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ORIGINAL PAPER

Dithiols as more effective than monothiols in protecting biomacromolecules from free-radical-mediated damage: in vitro oxidative degradation of high-molar-mass hyaluronan

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Oxidative stress and the resulting damage to cellular and extracellular components has been observed in a variety of degenerative processes, including degenerative joint disorders, where high-molar-mass hyaluronan (HA) is often found to be massively degraded. The present study sought to test the hypothesis that dithiols are more effective in protecting biomacromolecules from free-radical-mediated damage than monothiols. The materials/thiols tested included bucillamine (BUC), dithioerythritol (DTE), dithiothreitol (DTT) and glutathione (GSH), as a reference, for their effectiveness in protecting HA from oxidative degradation induced in vitro. Since HA degradation results in a decrease in its dynamic viscosity, rotational viscometry was applied to follow HA oxidative degradation. The free-radical-scavenging activities of the thiols tested were determined by 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assays. It was found that all the dithiols in the concentration range tested protected HA from the oxidative degradation. On the other hand, monothiol GSH exerted protection only at high concentrations (10 μmol L^{-1} and 100 μmol L^{-1}) and 1 μmol L^{-1} of GSH even exhibited a pro-degradative effect. The ABTS assay revealed free-radical scavenging activities in the following order: BUC, DTT, DTE, GSH, and that of the DPPH assay: BUC, DTE, DTT, GSH. In conclusion, it was demonstrated that dithiols may be more effective than monothiols in affording biomacromolecule protection from oxidative degradation.

Keywords: ABTS, DPPH, oxidative stress, rotational viscometry

Introduction

Hyaluronan (hyaluronic acid, HA; Fig. 1) is a unique glycosaminoglycan composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ glycosidic bonds (Hrabárová et al., 2007). HA was discovered, identified and first isolated from the eye vitreous body in 1934 by Meyer and Palmer (1934). Subsequently, HA was found to be present in a number of tissues, including skin, umbilical cord, tumour tissue and rooster comb (Ziouti et al., 2004).

HA is a major component of the extracellular matrix (ECM) and is abundant in synovial fluid (SF) and cartilage. In the ECM of cartilage, HA intertwined among collagen fibrils serves as a backbone for the attachment of proteoglycans. Glycosaminoglycans possess chondroitin-sulphate and keratin-sulphate-rich regions and link proteins that facilitate the binding of aggrecans to HA.

Oxidative stress has repeatedly been reported as being involved in several human diseases (Giles et al., 2002; Rahal et al., 2014), including the degenerative processes of the synovial joint affecting its integral components including synovial fluid and HA in particular (Afonso et al., 2007). Three dithiol compounds, namely dithiothreitol (DTT), dithioerythritol (DTE) and bucillamine (BUC), a structural analogue of cys-

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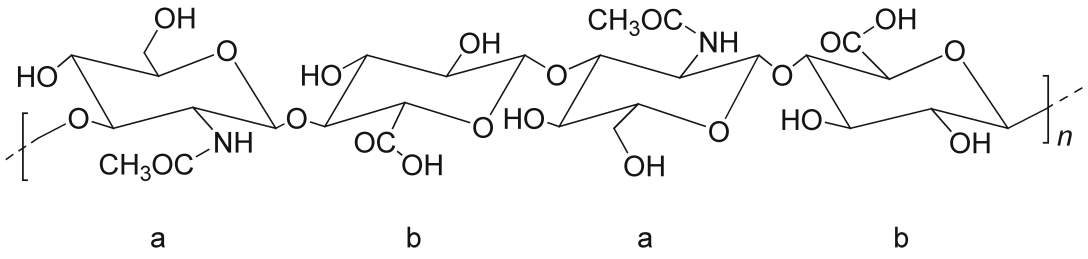

Fig. 1. Chemical structure of repeating disaccharide units of hyaluronan: a – *N*-acetyl-D-glucosamine; b – D-glucuronic acid.

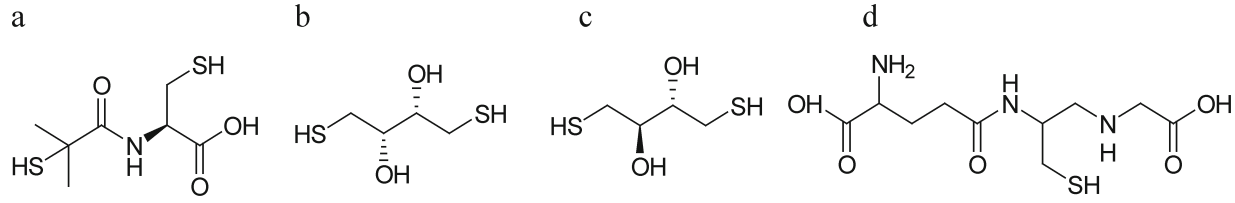

Fig. 2. Chemical structure of BUC (a), dithiothreitol (b), dithioerythritol (c) and glutathione (d).

teine (Figs. 2a–2d), were tested for their potential to protect HA against its oxidative degradation induced in vitro. Monothiol glutathione (GSH) was used as a reference. Significantly, in Asia BUC is prescribed for the treatment of rheumatoid arthritis (Kładna et al., 2006). DTT, a redox compound known as Cleland's reagent, is used to reduce disulphide bonds of proteins (Chang et al., 1997; Scigelova et al., 2001). DTE, an epimeric compound of DTT, has hydroxyl groups in *cis* orientation, while in the *trans* position in DTT. GSH (γ-L-glutamyl-L-cysteinylglycine) is present in its reduced form in millimolar concentrations within the cell. As an endogenous antioxidant, GSH plays an important role in protecting the organism from oxidative stress (Moskaug et al., 2005). However, it should be noted that, in the ECM, GSH is only in the micromolar concentration (Govindaraju et al., 2003).

The present study sought to test the original hypothesis that dithiols are more effective than monothiols in protecting biomacromolecules from free-radical-mediated damage. Accordingly: (i) the free-radical scavenging activity of the selected dithiols BUC, DTE and DTT as well as that of reference monothiol GSH was evaluated in the ABTS and DPPH assays and (ii) these compounds were assessed for their potential to protect HA from oxidative degradation induced in vitro.

Experimental

General

Five different native hyaluronans, used throughout the study, were either kindly donated or purchased from the following HA manufacturers: P0207-1, P9710-2, P9706-6 and 1-9100-1 from Lifecore Biomedical (USA) and 35H0439 from Sigma–Aldrich (USA).

NaCl and $CuCl_2 \cdot 2H_2O$ (analytical purity grade) were purchased from Slavus (Slovakia), ascorbate and potassium persulphate from Merck (Germany), ABTS (>99 %) from Fluka (Germany), DPPH and glutathione from Sigma–Aldrich, DTE from Fluka, DTT from Aldrich (Belgium). BUC was kindly donated by Dr. Fumio Tsuji (Santen Pharmaceutical Japan).

Deionised high-purity grade water, with conductivity of $\leq$0.055 μS cm^{-1}, was produced using the TKA water purification system (Water Purification Systems, Germany).

The hyaluronan samples (20.0 mg) were dissolved in 0.15 mol L^{-1} of aqueous NaCl solution in darkness for 24 h. HA sample solutions were prepared in two steps: first, 4.0 mL and after 6 h, 3.85 mL of 0.15 mol L^{-1} NaCl were added. Solutions of ascorbate (16 mmol L^{-1}), BUC, DTE, DTT, GSH, (each in 16.00 mmol L^{-1}, 1.60 mmol L^{-1}, 0.16 mmol L^{-1}) and cupric chloride (16.00 mmol L^{-1} diluted to a 160 μmol L^{-1} solution) were also prepared in 0.15 mol L^{-1} aqueous NaCl.

ABTS and DPPH assays

The standard ABTS decolorisation assay was performed as previously reported (Re et al., 1999; Cheng et al., 2006). In brief, an aqueous solution of the $ABTS^{\bullet+}$ cation radical was prepared 24 h prior to the measurements at ambient temperature as follows: the ABTS aqueous stock solution (7 mmol L^{-1}) was mixed with an equal volume of the $K_2S_2O_8$ aqueous solution (2.45 mmol L^{-1}). The following day, 1.1 mL of the resulting solution was diluted with 96 vol. % of ethanol to the final volume of 50 mL. The ethanol–aqueous reagent $ABTS^{\bullet+}$ with a concentration of 101 mmol L^{-1} in the volume of 250 μL was added to 2.5 μL of the ethanol solutions of BUC, DTE,

DTT and GSH. The concentration of each stock solution ranged from 0.808 mmol L^{-1} to 101 mmol L^{-1}. The concentration of each substance in the plates ranged from 8.08 μmol L^{-1} to 1.01 mmol L^{-1}. Absorbance (734 nm) of the samples was recorded at the 6^{th} min after onset of the reaction.

For the DPPH decolorisation assay, DPPH (1.1 mg) was dissolved in 50 mL of distilled methanol to generate $DPPH^{\bullet}$. The $DPPH^{\bullet}$ radical solution with a concentration of 55 μmol L^{-1} in the volume of 225 μL was added to 25 μL of the methanol solution of BUC, DTE, DTT and GSH, all in the concentration range of 0.078–10 mmol L^{-1}. The concentration of each substance in the plates ranged from 0.0078 to 1.0 mmol L^{-1}. Absorbance (517 nm) of the samples was recorded after 30 min (Valachová et al., 2010).

Measurements were performed in quadruplicate in 96-well Greiner UV-Star microplates (Greiner-Bio-One, Germany) by using the Tecan Infinite M 200 reader (Tecan, Austria).

Oxidative degradation of HA – rotational viscometry

HA degradation was induced in vitro by Weissberger's biogenic oxidative system (WBOS) comprising biogenic compounds in the respective physiological concentrations, specifically 100 μmol L^{-1} ascorbate plus 1 μmol L^{-1} $CuCl_2 \cdot 2H_2O$, applied under air atmosphere conditions. The procedure was as follows: 50 μL of $CuCl_2 \cdot 2H_2O$ (160 μmol L^{-1}) was added to the HA solution (7.90 mL). After 30 s stirring, the reaction mixture was allowed to stand for 7.5 min at ambient temperature, then 50 μL of ascorbate (16 mmol L^{-1}) was added and the mixture was again stirred for 30 s. The resulting 8 mL of the reaction mixture containing HA was transferred into the Teflon® cup reservoir of a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., USA) and changes in the dynamic viscosity of the HA solution were recorded at (25.0 ± 0.1)℃ at 3 min intervals for 2 h. The viscometer Teflon® spindle rotated at 180 min^{-1}, corresponding to a shear-rate of 237.6 s^{-1}.

Two experimental arrangements were applied in testing the potential protection by BUC, DTE, DTT and GSH of the high-molar-mass HA from its free-radical-mediated degradation: (i) the compounds tested were added to the mixture at the beginning of the HA oxidative degradation, specifically 30 s prior to the reaction being started by the addition of ascorbate; (ii) in the second experimental setup, the compounds were added to the reaction mixture one hour after the onset of the HA oxidative degradation and stirred for 30 s. Subsequently, the HA mixture was transferred into the viscometric Teflon® cup reservoir and its degradation was recorded.

Table 1. IC_{50} values of compounds tested determined by ABTS and DPPH decolorisation assays ($n = 4$; data expressed as mean ± s.e.m.)

Substance	ABTS IC_{50}	DPPH IC_{50}
	μmol L^{-1}	
Bucillamine	(4 ± 0.4) n.s.	(9.0 ± 0.4)***
DTT	(16.5 ± 0.4)***	(14.8 ± 0.3)***
DTE	(26 ± 0.5)***	(12.6 ± 0.6)***
GSH	(24 ± 0.3)***	n.e.
Quercetin	2.9 ± 0.2	4.4 ± 0.2

n.s. – Not significant; n.e. – not effective in concentration range tested; significance of differences was determined by the one-way ANOVA test: *** $p < 0.001$ compared to the quercetin value.

Statistical analysis

All the analyses were run in quadruplicate and the results were expressed as mean ± standard error of the mean (s.e.m.). The Origin statistic software package was used for the statistical analysis. Differences were determined by the one-way ANOVA test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results and discussion

ABTS and DPPH decolorisation assays

Table 1 shows the IC_{50} values of the compounds tested. The values were derived from the respective dose response inhibition curves for the ABTS cation radical and DPPH radical. The IC_{50} values were compared to that of quercetin (Valachová et al., 2010), used as a standard antioxidant. On the basis of its free-radical scavenging effects (IC_{50} 4 μmol L^{-1} and 9 μmol L^{-1} for ABTS and DPPH, respectively), BUC was found to possess a similar antioxidative activity. On the other hand, the other compounds tested, namely DTT, DTE and GSH, exerted higher IC_{50} values in comparison with BUC, indicating a lower antioxidant activity than BUC.

Rotational viscometry

The dynamic viscosity of five native HA solutions with different molar masses was monitored for 5 h (Fig. 3a). From the dynamic viscosity values, the molar mass of HA samples can be estimated (Fig. 3b).

To evaluate the effects of the compounds tested, two distinct experimental arrangements were used: (i) when the compound tested was applied immediately prior to the onset of HA degradation (i.e. preventative mode) and; (ii) when the compound tested was applied one hour after initiation of the HA degradation (treating mode). Figs. 4–6 illustrate that the dithi-

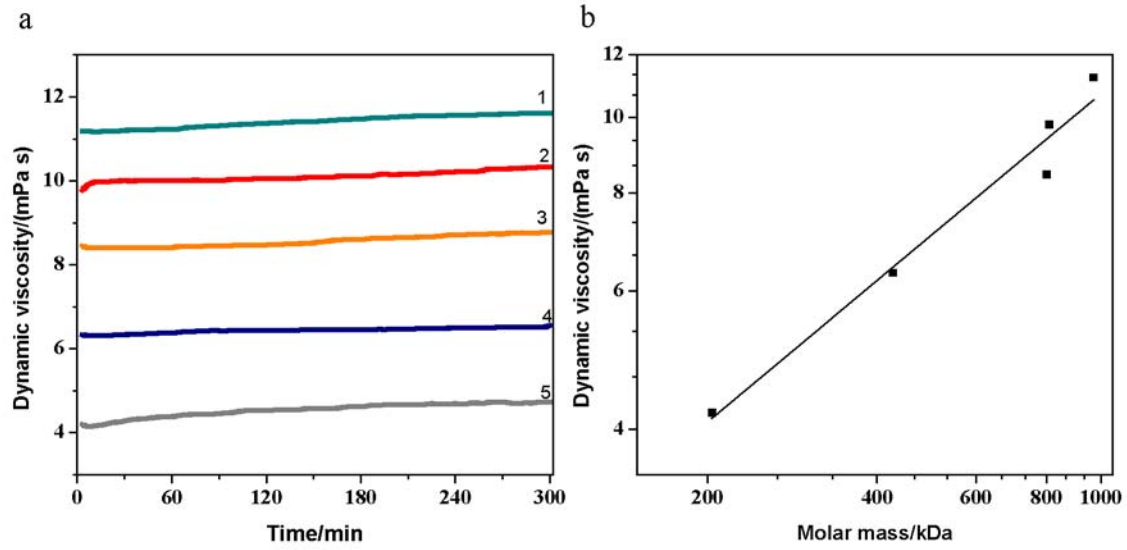

Fig. 3. Characteristics of five native hyaluronans: time-dependences of dynamic viscosity of five native HA solutions over 5 h (a); 1 – HAs: P0207-1, 2 – P9710-2, 3 – P9706-6, 4 – 35H0439, 5 – 1-9100-1; and double-decalogarithmic dependence of molar mass on dynamic viscosity for the native HAs (b).

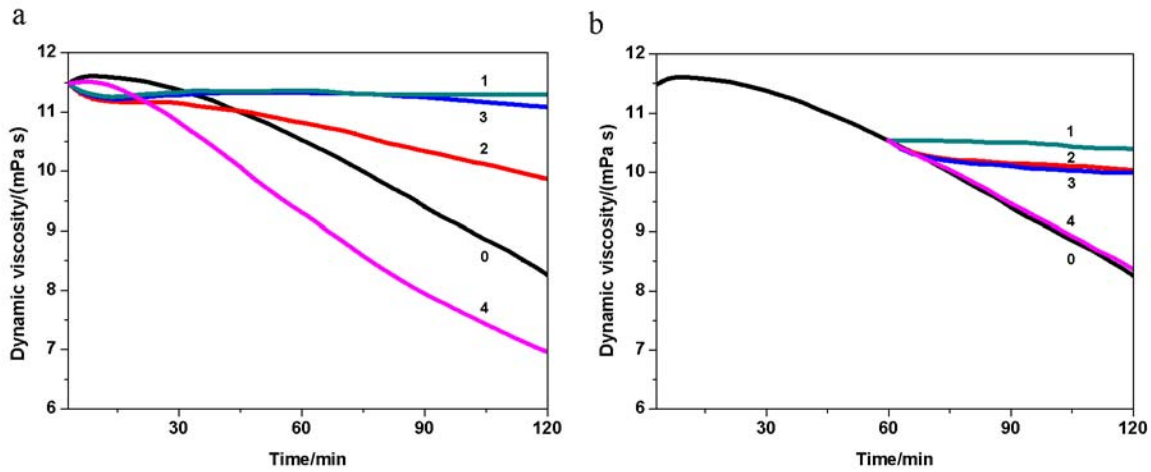

Fig. 4. Effects of 1 μmol L^{-1} thiols on in vitro HA degradation by WBOS: representative tracings of changes in dynamic viscosity of HA during its oxidative degradation: 0 – WBOS only, 1 – WBOS + BUC, 2 – WBOS + DTE, 3 – WBOS + DTT or 4 – WBOS + GSH. Compounds tested were applied immediately prior to onset of HA degradation (a); and 1 h after onset of HA degradation (b).

ols significantly inhibited the oxidative degradation of HA, indicated by no decrease or only a partial decrease in HA dynamic viscosity.

BUC (1 μmol L^{-1}) applied in preventative or treating mode fully preserved the HA viscosity, indicating its effective protection of HA from its oxidative degradation (Figs. 4a and 4b). For DTE and DTT, the preservation of HA viscosity was somewhat lower than for BUC. On the other hand, monothiol GSH (1 μmol L^{-1}) promoted a decrease in HA viscosity in preventative mode (Fig. 4a) and the inhibition of the HA viscosity decrease was found not to be significant in treating mode (Fig. 4b).

Applied in preventative mode, DTE (10 μmol L^{-1}) completely preserved viscosity of HA attacked by WBOS, while DTT, BUC and GSH (all in 10 μmol L^{-1}) exerted 87 %, 93 % and 86 % protection respectively (Fig. 5a). In treating mode (10 μmol L^{-1}), BUC exerted 100 % preservation, DTE and DTT approximately 70 %, and GSH approximately 40 % preservation of HA viscosity (Fig. 5b).

The results on the compounds tested in preventative mode, showed that BUC and DTE (both at 100 μmol L^{-1}) completely preserved the viscosity of HA attacked by WBOS, while GSH and DTT (both at 100 μmol L^{-1}) exerted about 90 % and 85 % preservation, respectively (Fig. 6a). When tested in treating mode, BUC exerted 100 % preservation of the viscosity of HA, while the three other compounds exerted approximately 80 % preservation (Fig. 6b).

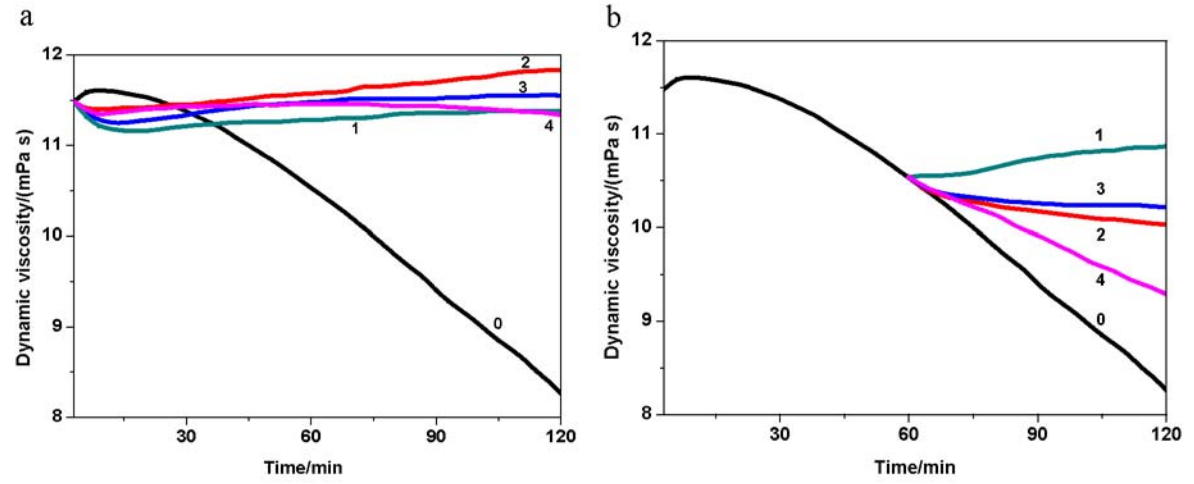

Fig. 5. Effects of 10 μmol L^{-1} thiols on in vitro HA degradation initiated by WBOS: representative tracings of changes in dynamic viscosity of HA during its oxidative degradation; 0 – WBOS only, 1 – WBOS + BUC, 2 – WBOS + DTE, 3 – WBOS + DTT or 4 – WBOS + GSH. Compounds tested were applied immediately prior to onset of HA degradation (a) and 1 h after onset of HA degradation (b).

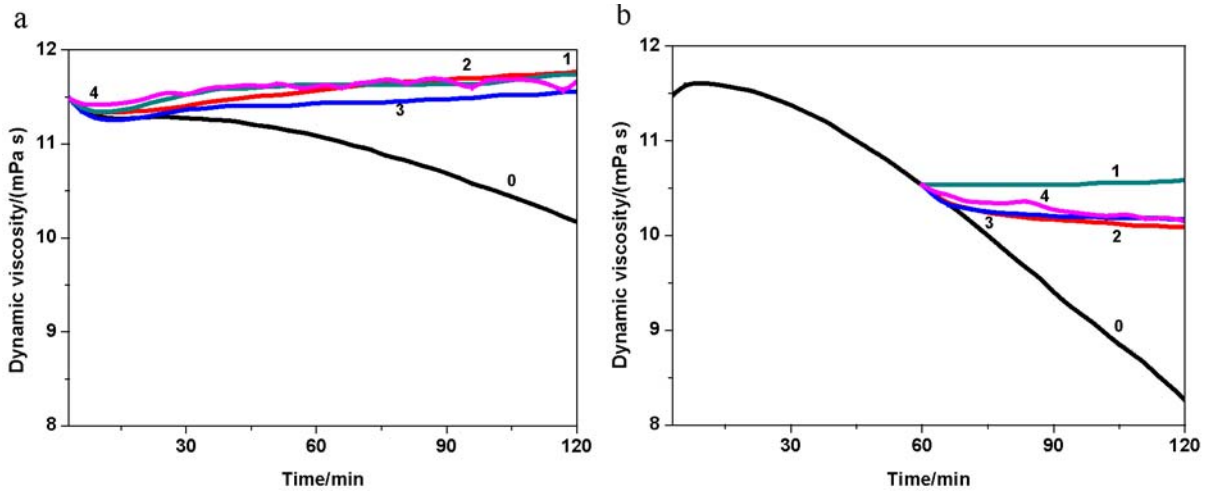

Fig. 6. Effects of 100 μmol L^{-1} thiols on in vitro HA degradation initiated by WBOS: representative tracings of changes in dynamic viscosity of HA during its oxidative degradation; 0 – WBOS only, 1 – WBOS + BUC, 2 – WBOS + DTE, 3 – WBOS + DTT or 4 – WBOS + GSH. Compounds tested were applied immediately prior to onset of HA degradation (a) and 1 h after onset of HA degradation (b).

Due to their antioxidative potential, endogenous low-molar-mass thiol-containing compounds such as GSH play an important role in protecting essential biomacromolecules from free-radical-mediated damage occurring in vivo under conditions of oxidative stress. The present study tested the original hypothesis that dithiols, containing two functional groups with intrinsic antioxidant activity, are more effective in protecting biomacromolecules from oxidative degradation than GSH. HA, used as a model macromolecule, was exposed to oxidative damage in vitro. Free-radical-mediated degradation of HA was induced by the WBOS-comprising endogenous substances in appropriate pathophysiological concentrations. WBOS actually imitates the conditions of an early stage of acute inflammation in the synovial joint – under aerobic conditions, ascorbate plus cupric ions generate hydrogen peroxide. In brief, cuprous ions (1 μmol L^{-1}) undergo a charge stabilisation by an excess of ascorbate (100 μmol L^{-1}): $AscH^- + Cu(I) \rightarrow [AscH^- \cdots Cu(I)]$. The resulting intermediate is then involved in the bi-electron reduction of molecular oxygen, affording hydrogen peroxide (Buettner & Schafer, 2002). Next, hydrogen peroxide is decomposed by Cu(I) in the Fenton-like reaction, and $^{\bullet}OH$ is formed (Fig. 7). Finally, $^{\bullet}OH$ radicals initiate the free-radical-mediated oxidative degradation of high-molar-mass HA.

In the present study, the antioxidative potential of the selected dithiols was tested (specifically, electron-donor activity) using ABTS and DPPH assays as well as their potential to protect HA (due to proton-donor

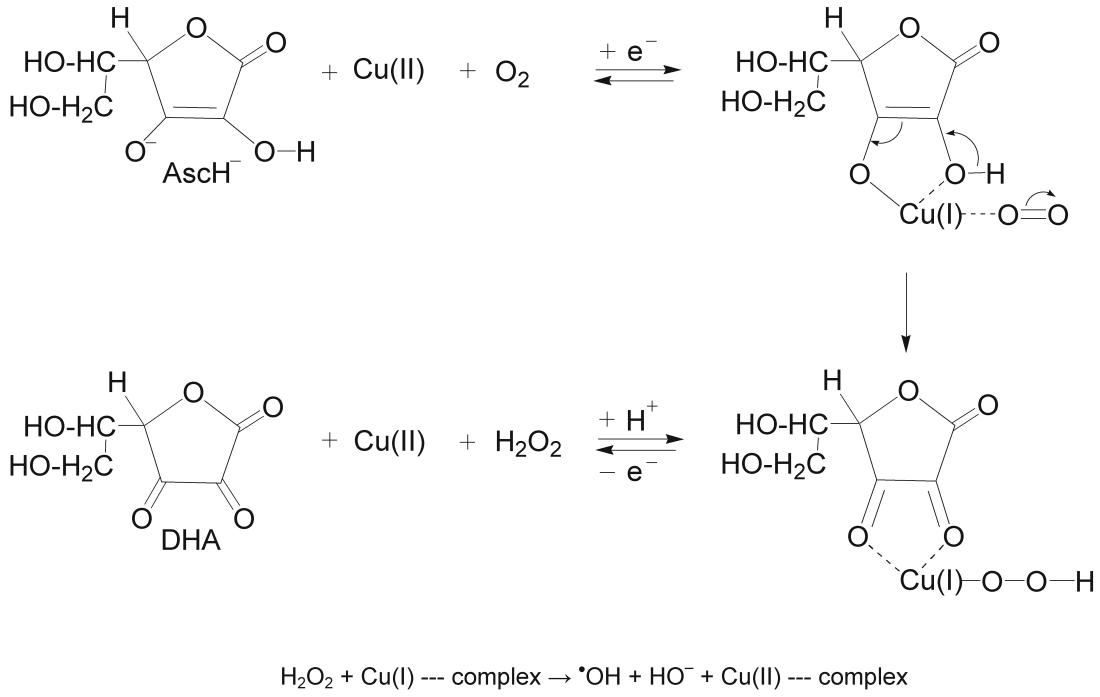

Fig. 7. Weissberger biogenic oxidative system; DHA – dehydroascorbic acid.

activity) from its oxidative degradation via rotational viscometry.

To assess the electron-donor activity of the dithiols tested (DTT, DTE and BUC), decolorisation assays of ABTS and DPPH were used (Magalhães et al., 2008). One-electron reduction of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals yielding the respective products ABTS and DPPH is accompanied by a change in their specific absorbance spectra (Apak et al., 2013).

The IC_{50} values of the thiols tested were taken from the respective inhibitory ABTS and DPPH curves and compared with the IC_{50} of the standard quercetin (Table 1). Based on their IC_{50} values (4 μmol L^{-1} and 9 μmol L^{-1} for ABTS and DPPH, respectively), BUC may be classified as a potent antioxidant. On the other hand, the IC_{50} values of DTT, DTE and GSH were higher than for BUC, indicating that these three compounds possess lower antioxidative activity.

A previous study demonstrated that $^{\bullet}OH$ radicals were generated predominantly within the first hour of HA degradation, initiated by WBOS (Šoltés et al., 2006). Subsequently, after one hour of HA oxidative degradation, mostly alkoxy- and peroxy-radicals are produced. Hence, two modes for application of the tested compounds were chosen for the rotational viscometry assay. The preventative and treating modes make it possible to follow the proton-donating activity of the compounds eliminating $^{\bullet}OH$ and/or alkoxy- and peroxy-radicals within the first hour and after one hour, respectively.

Under the above experimental conditions, the dithiols tested were found to be more potent than the monothiol GSH in inhibiting the dynamic viscosity decrease of HA prompted by WBOS (Figs. 4–6). As dithiols possess two thiol groups, readily donating $^{\bullet}H$ radicals (Fig. 2), they may be more effective in scavenging $^{\bullet}OH$ or $ROO^{\bullet}$ radicals. From the present findings, dithiols may be classified as preventative and/or chain-breaking antioxidants. On the other hand, it cannot be excluded that dithiols may also act via the mechanism of sequestering transition metals, as previously reported (Lima et al., 2006; Mazor et al., 2006). Hence, dithiols would sequester the copper ions of the Weissberger system thereby preventing $^{\bullet}OH$ radical production and, thus, initiation of the oxidative HA degradation.

This study was carried out on the assumption that the antioxidative potential of low-molar-mass endogenous thiols, such as GSH, is involved in the in vivo protection of essential biomacromolecules from their undesired oxidative modification. The antioxidative activity of selected thiol-containing compounds, namely BUC, DTE, DTT and GSH, was assessed using in vitro ABTS and DPPH assays. The scavenging of the $^{\bullet}OH$ radical is probably the most crucial event, as this radical is involved in the initiation of oxidative HA degradation. The ability of the compounds to protect HA from the oxidative degradation induced in vitro was then tested.

Conclusions

From these findings, it may be concluded that dithiols, specifically BUC, DTE and DTT in all the concentrations tested, were more effective in protect-

ing HA from its oxidative degradation than monothiol GSH (Figs. 4–6). The free-radical scavenging activity of dithiols, particularly that of BUC as used clinically in Japan and South Korea, may be involved, at least in part, in the mechanism of protecting high-molar-mass HA from oxidative-stress-mediated damage, often occurring during degenerative synovial joint disorders.

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References

Afonso, V., Champy, R., Mitrovic, D., Collin, P., & Lomri, A. (2007). Reactive oxygen species and superoxide dismutases: Role in joint diseases. *Joint Bone Spine*, *74*, 324–329. DOI: 10.1016/j.jbspin.2007.02.002.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., & Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry*, *85*, 957–998. DOI: 10.1351/pac-rep-12-07-15.

Buettner, G. R., & Schafer, F. Q. (2002). Ascorbate (vitamin C), its antioxidant chemistry. The virtual free radical school for oxygen society. Retreived March, 22, 2014, from http://www.healthcare.uiowa.edu/corefacilities/esr/publications/buettnerpubs/pdf/Buettner-Ascorbate-Chemistry-1.pdf

Chang, J. Y. (1997). A two-stage mechanism for the reductive unfolding of disulfide-containing proteins. *Journal of Biological Chemistry*, *272*, 69–75. DOI: 10.1074/jbc.272.1.69.

Cheng, Z. H., Moore, J., & Yu, L. L. (2006). High-throughput relative DPPH radical scavenging capacity assay. *Journal of Agricultural and Food Chemistry*, *54*, 7429–7436. DOI: 10.1021/jf0611668.

Giles, G. I., Tasker, K. M., & Jacob, C. (2002). Oxidation of biological thiols by highly reactive disulfide-S-oxides. *General Physiology and Biophysics*, *21*, 65–72.

Govindaraju, K., Govindaraju, V., & Eidelman, D. H. (2003). A analysis of glutathione in rat airway surface liquid by capillary zone electrophoresis with conductivity detection. *Journal of Chromatography B*, *788*, 369–376. DOI: 10.1016/s1570-0232(03)00061-8.

Hrabárová, E., Gemeiner, P., & Šoltés, L. (2007). Peroxynitrite: in vivo and in vitro synthesis and oxidant degradative action on biological systems regarding biomolecular injury and inflammatory processes. *Chemical Papers*, *61*, 417–437. DOI: 10.2478/s11696-007-0058-8.

Kładna, A., Aboul-Enein, H. Y., Kruk, I., Michalska, T., & Lichszteld, K. (2006). Anti-oxidant and pro-oxidant behaviour of bucillamine. *Luminescence*, *21*, 90–97. DOI: 10.1002/bio.890.

Lima, A. I. G., Corticeiro, S. C., & Figueira, E. M. A. P. (2006). Glutathione-mediated cadmium sequestration in *Rhizobium leguminosarum*. *Enzyme and Microbial Technology*, *39*, 763–769. DOI: 10.1016/j.enzmictec.2005.12.009.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, *613*, 1–19. DOI: 10.1016/j.aca.2008.02.047.

Mazor, D., Greenberg, L., Shamir, D., Meyerstein, D., & Meyerstein, N. (2006). Antioxidant properties of bucillamine: possible mode of action. *Biochemical and Biophysical Research Communications*, *349*, 1171–1175. DOI: 10.1016/j.bbrc.2006.08.155.

Meyer, K., & Palmer, J. W. (1934). The polysaccharide of the vitreous humor. *Journal of Biological Chemistry*, *107*, 629–634.

Moskaug, J. Ø., Carlsen, H., Myhrstad, M. C. W., & Blomhoff, R. (2005). Polyphenols and glutathione synthesis regulation. *American Journal of Clinical Nutrition*, *81*, 277S–283S.

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: The interplay. *BioMed Research International*, *2014*, 1–19. DOI: 10.1155/2014/761264.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*, 1231–1237. DOI: 10.1016/s0891-5849(98)00315-3.

Scigelova, M., Green, P. S., Giannakopulos, A. E., Rodger, A., Crout, D. H. G., & Derrick, P. J. (2001). A practical protocol for the reduction of disulfide bonds in proteins prior to analysis by mass spectrometry. *European Journal of Mass Spectrometry*, *7*, 29–34.

Šoltés, L., Stankovská, M., Brezová, V., Schiller, J., Arnhold, J., Kogan, G., & Gemeiner, P. (2006). Hyaluronan degradation by copper(II) chloride and ascorbate: rotational viscometric, EPR spin-trapping, and MALDI-TOF mass spectrometric investigations. *Carbohydrate Research*, *341*, 2826–2834. DOI: 10.1016/j.carres.2006.09.019.

Valachová, K., Hrabárová, E., Dráfi, F., Juránek, I., Bauerová, K., Priesolová, E., Nagy, M., & Šoltés, L. (2010). Ascorbate and Cu(II)-induced oxidative degradation of high-molar-mass hyaluronan. Pro- and antioxidative effects of some thiols. *Neuroendocrinology Letters*, *31*, 101–104.

Ziouti, N., Triantaphyllidou, I. E., Assouti, M., Papageorgakopoulou, N., Kyriakopoulou, D., Anagnostides, S. T., & Vynios, D. H. (2004). Solid phase assays in glycoconjugate research: applications to the analysis of proteoglycans, glycosaminoglycans and metalloproteinases. *Journal of Pharmaceutical and Biomedical Analysis*, *34*, 771–789. DOI: 10.1016/s0731-7085(03)00565-x.