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Reduction of DNA Fragmentation and Hydroxyl Radical Production by Hyaluronic Acid and Chondroitin-4-sulphate in Iron Plus Ascorbate-induced Oxidative Stress in Fibroblast Cultures

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Glycosaminoglycans (GAGs), components of extracellular matrix, are thought to play important roles in cell proliferation and differentiation in the repair process of injured tissue. Oxidative stress is one of the most frequent causes of tissue and cell injury and the consequent lipid peroxidation is the main manifestation of free radical damage. It has been found to play an important role in the evolution of cell death. Since several reports have shown that hyaluronic acid (HYA) and chondroitin-4-sulphate (C4S) are able to inhibit lipid peroxidation during oxidative stress, We investigated the antioxidant capacity of these GAGs in reducing oxidative damage in fibroblast cultures.

Free radicals production was induced by the oxidizing system employing iron (Fe^{2+}) plus ascorbate. We evaluated cell death, membrane lipid peroxidation, DNA damage, protein oxidation, hydroxyl radical ($OH^{\cdot}$) generation and endogenous antioxidant depletion in human skin fibroblast cultures.

The exposition of fibroblasts to $FeSO_4$ and ascorbate caused inhibition of cell growth and cell death, increased $OH^{\cdot}$ production determined by the aromatic trap method; furthermore it caused DNA strand breaks and protein oxidation as shown by the DNA fragments analysis and protein carbonyl content, respectively. Moreover, it enhanced lipid peroxidation evaluated by the analysis of conjugated dienes (CD) and decreased antioxidant defenses assayed by means of measurement of superoxide dismutase (SOD) and catalase (CAT) activities.

When fibroblasts were treated with two different doses of HYA or C4S a protective effect, following oxidative stress induction, was shown. In fact these GAGs were able to limit cell death, reduced DNA fragmentation and protein oxidation, decreased $OH^{\cdot}$ generation, inhibited lipid peroxidation and improved antioxidant defenses.

Our results confirm the antioxidant activity of HYA and C4S and this could represent a useful step in the understanding of the exact role played by GAGs in living organisms.

Keywords: Glycosaminoglycans; Lipid peroxidation; Fibroblasts; Oxidative stress; Aromatic trap; Free radicals

INTRODUCTION

Proteoglycans (PGs) consist of a protein portion and long, unbranched polysaccharides, the glycosaminoglycans (GAGs). GAGs are a family of sulphated polysaccharides that display a variety of fundamental biological roles.[1] They possess a high negative charge, owing to the presence of acidic sugar residues and/or modification by sulphate groups. The acidic sugar alternates with an amino sugar in repeated disaccharide units. There are two major classes of sulphated GAGs distinguishing by the content of uronic acid and hexosamine units: (a) the heparan sulphate (HS) family that includes various types of HS; and (b) the chondroitin

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sulphate family including chondroitin sulphates (C4S and C6S), and dermatan sulphate (DS). Keratan sulphate is yet another sulphated GAG, containing galactose (instead of uronic acid) and *N*-acetyl-glucosamine. The exception is hyaluronic acid (HYA), a non-sulphated GAG, which exists as a protein-free polysaccharide on cell surfaces and in the extracellular matrix (ECM).[1] GAGs adopt an extended conformation, attract cations and bind water. The particular sulphation patterns in the GAG chains allow different electrostatic interactions, with a number of biological constituents like growth factors and many other different molecules with different specific interactions at the same time.[2]

Reactive oxygen species (ROS) are known to play multiple roles in physiological and pathological states and are constantly produced in living organisms.[3] Internal physiological sources of ROS in aerobic mammalian cells are the mitochondrial electron carriers and enzymes. The highly reactive superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) may be toxic to cell by direct attack at the molecular level or indirectly by generating, via Haber–Weiss or Fenton's reaction, a secondary reactive species such as the detrimental $OH^{\cdot}$.[3]

DNA is under constant attack from these reactive species. Interaction of ROS with DNA can induce a multiplicity of products of varying structures and with differing biological impacts.[4] These products include oxidation of all four constituent bases, although guanine is, by far, the most readily oxidized. Ring fragmentation, sugar modification, covalent cross links with amino acids/other DNA bases and strand breaks have also been shown to occur.[5,6] Several data also indicate that ROS-induced lipid peroxidation of cell membranes is thought to be another different mechanism in the evolution of cell injury.[7] The amount of radicals actually reaching the DNA and cell membranes, and producing damage, is limited by cellular antioxidants. Enzymic and non-enzymic antioxidants intercept ROS and can prevent damage being induced. When the balance between these pro-oxidant and antioxidant processes is shifted in favor of the former, oxidative DNA damage and membrane lipid peroxidation occurs.[7,8]

Recently, a number of reports have demonstrated antioxidant activity of HYA and chondroitin-4-sulphate (C4S) both *in vitro* and *in vivo* experimental models.[9,11] These antioxidant properties are probably due to their capacity to chelate transition metals like Cu^{++} or Fe^{++} that are in turn responsible of the initiation of Haber–Weiss and Fenton's reaction.[12,13]

According to these previous data, we investigated the effects of HYA and C4S in limiting $OH^{\cdot}$ generation, with the consequent changes of membrane lipid peroxidation and DNA injury in a model of iron-induced oxidative damage in human skin fibroblast cultures.

MATERIALS AND METHODS

Materials

Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, 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). Ascorbic acid, iron (II) sulphate, sucrose, ethylenediamine-tetracetic acid (EDTA), potassium phosphate, butylated hydroxytoluene (BHT), trypan blue, reduced nicotinamide adenine dinucleotide (NADH), guanidine hydrochloride, 2,4-dinitrophenylhydrazine (DNPH), sodium pyruvate, sodium salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), RNase, proteinase K, protease inhibitor cocktail, sodium dodecylsulphate (SDS), HYA, C4S 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, UK). 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, UK) at 37°C in humidified air with 5% CO_2. Cells were used between the 11th 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. Twelve hours 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 the vehicle in which GAGs were dissolved, or HYA, or C4S in concentrations of 1.0 and 2.0 mg/ml. After 4 h of incubation, oxidative stress was induced in the cells in the following way: 10 μl of 400 μM $FeSO_4$ was added in a series of wells (final concentration 2.0 μM) pretreated with GAGs. Then, after 15 min, 10 μl of 200 mM of ascorbic acid was added for free radical production.[14] After 1.5 h, in all experiments, the medium was discarded and

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replaced by 2 ml of the same fresh medium. Twenty four hours later cells were subjected to morphological and biochemical evaluation.

Cell Viability Assay

Twenty four hours after oxidative stress, cell viability was determined under photozoom invertite microscope (Optech GmbH, Munchen, Germany) connected with a digital camera (mod. Coolpix 4500, Tokyo, Japan). The exact number of surviving cells was then evaluated by Trypan blue dye exclusion test.[15] Briefly, after 5 min incubation live cells excluded the dye, whereas dead ones were stained; the number of cells excluding the dye was expressed as a percentage counted from several randomly chosen areas of each well.

Lipid Peroxidation Analysis

Estimation of conjugated dienes (CD) in the cell lysate samples was performed to estimate the extension of lipid peroxidation in the fibroblast cultures. Cell samples, $4-5 \times 10^6$, were obtained 24 h after oxidative stress induction, collected in 500 μl of PBS containing 200 μM BHT and were frozen at −80°C until the assay. The day of analysis, after thawing, cell samples were centrifuged at 500*g* for 5 min at 4°C. The pellet was resuspended and sonicated in 500 μl of sterile H_2O (Transsonic Model 420, Elma instrumentation, Germany). For assay of CD lipid were extracted from cell lysate samples by chloroform/methanol (2:1) and dried under nitrogen atmosphere and then dissolved in cyclohexane. The cell evaluation content of CD was performed at 232 nm (Uvikon mod. 860, Kontron instruments, Everett) by using a spectrophotometric technique.[16] The amount of CD was expressed as ΔABS/well.

DNA Strand Breaks Determination

DNA extraction was performed on $4-5 \times 10^6$ cell samples obtained 24 h after oxidative stress induction.[17] Cells were collected in 500 μl of PBS and DNA was extracted with a buffer consisting of 10 mM Tris–HCl, pH 8.0, 0.1 M EDTA, 20 μg/ml RNase and 0.5% SDS. Then, fibroblasts were digested with 100 μg/ml proteinase K overnight at 37°C, followed by double phenol/chloroform/isoamyl alcohol (1:24:1) purification and precipitation by 2.5 volumes of cold ethanol. DNA samples were applied to 0.8% agarose gels in a 1× Tris–HCl–Acetic acid (TAE) buffer system. Following electrophoresis, gels were stained with ethidium bromide and recorded, under UV illumination, by a digital camera (mod. Coolpix 4500, Tokyo, Japan), acquired by the computer and elaborated by a specific data acquisition program (Nikon view 5). Then, each portion of the gel delimiting DNA bands was cut out and the total DNA content in the band was eluted with a specific commercial kit (Jet quick, cod.400250, Genomed GmbH, Bad Oeynhausen, Germany) and assayed spectrophotometrically at 260 nm.

Carbonyl Content Measurement

The protein carbonyl content was determined in order to evaluate the extension of protein oxidation. The analysis was carried out spectrophotometrically using the DNPH-labeling procedure.[18] Briefly, fibroblasts (4–5 × 106) were incubated, after addition of 400 μl 0.2% DNPH in 2 M HCl, for 1 h at 37°C. The protein hydrazone derivatives were sequentially extracted with 10% (w:v) trichloroacetic acid, then were treated with ethanol/ethyl acetate 1:1 (v:v), and reextracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride and the absorbance was measured in a spectrophotometer at 360 nm. The difference in absorbance between DNPH treated samples and samples treated in parallel with HCl alone without DNPH was used to calculate the carbonyl content using the molar absorption coefficient of 22,000 /M/cm for aliphatic hydrazones. Results were expressed as nmol of DNPH incorporated per well.

Aromatic Trap Analysis

The aromatic trap technique[19] was used in order to quantify $OH^{\cdot}$ production in fibroblast cultures after oxidative stress. Sodium salicylate may be used as a specific trap for hydroxyl radicals because it can react chemically with $OH^{\cdot}$ radicals produced, yielding 2,5-DHBA, 2,3-DHBA and catechol as its hydroxylation derivatives in an approximate proportion of 40, 49 and 11%, respectively.[20,21] In this study, we measured both 2,5-DHBA and 2,3-DHBA. To allow the chemical reaction, fibroblasts received sodium salicylate to a final concentration of 0.5 mM added directly in the culture medium before GAGs and iron plus ascorbate treatment. The medium was assayed before and 15 min after oxidative stress induction (basal). The medium samples were collected in polyethylene tubes and after centrifugation at 3000*g* for 10 min at 4°C were frozen at −80°C until the assay. To measure 2,5-DHBA and 2,3-DHBA formation, a HPLC method, with some modification, was used.[22] Briefly, 500 μl of medium was treated with 10 μl of 100 μM 2,4-DHBA, which has been used as internal standard. Then samples were extracted with 2.5 ml HPLC grade diethylether and mixed on a vortex for 2 min. After centrifugation for 15 min at 15,000*g* at 4°C, the diethylether layer was separated and then evaporated in a vacuum concentrator system

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(Rotating evaporator, mod. Strike 102/202, Steroglass, Perugia, Italy). The residue obtained was dissolved in 30 μl of 0.1N HCl and 32.5 μl of mobile phase, and 50 μl of the solution was injected into the HPLC apparatus. The HPLC equipment consisted of a dual reciprocating pump (Mod. LC-10Advp, Shimadzu, Kyoto, Japan) coupled to a manual injector (Rheodyne, Mod. 7725i) with a 50 μl fixed loop, and to a programmable UV/VIS monitor (Mod. 1706, BioRad Laboratories, Hercules, CA) connected to an automatic integrator (Mod. CR-3A, Shimadzu). The column used was a Ultrasphere ODS, 5 μ 250 × 4.6 mm (Beckman Instruments, San Ramon), attached to a precolumn (Guard column Water-Millipore, Milford). The mobile phase was 80% 0.03 M citric acid, 0.03 M acetic acid buffer (pH = 3.6) and 25% methanol at flow rate of 1.3 ml/min. The detector was set at a wavelength of 315 nm. The concentrations of 2,3-DHBA and 2,5-DHBA were expressed in μM.

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Superoxide Dismutase (SOD) Evaluation

Fibroblasts (4–5 × 10^6) obtained 24 h after oxidative stress induction, were collected in 500 μl of PBS containing 50 μl of protease inhibitor cocktail and centrifuged at 1000*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*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. no. Sd 125, Randox Laboratories, Crumlin, UK). 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 xanthine and 0.025 mM iodonitrotetrazolium chloride. After mixing for 5 s, 250 μl of xanthine oxidase solution (80 U/l) were added. Then, initial absorbance was read and the final absorbance was read after additional 3 min. A standard curve was made up with commercial SOD solution (from 0 to 320 U/ml) 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.

Catalase Activity Measurement

Fibroblasts (4–5 × 10^6) obtained 24 h after oxidative stress induction, were collected in 500 μl of PBS containing 50 μl of protease inhibitor cocktail and centrifuged at 1000*g* for 5 min at 4°C. Then, the pellet was resuspended and sonicated in 250 μl ice-cold 0.05 M potassium phosphate buffer, pH = 7.4 containing 0.1 mM ethylenediamine tetraacetic acid sodium salt. Then, each sample was centrifuged at 20,000*g* for 20 min at 4°C. The supernatant was aspired and the CAT activity was assayed spectrophotometrically at 520 nm by using a commercial kit (Catalase-520, cat. no. 21042, OxisResearch, Portland, OR). Briefly, 30 μl of diluted samples (1:10, v:v with 0.01 M potassium phosphate buffer, pH = 7.4) were mixed with 500 μl of 10 mM H_2O_2 and incubated for 1 min at room temperature. Then the reaction was stopped by adding 500 μl of sodium azide. After mixing, 20 μl of each reaction mixture were added to 2 ml of a solution of 4-aminophenazone, 3,5-dichloro-2-hydroxybenzenesulfonic acid in phosphate buffer. After mixing and incubation for 10 min the absorbance was read at 520 nm. A standard curve of CAT activity (from 5 to 120 U/ml) was run for quantitation. CAT values were expressed as units/mg protein.

Protein Determination

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Lab., Richmond, CA) and bovine serum albumin as a standard according to the published method.[23]

Statistical Analysis

Data are expressed as means ± SD of at list 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 $p < 0.05$.

RESULTS

Effects of GAGs on Cell Viability

The exposition of fibroblasts to $FeSO_4$ plus ascorbate produced a large mortality and growth inhibition as showed in Fig. 1. In particular, the percent of cell viability ranged about 9%. The treatment with HYA or C4S exerted a protective effect in a dose-dependent way. The maximum protection was exerted by C4S and ranged about 80% with the maximum dose while HYA protected about 70% of fibroblasts with the maximum dose. The treatment of cells with C4S at the dose of 1.0 mg/ml limit death of about 55% fibroblasts, while HYA reduced mortality of about 42% cells. No variations were obtained by treating fibroblasts with HYA or C4S only (Fig. 1).

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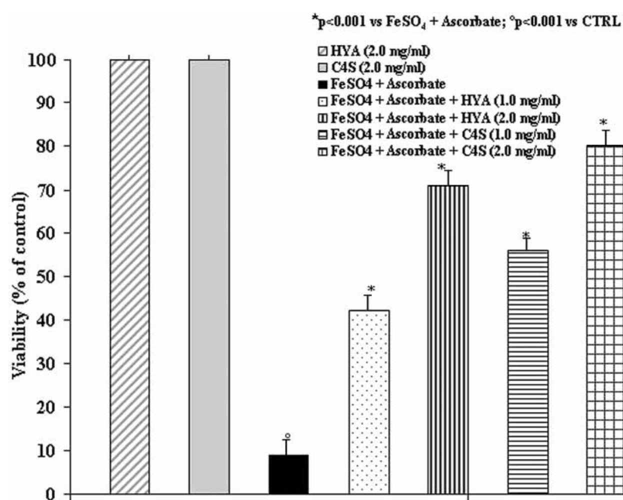

FIGURE 1 Effect of HYA or C4S on fibroblast viability (% of control) in the considered model of oxidative stress. Values are the mean ± SD of seven experiments.

CD Evaluation

Figure 2 shows the changes in CD concentrations of cell cultures after oxidative stress. Low levels of CD were found in the CTRL wells and these values were considered physiological. In contrast, a significant increase in CD production was seen in wells treated only with iron plus ascorbate. Also for this parameter both HYA and C4S exerted an evident protection in a dose-dependent manner. The maximum effect on the inhibition of lipid peroxidation was obtained after the treatment with C4S at the dose of 2.0 mg/ml, while the lowest was exerted by HYA administration at the dose of 1.0 mg/ml (Fig. 2).

DNA Damage

DNA strand breaking was evaluated as indicative marker of oxidative damage. As shown in Fig. 3A,B, more fragmented DNA strands were observed in fibroblasts after exposure to $FeSO_4$ plus ascorbate in comparison with normal cells. In contrast, the overall DNA appeared to be almost intact in fibroblasts after exposure to the oxidant and when treated with HYA and C4S at the dose of 2.0 mg/ml. Also in this case the attenuation of DNA damage was exerted by GAGs in a dose-dependent way (Fig. 3A,B).

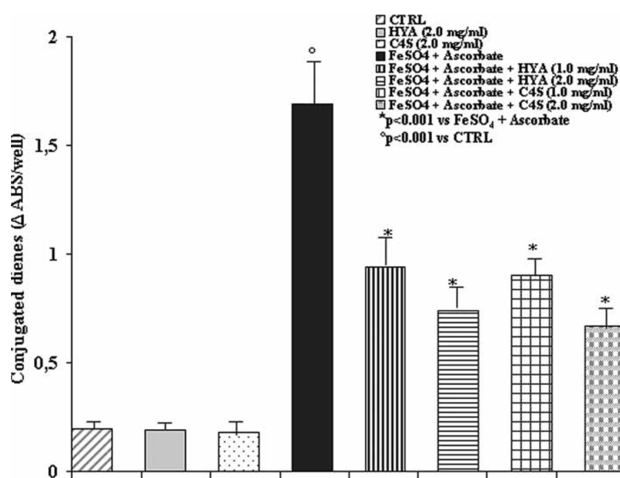

FIGURE 2 Effect of HYA or C4S on fibroblast lipid peroxidation in the considered model of oxidative stress. Values are the mean ± SD of seven experiments.

Protein Carbonyl Content

Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins.[18] Fibroblasts elicited an approximately 5-fold increase of carbonyl groups, compared to untreated cells when $FeSO_4$ plus ascorbate was added (Fig. 4). Although the carbonyl content of cells treated with HYA or C4S increased with the induction of oxidative stress, the increase was significantly lower than in cell untreated with GAGs. In fact the maximum effect on the inhibition of protein oxidation was exerted by the highest dose of C4S while the lowest was exerted by HYA with the dose of 1.0 mg/ml (Fig. 4).

$OH^{\cdot}$ Generation

Figure 5 shows the variation of 2,3-DHBA and 2,5-DHBA produced in fibroblast cultures and measured under basal conditions and 15 min after the exposition to $FeSO_4$ plus ascorbate. Very low amounts of the acids were detected before oxidative stress induction. (< 0.7 μM for 2,3-DHBA and < 2.0 μM for 2,5-DHBA). While high levels of both acids were seen 15 min after oxidative damage induction. Treatment of fibroblasts with HYA or C4S showed a significant reduction in $OH^{\cdot}$ generation with both used doses (Fig. 5A,B).

Because salicylic acid makes the stable derivatives by trapping $OH^{\cdot}$, it may theoretically be considered as a scavenger of $OH^{\cdot}$. Therefore, in an additional experiment, we compared the wells exposed to $FeSO_4$ plus ascorbate or $FeSO_4$ plus ascorbate/HYA/C4S treated with sodium salicylate against other wells not treated with sodium salicylate with the aim to study any possible direct action of this acid on the considered parameters. We did not observe any interaction between salicylate and HYA or C4S, and its addition failed to exert any beneficial effect after $FeSO_4$ plus ascorbate exposition (data not shown). This suggests that salicylate does not have a significant scavenging effect because it can trap only a small portion (< 11%) of produced $OH^{\cdot}$.[20]

Antioxidant Defenses

SOD and CAT activities were assayed in order to evaluate the antioxidant balance after free radical

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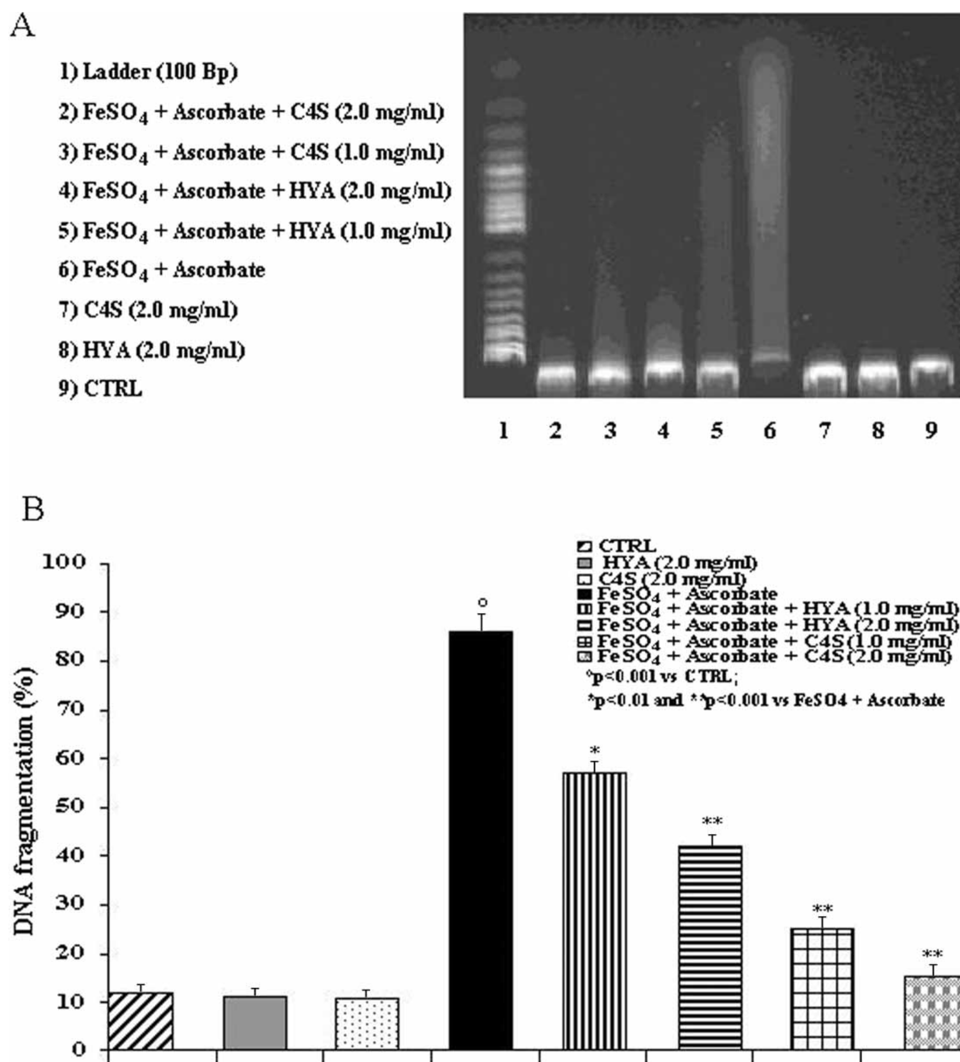

FIGURE 3 Effect of HYA or C4S on DNA strand breaks in the considered model of oxidative stress. (A) Visualization of DNA fragmentation. (B) Quantification of DNA damage. Values are the mean ± SD of seven experiments.

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production (Fig. 6A,B), respectively. In the CTRL wells, SOD and CAT ranged between 25.0–32.0 and 40.0–50.0 U/mg protein, respectively, and these values were considered physiological. In contrast, a significant reduction in both antioxidants was observed in fibroblasts treated with $FeSO_4$ plus ascorbate only. Also in this case HYA or C4S restored significantly the two antioxidants and protect cells from the injury induced by the transition metal. The maximum effect was produced by C4S at dose of 2.0 mg/ml, while the lowest was exerted by HYA at dose of 1.0 mg/ml (Fig. 6A,B).

DISCUSSION

ROS are part of normal regulatory circuits of living organisms. Nevertheless, imbalance or loss of cellular redox homeostasis results in oxidative stress causing severe damage of cellular components.[24] ROS produce permanent genetic changes by involving protooncogenesis and tumor suppressor genes, and they are able to activate cytoplasmic signal transduction pathways that are related to growth, differentiation, senescence, and tissue degradation.[25] In fact, ROS have been implicated to play a causal role in cancer, aging and other degenerative diseases like arteriosclerosis, osteoarthritis, neurodegeneration and impaired wound healing. These pathological states share unique features and are all characterized by a dysregulated, localized or diffuse connective tissue breakdown.[26–31]

Lipid peroxidation is a complex radical chain reaction whereby unsaturated membrane lipids are oxidized. This process directly results in membrane damage and indirectly in protein and DNA modifications provoked by reactive lipid peroxidation products like hydroperoxides.[7,32]

Transition metals like iron and copper are able to enhance lipid peroxidation. They act as initiators of this mechanism as well as catalysts of

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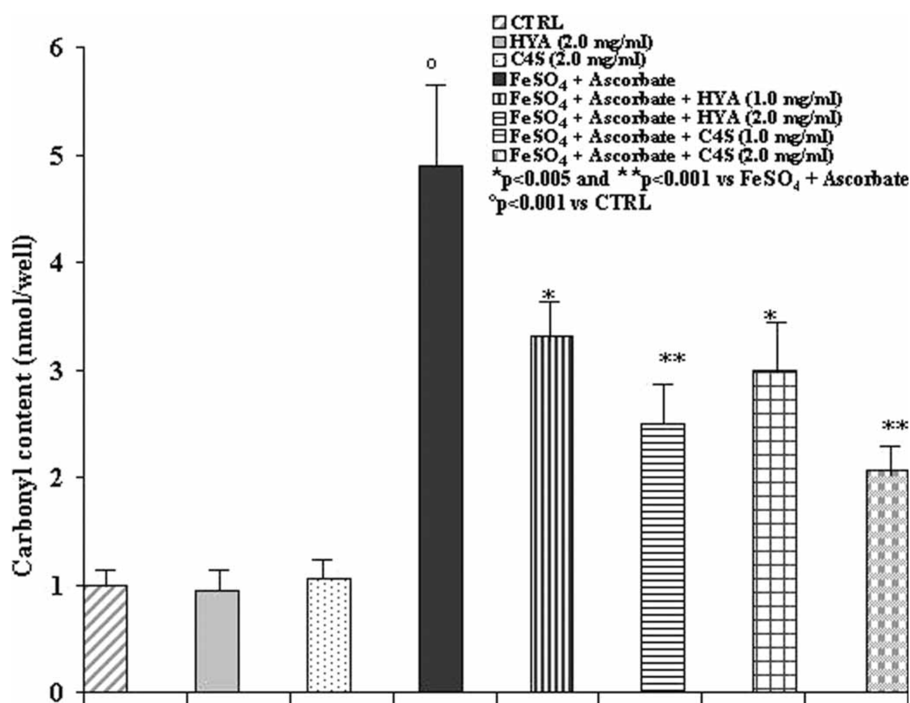

FIGURE 4 Effect of HYA or C4S on fibroblast protein carbonyl content in the considered model of oxidative stress. Values are the mean ± SD of seven experiments.

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the propagation steps of the chain reaction. Lipid hydroperoxides which are formed as primary products of LPO react rapidly with transition metals like iron and copper ions to generate alkoxyl or peroxyl radicals.[33]

HYA is a high molecular weight, non-sulphated GAG component of the ECM present in many tissues, such as skin, synovial joints and connective tissues. HYA has many structural, rheological and physiological functions within tissues, including ECM and cellular interaction, growth factor interaction and in the regulation of osmotic pressure and tissue lubrication.[34] HYA is also a key component of chronic wounds during each stage of the wound healing process, including the inflammatory, granulation and reepithelialization stages.[35] A number of evidences have shown that HYA possesses several roles in the activation and modulation of the inflammatory response, including the antioxidant scavenging of ROS derived from polymorphonuclear leukocytes (PMNs) and other sources.[10,11,36–38] Chondroitin sulphates (CS) are the GAGs more abundant in human organism, and they are localized wherever in connective tissues. Moreover, CS are the more representative components of circulating GAGs, and they also are constituents of normal urine, and are present in granulocytes, platelets and Kurloff cells. These molecules may be distinguished through their degree of sulphation. In fact they are sulphated at the C-4 or C-6 positions of galactosamine.[1] In the last years, several findings have reported an antioxidant activity of C4S capable to inhibit lipid peroxidation and to protect cells from ROS damage.[10–12,39,40] While C6S have shown no antioxidant properties in a model of High-density lipoprotein (HDL) peroxidation induced by transition metals.[12,39,40] The reason of this lack in antioxidant activity of C6S seems to be due to the different position of sulphate group.[12,39,40]

In the present study, we have investigated the protective effects of HYA or C4S treatment in a simple culture system of fibroblasts following exposure to the prooxidant $FeSO_4$ plus ascorbate. The data obtained by treating fibroblasts with these two natural compounds showed positive effects in all considered parameters and in a dose-dependent way.

Lipid peroxidation is considered a critical mechanism of injury occurring in cell during oxidative stress.[41] The evidence supporting these biochemical changes is based on analysis of a wide number of intermediate products.[42] An indicative method extensively used for evaluating lipid peroxidation is CD analysis.[42] The increment of CD levels found in the fibroblasts exposed to the oxidant agent is consistent with the occurrence of a free-radical-mediated cell damage. The treatment with HYA or C4S limited membrane lipid peroxidation and consequently cell death as reported by cell viability data.

Interaction of ROS with DNA can induce a multiplicity of products of varying structures and with differing biological impacts. The antioxidant

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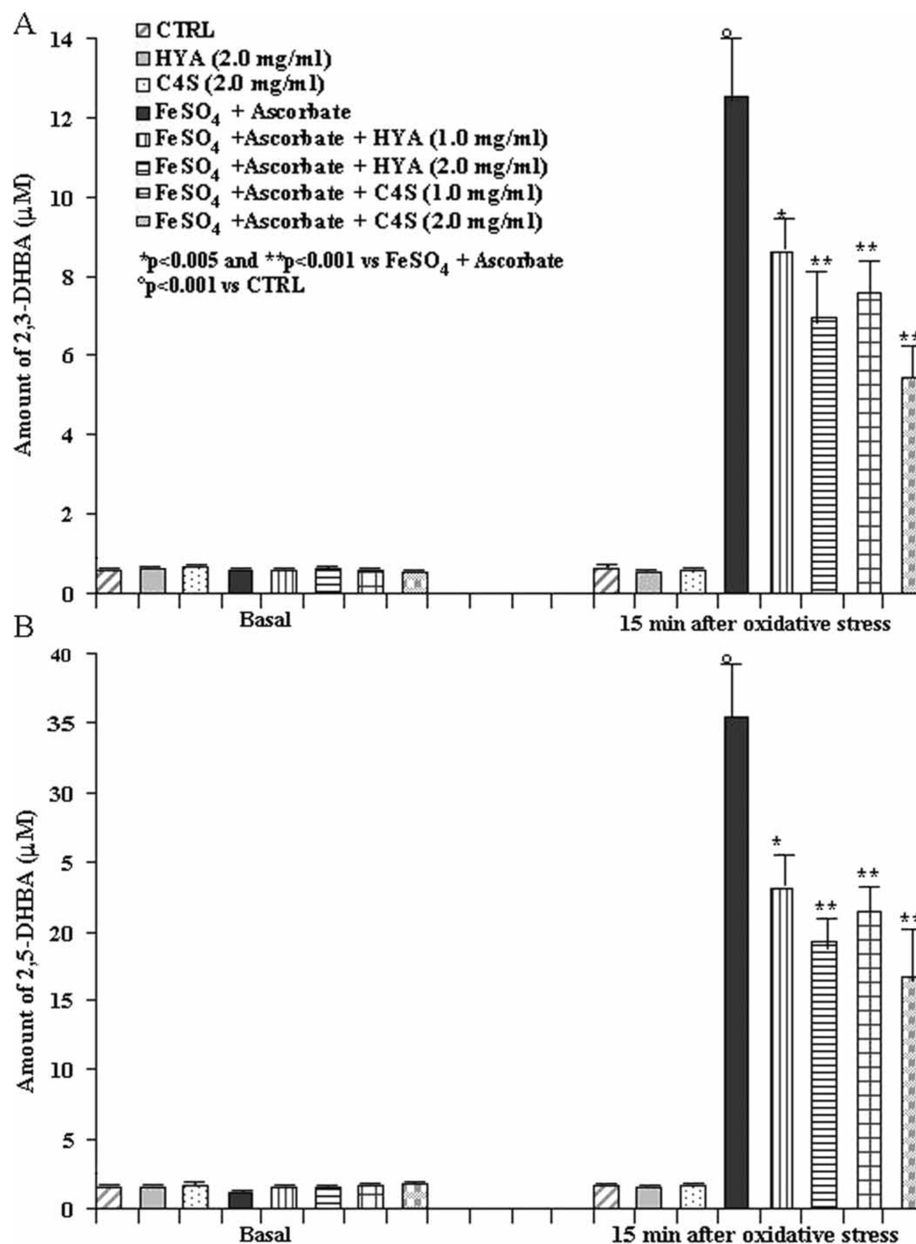

FIGURE 5 Effect of HYA or C4S on 2,3-DHBA and 2,5-DHBA production evaluated in basal conditions and after 15 min of stress induction in the fibroblast cultures. Values are the mean ± SD of seven experiments.

cell defense system intercepts ROS and normally inhibits cellular and nuclear damage. When the amount of ROS produced overwhelms these endogenous defenses, an increase in oxidative DNA injury occurs.[7] The data obtained have shown that the marked fragmentation of DNA strand observed in the fibroblasts exposed to $FeSO_4$ plus ascorbate was significantly reduced by HYA or C4S treatment.

As indicative marker of oxidative damage to cells, the occurrence of protein oxidation was evaluated.[18] The increase of carbonyl content observed in cell culture subjected to oxidative damage was decreased by adding the two GAGs.

The detection of 2,3-DHBA and 2,5-DHBA is usually considered a bona fide reporter for the flux of hydroxyl radicals which reacted with salicylic acid during oxidative stress both *in vivo* and *in vitro* models.[20,43,44] The results obtained show that high levels of $OH^{\cdot}$ radical were produced in fibroblasts after the exposition to $FeSO_4$ plus ascorbate. HYA and C4S reduced significantly both the acids 2,3-DHBA and 2,5-DHBA formation by inhibiting $OH^{\cdot}$ production and the consequent formation of the two acids.

The high generation of reactive species that occurs in fibroblast cultures decreased SOD and CAT concentrations as a consequence of their

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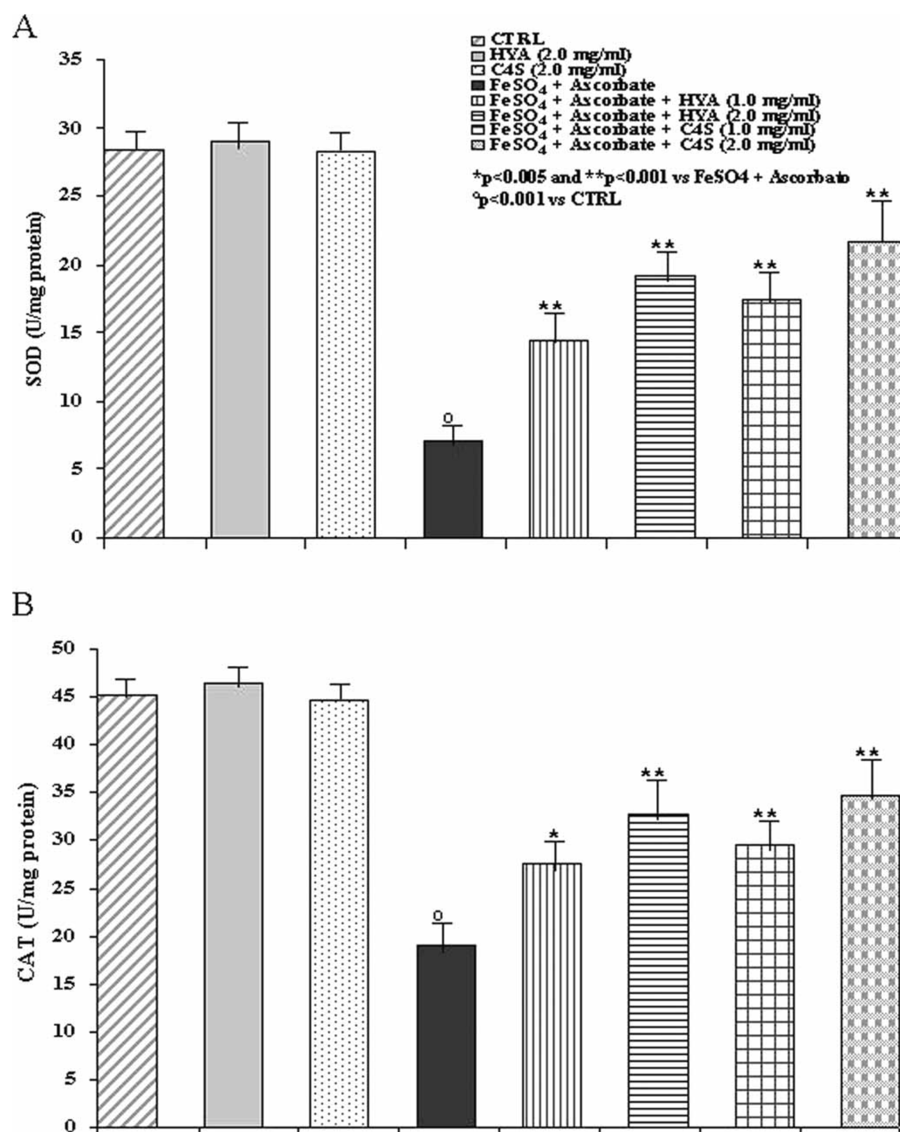

FIGURE 6 Effect of HYA or C4S on SOD and CAT activities in the considered model of oxidative stress. Values are the mean ± SD of seven experiments.

consumption during oxidative damage.[45] This reduction contributes to increased cellular destruction by favoring free radical attack. Also in this case the treatment of cells with HYA or C4S limited SOD and CAT consumption by the reduction of free radical generation.

The antioxidant mechanism of HYA and C4S is due to their particular chemical structure. In fact, as other GAGs, HYA and C4S are linear polymers formed by alternating hexuronic acid and hexosamine units that are different in the different GAGs. HYA is the only non-sulphated compound, whereas C4S is sulphated in the position 4 of the aminosugar. The explanation of the antioxidant activity for HYA and C4S is the presence, in their structure, of carboxylic group in the same position and for C4S the presence of sulphated group in position 4 in the opposite side of carboxylic group. These charged groups may interact with the transition metals ions like Cu^{++} or Fe^{++} that are in turn responsible of the initiation of Fenton's reaction. The ability of these compound to chelate different ions and transition metals was extensively reported by several authors.[12,13,39,40,46,47]

Ion binding by acid GAGs is reasonably predictable, given that they are linear polyanions, with varying ratios of carboxylic and sulphate groups. Cation positions have been elucidated for structures containing calcium ions. The co-ordination of the calcium ion which bridges carboxylate groups in separate chains and also bridges the carboxylate and sulphate group within a single chain of C4S was shown.[48]

C4S binds Cu^{++} ions more strongly than it binds calcium ions.[49] Furthermore, solid complexes of Iron(III) with HYA are very stable and show binding

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constants log K of value about 8.0.[47] A species of composition CuL_2, L being a disaccharide unit of HYA, showed a stability constant log K of 3.47.[50,51] All these data strongly suggest that GAGs as HYA and C4S are able to bind iron and cupper cations in solutions. That would certainly decrease their availability for oxidation processes.

Alternatively, another antioxidant mechanism may be the directly scavenge effect of HYA or C4S on free radical molecules especially the detrimental $OH^{\cdot}$ or other Fenton's reaction intermediates like $O_2^{\bullet -}$.

In conclusion, the results obtained in this experiment confirm the antioxidant activity of HYA and C4S and this could represent a useful step in the understanding the exact role played by GAGs in living organisms. Data, indeed, suggest that a physiological increase in GAG production following oxidative stress may be a natural defense able to reduce cell damage and DNA oxidation.

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