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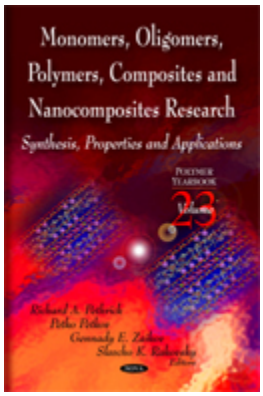

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Chapter 3

OXIDATIVE DEGRADATION OF HYALURONAN: IS MELATONIN AN ANTIOXIDANT OR PROOXIDANT?*

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ABSTRACT

The model of hyaluronan (HA) degradation was used in the study of the action of melatonin (MEL) as a scavenger of $^{\bullet}OH$ radicals and/or hypochlorite. The study showed that in systems with hydrogen peroxide or hypochlorite MEL in the concentrations used (< 77.8 μg ml^{-1}) exerted only a weak effect as a "chain-breaking antioxidant" and failed to trap $^{\bullet}OH$ radicals. When added to the system with HOCl/ClO^{-}, MEL had an even prooxidative effect on HA degradation.

Keywords: hyaluronan, melatonin, reactive oxygen species, H_2O_2, Cu(II), ascorbic acid, $^{\bullet}OH$ radicals, hypochlorite

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Dedicated to Prof. Russel J. Reiter, Department of Cellular & Structural Biology, UT Health Science Center, San Antonio, TX, U.S.A.

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INTRODUCTION

Melatonin (MEL) is effective in scavenging most of the reactive oxygen and nitrogen species (ROS and RNS). The rate constant, k, of the reaction between MEL and $O_2^{\bullet-}$ is equal to 0.60×10^{11} $M^{-1}s^{-1}$. The k values of the reaction of MEL and peroxynitrite = 5.0 $M^{-1}s^{-1}$, nitrogen oxide = 3.0×10^{7} $M^{-1}s^{-1}$, singlet oxygen = 2.2×10^{7} $M^{-1}s^{-1}$, hydrogen peroxide = $(2.52\pm0.19)\times10^{5}$ $M^{-1}s^{-1}$, and HOCl = 7.0×10^{3} $M^{-1}s^{-1}$ [1–5]. Besides its direct radical scavenging activity, MEL protects the cells by cooperating with further antioxidants, e.g., ascorbic acid, vitamin E [4], and it also affects the activity of several enzymes e.g., superoxidismutase, glutathionreductase, and catalase [6]. MEL is known to modulate several biological functions in the body, such as sleep, thermoregulation, and immune system, and it activates human monocytes and stimulates the production of interleukins, exerts its effect on T-lymphocytes and inhibits apoptosis of B-cells [7]. MEL has a protective effect on lymphocytic chromosomes injured by radiation [8]. The reactions of MEL with various free radicals and oxidants were reviewed in detail by Allegra et al. [5].

Although MEL is one of the most effective antioxidants, its presence is adverse in patients with autoimmune diseases, as e.g., with rheumatoid arthritis (RA). The synovial fluid (SF) of patients with RA contains a higher concentration of MEL 79.8±38.9 pg ml^{-1}. The increased level of MEL found in these patients is presumably produced directly (*in situ*) in the joint [9]. Macrophages in synovial membrane possess a specific binding site for MEL. MEL-induced stimulation of macrophages is accompanied by the production of cytokines and nitric oxide [10]. The association of MEL and RA may particularly explain the complaint of arthritic patients to have the greatest pain and edemas early in the morning when the highest production of pro-inflammatory cytokines has been demonstrated [10,11].

Exogenous MEL administration was found to have positive effects in sleeplessness, chronic sleep disorder, problems associated with jet-leg, and some forms of cancer [12,13]. MEL partially inhibited phenytoin-induced biochemical changes in experimental animals [14]. Despite the extremely low toxicity of MEL, developmental administration of this xenobiotic was reported to cause subtle neurobehavioral changes in rat offspring [15].

In the human body, MEL is generated by the pineal gland in the course of sleep (approximately between 8:00 p.m. and 8:00 a.m.), with the maximal production occurring at about 4:00 a.m. Further sites of increased production of MEL are the retina and gastrointestinal tract. Overnight, MEL is distributed in the whole body by the bloodstream, entering all tissues. Since the physical activity of people exhibits a daytime and nocturnal phase, several authors attempted to find a relation between the changes of MEL concentration in the joints during sleep and daytime activity. Although MEL produced by the pineal gland is effectively distributed by the bloodstream to all organs/tissues and crosses easily the cell-wall barriers, the production of MEL directly in the joints has also been documented. The content of MEL in SF of healthy individuals amounts to an average of 20 pg ml^{-1}.

Hyaluronan (HA; also called hyaluronic acid, hyaluronate, see Scheme 1), is a polyelectrolyte component of SF. The concentration of HA in human SF is 2.5 mg ml^{-1} on average. In SF of healthy subjects, the HA molar mass is of the order of several megaDaltons. In patients suffering from RA, the mean HA molar mass is, however, significantly reduced, resulting in deterioration of viscoelastic properties of SF [16]. The fragments of the degraded HA exert at the same time proinflammatory and immunostimulatory actions in the joint.

The observed reduction of the molar mass of HA in SF of patients suffering from RA led to *in vitro* studies of the degradation of this biopolymer by different ROS and RNS. The earliest investigations go back to the 1940s; since then, numerous studies of this phenomenon have been reported, reviewed by [17]. Although different species affect the HA macromolecule at different targets, the action of ROS or RNS indicated in Scheme 1 finally results in degradation of the biopolymer, i.e., in reduction of its molar mass.

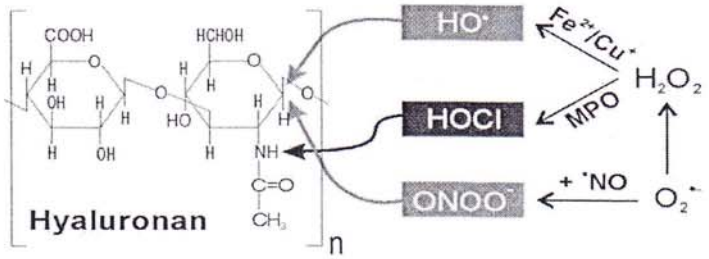
As evident from the proposed scheme, the generation of $^{\bullet}OH$ radicals is mainly due to H_2O_2 decomposition by transition metal ions in reduced oxidation state, i.e., Fe^{2+}, Cu^{+}.

It is well known that high-molar-mass HA is degraded in the synovial joints with a half-life of about 12 hours, and that despite the fact that SF does not exert any activity of hyaluronidase enzymes. Thus it can be assumed that the rapid catabolism of HA in the joint must be caused by ROS (and possibly also by RNS). This is one of the reasons why the relationship between oxidative degradation of HA by the action of ROS and the involvement of MEL appears to provide an intriguing study goal. To expand the knowledge on the action of MEL during oxidative degradation of high-molar-mass HA, in the present study, we applied the recently established method of rotational viscometry [18].

EXPERIMENTAL

Chemicals

NaCl and $CuCl_2 \cdot 2H_2O$, analytical purity grade, were from Slavus Ltd., Bratislava, Slovakia; H_2O_2 (30%) was the product of Chemapol (Prague, Czech Republic); L-ascorbic acid was from Merck KGaA, Darmstadt, Germany. Aqueous solution of NaOCl (≈ 1 M) was the product of Riedel de Haen AG, Seelze, Germany. MEL, batch No. 13961-081, was purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). High-molar-mass HA used throughout the study was a generous gift of Dr. K. Thacker from Lifecore Biomedical Inc., Chaska, MN, USA (sample Lifecore P9710-2, M_w =1215 kDa; M_w/M_n = 1.79). The presence of 13 ppm of iron and 4 ppm of copper ions in this sample was claimed ["Certificate of Analysis" (Lifecore Biomedical Inc., Chaska, MN, USA)]. Water used was of redistilled de-ionized quality grade.



Scheme 1. Degradation and/or chemical modification of hyaluronan by various ROS and RNS [http://pubs3.acs.org/acs/journals/doilookup?in_doi=10.1021/bm050867v].

Methods

Preparation of Working Solutions

The solution of hydrogen peroxide (8.82 M) was prepared by dissolving NaCl in commercial H_2O_2 solution to a salt concentration of 0.15 M. Similarly, the solution of cupric chloride (16.0 μM) and that of ascorbic acid (16.0 mM) were prepared in 0.15 M water solution of NaCl. The solution of sodium hypochlorite (392 mM) was prepared by dilution of the commercial NaOCl solution in redistilled de-ionized water. (Dissociation of NaOCl at neutral pH due to the value of its pK_a = 7.53 results in nearly equimolar concentration of undissociated acid (HOCl) and the ions of ClO^-).

Preparation of Degradative Systems

For rheological measurements, 20 mg of high-molar-mass HA [Lifecore P9710-2] was dissolved in 0.15 M aqueous NaCl overnight in dark, at room temperature, in two steps: First, 4.0 ml solvent was added in the morning. The next portion of the solvent was added after six hours. Three different degradative reaction systems were employed in the studies of degradation of the HA sample Lifecore P9710-2: (a) containing HA + 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + hydrogen peroxide at the final concentration of 50 μM, (b) containing HA + 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 2 mM sodium hypochlorite, and (c) containing HA + 0.1 μM $CuCl_2$ and 100 μM ascorbic acid. The final concentration of hyaluronan was 2.5 mg ml^{-1}.

Preparation of Inhibitory Systems

Two different arrangements were employed in the inhibitory studies of degradation of the Lifecore P9710-2 sample in the above mentioned systems: (i) containing MEL added to the system one hour after the reaction onset at final concentrations 0.778, 7.78, and 77.8 μg ml^{-1} (in the system c); (ii) containing MEL added to the system before the reaction onset to the final concentrations 0.778, 7.78, and 77.8 μg ml^{-1} (in the system c).

Rotational Viscometry

The resulting solution (8.0 ml) was immediately transferred into the Teflon® cup reservoir of the rotational viscometer. The record of the viscometer output parameters started two minutes after the experiment onset. The changes of dynamic viscosity (η) and torque were monitored at 25 °C using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs, Inc., Middleboro, MA, USA) equipped with a cape-spindle pair built of Teflon® designed in our laboratory. At the spindle rotational speed of 180 rpm, the shear rate equaled 237.6 s^{-1}. The Lifecore P9710-2 sample degradation was monitored until the nominal η value 5.8 mPa·s was reached (degradation study) or the kinetics of inhibition were monitored over 180 minutes (inhibition study).

Results

Figure 1 represents the effect of MEL to HA degradation by a system of 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 50 μM H_2O_2. MEL added into the system one hour after the start of HA degradation (fig. 1, panel A) resulted in a mild inhibition of the degradation of HA, which was counted at 25th minute after addition of MEL (7.78 pg ml^{-1}) into the system (curve coded

1) or at 106th minute when 77.8 pg ml^{-1} MEL was applied (curve coded 2). It should be however pointed out that the shapes of both curves (cf. fig. 1, panel A, curves coded 1 and 2) are practically identical.

Panel B in Figure 1 illustrates the kinetics of HA degradation when MEL was added into the reaction mixture exactly at the reaction start. It is indicative that even the highest MEL dosage (77.8 pg ml^{-1}) did not influence the shape of the curve (cf. fig. 1, panel B, curve coded 2).

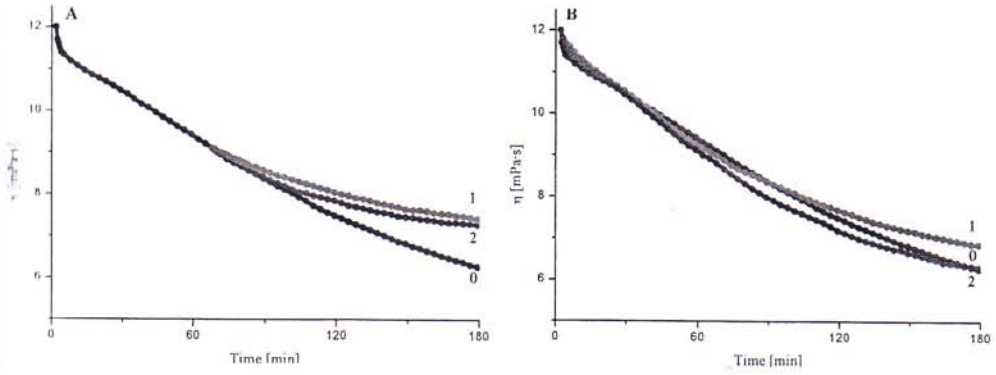


Figure 1. MEL effect on HA degradation by a system of 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 50 μM H_2O_2. MEL added to the system 1 h after reaction start (A), or immediately before reaction initiation (B): control – no MEL addition (0); with 7.78 pg ml^{-1} (1) or 77.8 pg ml^{-1} (2) of MEL added into the system.

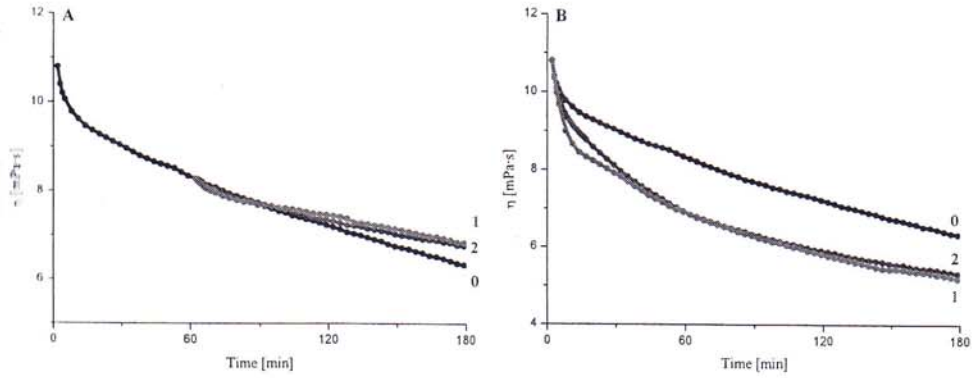


Figure 2. MEL effect to HA degradation by a system of 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 2 mM NaOCl. MEL added to the system after 1 h of reaction start (A), or immediately before the reaction initiation (B): control – no MEL addition (0); with 7.78 pg ml^{-1} (1) or 77.8 pg ml^{-1} (2) of MEL added into the system.

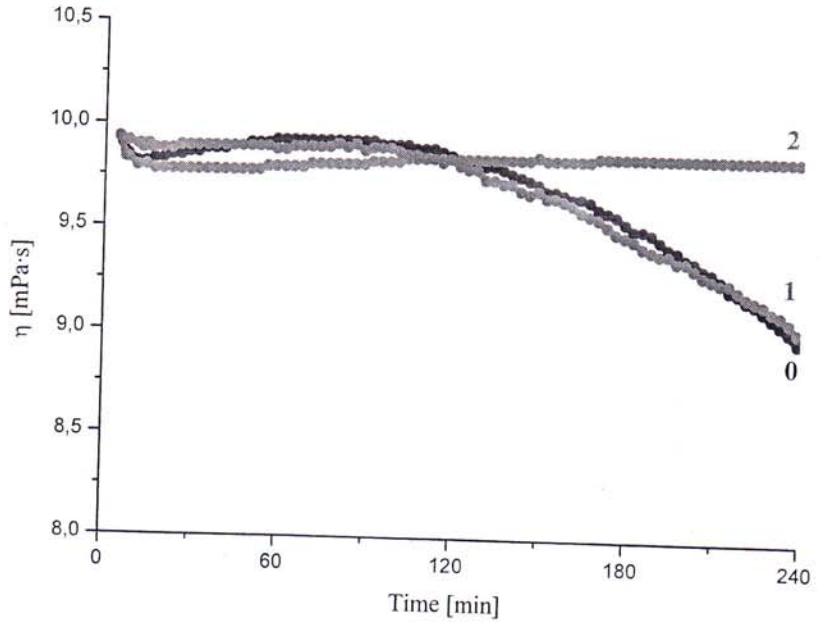


Figure 3. MEL effect to HA degradation by a system of 0.1 μM $CuCl_2$ + 100 μM ascorbic acid. MEL added into the system before the reaction initiation: control – no MEL addition (0); with 0.778 or 7.78 μg ml^{-1} (both dependences coded 1 are identical), or 77.8 μg ml^{-1} (2) of MEL added into the system.

The influence of the addition of MEL into the system 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 2 mM NaOCl is shown in Figure 2. As evident, MEL added one hour after starting the HA degradation in both selected concentrations (7.78 or 77.8 pg ml^{-1}) has practically no effect on the reaction kinetics (cf. fig. 2, panel A). Yet addition of MEL at starting HA degradation indicates a significant acceleration of the degradation of the polymer macromolecules first appr. at 20 minutes. Thereafter, the dependences were practically parallel with the course of the sample without MEL (cf. fig. 2, panel B).

Figure 3 represents the effect of MEL addition at the start of HA degradation by applying the system of 0.1 μM $CuCl_2$ + 100 μM ascorbic acid. As evident, MEL (0.778 or 7.78 μg ml^{-1}, curve coded 1) has no effect. The shape of the curves (coded 1) is identical with that coded 0 where no MEL was applied. Yet addition of MEL in the highest concentration 77.8 μg ml^{-1} resulted in total inhibition of HA degradation (curve 2).

Discussion

Although the etiopathogenesis of RA has not yet been fully elucidated, it is assumed that a negative involvement of free radicals may participate in the origin and development of this autoimmune disease. Due to the action of these radicals/oxidants ($^{\bullet}OH$, HOCl, $ONOO^-$), produced by infiltrating polymorphonuclear leukocytes, important functional components of the joints may be impaired.

MEL is considered to be an effective scavenger of free radicals and oxidants [1–5]. Since the SF of patients suffering from RA contains an increased concentration of MEL and since

this xenobiotic is considered to be a very effective antioxidant, we decided to assess its effect on HA degradation *in vitro*.

In the concentrations used, MEL was effective in both systems (with hydrogen peroxide and hypochlorite) only as a weak "chain-breaking antioxidant" (figs. 1 and 2). Due to the poor solubility of MEL in the aqueous NaCl solution, the maximal applicable MEL concentration was 77.8 μg ml^{-1}. To date, it is a controversial issue whether MEL affects already the proceeding radical process. While one source [19,20] failed to find inhibition of lipid peroxidation, Pieri et al. [21] reported an inhibitory effect.

The weak inhibitory effect of MEL exerted on HA degradation in both our systems used may have been caused by scavenging $^{\bullet}OH$ radicals in the case when MEL was added in the course of the proceeding degradation. In both systems, a small amount of $^{\bullet}OH$ radicals may be generated during the whole degradation process. This presumption, however, could not been confirmed since MEL added to the system with H_2O_2 at the very beginning of the process failed to affect HA degradation (fig. 1). Despite the fact that MEL is considered an excellent $^{\bullet}OH$ scavenger, its effect depends most probably on the ratio of MEL and $^{\bullet}OH$ radical concentrations. A weak inhibitory effect of high MEL concentration (1 mM) on HA degradation in the system Fe^{2+}-EDTA + H_2O_2 was confirmed by Štetinová et al. [20].

On adding MEL at the beginning of the HA degradation process to the system containing NaOCl, MEL exerted a prooxidative effect (fig. 2). MEL showed a weak trapping of hypochlorite ($k = 7.0 \times 10^3$ $M^{-1}s^{-1}$) [8]. Kladna et al. [22] reported a prooxidative effect of MEL applied in a higher concentration (0.5–1 mM) in the system Cu^{2+} + H_2O_2 on using the method of chemiluminiscence. The authors, however, failed to indicate the substance in which MEL was dissolved. They assumed that *N*-acetyl-*N*-formyl-5-methoxykynuramine, which was generated after the reaction of MEL with $^{\bullet}OH$, may under aerobic conditions yield singlet oxygen ($^1O_2$). $^1O_2$ is also able to degrade HA [23]. The appearance of this reactive molecule was confirmed by inhibition of the intensity of the chemiluminescence signal, which occurred after the scavenger of $^1O_2$ was added to the system. Contrary to the above literary findings, we failed to observe any inhibition when testing the effect of MEL on HA degradation in the systems Cu^{2+} + ascorbic acid + H_2O_2 (fig. 1).

MEL added to the system Cu^{2+} + ascorbic acid completely inhibited HA degradation only when applied in a higher concentration (77.8 μg ml^{-1} i.e. 0.335 mM). Such a concentration of MEL in the resulting degradation system could not be accomplished due to the poor solubility of MEL in aqueous NaCl solution. We managed to reach the given concentration of MEL in the resulting HA solution only when we dissolved HA in 0.15 M NaCl over 24 hours, on previously dissolving MEL in the given physiological solution. Since such a high MEL concentration is absolutely non-physiologic, we did not use it in other HA degradation systems.

COMMENTARIES

Melatonin as Antioxidant

MEL can be obtained in the form of tablets as a nutrition additive. It occurs naturally as an ingredient in some plants (mustard seed, fennel, morello cherry, banana). MEL tablets are

used not only as an additive with antioxidative properties, but also by flight passengers traveling between time-zones in order to alleviate the jet-lag syndrome, and it is also used against insomnia.

Melatonin as Prooxidant

The content of MEL is higher in the SF of patients with RA (79.8±38.9 pg ml^{-1}) than in SF of healthy individuals (≈ 20 pg ml^{-1}). On the one hand, MEL is an excellent antioxidant, yet patients with autoimmune diseases, such as RA, are not recommended to take MEL due to its pro-inflammatory action. Our presented findings corroborate the thesis of the prooxidative action of MEL on high-molar-mass HA degradation initiated by $^{\bullet}OH$ radicals themselves as well as in combination with hypochloride.

Conclusions

Melatonin is an antioxidant with positive effects on several physiological processes in the body; nevertheless, patients with autoimmune diseases (e.g., RA) should not use it as a nutritional additive. Neither is MEL recommended during pregnancy. In patients with asthma, MEL may be involved in the generally observed impairment of manifestation of asthma during the night [24]. MEL reduces the function of thyroidal glands [25]. In several countries, including Slovakia, MEL can be obtained over the counter.

Acknowledgments

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