserving the redox status of cells and protecting tissues against damages caused by the elevated reactive oxygen/nitrogen species (ROS/RNS) levels by which oxidative stress might be indicated.

Soltes and his coworkers examine the effect of several thiol compounds on inhibition of the degradation kinetics of a high-molecular-weight HA in vitro. High molecular weight hyaluronan (HA) samples were exposed to free-radical chain degradation reactions induced by ascorbate in the presence of Cu (II) ions the so called Weissberger's oxidative system. The concentrations of both reactants [ascorbate, Cu (II)] were comparable to those that may occur during an early stage of the acute phase of joint inflammation (see Figure 5). [75-80].

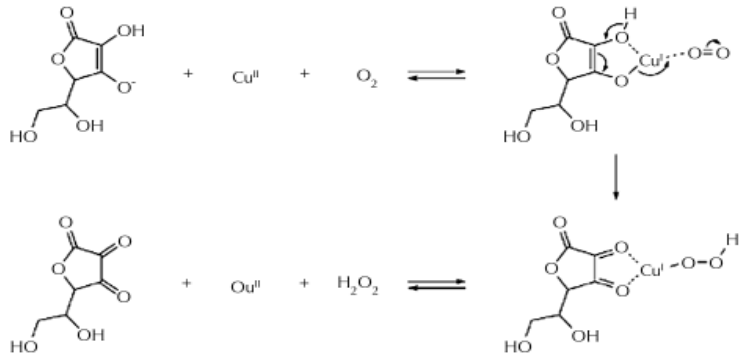


FIGURE 5 Scheme. Generation of H_2O_2 by Weissberger's System from Ascorbate and Cu (II) ions under Aerobic Conditions (Valachova K. et al., 2011).

Figure (6) illustrate the dynamic viscosity of hyaluronan solution in presence and absent of bucillamine, D-penicillamine and L-cysteine as inhibitors for free radical degradation of HA. The study shows that bucillamine has both a preventive and chain-breaking antioxidant. In the other hand D-penicillamine, and L-cysteine, dose dependently, acts as a scavenger of ˙OH radicals within the first 60 min then, however, its inhibition activity is lost and degradation of hyaluronan takes place[81-82]

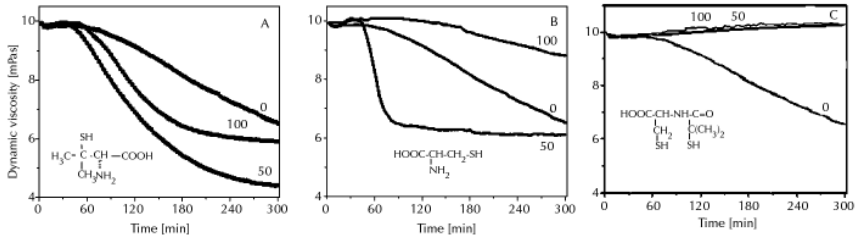


FIGURE 6 Effect of A) L-Penicillamine, B) L-cysteine and C) Bucillamine with different concentration (50,100 μM) on HA degradation induced by the oxidative system containing 1.0 μM $CuCl_2$ + 100 μM ascorbic acid. (Valachova K et al., 2011).

L-Glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine; a ubiquitous endogenous thiol, maintains the intracellular reduction oxidation (redox) balance and regulates signaling pathways during oxidative stress/conditions. GSH is mainly cytosolic in the concentration range of ca. 1–10 mM; however, in the plasma as well as in SF, the range is only 1–3 μM [83]. This unique thiol plays a crucial role in antioxidant defense, nutrient metabolism, and in regulation of pathways essential for the whole body homeostasis. Depletion of GSH results in an increased vulnerability of the cells to oxidative stress [84].

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. [85]

Figure (7) the dynamic viscosity versus time profiles of HA solution stressed to degradation with Weissberger's oxidative system. As evident, addition of different concentration of GSH resulted in a marked protection of the HA macromolecules against degradation. The greater the GSH concentration used, the longer was the observed stationary interval in the sample viscosity values. At the lowest GSH concentration used, i.e. 1.0 μM (Figure 7), the time-dependent course of the HA degradation was more rapid than that of the reference experiment with the zero thiol concentration. Thus, one could classify GSH traces as functioning as a pro-oxidant.

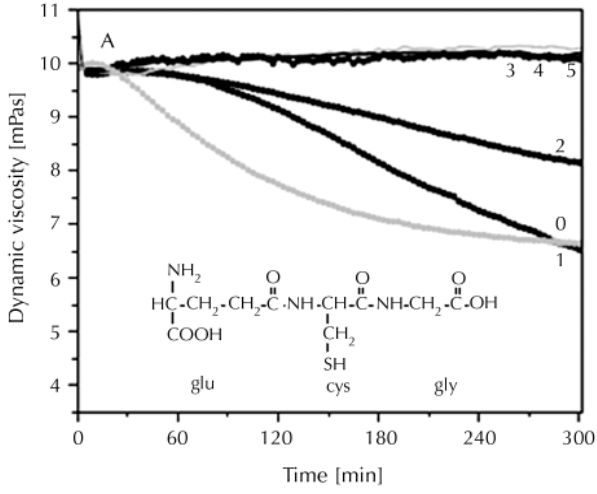


FIGURE 7 Comparison of the effect of L-glutathione on HA degradation induced by the system containing 1.0 mM $CuCl_2$ plus 100 μM L-ascorbic acid. Concentration of L-glutathione in μM: 1–1.0; 2–10; 3, 4, 5–50, 100, and 200, respectively. Concentration of Reference experiment: 0–nil thiol concentration (Hrabarova et al., 2009, Valachova et al., 2010a).

The effectiveness of antioxidant activity of 1, 4-dithioerythritol expressed as the radical scavenging capacity was studied by a rotational viscometry method [86]. L, 4-dithioerythritol widely accepted and used as an effective antioxidant in the field of enzyme and protein oxidation, is a new potential antioxidant standard exhibiting very good solubility in a variety of solvents. Figure (8) describe effect of 1, 4-dithioerythritol on degradation of HA solution under free radical stress [87].

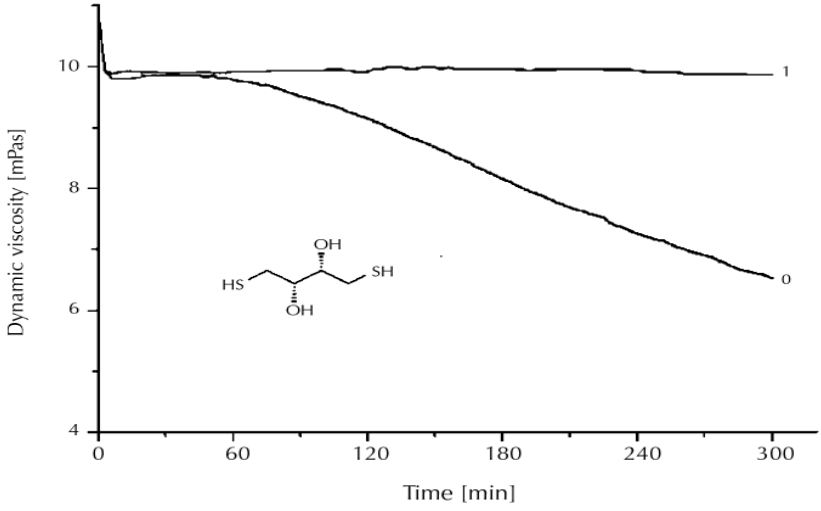


FIGURE 8 Effect of 1, 4-dithioerythritol on HA degradation induced by Weissberger's oxidative system containing (Hrabarova E et al 2010).

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 (Soloveva M. E et al 2007, Thibodeau P. A., et al 2001). At low concentrations, it is a powerful protector of α-1-antiproteinase against the enzyme inactivation by HOCl. NAC reacts with $HO^{\cdot}$ radicals and slowly with H_2O_2; however, no reaction of this endobiotics with superoxide anion radical was detected [88].

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 [89].

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 $O_2^{\cdot -}$ (Soloveva M. E et al 2007, Thibodeau P. A., et al 2001). 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 (Figure 9 (a)). Subsequently, NAC exerted a modest pro-oxidative effect, more profound at 25-μM than at 100-μM concentration (Figure 9 (a)). Application of NAC 1 h after the onset of the reaction (Figure 9 (b)) revealed its partial inhibitory effect against formation of the peroxy-type radicals, independently from the concentration applied [90].

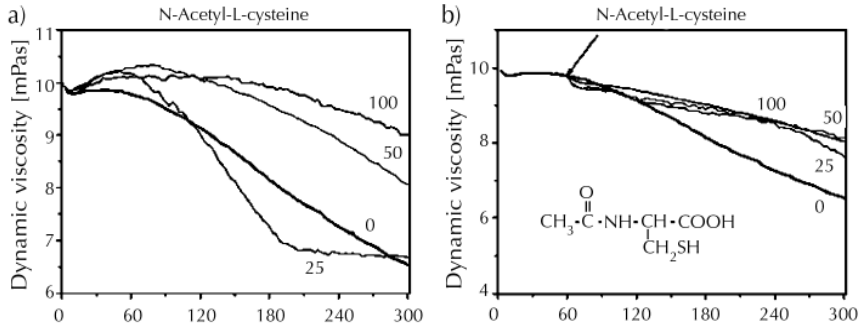


FIGURE 9 Evaluation of antioxidative effects of N-acetyl-l-cysteine against high-molar-mass hyaluronan degradation in vitro induced by Weissberger_s oxidative system. Reference sample (black): 1 μM Cu (II) ions plus 100 μM ascorbic acid; nil thiol concentration. N-Acetyl-l-cysteine addition at the onset of the reaction (a) and after 1 h (b) (25, 50,100 μM). (Hrabarova E, et al 2012).

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 (Figure 7 (a), and Figure 10 (a)). Moreover, the same may be concluded, when applied 1 h after the onset of the reaction (Figure 10 (b)), at the two concentrations (50 and 100 μM), suggesting that CAM may be an excellent scavenger of peroxy radicals generated during the per oxidative degradation of HA [91].

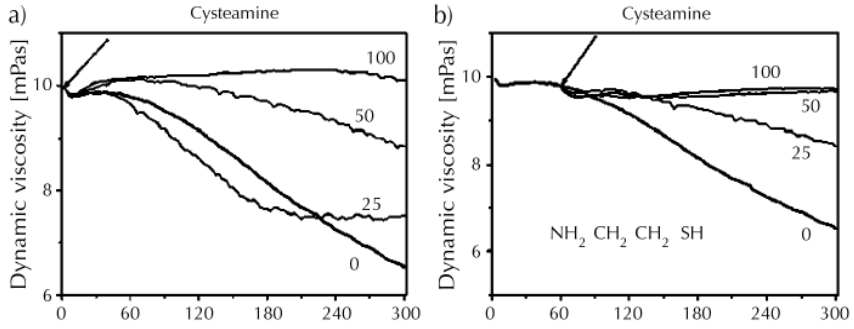


FIGURE 10 Evaluation of antioxidative effects of cysteamine against high-molar-mass hyaluronan degradation in vitro induced by Weissbergers oxidative system. Reference sample (black): 1 mM CuII ions plus 100 μM ascorbic acid; nil thiol concentration. Cysteamine addition at the onset of the reaction (a) and after 1 h (b) (25, 50,100 μM). (Hrabarova E, et al 2012).

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KEYWORDS

- **Antioxidants**
- **Hyaluronan**
- **Oxidation stress**

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