Glycoconjugate Journal 20, 133–141, 2004

Glycosaminoglycans reduce oxidative damage induced by copper (Cu^{+2}), iron (Fe^{+2}) and hydrogen peroxide (H_2O_2) in human fibroblast cultures

Giuseppe M. Campo[1], Angela D'Ascola[1], Angela Avenoso[1], Salvatore Campo[1], Alida M. Ferlazzo[2], Carmelo Micali[3], Laura Zanghì[1] and Alberto Calatroni[1]

[1] *Department of Biochemical, Physiological and Nutritional Sciences, School of Medicine, University of Messina, Policlinico Universitario, 98125 Messina, Italy,* [2] *Department of Morphology, Biochemistry, Physiology and Animal Production, School of Veterinary Medicine, University of Messina, Contrada Annunziata, 98168 Messina, Italy,* [3] *Haematologic Operative Unit, School of Medicine, University of Messina, Policlinico Universitario, 98125 Messina, Italy*

Acid glycosaminoglycans (GAGs) antioxidant activity was assessed in a fibroblast culture system by evaluating reduction of oxidative system-induced damage.

Three different methods to induce oxidative stress in human skin fibroblast cultures were used. In the first protocol cells were treated with $CuSO_4$ plus ascorbate. In the second experiment fibroblasts were exposed to $FeSO_4$ plus ascorbate. In the third system H_2O_2 was utilised.

The exposition of fibroblasts to each one of the three oxidant systems caused inhibition of cell growth and cell death, increase of lipid peroxidation evaluated by the analysis of malondialdehyde (MDA), decrease of reduced glutathione (GSH) and superoxide dismutase (SOD) levels, and rise of lactate dehydrogenase activity (LDH).

The treatment with commercial GAGs at different doses showed beneficial effects in all oxidative models. Hyaluronic acid (HA) and chondroitin-4-sulphate (C4S) exhibited the highest protection. However, the cells exposed to $CuSO_4$ plus ascorbate and $FeSO_4$ plus ascorbate were better protected by GAGs compared to those exposed to H_2O_2.

These outcomes confirm the antioxidant properties of GAGs and further support the hypothesis that these molecules may function as metal chelators.

Published in 2004.

Keywords: **glycosaminoglycans, lipid peroxidation, fibroblasts, oxidative stress, free radicals, cellular cultures, antioxidants**

Introduction

Glycosaminoglycans (GAGs) are a family of acid polysaccharides that display a variety of fundamental biological roles [1,2]. The typical GAGs structure consists of alternating units of uronic acid and hexosamine. Except for hyaluronic acid (HA), GAGs also contain sulphate groups. There are two major classes of sulphated GAGs distinguishing by the nature of hexosamine units: (a) the glucosamine-containing heparan sulphate family that includes heparan sulphates (HS); and (b) the galactosamine-containing chondroitin sulphate family including chondroitin sulphates (C4S and C6S) and dermatan sulphate (DS). A further GAG is keratan sulphate (KS) containing galactose (instead of uronic acid) and *N*-acetylglucosamine. Except for unsulphated HA, the GAG structural complexity is further compounded by sequence heterogeneity, caused primarily by the variation of degree and position of sulphate groups and by covalent binding to different core proteins to give proteoglycans (PGs) [1,2]. The structural diversity of GAGs and PGs poses significant challenges in the field of glycobiology. The polysaccharides affect proteoglycan core proteins interaction and are responsible for many aspects of their biological activity. GAGs are located on the surface of all higher animal cells, in the extracellular matrix of connective tissue and in basement membranes. GAGs function both as structural molecules

To whom correspondence should be addressed: Giuseppe M. Campo, PhD Department of Biochemical, Physiological and Nutritional Sciences, School of Medicine, University of Messina, Policlinico Universitario, Torre Biologica, 5° piano, Via C. Valeria, 98125 Messina, Italy. Tel: +39 90 221 3334; Fax: +39 90 221 3330; E-mail: gcampo@unime.it

and as scaffold structures binding a wide variety of protein ligands through GAG-protein and protein-protein interactions [3].

Significant increases with respect to normal values of plasma GAG concentration were observed in patients with systemic lupus erythematosus [4], rheumatoid arthritis [5], and liver disease [6]. The obvious explanation is that GAGs originate from the metabolism of inflamed tissues. Nevertheless, the exact meaning of their rise is at the moment unclear.

It is widely known that the generation of free radicals and other reactive oxygen species (ROS) play a key role in a large number of pathologies, including rheumatoid arthritis [7], diabetes [8], ischaemia and reperfusion [9], ulcerative colitis [10], liver disease [11], atherosclerosis [12] etc. These reactive molecules are formed during normal aerobic metabolism in cells, and following phagocyte activation during infection/inflammation; a consequence of uncontrolled production of free radicals is damage to biomolecules leading to altered function and disease [13]. Endogenous defence mechanisms have been identified which use antioxidants or free radical scavengers to neutralise reactive species-generated lipid peroxidation; however, the extensive generation of free radicals appears to overwhelm the natural defence mechanisms, dramatically reducing the levels of endogenous antioxidants [14].

In the last years, many findings evidenced antioxidant properties of GAGs (particularly for HA and CS) both *in vitro* and *in vivo* experimental models [15–18]. A plausible hypothesis about this antioxidant mechanism of GAGs is that they may bind the transition metal ions as Cu^{++} or Fe^{++} that are in turn responsible for the initiation of Fenton's reaction [15,16].

Starting from these assumptions, the aim of this study was to evaluate the ability of commercial GAGs in limiting cell damage in three different models of oxidative stress in human skin fibroblast cultures.

Materials and methods

Materials

Dulbecco's Minimal Essential Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomicyn, trypsin-EDTA solution and phosphate buffered saline (PBS) were obtained from GibcoBRL (Grand Island, NY, USA). All cell culture plastics were obtained from Falcon (Oxnard, CA, USA). Bathocuproine disulphonate (BCS), Deferoxamine mesylate (DFOM), Ascorbic acid, CuSO4, $FeSO_4$, Sucrose, ethylenediaminetetracetic acid (EDTA), Potassium phosphate, Butylated hydroxytoluene (BHT), Trypan blue, Catalase, Reduced nicotinamide adenine dinucleotide (NADH), Sodium pyruvate, Hyaluronic acid, Chondroitin-4-sulphate, Chondroitin-6-sulphate (C6S), Heparan sulphate, Dermatan sulphate, Keratan sulphate and all other general laboratory chemicals were obtained from Sigma-Aldrich S.r.l. (Milan, Italy).

Cell culture

Normal human skin fibroblasts type CRL 2056 were obtained from American Type Culture Collection (Promochem, Teddington U.K.). Fibroblasts were cultured in 75 cm^2 plastic flasks containing 15 ml of DMEM supplemented with 10% FBS, L-glutamine (2.0 mM) and penicillin/streptomycin (100 U/ml, 100 μg/ml), and incubated in an incubator (mod. Galaxy B, RS Biotech, U.K.) at 37°C in humidified air with 5% CO_2. Cells were used between the eleventh and the 20th passage. Their population doubling time and their plate efficiency were about 48 h and 80% respectively.

Oxidative stress

Fibroblasts were cultured into six-well culture plates at a density of 1.3×10^5 cells/well. 12 h after plating (time 0), when cells were firmly attached to the substratum (about 1×10^5 cells/well), the culture medium was replaced by 2 ml of the same fresh medium containing HA, or C4S, or C6S, or HS, or DS, or KS in concentrations of 0.5, 1.0 and 2.0 mg/ml. Fibroblasts were also incubated with BCS, DFOM or catalase in the same way as with GAGs. After 4 h of incubation, oxidative stress was induced in the cells by three different ways: (1) In the first experiment 10 μl of 100 μM $CuSO_4$ were added in a series of wells (final concentration 0.5 μM) pretreated with GAGs as described above. Then, 15 min after, 10 μl of 200 mM ascorbic acid were added, to final concentration of 1.0 mM, in order to induce free radical production [19]. 10 μl of 400 μM BCS were added (final concentration 2.0 μM) as chelating agent for Cu^{++} [20]. (2) In the second experiment 10 μl of 400 μM $FeSO_4$ were added in an other series of wells (final concentration 2.0 μM) pretreated with GAGs. Then, 15 min after, 10 μl of 200 mM ascorbic acid were added for free radical production [21]. 10 μl of 1.0 mM DFOM were used (final concentration 5.0 μM) as chelating agent for Fe^{++}. (3) In the third experiment 10 μl of 400 mM H_2O_2 were added to a further series of wells (final concentration 200 μM) in order to induce directly the oxidative cell damage [22]. 10 μl containing 2,000 U/ml of catalase were used as a scavenger agent for H_2O_2. After 1.5 h, in all experiments, the medium was discarded and replaced by 2 ml of the same fresh medium. 24 h later cells were subjected to morphological and biochemical evaluation.

Cell viability assay

24 h after oxidative stress, cells viability was determined under photozoom invertite microscope (Cambridge Instruments, U.K.) connected with a digital camera (mod. X-300, Minolta, Osaka, Japan). The exact number of surviving cells was then evaluated by Trypan blue dye exclusion test [23]. Briefly, after 5 min incubation live cells excluded the dye, whereas dead cells were stained; the number of cells excluding the dye was expressed as a percentage counted from several randomly chosen areas of each well.

Malondialdehyde determination

Measurement of malondialdehyde in the cell lysate samples was performed to estimate the extension of lipid peroxidation in the fibroblast cultures. 4–5 $\times$ 10^6 cell samples obtained 24 h after oxidative stress induction were collected in 500 μl of PBS containing 200 μM butylated hydroxytoluene and were frozen at -80°C until the assay. The day of analysis, after thawing, cell samples were centrifuged at 500 $\times$ g for 5 min at 4°C. The pellet was resuspended and sonicated in 250 μl of sterile H_2O (Transsonic Model 420, Elma instrumentation, Germany). Lipid peroxidation evaluation was carried out according to the manufacturer's protocol of a colorimetric commercial kit (Lipid peroxidation assay kit, cat.n°437634, Calbiochem-Novabiochem Corporation, USA). Briefly, 0.65 ml of 10.3 mM *N*-methyl-2-phenyl-indole in acetonitrile were added to 0.2 ml of sonicated pellet. After vortexing for 3–4 s and addition 0.15 ml of 37% HCl, samples tubes were carefully mixed, closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard malondialdehyde solution (from 0 to 64 nmol/ml) was also run for quantification. The concentration of malondialdehyde in cell samples was expressed as nmol/mg protein.

Reduced glutathione assessment

Fibroblasts (4–5 $\times$ 10^6), obtained 24 h after oxidative stress induction, were collected in 500 μl of PBS and frozen at -80°C until the assay. The biochemical analysis was performed by using a specific colorimetric assay (Bioxytech GSH-400 assay kit, cat n° 21011, OxisResearch, Portland, OR, USA). Briefly, after thawing, cell samples were centrifuged at 2,500 $\times$ g for 5 min at 4°C. The pellet was resuspended and sonicated in 500 μl of 5% metaphosphoric acid, at 4°C. Then, each sample was mixed and centrifuged at 3,000 $\times$ g for 10 min at 4°C. An aliquot of supernatant (0,2 ml) was added in polyethylene tube containing 0.7 ml of potassium phosphate buffer containing diethylenetriamine pentaacetic acid and lubrol. After vortexing, 50 μl of 4-chloro-1-methyl-7-trifluoromethyl-quinolinum methylsulfate in HCl were added. The samples were vortexed again and 50 μl of 30% NaOH were added. After vortexing the samples were incubated in the dark for 10 min at 25°C. Then the absorbance was read at 400 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of glutathione. The amount of fibroblast glutathione was expressed as nmol/mg protein.

Superoxide dismutase evaluation

Fibroblasts (4–5 $\times$ 10^6), obtained 24 h after oxidative stress induction, were collected in 500 μl of PBS and centrifuged at 1,000 $\times$ g for 5 min at 4°C. Then, the pellet was resuspended and sonicated in 250 μl ice-cold 0.25 M sucrose containing 1 mM diethylenetriamine pentaacetic acid. After centrifugation at 20,000 $\times$ g for 20 min at 4°C, the supernatant of each sample was collected and the total SOD activity was assayed spectrophotometrically at 505 nm by using a commercial kit (Ransod assay kit, cat. N° Sd 125, Randox Laboratories, Crumlin, U.K.). Briefly, 50 μl of diluted samples (1:10, v:v with 0.01 M potassium phosphate buffer, pH 7.0) were mixed with 1.7 ml of solution containing 0.05 mM xantine and 0.025 mM iodonitrotetrazolium chloride. After mixing for 5 s, 250 μl of xantine oxidase (80 U/l) were added. Then, initial absorbance was read and the final absorbance was read after additional 3 min. A standard curve of commercial SOD solution (from 0 to 320 U/ml) was run for quantitation. All standards and diluted sample rates were converted into percentage of buffer diluent rate and subtracted from 100% to give a percentage inhibition. Sample SOD activities were obtained from a plotted curve of the percentage inhibition for each standard. SOD values were expressed as units/mg protein.

Lactate dehydrogenase assay

24 h after oxidative stress induction, the culture medium was collected, centrifuged at 10,000 $\times$ g for 10 min at 4°C in order to remove debris and then frozen at -80°C until assay. In order to estimate total LDH, cells (4–5 $\times$ 10^6) were also collected in 500 μl of PBS, and after centrifugation at 500 $\times$ g for 5 min at 4°C, were sonicated in Triton X-100. An aliquot of the supernatant was used for the assay. LDH evaluation was performed by using a published method [24] with some modifications. Briefly, after thawing, 50 μl of sample were mixed with 100 μl of 2.0 mM NADH and 850 μl of 20 mM phosphate buffer pH 7.4. After mixing for 5 s, duplicate aliquots (200 μl) of each sample were placed into 96-well plates at room temperature and reaction was initiated by addition of 20 μl of 3.3 mM sodium pyruvate. The rate of disappearance of NADH was measured at 340 nm by using a plate reader (DAS srl, Rome, Italy). The values of unknown samples were drawn from a standard curve plotted by assaying different known concentration of LDH. The percentage of release was determined by dividing the LDH activity in the medium by total LDH activity.

Protein determination

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Lab., Richmond, CA, USA) and bovine serum albumin as a standard according to the published method [25].

Statistical analysis

Data are expressed as means $\pm$ S.D. of at least seven experiments for each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was performed by one-way analysis of variance (ANOVA). The statistical significance of differences was set at $p < 0.05$.

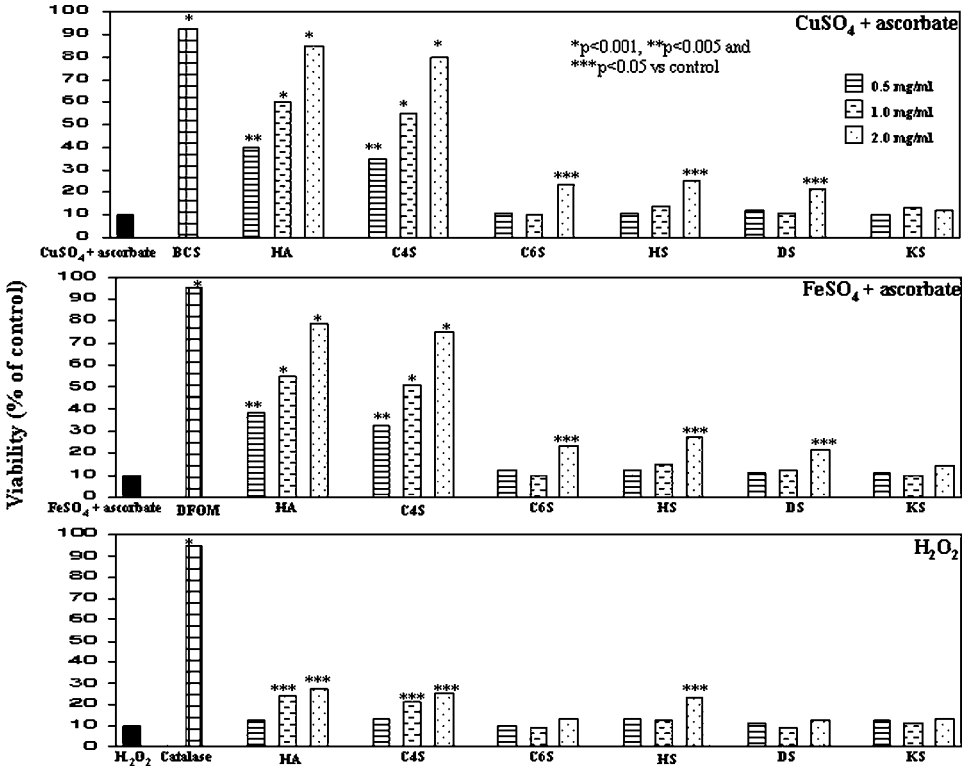

Figure 1. Effect of GAGs on fibroblast viability (% of control) in the three considered models of oxidative stress. Values are the mean ± S.D. of seven experiments.

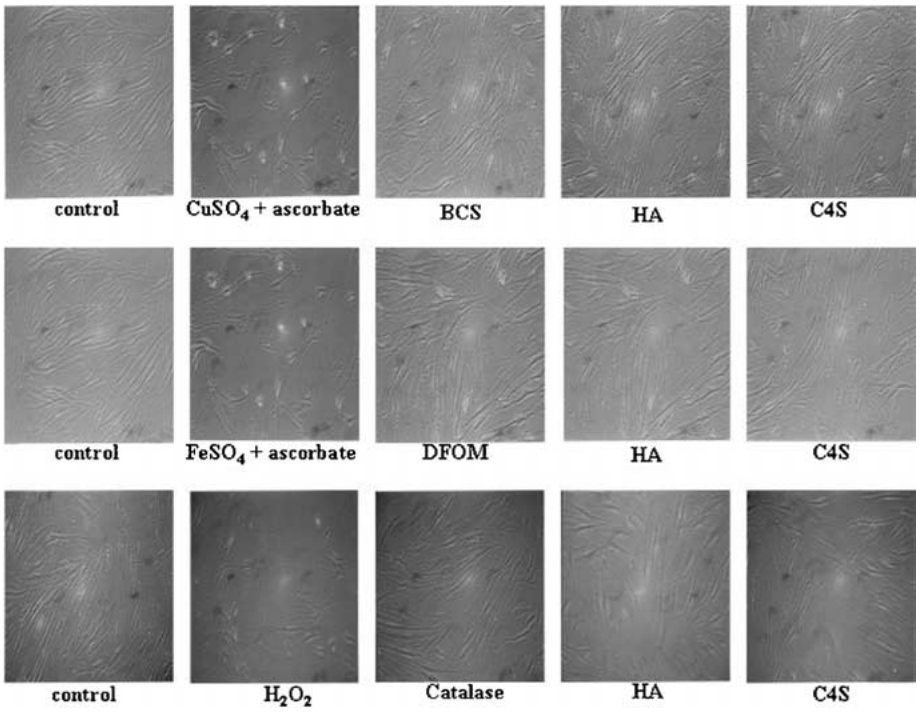

Figure 2. Microscopic analysis of surviving fibroblasts in wells exposed to the three oxidative models and effects of GAG treatment. Pictures reported are related to the treatment with the highest dose of GAGs.

Table 1. Effect of GAGs on fibroblast lipid peroxidation (malondialdehyde) in the three considered models of oxidative stress

Treatment		*0.5 mg/ml*	*1.0 mg/ml*	*2.0 mg/ml*
Control	0.12 ± 0.08			
$CuSO_4$ + ascorbate + vehicle	$2.51 \pm 0.46^{\circ}$			
$CuSO_4$ + ascorbate + BCS	$0.64 \pm 0.15^{*}$			
$CuSO_4$ + ascorbate + HA		$1.41 \pm 0.23^{*}$	$1.36 \pm 0.25^{*}$	$1.13 \pm 0.27^{*}$
$CuSO_4$ + ascorbate + C4S		$1.62 \pm 0.26^{*}$	$1.41 \pm 0.24^{*}$	$1.32 \pm 0.31^{*}$
$CuSO_4$ + ascorbate + C6S		2.53 ± 0.53	2.35 ± 0.48	$1.83 \pm 0.31^{**}$
$CuSO_4$ + ascorbate + HS		2.24 ± 0.42	2.15 ± 0.47	$1.80 \pm 0.36^{**}$
$CuSO_4$ + ascorbate + DS		2.52 ± 0.58	2.43 ± 0.62	$1.85 \pm 0.30^{**}$
$CuSO_4$ + ascorbate + KS		2.44 ± 0.53	2.35 ± 0.49	2.48 ± 0.52
Control	0.13 ± 0.06			
$FeSO_4$ + ascorbate + vehicle	$2.62 \pm 0.53^{\circ}$			
$FeSO_4$ + ascorbate + DFOM	$0.74 \pm 0.23^{*}$			
$FeSO_4$ + ascorbate + HA		$1.61 \pm 0.26^{*}$	$1.52 \pm 0.27^{*}$	$1.35 \pm 0.34^{*}$
$FeSO_4$ + ascorbate + C4S		$1.68 \pm 0.21^{*}$	$1.62 \pm 0.25^{*}$	$1.51 \pm 0.28^{*}$
$FeSO_4$ + ascorbate + C6S		2.43 ± 0.43	2.31 ± 0.48	$1.90 \pm 0.28^{**}$
$FeSO_4$ + ascorbate + HS		2.58 ± 0.54	2.33 ± 0.56	$1.94 \pm 0.22^{**}$
$FeSO_4$ + ascorbate + DS		2.66 ± 0.46	2.57 ± 0.53	$1.93 \pm 0.19^{**}$
$FeSO_4$ + ascorbate + KS		2.78 ± 0.48	2.64 ± 0.51	2.51 ± 0.39
Control	0.11 ± 0.06			
H_2O_2 + vehicle	$2.73 \pm 0.61^{\circ}$			
H_2O_2 + Catalase	$0.64 \pm 0.24^{*}$			
H_2O_2 + HA		2.51 ± 0.46	$1.95 \pm 0.22^{**}$	$1.86 \pm 0.32^{**}$
H_2O_2 + C4S		2.70 ± 0.51	$1.98 \pm 0.20^{**}$	$1.96 \pm 0.21^{**}$
H_2O_2 + C6S		2.64 ± 0.63	2.75 ± 0.54	2.58 ± 0.56
H_2O_2 + HS		2.53 ± 0.47	2.46 ± 0.41	$2.00 \pm 0.11^{**}$
H_2O_2 + DS		2.73 ± 0.38	2.56 ± 0.42	2.68 ± 0.52
H_2O_2 + KS		2.51 ± 0.36	2.43 ± 0.39	2.44 ± 0.28

Values are the mean ± S.D. of 7 different experiments and are expressed as nmol/mg protein. $^{\circ}p < 0.001$ vs. control; $^{*}p < 0.001$ and $^{**}p < 0.01$ vs. vehicle.

Results

Effects of GAGs on cell viability

The exposition of fibroblasts to $CuSO_4$, $FeSO_4$ and H_2O_2 produced a large mortality and growth inhibition as showed in Figures 1 and 2. The percent of cell viability ranged about 10% in all used models (Figure 1). The treatment with GAGs exerted a protective effect in the two models in which free radicals production was induced by using the transition metals. HA and C4S protected cells in a dose-dependent way (Figures 1 and 2 with the highest dose); C6S, HS and DS exerted a slight effect with the highest dose only (Figure 1 with the highest dose); no significant protection was observed by treatment with KS (Figure 1). In the model exposed to H_2O_2, only a slight protective effect was found to be exerted by HA, C4S (with the dose of 1.0 and 2.0 mg/ml) and by HS (with the dose of 2.0 mg/ml) treatment (Figure 1). The use of chelating agents (BCS and DFOM) or a natural scavenger agent (catalase) inhibited the production of free radicals and prevented cell destruction (Figures 1 and 2). In fact, both BCS and DFOM that bound Cu^{++} and Fe^{++} ions, respectively and catalase that neutralises H_2O_2 protected about 80% of cells (Figure 1).

Lipid peroxidation analysis

Determination of malondialdehyde was performed to estimate the degree of free radical production on cell culture (Table 1). Low levels of malondialdehyde were found in the control wells and these values were considered physiological. In contrast, a significant increase in malondialdehyde production was seen in all considered models. In the wells exposed to the transition metals, HA and C4S exerted the better protection in a dose dependent manner, while C6S, HS and DS protected fibroblasts with the highest dose only (Table 1). The cells exposed to H_2O_2 were slightly protected by HA, C4S and HS. No effect was found in all models by the treatment with KS. The maximum effect was achieved with the use of the neutralising substrates BCS, DFOM and catalase.

Antioxidant status

The concentration of glutathione and SOD were assayed in order to evaluate the antioxidant balance after free radical production (Tables 2 and 3, respectively). In the control wells, glutathione and SOD ranged between 5.0–9.0 nmol/mg protein, and 21.0–36.0 U/mg protein, respectively, and these values were

Table 2. Effect of GAGs on fibroblast glutathione activity in the three considered models of oxidative stress

Treatment		*0.5 mg/ml*	*1.0 mg/ml*	*2.0 mg/ml*
Control	7.14 ± 2.17			
$CuSO_4$ + ascorbate + vehicle	2.60 ± 0.53°			
$CuSO_4$ + ascorbate + BCS	6.45 ± 2.21*			
$CuSO_4$ + ascorbate + HA		4.53 ± 1.04*	4.63 ± 1.10*	4.72 ± 1.15*
$CuSO_4$ + ascorbate + C4S		4.42 ± 0.92*	4.51 ± 0.98*	4.60 ± 1.08*
$CuSO_4$ + ascorbate + C6S		3.32 ± 0.84	3.26 ± 0.91	3.52 ± 0.68**
$CuSO_4$ + ascorbate + HS		3.21 ± 0.76	3.24 ± 0.81	3.56 ± 0.76**
$CuSO_4$ + ascorbate + DS		3.11 ± 0.71	3.21 ± 0.76	3.61 ± 0.82**
$CuSO_4$ + ascorbate + KS		3.23 ± 0.85	3.12 ± 0.78	3.25 ± 0.83
Control	6.92 ± 1.87			
$FeSO_4$ + ascorbate + vehicle	2.51 ± 0.47°			
$FeSO_4$ + ascorbate + DFOM	5.89 ± 1.53*			
$FeSO_4$ + ascorbate + HA		4.38 ± 1.03*	4.47 ± 1.10*	4.56 ± 1.08*
$FeSO_4$ + ascorbate + C4S		4.35 ± 0.96*	4.40 ± 1.05*	4.48 ± 1.09*
$FeSO_4$ + ascorbate + C6S		3.11 ± 0.84	3.24 ± 0.88	3.48 ± 0.75**
$FeSO_4$ + ascorbate + HS		3.21 ± 0.94	3.15 ± 0.87	3.53 ± 0.86**
$FeSO_4$ + ascorbate + DS		3.11 ± 0.89	3.18 ± 0.89	3.42 ± 0.70**
$FeSO_4$ + ascorbate + KS		3.04 ± 0.91	2.94 ± 0.78	3.12 ± 0.87
Control	6.97 ± 1.56			
H_2O_2 + vehicle	2.67 ± 0.61°			
H_2O_2 + Catalase	6.22 ± 1.76*			
H_2O_2 + HA		3.82 ± 1.37	3.96 ± 1.38**	4.08 ± 1.42**
H_2O_2 + C4S		3.73 ± 1.31	4.16 ± 1.45**	4.20 ± 1.40**
H_2O_2 + C6S		3.21 ± 1.24	3.37 ± 1.15	3.69 ± 1.32
H_2O_2 + HS		3.27 ± 1.09	3.46 ± 1.21	3.85 ± 1.26**
H_2O_2 + DS		3.05 ± 1.06	3.16 ± 1.11	3.32 ± 1.24
H_2O_2 + KS		3.04 ± 1.14	2.94 ± 0.97	3.13 ± 1.02

Values are the mean ± S.D. of 7 different experiments and are expressed as nmol/mg protein. °$p < 0.001$ vs. control; *$p < 0.001$ and **$p < 0.05$ vs. vehicle.

considered physiological. In contrast, a significant reduction in both antioxidants was observed in all used models. Also in this case HA and C4S restored the two endogenous antioxidants in wells where free radicals production was induced by transition metals. C6S, HS and DS slightly restored glutathione and SOD with the highest dose. In the model in which fibroblasts were exposed to H_2O_2, HA and C4S spared antioxidants with the dose of 1.0 and 2.0 mg/ml, while HS was effective with the highest dose only. The treatment with KS did not produce any beneficial effect. Also in this case, the maximum effect was achieved with the use of the neutralising substrates BCS, DFOM and catalase.

Cytotoxicity evaluation

LDH activity was determined as index of cytotoxicity in fibroblasts cultures (Table 4). The release of LDH in unexposed wells was about 10% and this percentage was considered physiological. In contrast, a marked increase in this enzyme was observed in the three considered models. The treatment with HA and C4S reduced cytotoxicity in wells where free radicals production was induced by transition metals. C6S, HS and DS slightly decreased LDH activity with the highest dose. In the model where fibroblasts were exposed to H_2O_2, HA and C4S showed significant effects with the dose of 1.0 and 2.0 mg/ml, while HS was effective with the dose of 2.0 mg/ml only. BCS, DFOM and catalase blunted the detrimental effect of $CuSO_4$, $FeSO_4$ and H_2O_2, respectively.

Discussion

There is now considerable evidence of the participation of reactive oxygen species and other free radicals in human disease states [26,27]. Oxidative stress in cells and tissues is thought to be a direct result of increased generation of superoxide radicals. When excess superoxide reacts with SOD, large amounts of intracellular hydrogen peroxide are produced. Neither superoxide nor H_2O_2 is highly toxic; however, in the presence of metal ion these species are converted to hydroxyl radicals through a Fenton's reaction or Haber-Weiss reaction [28]. The presence of ascorbate also contributes to enhance the production of the detrimental $OH^{\bullet}$ [29]. Moreover, lipid hydroperoxides which are formed as primary products of lipid peroxidation react rapidly with transition metals like iron and copper ions to generate alkoxyl or peroxyl radicals [30]. GAGs are linear acid polysaccharides composed of alternating hexuronic acid

Table 3. Effect of GAGs on fibroblast SOD activity in the three considered models of oxidative stress

Treatment		*0.5 mg/ml*	*1.0 mg/ml*	*2.0 mg/ml*
Control	30.04 ± 6.71			
$CuSO_4$ + ascorbate + vehicle	11.06 ± 3.13°			
$CuSO_4$ + ascorbate + BCS	23.10 ± 4.25*			
$CuSO_4$ + ascorbate + HA		18.14 ± 3.72**	18.95 ± 4.15**	20.00 ± 4.43**
$CuSO_4$ + ascorbate + C4S		17.19 ± 3.31**	18.01 ± 3.87**	19.33 ± 4.27**
$CuSO_4$ + ascorbate + C6S		10.16 ± 2.83	11.72 ± 2.65	15.11 ± 3.58***
$CuSO_4$ + ascorbate + HS		11.62 ± 2.74	12.36 ± 2.89	14.84 ± 3.29***
$CuSO_4$ + ascorbate + DS		12.63 ± 2.48	12.01 ± 2.75	14.95 ± 3.17***
$CuSO_4$ + ascorbate + KS		12.17 ± 2.88	11.42 ± 2.75	10.86 ± 2.47
Control	29.13 ± 6.48			
$FeSO_4$ + ascorbate + vehicle	7.85 ± 2.82°			
$FeSO_4$ + ascorbate + DFOM	21.42 ± 4.87*			
$FeSO_4$ + ascorbate + HA		13.37 ± 3.09**	14.54 ± 3.38**	15.43 ± 3.81**
$FeSO_4$ + ascorbate + C4S		13.34 ± 2.89**	14.71 ± 3.24**	15.37 ± 3.97**
$FeSO_4$ + ascorbate + C6S		7.56 ± 2.21	7.92 ± 2.37	11.24 ± 1.88***
$FeSO_4$ + ascorbate + HS		7.73 ± 1.94	7.21 ± 1.87	10.96 ± 1.71***
$FeSO_4$ + ascorbate + DS		7.43 ± 2.11	8.36 ± 1.91	10.82 ± 1.63***
$FeSO_4$ + ascorbate + KS		7.48 ± 1.59	6.83 ± 1.74	7.64 ± 1.83
Control	26.89 ± 5.87			
H_2O_2+ vehicle	6.53 ± 2.11°			
H_2O_2 + Catalase	22.38 ± 4.75*			
H_2O_2 + HA		7.22 ± 1.78	8.87 ± 1.29***	9.34 ± 1.81***
H_2O_2 + C4S		7.11 ± 1.56	8.62 ± 1.32***	9.53 ± 1.96***
H_2O_2 + C6S		6.37 ± 1.54	6.71 ± 1.61	7.13 ± 1.29
H_2O_2 + HS		5.81 ± 1.14	7.34 ± 1.51	9.38 ± 1.51**
H_2O_2 + DS		6.43 ± 1.26	6.97 ± 1.18	7.34 ± 1.56
H_2O_2 + KS		5.73 ± 1.43	6.94 ± 1.49	7.27 ± 1.38

Values are the mean ± S.D. of 7 different experiments and are expressed as U/mg protein. $^{\circ}p < 0.001$ vs. control; $^{*}p < 0.001$, $^{**}p < 0.005$ and $^{***}p < 0.05$ vs. vehicle.

and hexosamine units. These compounds interact with a number of proteins, and act as cellular organizers [31]. The GAG molecules that are present in blood could function as carriers/modulators for agents that regulate the functions of adjacent cells. Since some GAGs possess antioxidant activity capable of inhibiting lipid peroxidation [15–18] and the use of these compound as therapeutic agents showed some positive outcomes [32–34], in the present study we investigated the antioxidant effect of GAGs in a simple culture system of human fibroblasts where oxidative stress is induced.

The data obtained in fibroblasts treated with different GAGs showed positive effects in all parameters evaluated in order to measure oxidative damage. Only KS treatment did not exert any significant effect, while HA and C4S treatments were effective also at low doses. In both models following exposure to $CuSO_4$ and $FeSO_4$, only cells receiving the highest dose of C6S, HS or DS exhibited a slightly significant effect. Moreover, in the model where cells were exposed to H_2O_2, the effect exerted by HA and C4S was less marked than the effect exerted in the two other models. In this case, C6S and DS failed to produce any protective effect, while only the highest dose HS reduced cellular damage at low significance level.

Lipid peroxidation was evaluated by means of the widely used malondialdehyde analysis [35]. The increase of malondialdehyde levels found in the three models of cell exposition to oxidant agents is consistent with a general occurrence of a free-radical-mediated cell damage. The treatment with GAGs limited membrane lipid peroxidation and consequently cell death as reported by cell viability data.

The massive production of reactive species that occurs in fibroblast cultures decreases glutathione and SOD concentrations as a consequence of their consumption during oxidative damage [36]. This reduction contributes to cellular destruction by favouring free radical attack. The treatment of cells with GAGs limited glutathione and SOD consumption suggesting reduction of free radical generation.

High LDH activity levels in the medium may be interpreted as a progression of cell injury because of its intracellular localisation. The decrease of LDH release after GAG treatment may be a consequence of free radical reduction.

The way by which GAGs reduce cellular damage against free radical overproduction is similar to that exerted by typical chelating agents. A chelating mechanism is then suggested. Among the most effective GAGs, HA, the only non-sulphated

Table 4. Effect of GAGs on fibroblast LDH activity in the three considered models of oxidative stress

Treatment		*0.5 mg/ml*	*1.0 mg/ml*	*2.0 mg/ml*
Control	9.81 ± 2.45			
$CuSO_4$ + ascorbate + vehicle	91.12 ± 13.36°			
$CuSO_4$ + ascorbate + BCS	35.13 ± 6.28*			
$CuSO_4$ + ascorbate + HA		68.73 ± 9.34**	54.62 ± 8.24*	50.01 ± 7.87*
$CuSO_4$ + ascorbate + C4S		70.11 ± 8.31**	68.19 ± 9.63**	57.66 ± 7.21*
$CuSO_4$ + ascorbate + C6S		89.23 ± 9.24	84.21 ± 9.36	76.13 ± 8.46***
$CuSO_4$ + ascorbate + HS		85.44 ± 9.18	83.89 ± 9.87	75.68 ± 7.93***
$CuSO_4$ + ascorbate + DS		92.32 ± 10.31	85.15 ± 9.42	77.09 ± 8.17***
$CuSO_4$ + ascorbate + KS		87.45 ± 9.39	86.19 ± 9.45	91.52 ± 10.16
Control	10.87 ± 2.93			
$FeSO_4$ + ascorbate + vehicle	90.65 ± 12.87°			
$FeSO_4$ + ascorbate + DFOM	33.72 ± 5.42*			
$FeSO_4$ + ascorbate + HA		66.33 ± 9.76**	58.94 ± 8.17*	55.12 ±7.45*
$FeSO_4$ + ascorbate + C4S		67.72 ± 9.89**	61.65 ± 7.39*	57.64 ± 7.46*
$FeSO_4$ + ascorbate + C6S		88.76 ± 10.21	89.51 ± 9.74	78.27 ± 7.17***
$FeSO_4$ + ascorbate + HS		91.08 ± 10.51	90.15 ± 10.47	77.21 ± 7.81***
$FeSO_4$ + ascorbate + DS		92.36 ± 11.24	89.52 ± 10.35	78.13 ± 6.94***
$FeSO_4$ + ascorbate + KS		90.21 ± 10.47	93.10 ± 10.39	89.45 ± 11.13
Control	10.17 ± 2.68			
H_2O_2 + vehicle	89.32 ± 13.15°			
H_2O_2 + Catalase	27.86 ± 5.19*			
H_2O_2 + HA		87.39 ± 10.43	76.64 ± 7.12***	77.31 ± 6.95***
H_2O_2 + C4S		86.15 ± 10.23	76.17 ± 7.01***	75.24 ± 7.82***
H_2O_2 + C6S		86.83 ± 9.15	90.95 ± 8.39	89.15 ± 7.93
H_2O_2 + HS		90.88 ± 9.47	88.37 ± 9.31	76.45 ± 6.94***
H_2O_2 + DS		92.56 ± 10.81	90.63 ± 9.34	87.94 ± 8.65
H_2O_2 + KS		85.52 ± 9.72	83.56 ± 9.34	82.44 ± 10.21

Values are the mean ± S.D. of 7 different experiments and are expressed as % of the total LDH activity. $^{\circ}p < 0.001$ vs. control; $^{*}p < 0.001$, $^{**}p < 0.005$ and $^{***}p < 0.05$ vs. vehicle.

compound, and C4S bearing an ester sulphate group on the 4 position of the aminosugar. However, although so different, the two GAGs have a similar secondary structure, with carboxylic groups in the same spatial position; these charged groups may interact with the transition metals ions like Cu^{++} or Fe^{++} [37,38] that are in turn responsible of the initiation of Fenton's reaction in the same way in both GAGs. The interaction limits, likely by a chelation mechanism, the availability of dangerous cations. In C4S chelating activity may be reinforced by the presence of sulphated group on the 4 position in the opposite side of the carboxylic group. In the other GAGs, the different orientation of sulphated groups (C6S contains a ester sulphate group at position 6 of the aminosugar, and HS contains a *N*-sulphated group at position 2 of the aminosugar), or of carboxylic groups (DS, that contains a sulphated group in position 4 of the aminosugar as C4S, also contains L-iduronic acid, that is the epimer at carbon 5 of D-glucuronic acid) clearly limits the interaction with cations. The slight effect obtained with C6S and DS, in the model exposed to $CuSO_4$ and $FeSO_4$, may be due to the presence of short segments of C4S chains contained in their structure. GAGs are indeed copolymers, that contain large segments of repeats of the typical disaccharide (hexuronic acid and hexosamine) unit that alternate with shorter segments of repeats of disaccharide units typical of other GAG chains. In fact, C6S and DS contain small amounts of C4S chain segments. Moreover, the treatment of fibroblasts with KS did not exert any significant effect since KS lacks carboxylic groups. The limited effect obtained after GAG treatment in the fibroblast cultures exposed to H_2O_2, may be due to the different mechanism that primes free radical production, not based on metal cations. This evidence gives further support to the hypothesis that GAGs act by chelating metal ions. The slight effect exerted by HA, C4S and HS could be due to a low scavenger activity of these GAG molecules.

In conclusion the data obtained by these experiments suggest that GAGs may function as metal chelators like the antioxidant BCS and DFOM, but further investigations are needed to confirm this hypothesis.

Acknowledgments

This study was supported by a grant ex 40% (COFIN 2000) of the MIUR, Italy.

References

1 Prydz K, Dalen KT, Synthesis and sorting of proteoglycans, *J Cell Sci* **113**, 193–205 (2000).
2 Templeton DM, Proteoglycans in cell regulation, *Crit Rev Clin Lab Sci* **29**, 141–84 (1992).
3 Iozzo RV, Matrix proteoglycans: From molecular design to cellular function, *Annu Rev Biochem* **67**, 609–52 (1998).
4 Parildar Z, Uslu R, Tanyalcin T, Doganavsargil E, Kutay F, The urinary excretion of glycosaminoglycans and heparan sulphate in lupus nephritis, *Clin Rheumatol* **21**, 284–8 (2002).
5 Shigemori M, Takei S, Imanaka H, Maeno N, Hokonohara M, Miyata K, Diagnostic significance of increased serum hyaluronic acid in juvenile rheumatoid arthritis, *Ped Intern* **44**, 394–9 (2002).
6 Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RWH, Zarski JP, Drouet E, Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption, *J Med Virol* **68**, 206–15 (2002).
7 Droge W, Free radicals in the physiological control of cell function, *Physiol Rev* **82**, 47–95 (2002).
8 Davison GW, George L, Jackson SK, Young IS, Davies B, Bailey DM, Peters JR, Ashton T, Exercise, free radicals, and lipid peroxidation in type 1 diabetes mellitus, *Free Rad Biol Med* **33**, 1543–51 (2002).
9 Anaya-Prado R, Toledo-Pereyra LH, Lentsch AB, Ward PA, Ischemia/reperfusion injury, *J Surg Res* **15**, 248–58 (2002).
10 Nieto N, Torres MI, Fernandez MI, Giron MD, Rios A, Suarez MD, Gil A, Experimental ulcerative colitis impairs antioxidant defense system in rat intestine, *Dig Dis Sci* **45**, 1820–7 (2000).
11 Albano E, Free radical mechanisms in immune reactions associated with alcoholic liver disease, *Free Rad Biol Med* **32**, 110–4 (2002).
12 Poredos P, Endothelial dysfunction in the pathogenesis of atherosclerosis, *Int Angiol* **21**, 109–16 (2002).
13 Evans P, Halliwell B, Free radicals and hearing. Cause, consequence, and criteria, *Ann N Y Acad Sci* **884**, 19–40 (1999).
14 Knight JA, Review: Free radicals, antioxidants, and the immune system, *Ann Clin Lab Sci* **30**, 145–58 (2000).
15 Presti D, Scott JE, Hyaluronan-mediated protective effect against cell damage caused by enzymatically produced hydroxyl (OH) radicals is dependent on hyaluronan molecular mass, *Cell Biochem Funct* **12**, 281–8 (1994).
16 Albertini R, De Luca G, Passi A, Moratti R, Abuja PM, Chondroitin-4-sulfate protects high-density lipoprotein against copper-dependent oxidation, *Arch Biochem Biophys* **365**, 143–9 (1999).
17 Arai H, Kashiwagi S, Nagasaka Y, Uchida K, Hoshii Y, Nakamura K, Oxidative modification of apoliproprotein E in human very-low-density lipoprotein and its inhibition by glycosaminoglycans, *Arch Biochem Biophys* **367**, 1–8 (1999).
18 Sela S, Shurtz-Swirski R, Shapiro G, Nasser L, Hamzi M, Shasha SM, Kristal B, Oxidative stress during hemodialysis: Effect of heparin, *Kidney Int* **78**, S153–S63 (2001).
19 Cuypers A, Vangronsveld J, Clijsters H, The chemical behavious of heavy metals plays a prominent role in the induction of oxidative stress, *Free Rad Res* **31**, S39–S43 (1999).
20 Sayre LM, Alzheimer's precursor protein and the use of bathocuproine for determining reduction of copper (II), *Science* **274**, 1933–4 (1996).
21 Collis CS, Yang M, Peach SJ, Diplock AT, Rice-Evans C, The effects of ascorbic acid and iron co-supplementation on the proliferation of 3T3 fibroblasts, *Free Rad Res* **25**, 87–93 (1996).
22 Gille JJ, Joenje H, Cell culture models for oxidative stress: Superoxide and hydrogen peroxide versus normobaric hyperoxia, *Mutat Res* **275**, 405–14 (1992).
23 Krischel V, Bruch-Gerharz D, Suschek C, Kroncke KD, Ruzicka T, Kolb-Bachofen V, Biphasic effect of exogenous nitric oxide on proliferation and differentiation in skin derived keratinocytes but not fibroblasts, *J Invest Dermatol* **111**, 286–91 (1998).
24 Bergmeyer HU, Bernt E, Hess B, Lactate dehydrogenase. In *Method of Enzymatic Analysis*, edited by Bergmeyer HU (Academic press, New York, 1963), vol. 2, pp. 574–9.
25 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem* **72**, 248–54 (1976).
26 Esterbauer H, Ramos P, Chemistry and pathophysiology of oxidation of LDL, *Rev Physiol Biochem Pharmacol* **127**, 31–64 (1996).
27 Halliwell B, Gutteridge JMC, *Free Radicals in Biology and Medicine*, 3rd ed. (Clarendon Press Oxford, 1999).
28 Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YMW, Marsh JP, Mossman BT, Differential regulation of antioxidant enzymes in response to oxidants, *J Biol Chem* **266**, 24398–403 (1991).
29 Nappi AJ, Vass E, Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols, *Biochim Biophys Acta* **1336**, 295–301 (1997).
30 Halliwell B, Gutteridge JMC, *Free Radicals in Biology and Medicine*, 2nd ed. (Oxford University Press, London, 1989).
31 Ruoslahti E, Yamaguchi Y, Proteoglycans as modulators of growth factor activities, *Cell* **64**, 867–9 (1991).
32 Graf J, Neusel E, Schneider E, Niethard FU, Intra-articular treatment with hyaluronic acid in osteoarthritis of the knee joint: A controlled clinical trial versus mucopolysaccharide polysulfuric acid ester, *Clin Exp Rheumatol* **11**, 367–72 (1993).
33 Breborowicz A, Wieczorowska K, Martis L, Oreopoulos DG, Glycosaminoglycan chondroitin sulphate prevents loss of ultrafiltration during peritoneal dialysis in rats, *Nephron* **67**, 346–50 (1994).
34 Shankland 2nd WE, The effects of glucosamine and chondroitin sulfate on osteoarthritis of the TMJ: A preliminary report of 50 patients, *Cranio* **16**, 230–35 (1998).
35 Wills ED, Evaluation of lipid peroxidation in lipid and biological membranes. In *Biochemical Toxicology*; A Practical Approach, edited by Snell K, Mullock B (IRL Press, Oxford, 1987), pp. 127–52.
36 Knigth JA, Review: Free radicals, antioxidants, and the immune system, *Ann Clin Lab Sci* **30**, 145–58 (2000).
37 Albertini R, Passi A, Abuja PM, De Luca G, The effect of glycosaminoglycans and proteoglycans on lipid peroxidation, *Int J Mol Med* **6**, 129–36 (2000).
38 Volpi N, Tarugi P, Influence of chondroitin sulphate charge density, sulfate group position, and molecular mass on Cu^{2+}-mediated oxidation of human low-density lipoproteins: Effect of normal human plasma-derived chondroitin sulfate, *J Biochem* **125**, 297–304 (1999).

Received 6 August 2003; revised 21 November 2003;
accepted 25 November 2003