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In vitro screening of the action of non-steroidal anti-inflammatory drugs on hypochlorous acid-induced hyaluronan degradation

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Abstract

The antioxidative effect of two non-steroidal anti-inflammatory drugs was studied *in vitro* by measuring the kinetics of degradation of high-molecular weight hyaluronan (HA) in a system comprising hypochlorous acid + $CuCl_2$ + ascorbic acid using a Brookfield rotational viscometer equipped with a Teflon cup and spindle of coaxial cylindrical geometry. The changes in HA chemical structure were investigated by chemiluminometry. When sodium naproxen was added to the system during a running degradative process its inhibitory effect was clearly shown. The inhibition was dependent on the drug concentration. However, when this drug was added to the system before the initiation of HA degradation, no inhibition was seen even at the highest drug concentration tested. The inhibitory effect of acetylsalicylic acid was achieved with a relatively low concentration of the drug and was independent of the experimental model used.

Keywords: Hyaluronan degradation; Hypochlorous acid; Non-steroidal anti-inflammatory drugs; Rotational viscometry; Chemiluminometry

1. Introduction

Oxygen derived free radicals produced by polymorphonuclear leukocytes (PMNLs) are involved in tissue damage associated with inflammatory joint diseases. Rheumatoid arthritis is accompanied by a decrease in viscosity of the synovial fluid due to the degradation of high-molecular weight hyaluronan (HA) [1–4]. HA is degraded by hydroxyl radicals produced as a result of the Fenton reaction [5–10]. An alternative mechanism is the damaging effect on HA by hypochlorous acid (HOCl) generated by myeloperoxidase (MPO) released from stimulated PMNLs. MPO concentration was found in rheumatoid synovial fluid in the range of 16–29 μg/ml [11]. *In vitro* studies of HA degradation by HOCl revealed the decrease of molecular weight of HA and of the viscosity of synovial fluid solution [6,12,13].

The chemistry of hyaluronan degradation by HOCl was investigated by ^{1}H NMR spectroscopy [14,15] as well as by EPR spectroscopy [16–18]. Besides the direct attack of HOCl on *N*-acetyl groups of HA, hypochlorous acid can take part in a number of other reactions leading to further reactive species, *e.g.*:

$$HOCl + O_2^{\bullet -} \rightarrow {}^{\bullet}OH + O_2 + Cl^- \text{[19]}$$

$$HOCl + M^{n+} \rightarrow M^{(n+1)+} + {}^{\bullet}OH + Cl^- \text{[20]}$$

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Hypochlorous acid may also react with nitrite to form reactive nitryl chloride [21,22]:

$$HOCl + NO_2^- + H^+ \rightarrow NO_2Cl + H_2O$$

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the therapy of arthritis. As a group, these drugs exhibit analgetic, antipyretic, anti-oncogenic and anti-inflammatory actions. These drugs act by inhibiting prostaglandin synthesis and affecting immune reactions. However, there are several other potential sites of action for NSAIDs, including their capacity to prevent the release of free radicals and degradative enzymes from synovial macrophages and polymorphonuclear leukocytes [23,24]. Further they affect the mobility, chemotaxis and aggregation of PMNLs and macrophages [23,25]. NSAIDs are supposed to be direct scavengers of free radicals [26–30] and some NSAIDs are also supposed to have a weak capacity to bind iron ions.

One of the NSAIDs, naproxen, reacts with $^{\bullet}OH$ ($k = 2.4 \times 10^{10}\ M^{-1}\ s^{-1}$) [31]. It reacts with a considerably lower rate with $^1O_2$ ($k = 1.1 \pm 0.1 \times 10^5\ M^{-1}\ s^{-1}$) [32] and weakly with $O_2^{\bullet-}$ ($k = 7.3 \times 10^2\ M^{-1}\ s^{-1}$) [33]. Another NSAID, acetylsalicylic acid, also reacts very fast with $^{\bullet}OH$ ($k = 3.6 \times 10^{10}\ M^{-1}\ s^{-1}$) and weakly with H_2O_2 and $O_2^{\bullet-}$ [34].

Some studies [35–41] were aimed at testing NSAIDs as scavengers of HOCl. Most NSAIDs were not at all or only moderately affected by HOCl, however, the inhibitory effect on the MPO–H_2O_2–Cl^- system was striking. The increased level of copper ions [42] and reduction of the ascorbic acid level [43] in rheumatoid synovial fluid should also be taken into consideration. Studies of the antioxidative effect of NSAIDs on degradation of hyaluronan by the system sodium hypochlorite + ascorbic acid + Cu^{2+} have not yet been performed.

In the present study, we investigated HA degradation induced by the system NaOCl alone, NaOCl + ascorbic acid, as well as NaOCl + ascorbic acid + $CuCl_2$. The degradation was monitored by measuring the viscosity of HA solution. Chemiluminometry was used to reveal the differences between the intact and degraded hyaluronans. The inhibitory effect of naproxen and acetylsalicylic acid was tested in the degradative system with hypochlorous acid + copper ions + ascorbic acid.

2. Experimental

2.1. Materials

High-molecular weight HA used throughout the study was a generous gift of Dr. K. Thacker from Lifecore Biomedical Inc., Chaska, MN, USA (sample Lifecore P9710-2, $M_w = 1215$ kg/mol; $M_w/M_n = 1.79$) and 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)]. NaCl and $CuCl_2 \cdot 2H_2O$, analytical purity grade, were from Slavus Ltd., Bratislava, Slovakia; ascorbic acid was from Merck KGaA, Darmstadt, Germany. Aqueous solution of NaOCl (≈ 1 M) was the product of Riedel de Haen AG, Seelze, Germany. Sodium naproxen was a generous gift from Matrix Laboratories Limited, India. Acetylsalicylic acid was of pharmacopeal quality.

2.2. Preparation of working solutions

The stock Cu(II) chloride solution (16.0 μM) and that of ascorbic acid (16.0 mM) were prepared in 0.15 M NaCl. The stock 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 $pK_a = 7.53$ [44] results in nearly equimolar concentration of undissociated acid (HOCl) and ClO^- ions.)

These solutions were freshly prepared each day and appropriately diluted with 0.15 M NaCl. The actual concentration of NaOCl solution was determined at alkaline pH using $\varepsilon_{290} = 350\ M^{-1}\ cm^{-1}$ for ClO^- [44].

Stock solutions of sodium naproxen (100 mM) and acetylsalicylic acid (13.9 mM) were prepared in 0.15 M water solution of NaCl. Acetylsalicylic acid dissolution was speeded up by stirring with mild heating.

2.3. Preparation of degradative systems

For rheological measurements, 20.0 mg of high-molecular weight 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. Next, 4.0 ml portion of the solvent was added after 6 h.

Three different degradative reaction systems were employed in the studies of degradation of the high-molecular weight HA sample Lifecore P9710-2: (a) containing HA + sodium hypochlorite at the final concentration of 10 mM, (b) containing 10 mM sodium hypochlorite + 100 μM ascorbic acid and (c) containing 0.1 μM $CuCl_2$ and 100 μM ascorbic acid, followed by the addition of sodium hypochlorite at the final concentrations 10 mM, 8 mM, 2 mM, and 0.5 mM.

2.4. Preparation of inhibitory systems

Four different arrangements were employed in the inhibitory studies of degradation of the high-molecular weight HA sample Lifecore P9710-2 in the system 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 2 mM NaOCl: (a) containing sodium naproxen added to the system 1 h after the reaction onset at final concentrations 0.0 mM, 0.143 mM, 1.43 mM and 10 mM; (b) containing sodium naproxen added to the system before the reaction onset to the final concentrations 0.0, 1.43 and 10 mM; (c) containing acetylsalicylic acid added to the system 1 h after the reaction onset at final concentrations 0.0 mM, 0.0143 mM, 0.143 mM and 1.43 mM; (d) containing acetylsalicylic acid added to the system before the reaction onset to the same final concentrations as in system (c).

2.5. 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 2 min after the experiment onset. The changes of dynamic viscosity (η) and torque were monitored at 25 ± 0.1 °C using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs, Inc., Middleboro, MA, USA) equipped with a cup–spindle pair built of Teflon® 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 min (inhibition study).

2.6. Preparation of samples for chemiluminometry

The reservoir content (at the nominal η value 5.8 mPa s) was poured into 20 ml ethanol, which led to precipitation of the polymer. On the following day, the polymer precipitate was washed out with 20 ml ethanol, centrifuged, and dried in a dessicator. The pellet prepared from the dried polymer obtained from the degradation system involving sodium hypochlorite was brittle, and the odor of chlorine was detectable even after the precipitation of the degraded sample.

2.7. Chemiluminometry

Chemiluminescence measurements were performed with a photon-counting instrument Lumipol 3 manufactured at the Polymer Institute of the Slovak Academy of Sciences. The sample was placed on an aluminum pan in the sample compartment. The gas flow (pure oxygen or nitrogen) through the sample cell was 3.0 l/h. The temperature in the sample compartment of the apparatus was raised from 40 up to 220 °C, with a linear gradient of 2.5 °C min^{-1}. The signal from the photocathode was recorded at 10 s data collection interval. The amount of samples used for each measurement ranged from 0.98 to 1.62 mg.

3. Results

3.1. Degradation of hyaluronan by sodium hypochlorite

Fig. 1A shows changes in viscosity of HA solution during sample treatment with three different systems – in the absence of NaOCl (0), with addition of 10 mM NaOCl (1) and with addition of 10 mM NaOCl + 100 μM ascorbic acid (2). We observed a moderate time-dependent increase of viscosity of hyaluronan in the absence of NaOCl during a 3-h measurement (0). Sodium hypochlorite (10 mM) added to the HA solution resulted in a rapid decrease of the dynamic viscosity. The magnitude of $\eta = 5.8$ mPa s was reached after 134 min (1). The addition of both NaOCl (10 mM) and ascorbic acid (100 μM) resulted in an even more accelerated degradation of hyaluronan. The decrease of the η value to 5.8 mPa s was achieved in 67 min (2).

However, introduction of 0.1 μM $CuCl_2$ together with 100 μM ascorbic acid resulted in a very fast degradation of hyaluronan, and the decrease of the η value to 5.8 mPa s was achieved in 12 min (Fig. 1B, curve 1). The rate of HA degradation was hypochlorous acid concentration-dependent. At the concentration of 0.5 mM NaOCl, only minimal decrease of viscosity was observed (Fig. 1B, curve 4).

Figs. 2 and 3 show the chemiluminescence runs in profiles obtained upon heating of non-degraded HA and oxidative modified HA samples in the presence of oxygen (Fig. 2) or nitrogen (Fig. 3) gas in the chemiluminometric approach.

It is important that the distinct chemiluminescence signal for intact HA sample may be observed even in nitrogen. This is a demonstration that each intact sample contains initially a certain initial concentration of hydroperoxides which reflects its history, previous treatment and conditions of storage [49]. As shown in Table 1, the signal intensity in nitrogen is lower than that in oxygen, which corresponds to lower stationary concentrations of hydroperoxides in the former case.

In the presence of HOCl, the maximum chemiluminescence intensity is reduced almost by a factor of 5 in both nitrogen and oxygen atmosphere. At the same time, the inflexion point of experimental curves is shifted to lower temperatures, *i.e.* the sample becomes less stable after the degradation cycle. Ascorbic acid and copper ions have the tendency to shift the inflexion point back to higher temperature which is, however, still lower than that for intact HA. This becomes more obvious on the panel B of Figs. 2 and 3 where experimental runs were normalized to 1.

3.2. Inhibitory study of hyaluronan degradation

Measurements of viscosity changes of HA solution by HOCl (2 mM) were also performed in the presence of sodium naproxen. When the drug was added 1 h after the reaction onset, the highest applied drug concentration (10 mM) totally inhibited the degradation of HA (Fig. 4, panel A). Addition of lower concentrations of the naproxen resulted in only partial inhibition of HA degradation.

Fig. 4 (panel B) illustrates the effect of sodium naproxen on HA degradation when it was added to the system before the reaction starts. Even at 10 mM, the highest concentration applied, the kinetic course of degradation was not changed.

Replacement of sodium naproxen by acetylsalicylic acid yielded different results. Fig. 5 (panel A) illustrates the effect of acetylsalicylic acid on HA degradation added into the system during the running degradation. Almost complete inhibition was observed at the concentration of 1.43 mM, while the concentrations of 0.0143 mM and 0.143 mM exhibited a partial inhibition of HA degradation. The same results were found with this drug in the experimental model when the drug was added to the system before reaction onset (Fig. 5, panel B).

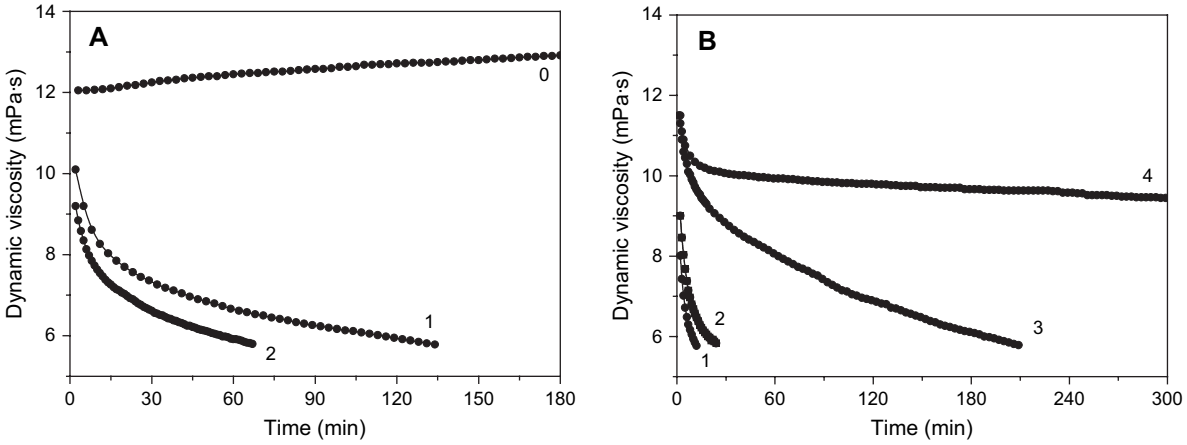

Fig. 1. (A) The dynamic viscosity *vs*. time profile of a 0.25% (w/v) solution of Lifecore P9710-2 sample incubated with sodium hypochlorite in the absence of NaOCl (0), with addition of 10 mM NaOCl (1) and with addition of 10 mM NaOCl + 100 μM ascorbic acid (2). (B) The dynamic viscosity *vs*. time profile of a 0.25% (w/v) solution of Lifecore P9710-2 sample in the system 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + NaOCl. Final concentrations of NaOCl are 10 mM (1), 8 mM (2), 2 mM (3) and 0.5 mM (4).

4. Discussion

In our study, hypochlorous acid was chosen to degrade HA since this strong oxidant is generated in a myeloperoxidase-driven reaction by PMNLs in inflamed joints [11,45,46]. Although HOCl attacks *N*-acetyl groups less intensively than amino-groups and sulfhydryl-groups of proteins [14], its possible damaging attack on HA should be taken into consideration.

4.1. Chemistry of the hyaluronan degradation by HOCl

Relatively high concentrations of HOCl (of the order of up to 10 mM) or longer-incubation times were used in several studies of HA degradation [6,12,14]. Our aim was to verify the degradative effect of a high concentration of HOCl on HA and to assess potential reactions of HOCl with a trace metal and a redox component − ascorbate − present in the inflamed joint. Thus, the action of HOCl on the HA degradation was investigated in the presence of Cu^{2+} and ascorbic acid.

According to our results, two main processes induced by HOCl may contribute to hyaluronan degradation.

(i) At the beginning, ascorbic acid continuously reduces copper ions Cu^{2+} to Cu^{+} with formation of ascorbyl radical. After addition of hypochlorous acid, the generation of hydroxyl radicals could proceed according to

$$Cu^{+} + HOCl \rightarrow Cu^{2+} + Cl^{-} + {}^{\bullet}OH$$

A similar reaction has been described for Fe^{2+} and HOCl [47,48].

Hydroxyl radicals attack the hyaluronan macromolecule with formation of alkyl (macro)radicals $A^{\bullet}$, having the free radical sites in the positions 1 and 4 of the D-glucuronate unit [49]. In the presence of dioxygen, these alkyl radicals are rapidly converted to peroxyl

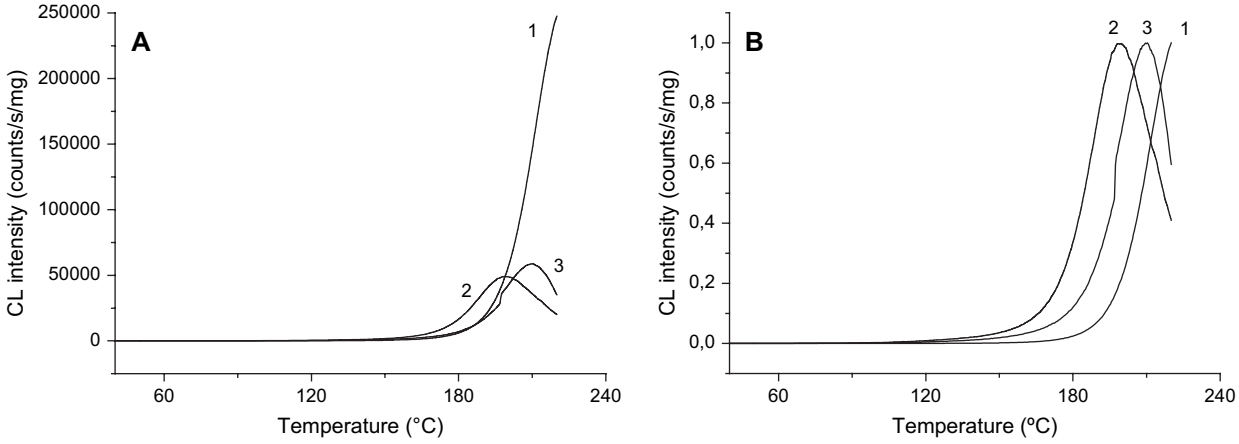

Fig. 2. Chemiluminescence (CL) for non-degraded Lifecore P9710-2 (1) and for degraded HA in systems 10 mM NaOCl (2) and 10 mM NaOCl + 0.1 μM $CuCl_2$ + 100 μM ascorbic acid (3). Measurement in oxygen (panel A) and curves from the panel A normalized to 1 by division of chemiluminescence intensity by its maximum value (panel B), the rate of heating being 2.5 °C min^{-1} (CL).

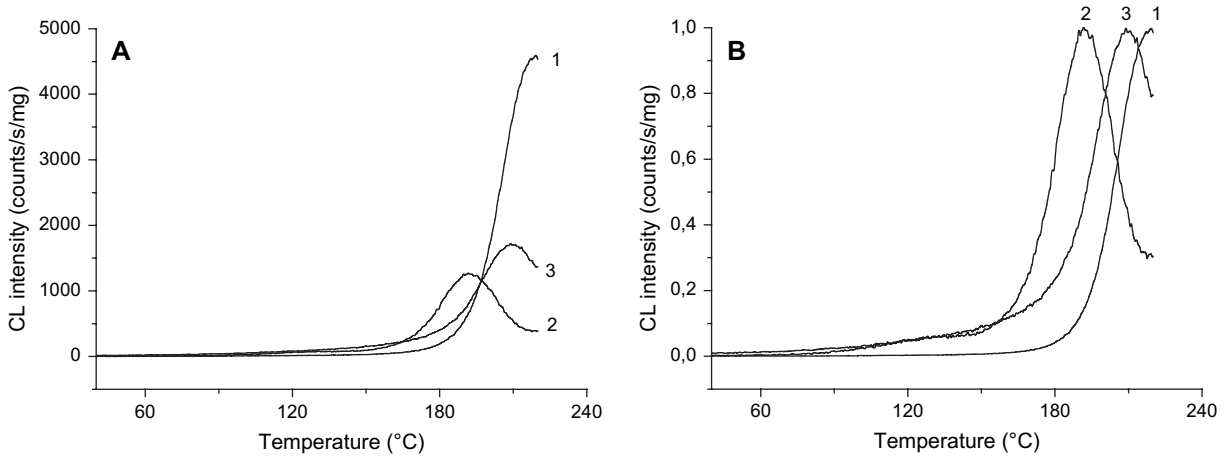

Fig. 3. Chemiluminescence (CL) for non-degraded Lifecore P9710-2 (1) and for degraded HA in systems 10 mM NaOCl (2) and 10 mM NaOCl + 0.1 μM $CuCl_2$ + 100 μM ascorbic acid (3). Measurement in nitrogen (panel A) and curves from the panel A normalized to 1 by division of chemiluminescence intensity by its maximum value (panel B), the rate of heating being 2.5 °C min^{-1}.

radicals, which can enter into reactions with HA, yielding hydroperoxides

$$HO^{\bullet} + HA \rightarrow H_2O + A^{\bullet}$$

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet}$$

$$AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$$

(ii) At the same time another reaction of HOCl is progressing

$$HOCl + HA \rightarrow HA{-}Cl \rightarrow A^{\bullet}$$

As stated by several groups [14–16], the reaction of HOCl with the *N*-acetyl group of *N*-acetyl-D-glucosamine moieties of HA leads to the formation of long-lived *N*-chloroamides (HA–Cl). The reaction then may proceed with the production of the *N*-centered amidyl radicals *via* both metal ion-dependent and ion-independent mechanisms. These *N*-centered radicals undergo rapid isomerization to give carbon-centered radicals at C-2 of the *N*-acetyl-D-glucosamine units (*via* a 1,2-hydrogen atom transfer) and at C-4 of the neighboring D-glucuronate moieties (*via* a 1,5-hydrogen atom transfer). In the presence of dioxygen, peroxyl radicals and hydroperoxides may be formed as well.

Table 1
The surface (*S*) under chemiluminescence (S_{CL}) (40–220 °C) curves for intact and degraded hyaluronans

Sample	Atmosphere	S_{CL} (counts/g)	S_{CLO}/S_{CLN}
Lifecore P9710-2	Oxygen (O)	8.37×10^{10}	40.4
	Nitrogen (N)	2.07×10^{9}	
10 mM NaOCl	Oxygen (O)	3.90×10^{10}	34.5
	Nitrogen (N)	1.13×10^{9}	
10 mM NaOCl + 0.1 μM $CuCl_2$ + 100 μM ascorbic acid	Oxygen (O)	3.64×10^{10}	25.1
	Nitrogen (N)	1.45×10^{9}	

S_{CLO} and S_{CLN} are the surfaces under chemiluminescence curves observed in the atmospheres of oxygen and nitrogen, respectively.

Thus, HOCl initiates two main pathways to degrade hyaluronan: (i) *via* the formation of hydroxyl radicals in the presence of free metal ions and ascorbic acid, and (ii) *via* a direct attack on *N*-acetyl groups of HA with the subsequent formation of hyaluronan radicals.

In order to understand the mechanism of NSAIDs on HA degradation, it is necessary to consider also other reactions of HOCl in the incubation cocktail and alterations in reactant concentrations.

The addition of Cu^{2+} to the incubation medium increases the rate of Cu^{2+} reduction by ascorbic acid and, thus, more Cu^{+} is available to react with HOCl to yield hydroxyl radicals. Data in Fig. 1 show clearly that the addition of 0.1 μM Cu^{2+} facilitates hyaluronan degradation in the presence of 100 μM ascorbic acid and 10 mM HOCl.

Hypochlorous acid is also known to react with ascorbic acid with the formation of non-radical products [20,50]. A rate constant of 6×10^{6} M^{-1} s^{-1} at pH 7.4 was found for this reaction [20]. In this way, the addition of HOCl causes a fast conversion of all ascorbic acid present in the incubation cocktail to non-radical products that are unable to take part in Cu^{2+}/Cu^{+} redox conversions. On the basis of this reaction, the dependence of hyaluronan degradation on HOCl concentration can be explained (see Fig. 1, panel B). At the lowest applied HOCl concentration at 0.5 mM, there is a slow process of HA degradation. Considering the complex chemistry of reaction of HOCl with ascorbic acid, both components are diminished considerably, and only few free HOCl remain that can be involved in HA degradation.

Thus, initiation of HA degradation by HOCl *via* the formation of hydroxyl radicals is only possible at the beginning of the incubation as long as free ascorbic acid is present to provide redox recycling of free metal ions. On the other hand, HA degradation by HOCl *via* attacks on *N*-acetyl groups of HA may be initiated during the whole incubation

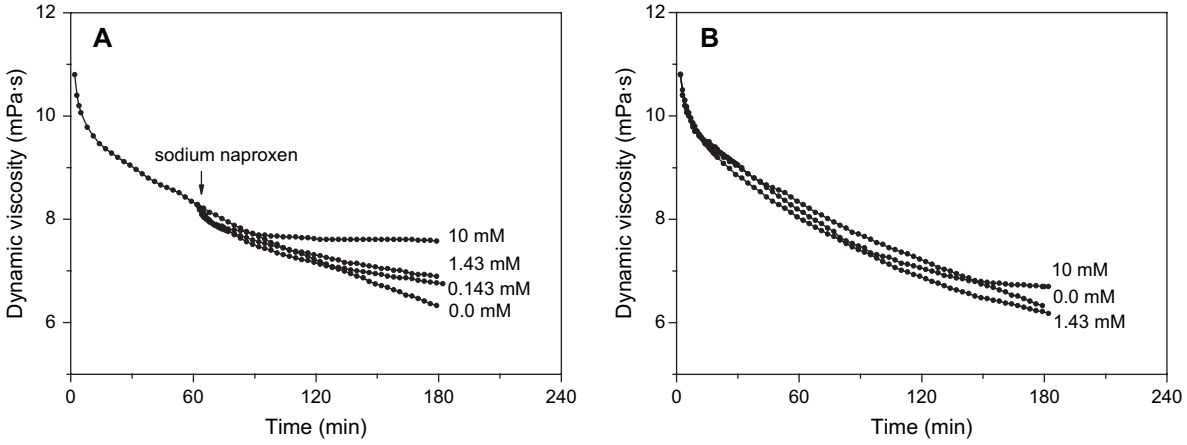

Fig. 4. Effect of sodium naproxen on HA degradation induced by the system 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 2 mM NaOCl. Concentration of sodium naproxen added into the system at 1 h (A) or before initiation of degradation (B) in mM: 0.0, 0.143, 1.43 and 10.

process. It is only necessary that enough unreacted HOCl is present.

All luminescence measurements were performed several hours after incubation of HA with oxidant systems. At that time, no redox conversions between Cu^{2+} and Cu^{+} should occur as all the ascorbic acid is inactivated by HOCl and there are only traces of HOCl in the system. Luminescence signals result from temperature-induced decomposition of instable intermediates of HA oxidation like hydroperoxides and long-lived *N*-chloroamides.

Decomposition of AOOH occurs either as monomolecular or bimolecular reaction or reaction induced by transition metal ions. It provides alkoxyl radicals, which are presumed intermediates of the main chain splitting and peroxyl radicals which in their recombination give light emission [51].

$$AOOH \rightarrow AO^{\bullet} + {}^{\bullet}OH$$

$$2AOOH \rightarrow AO_2^{\bullet} + AO^{\bullet} + H_2O$$

$$AOOH + Cu^{+} \rightarrow AO^{\bullet} + {}^{-}OH + Cu^{2+}$$

$$2AO_2^{\bullet} \rightarrow A{=}O^{*} + AOH + O_2^{*}$$

A═O* and O_2* are excited triplet states of carbonyl and singlet oxygen, respectively, which yield chemiluminescence on passing to their ground states.

N-Chloroamides formed from the reaction of HOCl with the *N*-acetyl group of *N*-acetyl-D-glucosamine moieties of HA will decay much faster under formation of free radicals at elevated temperatures.

The above schemes may qualitatively explain the results from chemiluminometry of intact and degraded HA. Apparently, the decrease in inflexion point in oxidized HA samples depends on the concentration of residual HOCl. The system HA + HOCl yielded a higher decrease of inflexion point than the system HA + HOCl + Cu^{2+} + ascorbic acid. Due to the reaction between HOCl and ascorbic acid, the concentration of residual HOCl should be higher in the first system. However, when calculating the rate constants of oxidation for different temperatures of non-isothermal chemiluminescence runs according to the procedure described elsewhere

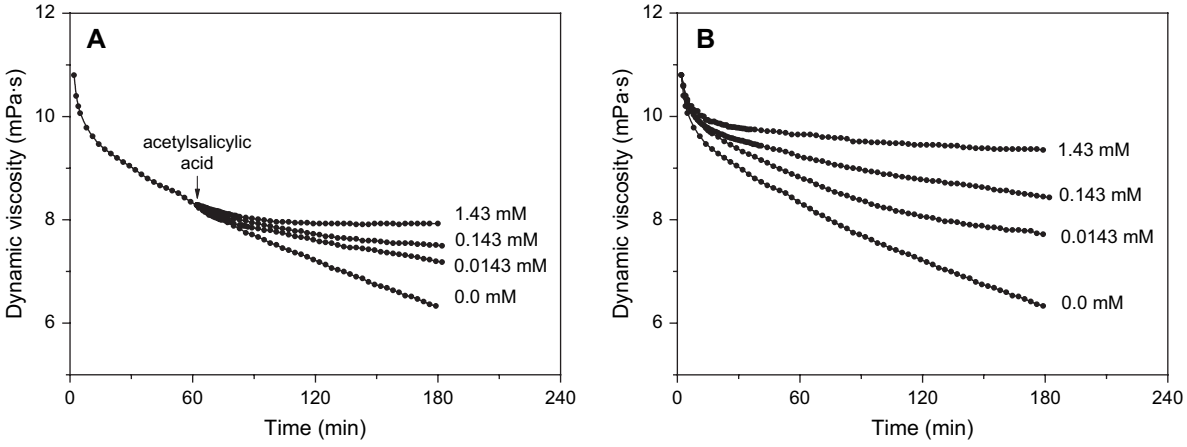

Fig. 5. Effect of acetylsalicylic acid on HA degradation in the system 0.1 μM $CuCl_2$ + 100 μM ascorbic acid + 2 mM NaOCl. Concentration of acetylsalicylic acid added into the system at 1 h (A), or before initiation of degradation (B) in mM: 0.0, 0.0143, 0.143, and 1.43.

[52] we see that at 100 °C and lower temperatures the second system gives a higher rate constant while opposite is true for *e.g.* 200 °C (1.1×10^{-3} s^{-1}; *cf.* Table 2). Apparently, an enhanced redox recycling of cuprous ions contributes to this effect.

4.2. Inhibitory study of HA degradation

The action of NSAIDs on hyaluronan degradation in the system with hypochlorous acid has not been studied so far. We tested the effect of drugs on HA degradation in two experimental arrangements: (i) addition of the drug to the system during the running degradative process and (ii) addition of the drug before initiation of HA degradation.

Tests of antioxidant effects of drugs on HA degradation are usually performed only by addition of drugs to the system before initiation of degradation. Our particular goal was to investigate the effect of drugs added to HA samples during the running degradative process. This approach reflects more precisely the pathophysiological situation in arthritis. No literature data have come to our attention showing whether NSAIDs can influence HA degradation under these conditions.

The initial concentrations of sodium naproxen (0.143 mM) and acetylsalicylic acid (0.0143 mM) used were the same as the maximum concentration of these drugs in synovial fluid (36 μg/ml) after a common therapeutic dose [53,54].

Naproxen showed a concentration-dependent inhibitory effect on HA degradation only if it was added during the degradation process, whereas acetylsalicylic acid inhibited HA degradation in both experimental arrangements.

Thus the formation of hydroxyl radicals by HOCl in the presence of metal ions and ascorbic acid, which is dominant only at the beginning of the incubation process, and all subsequent radical reactions in HA are unaffected by naproxen. Also, there is no direct interaction between HOCl and naproxen. The absence of any interaction between naproxen and HOCl was also demonstrated by the lack of any effects on oxidation of 5-thio-2-nitrobenzoic acid by HOCl [37,55]. However, this drug is apparently able to interfere with the HA system at the level of long-lived *N*-chloroamides or radicals derived from these products.

Acetylsalicylic acid inhibited HA degradation in both early and late applications. This effect can be explained by either direct interaction between acetylsalicylate and HOCl or by interaction with oxidized hyaluronan products (alkyl, peroxyl, alkoxyl radicals, hydroperoxides of hyaluronan) participating in both pathways of HA degradation. In contrast to salicylate and other salicylate derivatives, acetylsalicylic acid at micromolar concentrations caused only a minimal inhibition of myeloperoxidase [35]. All salicylate derivatives inhibited the chlorination cycle of this enzyme by accumulation of MPO enzyme but not by scavenging hypochlorous acid [35]. Neither acetylsalicylic acid nor naproxen affected HOCl-induced inactivation of α_1-proteinase inhibitor [39,56]. These data support the view that a direct interaction between HOCl and acetylsalicylic acid is unlikely. Acetylsalicylic acid is a very efficient scavenger for hydroxyl radicals [34]. Apparently, acetylsalicylic acid is also able to scavenge different hyaluronan radicals (Fig. 5A). Thus the differences in interactions of the drugs tested with radicals and intermediate components in HA oxidation may account for the different inhibitory profiles of naproxen and acetylsalicylic acid on HOCl-induced hyaluronan oxidation.

In conclusion, we consider high-molecular weight HA as a very good model for kinetic study of the effect of oxidants and free radicals, as well as for testing the inhibitory effect of natural and synthetic substances *in vitro*.

Table 2
The rate constants (in s^{-1}) of oxidation in oxygen for intact and degraded hyaluronans determined from non-isothermal chemiluminescence measurements

Sample	Temperature (°C)		
	40	100	200
Lifecore P9710-2	1.1×10^{-9}	8.3×10^{-8}	2.8×10^{-4}
10 mM NaOCl	1.7×10^{-8}	4.0×10^{-7}	2.0×10^{-3}
10 mM NaOCl + 0.1 μM $CuCl_2$ + 100 μM ascorbic acid	3.7×10^{-8}	1.7×10^{-6}	1.1×10^{-3}

Acknowledgements

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