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Aromatic Trap Analysis of Free Radicals Production in Experimental Collagen-induced Arthritis in the Rat: Protective Effect of Glycosaminoglycans Treatment

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Many findings demonstrated that Glycosaminoglycans (GAGs) and Proteoglycans (PGs) possess antioxidant activity. Collagen-induced arthritis (CIA) is an experimental animal model similar to human rheumatoid arthritis (RA) in which free radicals are involved. Sodium salicylate can be used as a chemical trap for hydroxyl radicals ($OH^{\bullet}$), the most damaging reactive oxygen species (ROS), yielding 2,5-dihydroxybenzoic acid), (2,5-DHBA) and 2,3-dihydroxybenzoic acid (2,3-DHBA). The measurement of these two acids in the plasma allows to indirectly assess the production of $OH^{\bullet}$ radicals.

The aim of the study was to investigate the effect of hyaluronic acid (HYA) (30 mg/kg i.p.) or chondroitin-4-sulphate (C4S) (30 mg/kg i.p.), on free radical production in Lewis rats subjected to CIA.

After the immunization with bovine collagen type II in complete Freund's adjuvant, rats developed an erosive hind paw arthritis, that produced high plasma $OH^{\bullet}$ levels assayed as 2,3-DHBA and 2,5-DHBA, primed lipid peroxidation, evaluated by analyzing conjugated dienes (CD) in the articular cartilage; decreased the concentration of endogenous vitamin E (VE) and catalase (CA) in the joint cartilage; enhanced macrophage inflammatory protein-2 (MIP-2) serum levels and increased elastase (ELA) evaluated as an index of activated leukocyte polymophonuclear (PMNs) accumulation in the articular joints.

The administration of HYA and C4S starting at the onset of arthritis (day 11) for 20 days, limited inflammation and the clinical signs in the knee and paw, reduced $OH^{\bullet}$ production, decreased CD levels, partially restored the endogenous antioxidants VE and CA, reduced MIP-2 serum levels and limited PMNs infiltration.

The results indicate that the GAGs HYA and C4S significantly reduce free radical production in CIA and could be used as a tool to investigate the role of antioxidants in RA.

Keywords: Glycosaminoglycans; Antioxidants; Aromatic trap; Hyaluronic acid; Chondroitin sulphate; Lipid peroxidation

INTRODUCTION

Rheumatoid arthritis (RA) is the most common disease of connective tissue of unknown origin.[1,2] There is no cure for RA. Several factors play a role in the onset and clinical course of the disease. The long-term outcome of this illness is characterized by significant morbidity, loss of functional capacity and increased mortality.[3] A genetic background is suspected, as well as a T cell receptor restricted presentation of an unknown "arthritogenic epitope". Various immunocompetent cells and proinflammatory cytokines are also involved in this erosive disease.[4]

Collagen-induced arthritis (CIA) in rats is a widely studied model of inflammatory polyarthritis that is mediated by an autoimmune response and presents many similarities to RA.[5]

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Reactive oxygen species (ROS) are thought to play a role in RA. An increased production of ROS was established in patients with inflammatory and autoimmune rheumatic disease.[6,7] Excessive phagocytic ROS production degrades the extracellular matrix and initiates the expression of leukocytic cell adhesion molecules and proinflammatory cytokines.[8,9] In normal conditions, antioxidant defence is provided by low-molecular-weight antioxidants including glutathione, β-carotene, uric acid, ascorbic acid, tocopherols and by antioxidant enzymes including glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase (CA). In these conditions, ROS may be neutralized in either a stoichiometric or a catalytic way.[10] However, in patients who suffer from rheumatic disease, the antioxidant defence systems are impaired and RA patients are thus exposed to oxidant stress and the consequent membrane lipid peroxidation.[11,12]

Several endogenous and exogenous antioxidants have been administered in an effort to restore a normal pool of ROS scavengers and modulate eicosanoic acid production, but although some positive results[13,14] have been obtained, a true therapy still does not exist.[15]

Glycosaminoglycans (GAGs), a large family of heterogeneous polysaccharides, are linear polymers composed of alternating hexuronic acid and hexosamine units that play important roles in all living organisms.[16] Their structure and degree of heterogeneity appear to be highly specific and the ability of several proteins to bind GAGs may reflect functional relationships and is likely to be exploited physiologically in a variety of different ways. Several reports show that during the progression of RA the physiological levels of blood GAGs are increased.[17,18] The obvious explanation is that GAGs originate from the joint cartilage erosion. Nevertheless, the exact meaning of their rise is at the moment unclear.

In the last years, several reports have shown antioxidant properties of GAGs (mainly for HYA and CS) both *in vitro* and *in vivo* experimental models.[19–21] They are able to inhibit lipid peroxidation.[19–21]

In the light of this evidence, the scope of this study was to evaluate the possible capacity of HYA and C4S in reducing inflammation and the joint cartilage damage in an experimental model of CIA in Lewis rats.

MATERIALS AND METHODS

Animals

Experiments were carried out on male Lewis rats with a mean weight of 175–200 g. Animals, purchased from Charles-River (Calco, Italy), were housed at a constant temperature of 22 ± 2°C under a 12 h light–dark cycle with unlimited access to standard laboratory rat chow and tap water. The health status of the animal colony was monitored according to the guidelines from the Italian Veterinary Board. Rats ware subdivided in the following groups: (1) CTRL; (2) CIA + Vehicle; (3) CIA + HYA (30 mg/kg); (4) CIA + C4S (30 mg/kg); (5) CIA + (HYA + C4S) (30 + 30 mg/kg).

Materials

Hyaluronic acid (HYA) from human umbilical cord, C4S from bovine trachea and bovine type II collagen were obtained from Sigma–Aldrich Srl, Milan, Italy; Complete Freund's Aduvant (CFA) was purchased from Difco Laboratories (Detroit, MI, USA). All the other reagents used were purchased from Fluka (division of Sigma–Aldrich Srl, Milan, Italy).

Induction of CIA

Arthritis was induced in rats ($n = 7,$) as previously described,[22] by multiple intradermal injections, at the base of the tail and into other 3–5 sites on the back, of 100 μg bovine type II collagen in 50 μl of 0.1 M acetic acid emulsified in an equal volume of CFA containing 2 mg/ml of mycobacterium tuberculosis H37 RA.[22] Seven days later, rats were rechallenged with the same antigen preparation. Before injections animals were anaesthetized with ether and injections were performed with a 15 gauge needle. Arthritis begin to develop 3–4 days after the second immunization (days 10–11).

HYA and C4S Administration

The treatment of rats initiated at day 10, that corresponded, approximately, with the onset of disease. HYA was given at dose of 30 mg/kg ($n = 7$), C4S at dose of 30 mg/kg ($n = 7$) and HYA plus C4S at dose of 30 + 30 mg/kg ($n = 7,$) GAGs were dissolved in physiological solution (NaCl 0.9%) and administered in a volume of 1 ml/kg, intraperitoneally once a day until the day 30th. At day 31, rats were sacrificed and specimens of blood and tissues were collected for biochemical and histological analysis.

Arthritis Evaluation

Assessment of joint inflammation was carried out blindly by an independent observer with no knowledge of the treatment protocol. The severity of the arthritis in each paw was quantified daily by a clinical score measurement[23] from 0 to 4 as follows: 0, no macroscopic signs of arthritis; 1, swelling of one

group of joints (i.e. wrist or ankle joints); 2, two groups of swollen joints; 3, three groups of swollen joints; 4, swelling of the entire paw. The maximum score for each rat was 12. Clinical severity of arthritis was also assessed by the quantification of the paw volume changes. Measurements were made by using a dial gauge caliper. Changes in body weight were monitored in order to determine the rate of the increment in each rat.

Aromatic Trap Measurement

The aromatic trap technique[24] was used in order to quantify $OH^{\bullet}$ production in the rats underwent to CIA. Sodium salicylate may be used as a specific trap for hydroxyl radicals because it can react chemically with $OH^{\bullet}$ 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.[25,26] In this study, we measured both 2,5-DHBA and 2,3-DHBA. To allow the chemical reaction, each group of animals received sodium salicylate (100 mg/kg i.p.)[27] (Sigma–Aldrich Srl, Milan, Italy) one hour before the first immunization and then, at the end of experiments, one hour before the sacrifice. Sodium salicylate by itself did not exert any protective effect in the injured joint cartilage. Samples of blood were drawn 5 min before the first immunization (basal) and immediately before the sacrifice (day 31st). The blood was collected in polyethylene tubes with previous addition of 20 μl of heparin solution (16,000 IU). The plasma obtained after centrifugation at 3000*g* for 10 min at 4°C was frozen at −80°C until the assay. To measure 2,5-DHBA and 2,3-DHBA formation, an HPLC method, with some modification, was used.[28] Briefly 250 μl of plasma was treated with 10 μl of 100 μM 2,4-dihydroxybenzoic acid (2,4-DHBA) (Janssen Chemical, Beerse, Belgium), which has been used as internal standard and 10 μl of 40% $HClO_4$. Then the plasma was 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 was then evaporated in a vacuum concentrator system (Heto Lab Equipment, Denmark). The residue obtained was dissolved in 30 μl of 0.1 N 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, USA) connected to an automatic integrator (Mod. CR-3A, Shimadzu, Kyoto, Japan). The column used was a Lichrosorb-10-RP_{18}, 10 μ 250 × 4.6 mm (Labservice Analytica, Milano, Italy), attached to a precolumn (Guard column Water-Millipore, Milford, USA). The mobile phase was 80% 0.03 M citric acid, 0.03 M acetic acid buffer (pH = 3.6) and 25% methanol (J.T. Baker, Deventer, Holland) 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.

Lipid Peroxidation Assessment

Estimation of conjugated dienes (CD) in the articular tissue was carried out in order to estimate the extension of lipid peroxidation in the damaged cartilage. At the end of the experiment hind limbs were removed and maintained cold in dry ice, then quickly the joint cartilage was separate from bone and muscular tissue and frozen at −80°C until the assay. The day of analysis, after thawing, cartilage samples were washed in ice-cold 20 mM Tris–HCl, pH = 7.4, and blotted on absorbent paper. Each sample was then minced in ice-cold 20 mM Tris–HCl, pH = 7.4 containing butylated hydroxytoluene (BHT) 1 mg/ml and homogenized in a ratio 1:10, w:v by using an ultra-turrax homogenizer. The biochemical assay of CD required previous lipid extraction from the omogenated samples by chloroform/methanol (2:1). The lipid layer was dried under nitrogen atmosphere and then dissolved in cyclohexane. Cartilage contents of CD was measured at 232 nm by using a spectrophotometric technique[29] with some modifications. The amount of tissue CD was expressed as ΔABS/g protein.

Vitamin E (α-tocopherol) Evaluation

VE levels in the articular tissue were measured in order to determine the oxidative state of the joints during arthritis. The assay was performed in cartilage tissue obtained at the end of experiments (day 31st). Articular specimens were stored at −80°C until the assay. The analysis was carried out by using a HPLC method.[30] Briefly, 0.2 ml of tissue homogenate, contained in polycarbonate tubes lined with aluminium foil, was treated with 200 μl of tocopherol acetate (25 μg/ml in ethanol), which has been used as an internal standard, and with 400 μl of butanol/ethyl acetate (1:1, v:v). After vortexing for 20 s, 20 mg of sodium sulphate were added and the sample was shaken on vortex mixer for additional 60 s. After Centrifugation at 15,000*g* for 5 min at 4°C, the organic layer was recovered and a 50 μl aliquot 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, USA) connected to an automatic

integrator (Mod. CR-3A, Shimadzu, Kyoto, Japan). The column used was a ultratechsphere C_{18}, 250 × 4.6 mm, 5 μ particle size (HPLC Technology LTD, Macclesfield, Cheshire, UK). Attached to a pre-column (Guard column, Water-Millipore, Milford, USA). The mobile phase was methanol/water (97:5, v:v) at a flow rate of 1 ml/min at room temperature. The detector was set at wavelength of 280 nm. VE levels were expressed as nmol/g protein.

Articular Catalase Analysis

CA activity was evaluated in order to estimate endogenous defence against free radicals. Specimens of joint cartilage obtained at the end of experiment were frozen at −80°C until assay. The day of analysis, each sample was washed with 0.9% NaCl and homogenized with an ultra-turrax homogenizer in 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 aspirated and the CA activity was assayed spectrometrically at 520 nm by using a commercial kit (Catalase-520, cat n° 21042, OxisResearch, Portland, OR, USA). Briefly, 30 μl of diluted samples (1:10, w: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 by inversion, 20 μl of each reaction mixture were added to 2 ml of a solution of 4-aminophenazone, 3,5-dichloro-2-hydroxybenzene-sulfonic acid in phosphate buffer. After mixing by inversion and incubation for 10 min the absorbance was read at 520 nm. A standard curve of CA solution (from 5 to 120 U/ml) was run for quantitation. CA values were expressed as units/g protein.

Serum MIP-2 Assay

Serum MIP-2 analysis was determined by using an ELISA commercial kit (Rat MIP-2 ELISA kit, cat. n° 22076, NBS Biologicals Ltd, Huntingdon, U.K.). Samples of blood (0.5 ml) were drawn, at the end of experiment, from a tail vessel. The blood was collected in polyethylene tubes and centrifuged at 3000*g* for 10 min at 4°C. The serum samples obtained were frozen at −80°C until the assay. Briefly, 50 μl of standard, samples and controls were added to each well of the precoated microplate. Then 50 μl of biotinylated anti-MIP-2 solution were added. After a gently mix, the plate was covered and incubated for 2 h at 37°C. Then, after decantation and elimination of the liquid, 100 μl of streptavidin-HRP working solution to each well were added. After an incubation for 60 min and a washing of the wells, 100 μl of chromogen were added. Then, the plate was covered and incubated in the dark for a further 30 min. Finally, 100 μl of stop solution were added and the absorbance was read spectrophotometrically at 450 nm. MIP-2 values were expressed as ng/ml.

Articular Elastase Content

EL levels in the articular joints were evaluated as an index of polymorphonuclear leukocyte (PMNs) accumulation and activation in the inflamed tissue because this enzyme is released from the stimulated granulocytes at the site of injury.[31] We measured ELA activity in the cartilage of articular joints by a specific assay for this enzyme.[32] Briefly, cartilage tissue samples were first diluted and homogenized in a solution containing 20 mM potassium phosphate buffer pH 7 in a ratio of 1:10 (w:v) and then centrifuged for 20 min at 10,000*g* at 4°C. An aliquot of each sample was incubated for 24 h at 37° C with 0.1 M Tris–Hcl buffer, pH = 8, containing 0.5 M NaCl and 1 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, that is a high specific synthetic substrate for neutrophil elastase (ELA). The amount of *p*-nitroanilide liberated was measured spectrophotometrically at 405 nm and was considered as neutrophil ELA activity. The ELA activity was converted and expressed as ng/g protein.

Histology

Rats were sacrificed at day 31 by ether narcosis. Knee joints were removed and fixed for 4 days in 4% formaldehyde. After decalcification in 5% formic acid, the samples were processed for paraffin embedding.[33] Tissue sections (7 μm thick) were stained with haematoxylin–eosin or safranin *O* for light microscope examination. Histopathological changes were scored by using the following parameters.

Infiltration of cells was scored on a scale from 0 to 3, depending on the amount of inflammation cells revealed in the synovial tissues. Inflammatory cells in the joint cavity were graded on a scale from 0 to 3 and expressed as exudates. Cartilage erosion was determined by using safranin O staining, and was scored on a scale from 0 to 3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. Bone erosion was on a scale ranging from 0 to 3, ranging from no abnormalities to complete loss of cortical and trabecular bone of the femoral head and patella. Histopathological changes in the knee joints were scored in the patella/femur region on 7 semiserial sections of the joint. Scoring was performed on decoded slides by two observers as previously reported.[33]

Statistical Analysis

Data are expressed as mean ± S.D. The difference between the means of two groups was evaluated with an ANOVA and was considered significant when $p < 0.05$. For incidence of arthritis data, statistical analysis was done with Fisher's exact probability test.

Statement of Animal Care

The studies reported in this manuscript have been carried out in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals.

RESULTS

Effect of GAGs on Clinical Manifestations of Arthritis

At day 11, rats started to show evidence of clinical inflammation in one or more hind paws. The first sign of disease was erythema of one or more ankle joints, followed by involvement of the metatarsal and interphalangeal joints. In Table I the data of CIA incidence during the 31-days study period are reported. The initial manifestations of arthritis in all group were evident at day 11 and the incidence was different but not significant among groups. In the Vehicle group incidence was 100% at day 17 and the same state was maintained until the end of the experiment. The treatment with GAGs exerted a significant amelioration in the incidence of CIA, 74% for HYA treatment, 65% for C4S treatment and 55% for HYA + CSA treatment (Table I).

The data of time-course of the development and progression of disease, as assessed by mean arthritis severity score and paw diameter, is shown in Table II and III. By 11 days, all animals showed evidence of disease, predominantly in the hind paws. The disease was always progressive with joint recruitment following the same pattern. The interphalangeal joints were never solely involved and inflammation in these joints was invariably associated with inflammation in the tarsal joint. The mean arthritis severity score in the Vehicle group was progressive from day 11 and achieved values over 10 day 23 (Table II). The same variations were observed in the hind paw diameter of the Vehicle group. In fact, at the end of the experiment, the increase in the hind paw diameter, due to oedema and inflammation, was 1.98 mm (Table III). Administration of HYA and C4S decreased both the mean arthritis severity score and the change in hind paw diameter (Table II and III).

Changes in Body Weight

In the first week, the absolute body weight increment was similar in all group considered, and no significant differences were seen among them. At the day 13 a significant loss in body weight was observed in the CIA + vehicle animals compared with the CTRL rats ($p < 0.001$). This weight loss, increased until end of the experiment (Table IV). Also in this case the treatment with GAGs ameliorated the body weight increment. The most effect was achieved after the treatment with both polymers ($p < 0.005$, Table IV).

Plasma $OH^{\bullet}$ Evaluation

Analysis of 2,3-DHBA and 2,5-DHBA was performed in order to indirectly assess the detrimental production of $OH^{\bullet}$ Radical. Table V reports the levels of 2,3-DHBA and 2,5-DHBA measured in each group under basal conditions and at the end of the experiment (day 31st). Very low amounts of both acids were detected before immunization in all considered groups (<0.5 mM for 2,3-DHBA and <1.0 for 2,5-DHBA). In contrast, a high level either of 2,3-DHBA or 2,5-DHBA was found at day 31 in the CIA plus vehicle group ($p < 0.001$). The treatment with HYA and C4S showed a marked reduction in the OH. Production (Table V).

Articular CD Assay

Determination of CD was carried out in the articular cartilage. Table VI reports that low levels of CD were observed in the CTRL group

TABLE I Effect of HYA and C4S on time-course of the development of arthritis. Cumulative incidence of arthritis (%) and day of onset of arthritis in rats during 31 days period after immunzation

	Days															
Experimental group	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31
CIA + Vehicle	0	0	0	0	0	46	65	85	100	100	100	100	100	100	100	100
CIA + HYA (30 mg/kg)	0	0	0	0	0	43	60	65*	70*	70*	75*	74*	77	75*	70*	74*
CIA + C4S (30 mg/kg)	0	0	0	0	0	35	48	53*	56**	54*	60**	58*	59*	62*	60*	65*
CIA + HYA + C4S (30 + 30 mg/kg)	0	0	0	0	0	30	45*	49*	44***	50**	52**	49**	49**	54**	52**	55**

Values are the mean of seven animals for each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$ vs. CIA + Vehicle.

TABLE II Effect of HYA and C4S on time-course of the development of arthritis. Arthritis severity scores and day of onset of arthritis in rats during 31 days period after immunization

Experimental group	Days										
	11	13	15	17	19	21	23	25	27	29	31
CIA + Vehicle	2.81 ± 0.46	4.01 ± 0.54	5.36 ± 0.95	6.41 ± 1.47	7.49 ± 1.51	9.76 ± 1.83	10.21 ± 2.51	10.34 ± 2.51	10.66 ± 2.42	10.81 ± 2.33	10.87 ± 2.17
CIA + HYA (30 mg/kg)	2.43 ± 0.34	3.54 ± 0.61	4.15 ± 1.02*	4.75 ± 1.08*	5.87 ± 1.15*	7.92 ± 1.17*	8.05 ± 1.23*	7.91 ± 1.36*	8.36 ± 1.24*	8.51 ± 1.10*	8.74 ± 1.22*
CIA + C4S (30 mg/kg)	2.57 ± 0.36	3.22 ± 0.58*	3.65 ± 0.87**	4.01 ± 1.02**	5.10 ± 1.00**	6.21 ± 1.43**	6.62 ± 1.50**	6.58 ± 0.97**	7.24 ± 1.13**	7.32 ± 1.06**	7.54 ± 1.27**
CIA + HYA + C4S (30 + 30 mg/kg)	2.67 ± 0.40	3.03 ± 0.65*	3.47 ± 0.81**	3.51 ± 1.26**	4.23 ± 1.22***	5.87 ± 1.51***	5.79 ± 1.48***	5.85 ± 1.02***	6.17 ± 0.93***	6.25 ± 1.01***	6.43 ± 1.49***

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$ vs CIA + Vehicle.

TABLE III Effect of HYA and C4S on time-course of the change in paw diameter in rats during 31 days period after immunization

Experimental group	Days										
	11	13	15	17	19	21	23	25	27	29	31
CIA + Vehicle	0.18 ± 0.03	0.32 ± 0.07	0.58 ± 0.13	0.87 ± 0.20	0.98 ± 0.20	1.12 ± 0.23	1.26 ± 0.24	1.35 ± 0.23	1.46 ± 0.25	1.73 ± 0.46	1.98 ± 0.49
CIA + HYA (30 mg/kg)	0.15 ± 0.04	0.28 ± 0.08	0.39 ± 0.11*	0.78 ± 0.12*	0.78 ± 0.12*	0.88 ± 0.14*	1.02 ± 0.14*	1.10 ± 0.17*	1.20 ± 0.19*	1.26 ± 0.21*	1.41 ± 0.23*
CIA + C4S (30 mg/kg)	0.16 ± 0.06	0.21 ± 0.06*	0.34 ± 0.09**	0.50 ± 0.11**	0.63 ± 0.15**	0.75 ± 01.14**	0.89 ± 0.13**	0.94 ± 0.20**	1.02 ± 0.22**	1.22 ± 0.24**	1.26 ± 0.18**
CIA + HYA + C4S (30 + 30 mg/kg)	0.13 ± 0.04*	0.19 ± 0.05**	0.31 ± 0.16***	0.38 ± 0.16***	0.51 ± 0.19***	0.64 ± 0.18***	0.78 ± 0.15***	0.87 ± 0.18***	0.95 ± 0.17***	1.06 ± 0.20***	1.12 ± 0.17***

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.05$, **$p < 0.005$ and ***$p < 0.001$ vs CIA + Vehicle.

TABLE IV Effect of HYA and C4S on time-course of body weight increment in rats during 31 days period after immunization

Experimental group	Days										
	11	13	15	17	19	21	23	25	27	29	31
CTRL	23.2 ± 5.23	36.5 ± 6.13	48.4 ± 7.15	73.6 ± 10.5	91.1 ± 15.8	102.4 ± 17.2	119.2 ± 20.3	131.7 ± 22.6	142.4 ± 27.9	153.1 ± 30.6	163.8 ± 28.5
CIA + Vehicle	20.6 ± 4.13	23.2 ± 5.12*	32.6 ± 6.33*	40.1 ± 7.17*	47.7 ± 7.21*	56.5 ± 8.47*	67.3 ± 9.31*	74.6 ± 10.1*	80.5 ± 11.7*	91.7 ± 17.8*	95.9 ± 21.4*
CIA + HYA (30 mg/kg)	21.5 ± 4.63	25.8 ± 6.31	36.2 ± 7.21	45.1 ± 5.25	51.3 ± 7.15	60.2 ± 10.8	73.0 ± 12.6	87.9 ± 11.5**	96.4 ± 12.6**	112.4 ± 12.1**	118.5 ± 14.3**
CIA + C4S (30 mg/kg)	219 ± 3.67	28.3 ± 5.85	37.5 ± 6.43	48.2 ± 6.01**	62.5 ± 7.28***	73.2 ± 9.17***	83.6 ± 11.1**	97.4 ± 13.2***	106.7 ± 14.8***	121.8 ± 13.1***	131.2 ± 15.6***
CIA + HYA + C4S (30 + 30 mg/kg)	22.1 ± 5.12	27.6 ± 4.23	40.8 ± 5.13**	54.4 ± 6.21***	66.5 ± 7.41****	76.3 ± 8.11****	90.7 ± 10.3****	101.5 ± 12.8****	112.6 ± 13.3****	128.9 ± 14.2****	140.1 ± 16.2****

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.001$ vs CTRL; **$p < 0.05$, ***$p < 0.005$ and ****$p < 0.001$ vs CIA + Vehicle.

(0.53 ± 0.07 ΔABS/g protein), and these values were considered normal. In contrast, a significant increase in CD formation was seen in the joint cartilage of CIA-rats given with vehicle (5.37 ± 1.46 ΔAbs/g protein). The administration of both GAGs reduced CD concentration by inhibiting lipid peroxidation in the articular tissue (Table VI).

Cartilage VE Content

Table VII reports cartilage VE levels assayed at the day 31st in the studied groups. In the CTRL rats, VE amounts ranged between 3.0 and 5.0 nmol/g protein, and these values were considered normal. Instead, a marked decrease in VE concentrations was found in the articular cartilage of CIA-rats treated with vehicle (0.75 ± 0.19 nmol/g protein). GAG treatment significantly inhibited the depletion in VE levels (Table VII).

Catalase Activity

Table VIII shows cartilage catalase activity evaluated at the day 31st in the joint cartilage of the considered groups. In the CTRL group CA activities ranged normally between 55.0 and 95.0 U/g protein. On the contrary, a substantial reduction in this enzyme was observed in the cartilage of CIA-rats given with vehicle (13.52 ± 4.85 U/g protein). Also in this case the treatment with HYA, C4S or both polymers significantly limited the decrease in CA activity (Table VIII).

Serum MIP-2 Evaluation

MIP-2 was assayed at day 31st in serum of rats underwent to CIA (Table IX). Very low levels of MIP-2 were seen in the serum of CTRL rats (0.05 ± 0.01 ng/ml). In contrast, a high amount of the cytokine was found in the CIA-vehicle group (2.64 ± 0.91). The treatment with HYA and C4S showed a significant reduction in MIP-2 release (Table IX).

Cartilage ELA Levels

Table X reports cartilage ELA activity assayed at the day 31st in the studied groups. Very low ELA concentrations were measured in the joint cartilage of CTRL rats (25.64 ± 4.35 ng/g protein). Instead, elevated levels of this enzyme were seen in the cartilage of CIA-rats treated with vehicle (475.37 ± 89.72 ng/g protein). Also in this case the administration of the two GAGs showed a marked reduction in neutrophil activation and infiltration (Table X).

TABLE V Effect of HYA and C4S on plasma amount of 2,3-DHBA and 2,5-DHBA analysed in basal conditions and at the day 31st in rats underwent to CIA

	2,3-DHBA (μM)		2.5-DHBA (μM)	
Experimental group	Basal	Day 31st	Basal	Day 31st
CTRL	0.30 ± 0.03	0.37 ± 0.03	0.97 ± 0.15	1.17 ± 0.18
CIA + Vehicle	0.28 ± 0.04	4.51 ± 1.18*	0.89 ± 0.21	13.15 ± 4.39*
CIA + HYA (30 mg/kg)	0.31 ± 0.03	3.38 ± 0.54**	1.04 ± 0.30	8.19 ± 3.13**
CIA + C4S (30 mg/kg)	0.33 ± 0.04	2.72 ± 0.67***	1.02 ± 0.27	6.43 ± 2.47***
CIA + HYA + C4S (30 + 30 mg/kg)	0.29 ± 0.04	2.26 ± 0.62****	0.95 ± 0.17	5.34 ± 1.42****

Values are the mean ± S.D. of 7 animals for each group *$p < 0.001$ vs CTRL; **$p < 0.05$, ***$p < 0.005$ and ****$p < 0.001$ vs CIA + Vehicle.

Histological Analysis

In Table XI and Fig. 1 the histological evaluations of the effect of HYA and C4S in joint of rats underwent to CIA at the end of experiment are reported. A high score was measured in the joint of CIA-rats given only with vehicle. In fact, a marked bone destruction (2.35 ± 0.39), cartilage destruction (2.75 ± 0.43), and white cell infiltration (3.00 ± 0.10) were identified in the joints of these animals at day 31st (Table XI and Fig. 1A). The chronic treatment of rats with HYA, C4S or both GAGs limited bone destruction, reduced cartilage destruction, and decreased the white cell infiltration (Table XI and Fig. 1 B, C, D). This result was also confirmed by ELA reduction (Table X).

DISCUSSION

Acid GAGs are present in blood, usually in proteoglycan form. The main GAG of normal human plasma in chondroitin-4-sulphate (C4S), that is mostly in low sulphate form.[34] Keratan sulphate, heparan sulphate and HYA are the other GAG structures usually detected in human plasma.[34] In animals, the total amounts of GAGs in plasma[35] is similar to those measured in humans.[36] Nevertheless, significant increased values of plasma GAG concentration were observed in a wide types of disease[37–39] including RA in humans[40,41] and experimental arthritis in rat and mouse.[42,43] These changes of circulating GAGs in RA are supposed to represent products of the connective tissue metabolism, and some circulating GAG structures are probably degradation products originating from articular cartilage.[44] However, the high levels of HYA and other GAGs found in RA[41] could not be explained only with the erosion endured by the articular cartilage. In fact, the biological role, the real sites of origin, and the metabolic fate of these aminosugar-containing polysaccharides are not clearly understood. Another approach to explain the increased presence of GAG in the plasma comes from the evidence that some GAGs possess antioxidant activity capable of inhibiting lipid peroxidation.[45–47] The use of these molecules as therapeutic agents showed some positive outcomes both in human and in experimental animal models.[48,49–53] The present study was designed to evaluate the effect of chronic treatment of HYA and C4S in a rat model of CIA. The choice of these two compound among the others GAGs, was done starting from the evidence that they showed an antioxidant activity and are able to inhibit lipid peroxidation.[48,49–53]

The data obtained in the groups treated with HYA, C4S or both GAGs were positive in all considered parameters. Nevertheless, the treatment with only HYA showed a minor protection, at limit of significance (Tables I–VIII,X), or not significant (Table IX and one parameter in Table XI), compared with C4S. In contrast the administration of both compounds showed the maximum effect on limiting the CIA damage in all parameters. The beneficial

TABLE VI Effect of HYA and C4S on articular cartilage Conjugated dienes content evaluated at the day 31st in joint of rats underwent to CIA

Experimental group	ΔABS/g protein
CTRL	0.53 ± 0.07
CIA + Vehicle	5.37 ± 1.46*
CIA + HYA(30 mg/kg)	3.85 ± 1.09**
CIA + C4S (30 mg/kg)	3.12 ± 0.83***
CIA + HYA + C4S (30 + 30 mg/kg)	2.54 ± 0.64****

Values are the mean ± S.D. of 7 animals for each group. *p < 0.001 vs CTRL; **$p < 0.05$, ***$p < 0.005$ and ****$p < 0.001$ vs CIA + Vehicle.

TABLE VII Effect of HYA and C4S on articular cartilage vitamin E content assayed at the day 31st in joint of rats underwent to CIA

Experimental group	nmol/g protein
CTRL	3.83 ± 0.91
CIA + Vehicle	0.75 ± 0.19*
CIA + HYA(30 mg/kg)	1.08 ± 0.32**
CIA + C4S (30 mg/kg)	1.35 ± 0.43***
CIA + HYA + C4S (30 + 30 mg/kg)	1.52 ± 0.47****

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.001$ vs CTRL; **$p < 0.05$, ***$p < 0.01$ and ****$p < 0.005$ vs CIA + Vehicle.

TABLE VIII Effect of HYA and C4S on articular cartilage Catalase content analysed at the day 31st in joint of rats underwent to CIA

Experimental group	U/g protein
CTRL	74.37 ± 19.22
CIA + Vehicle	13.52 ± 4.85*
CIA + HYA (30 mg/kg)	19.86 ± 5.42**
CIA + C4S (30 mg/kg)	22.92 ± 5.57***
CIA + HYA + C4S (30 + 30 mg/kg)	24.13 ± 6.31****

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.001$ vs CTRL; **$p < 0.05$, ***$p < 0.01$ and ****$p < 0.005$ vs CIA + Vehicle.

TABLE X Effect of HYA and C4S on articular cartilage Elastase content evaluated at the 31st in joint of rats underwent to CIA

Experimental group	ng/g protein
CTRL	25.64 ± 4.35
CIA + Vehicle	475.37 ± 89.72*
CIA + HYA (30 mg/kg)	372.31 ± 67.56**
CIA + C4S (30 mg/kg)	358.92 ± 51.34**
CIA + HYA + C4S (30 + 30 mg/kg)	315.78 ± 63.54***

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.001$ vs CTRL; **$p < 0.05$ and ***$p < 0.005$ vs CIA + Vehicle.

effect exerted by HYA and C4S was evident by measuring the incidence of CIA. Similar results were obtained for the mean arthritis severity score, the hind paw diameter and the body weight decrement.

The detection of 2,3-DHBA and 2,3-DHBA is usually considered a bona fide reporter for the flux of hydroxyl radicals which reacted with salicylic acid during oxidative stress.[25,54] The data obtained show that a large amount of $OH^{\bullet}$. Radical was generated in CIA-rats. The treatment with HYA and C4S reduced both the 2,3-DHBA and 2,5-DHBA formation, perhaps by directly trapping the $OH^{\bullet}$ radical and/or by inhibiting its production. Because salicylic acid makes the stable derivatives by trapping $OH^{\bullet}$, it may theoretically be considered as a scavenger of $OH^{\bullet}$. Therefore, we compared the effect of salicylic acid on the considered parameter against CIA-rats and CIA-treated rats (data not shown). Salicylic acid failed to exert any positive effect on the CIA-rats. This suggests that salicylic acid does not have a significant scavenging effect because it can trap only a small portion (11%) of produced OH.[55]

Lipid peroxidation is considered a critical mechanism of the injury occurring during RA.[12,56] The evidence supporting these biochemical changes is based on analysis of a wide number of intermediate products.[57] An indicative method extensively used of evaluating lipid peroxidation is tissue CD analysis.[57] The increment of CD levels found in the CIA + Vehicle group at day 31 is consistent with the occurrence of a free-radical-mediated damage. The treatment with the two GAGs limited membrane lipid peroxidation and tissue damage.

TABLE IX Effect of HYA and C4S on serum MIP-2 content assayed at the day 31st in rats underwent to CIA

Experimental group	ng/ml
CTRL	0.05 ± 0.01
CIA + Vehicle	2.64 ± 0.91*
CIA + HYA(30 mg/kg)	1.76 ± 0.58
CIA + C4S (30 mg/kg)	1.39 ± 0.41**
CIA + HYA + C4S (30 + 30 mg/kg)	1.20 ± 0.43***

Values are the mean ± S.D. of 7 animals for each group. *$p < 0.001$ vs CTRL; **$p < 0.01$ and ***$p < 0.005$ vs CIA + Vehicle.

The large production of ROS that occurs upon the arthritis development in the articular cartilage decreased tissue Vitamin E (VE) and CA concentrations as a consequence of their consumption during oxidative damage.[58,59] This reduction contributes to increased cellular destruction by favouring free radical attack. HYA and C4S limited the VE, and CA consumption, probably, by competing in ROS scavenging, or in the reduction of ROS generation.

Many findings have indirectly implicated MIP-2 as a contributor to the cellular damage in CIA.[60] The high levels of this cytokine may be interpreted as a progression of cartilage cell injury.[60] The antioxidant activity of GAGs may have reduced plasma MIP-2 values and consequently joint tissue injury.

ELA values showed that a marked decrease of activated PMNs occurred in joint cartilage. This reduction in PMNs infiltration and the other biochemical parameters were evaluated by histological analysis, thus confirming the protective effects of the two GAGs. We suggest that the decrease in neutrophil accumulation induced by GAGs may be due to inhibition of lipid peroxidation and the consequent reduction in the chemotactic peroxide production.[57]

In light of these outcomes, which could be the mechanism by which HYA and C4S protect the articular tissue against ROS overproduction? HYA and C4S are linear polymers formed by alternating hexuronic acid and hexosamine units. HYA is non-sulphated compound, whereas C4S is sulphated in the position 4 of the aminosugar. One plausible explanation of the antioxidant activity for HYA and C4S is the presence, in their structure, of carboxylic group that may bound the transition metals like Cu^{++} or Fe^{++}[47,61] that are in turn responsible of the initiation of Fenton's reaction. In this way these molecules may be function as metal chelators like the antioxidant deferoxamine or the calcium chelator ethylendiaminetetracetic acid (EDTA). Another antioxidant mechanism may be the directly scavenge effect of HYA and C4S on free radical molecules especially the detrimental hydroxyl radical ($OH^{\bullet}$) or other Fenton's reaction intermediates like

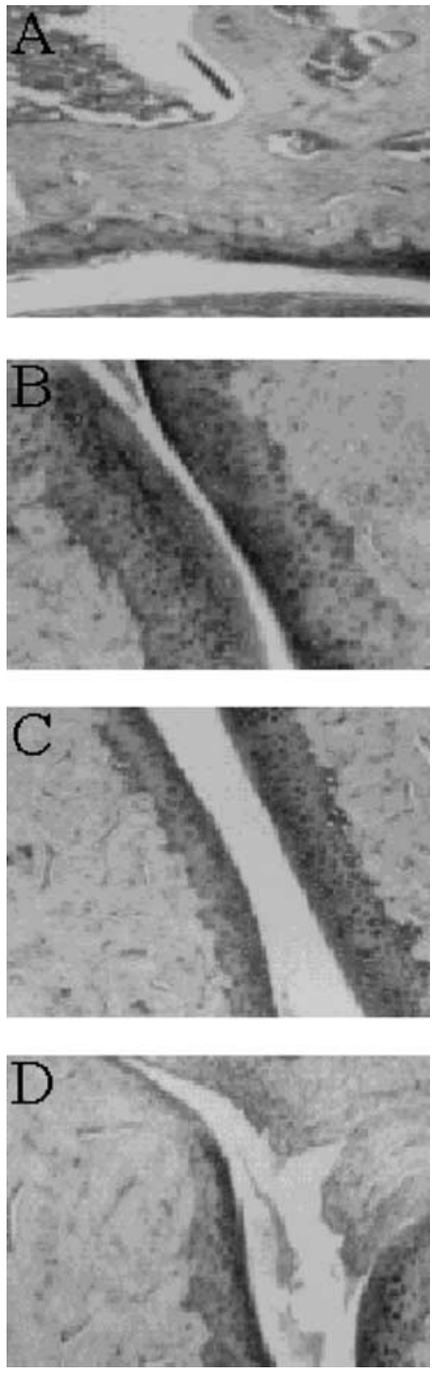

FIGURE 1 Histopathologic findings in joint sections of the considered experimental groups underwent to CIA with vehicle or with HYA, C4S or both glycosaminoglycans at the end of the experiment. (A) CIA + vehicle; (B) CIA + HYA; (C) CIA + C4S; (D) CIA + HYA + C4S.

the superoxide anion.[19,50] These hypotheses could explain the increased levels of GAGs during RA. In fact elevated circulating levels of GAGs may be a biological response against free radical production. The scope of our work was to increase the physiological levels of HYA and C4S by administering these endogenous compounds. In fact we assessed antioxidant parameters and free radical presence in the arthritic joint and the changes exerted by the GAG treatment never reported in previous works. Several findings have previously reported an increase in blood and tissue distribution of HYA and C4S after their administration in rats.[62,63] In our experiment, we evaluated *in vivo* HYA and C4S treatment by the intraperitoneal way, never used before. We suggest that, after the intraperitoneally administration, these polymers may be absorbed by the lymphatic and blood vessels and then achieve the sites of free radical production at high concentrations. Besides, the amount of the HYA and C4S may cause an increase in the total negative charge with a consequent inhibition of the lymphocyte interactions with the target cell surface. In this way GAGs could be exert a positive antiinflammatory effect.

These observations suggest a possible role of endogenous GAGs to limit/control the progression of this kind of disease and GAGs could be as useful tool to investigate the role of antioxidants in the RA.

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References

[1] Akil, M. and Amos, R.S. (1995) "ABC of rheumatology. Rheumatoid arthritis II: treatment", *British Medical J.* **310**, 587–590.
[2] Feldmann, M., Brennan, F.M. and Maini, R.N. (1996) "Rheumatoid arthritis", *Cell* **85**, 307–310.
[3] Doran, M.F., Pond, G.R., Crowson, C.S., O'Fallon, W.M. and Gabriel, S.E. (2002) "Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period", *Arthritis Rheum.* **46**, 625–631.
[4] VanderBorght, A., Geusens, P., Raus, J. and Stinissen, P. (2001) "The autoimmune pathogenesis of rheumatoid arthritis: role of autoreactive T cell and new immunotherapies", *Seminar Arthritis Rheum.* **31**, 160–175.
[5] Myers, L.K., Rosloniec, E.F., Cremer, M.A. and Kang, A.H. (1997) "Collagen-induced arthritis, an animal model of autoimmunity", *Life Sci.* **61**, 1861–1878.
[6] Lunec, J. (1990) "Free radicals: their involvement in disease processes", *Ann. Clin. Biochem.* **27**, 173–182.
[7] Bauerova, K. and Bezek, A. (1999) "Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis", *Gen. Physiol. Biophys.* **18**, 15–20.
[8] Jasin, H.E. (2000) "Mechanisms of joint destruction in rheumatoid arthritis", *J. Ortho. Sci.* **5**, 178–181.
[9] Wipke, B.T. and Allen, P.M. (2001) "Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis", *J. Immunol.* **167**, 1601–1608.
[10] Knight, J.A. (2000) "Review: free radicals, antioxidants, and the immune system", *Ann. Clin. Lab. Sci.* **30**, 145–158.
[11] Heliovaara, M., Knekt, P., Aho, K., Aaran, R.K., Alfthan, G. and Aromaa, A. (1994) "Serum antioxidants and risk of rheumatoid arthritis", *Ann. Rheum. Dis.* **53**, 51–53.

[12] Gambhir, J.K., Lali, P. and Jain, A.K. (1997) "Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis", *Clin. Biochem.* **30**, 351–355.
[13] Darlington, L.G. and Stone, T.W. (2001) "Antioxidants and fatty acids in the amelioration of rheumatoid arthritis and related disorders", *Br. J. Nutr.* **85**, 251–269.
[14] Ostrakhovitch, E.A. and Afanas'ev, I.B. (2001) "Oxidative stress in rheumatoid arthritis leukocytes: suppression by rutin and other antioxidants and chelators", *Biochem. Pharmacol.* **62**, 743–746.
[15] McLain, D.A. (2001) "Rheumatoid arthritis: update on new therapies", *South. Med. J.* **94**, 893–895.
[16] Iozzo, R.V. (1998) "Matrix proteoglycans: from molecular design to cellular function", *Annu. Rev. Biochem.* **67**, 609–652.
[17] Reddy, G.K. and Dhar, S.C. (1991) "Metabolism of glycosaminoglycans in tissue of arthritic rat", *Mol. Cell. Biochem.* **106**, 117–124.
[18] Caterson, B., Flanery, C.R., Hughes, C.E. and Little, C.B. (2000) "Mechanisms involved in cartilage proteoglycan cataboloism", *Matrix Biol.* **19**, 333–344.
[19] Presti, D. and Scott, J.E. (1994) "Hyaluronan-mediated protective effect against cell damage caused by enzymatically produced hydroxyl (OH.) radicals is dependent on hyaluronan molecular mass", *Cell Biochem. Function* **12**, 281–288.
[20] Cortivo, R., Brun, P., Cardarelli, L., O'Regan, M., Radice, M. and Abatangelo, G. (1996) "Antioxidant effects of hyaluronan and its alpha-methyl-prednisolone derivative in chondrocyte and cartilage cultures", *Semin. Arthritis Rheum.* **26**, 492–501.
[21] Albertini, R., De Luca, G., Passi, A., Moratti, R. and Abuja, P.M. (1999) "Chondroitin-4-sulfate protects high-density lipoprotein against copper-dependent oxidation", *Arch. Biochem. Biophys.* **365**, 143–149.
[22] Cremer, M. (1988) "Type II collagen-induced arthritis in rats", In: Greenwald, R.A. and Diamond, H.S., eds, Handbook of animal models for the rheumatic disease (CRC Press, Boca Raton, FL) **1**, pp. 17–27.
[23] Larsson, P., Kleinau, S., Holmdahl, R. and Klareskog, L. (1990) "Homologous type II collagen-induced arthritis in rats. Characterization of the disease and demonstration of clinically distinct forms of arthritis in two strains of rats after immunization with the same collagen preparation", *Arthritis Rheum.* **33**, 693–701.
[24] Floyd, R.A., Watson, J.J. and Wong, P.K. (1984) "Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and salicylate products", *J. Biochem. Biophys. Methods* **10**, 221–235.
[25] Powell, S.R. (1984) "Salicylate trapping of $OH^{\bullet}$ as a tools for studying post-ischemic oxidative injury", *Free Radic. Res.* **21**, 355–370.
[26] Halliwell, B., Kaur, H. and Ingleman-Sundberg, M. (1991) "Hydroxylation of salicylates as an assay for hydroxyl radicals: a cautionary note", *Free Radic. Biol. Med.* **10**, 439–441.
[27] Campo, G.M., Squadrito, F., Cecarelli, S., Calò, M., Avenoso, A., Campo, S., Squadrito, G. and Altavilla, D. (2001) "Reduction of carbon tetrachloride-induced rat liver injury by IRFI042, a novel dual vitamin E-like antioxidant", *Free Radic. Res.* **34**, 379–393.
[28] Onodera, T. and Ashraf, M. (1991) "Detection of hydroxyl radicals in the postischemic reperfused heart using salicylate as a trapping agent", *J. Mol. Cell. Cardiol.* **23**, 365–370.
[29] Corongiu, F., Lai, M. and Milai, A. (1983) "Carbon tetrachloride, bromotrichlormethane and ethane acute intoxication", *Biochem. J.* **212**, 625–631.
[30] Nieremberg, D.W. and Lester, D.C. (1985) "Determination of vitamin E and A in serum and plasma using a simplified clarification method and high-performance liquid chromatography", *J. Chromatogr.* **345**, 275–284.
[31] Momohara, S., Kashiwazaki, S., Inoue, K., Saito, S. and Nakagawa, T. (1997) "Elastase from polymorphonuclear leukocyte in articular cartilage and synovial fluids of patients with rheumatoid arthritis", *Clin. Rheumatol.* **16**, 133–140.
[32] Yoshimura, K., Nakagawa, S., Koyama, S., Kobayashi, T. and Homma, T. (1994) "Roles of neutrophil elastase and superoxide anion in leukotriene B4-induced lung injury in rabbit", *J. Appl. Physiol.* **76**, 91–96.
[33] Van de Berg, W.B., Joosten, L.A.B., Helsen, M.M.A. and van de Loo, F.A.J. (1994) "Amelioration of established murine collagen-induced arthritis with anti-IL-1 treatment", *Clin. Exp. Immunol.* **95**, 237–243.
[34] Calatroni, A., Donnelly, P.V. and Di Ferrante, N. (1969) "The glycosaminoglycans of human plasma", *J. Clin. Investig.* **48**, 332–343.
[35] Ferlazzo, A.M., Campo, S., Vinci, R., Ferrlazzo, A. and Calatroni, A. (1997) "Concentration and composition of serum and plasma glycosaminoglycans in domestic animal species", *Comp. Biochem. Physiol.* **118B**, 935–942.
[36] Calatroni, A., Vinci, R. and Ferlazzo, A.M. (1992) "Characteristics of the interactions between acid glycosaminoglycans and proteins in normal plasma as revealed by the behaviour of the protein-polysaccharide complexes in ultrafiltration and chromatographic procedures", *Clin. Chim. Acta* **206**, 167–180.
[37] Friman, C., Nordstrom, D. and Eronen, I. (1987) "Plasma glycosaminoglycans in systemic lupus erythematosus", *J. Rheumatol.* **14**, 1132–1134.
[38] Laurent, T.C., Laurent, U.B.G. and Fraser, J.R.E. (1996) "Serum hyaluronan as a disease marker", *Ann. Med.* **28**, 241–253.
[39] Radhakrishnamurthy, B., Tracy, R.E., Dalferes, Jr. and Berenson, G.S. (1998) "Proteoglycans in human coronary arterosclerotic lesions", *Exp. Mol. Pathol.* **65**, 1–8.
[40] Engstrom-Laurent, A. and Hallgren, R. (1985) "Circulating hyaluronate in rheumatoid arthritis: relationship to inflammatory activity and the effect of corticosteriod therapy", *Ann. Rheum. Dis.* **44**, 83–88.
[41] Roughley, P.J. (2001) "Articular cartilage and changes in arthritis: non-collagenous proteins and protoglycans in the extracellular matrix of cartilage", *Arthritis Res.* **3**, 342–347.
[42] Bjork, J., Kleinau, S., Tengblad, A. and Smedegard, G. (1989) "Elevated levels of serum hyaluronate and correlation with disease activity in experimental models of arthiritis", *Arthritis Rheum.* **32**, 306–311.
[43] Van der Kraan, P.M., de Lange, J., Vitters, E.L., van Beuningen, H.M., van Osch, G.J., van Lent, P.L. and van den Berg, W.B. (1994) "Analysis of changes in proteoglycan content in murine articular cartilage using image analysis", *Osteoarthritis Cartilage* **2**, 207–214.
[44] Thonar, E.J., Lenz, M.E., Klintworth, G.K., Caterson, B., Pachman, L.M., Glickman, P., Katz, R., Huff, J. and Kuettner, K.E. (1985) "Quantification of keratan sulfate in blood as a marker of cartilage catabolism", *Arthritis Rheum.* **28**, 1367–1376.
[45] Foschi, D., Castoldi, L., Radaelli, E., Abelli, P., Calderoni, G., Rastrelli, A., Mariscotti, C., Marazzi, M. and Trabucchi, E. (1990) "Hyaluronic acid prevents oxygen free-radical damage to granulation tissue: a study in rats", *Int. J. Tissue Reactivity* **12**, 333–339.
[46] Arai, H., Kashiwagi, S., Nagasaka, Y., Uchida, K., Hoshii, Y. and Nakamura, K. (1999) "Oxidative modification of apolipoprotein E in human very-low density lipoprotein and its inhibition by glycosamnoglycans", *Arch. Biochem. Biophys.* **367**, 1–8.
[47] Albertini, R., Passi, A., Abuja, P.M. and De Luca, G. (2000) "The effect of glycosaminoglycans and proteoglycans on lipid peroxiation", *Int. J. Mol. Med.* **6**, 129–136.
[48] Sakai, A., Hirano, T., Okazaki, R., Okimoto, N., Tanaka, K. and Nakamura, T. (1999) "Large dose ascorbic acid administration suppresses the development of arthritis in adjuvant-infected rats", *Arch. Orthopaed. Trauma Surg.* **119**, 121–126.
[49] Graf, J., Neusel, E., Schneider, E. and Niethard, F.U. (1993) "Intra-articular treatment with hyaluronic acid in osteoarthritis of the knee joint: a controlled clinical trial versus mucopolysaccharide polysulfuric acid ester", *Clini. Exp. Rheumatol.* **11**, 367–372.
[50] Breborowicz, A., Wieczorowska, K., Martis, L. and Oreopoulos, D.G. (1994) "Glycosaminoglycan chondroitin sulphate prevents loss of ultrafiltration during peritoneal dialysis in rats", *Nephron* **67**, 346–350.
[51] Shankland, 2nd, W.E. (1998) "The effects of glucosamine and chondroitin sulfate on osteoarthritis of the TMJ: a preliminary report of 50 patients", *Cranio* **16**, 230–235.

[52] Leffler, C.T., Philippi, A.F., Leffler, S.G., Mosure, J.C. and Kim, P.D. (1999) "Glucosamine, chondroitin, and manganese ascorbate for degenerative joint disease of the knee or low back: a randomized, double-blind, placebo-controlled pilot study", *Mil. Med.* **164**, 85–91.
[53] Beren, J., Hill, S.L., Diener-West, M. and Rose, N.R. (2001) "Effect of pre-loading oral glucosamine HCl/chondroitin sulfate/manganese ascorbate combination on experimental arthritis in rats", *Exp. Biol. Med.* **226**, 144–151.
[54] Grootveld, M. and Halliwell, B. (1986) "Aromatic hydroxylation as a potential measure of hydroxyl-radical formation *in vivo*: identification of hydroxylated derivatives of salicylate in human body fluid", *Biochem. J.* **237**, 499–504.
[55] Takemura, G., Onodera, T. and Ashraf, M. (1992) "Quantification of hydroxyl radical and its lack of relevance to myocardial injury during early reperfusion after graded ischemia in rat heart", *Circulation Res.* **71**, 96–105.
[56] Jira, W., Spiteller, G. and Richter, A. (1997) "Increased levels of lipid oxidation products in low density lipoproteins of patients suffering from rheumatoid arthritis", *Chem. Phys. Lipids* **87**, 81–89.
[57] Wills, E.D. (1987) "Evaluation of lipid peroxidation in lipid and biological membranes", In: Snell, K. and Mullock, B., eds, Biochemical Toxicology; A Practical Approach (IRL Press, Oxford), pp. 127–152.
[58] Kizilntuc, A., Cogalgil, S. and Cerrahoglu, L. (1998) "Carnitine and antioxidants levels in patients with rheumatoid arthritis", *Scand. J. Rheumatol.* **27**, 441–445.
[59] Hassan, M.Q., Hadi, R.A., Al-Rawi, Z.S., Padron, V.A. and Stohs, S.J. (2001) "The glutathione defense system in the pathogenesis of rheumatoid arthritis", *J. Appl. Toxicol.* **21**, 69–73.
[60] Kasamat, T., Strieter, R.M., Lukacs, N.W., Lincoln, P.M., Burdick, M.D. and Kunkel, S.L. (1995) "Interleukin-10 expression and chemokine regulation during the evolution of murine type II collagen-induced arthritis", *J. Clini. Investig.* **95**, 2868–2876.
[61] Volpi, N. and Tarugi, P. (1999) "Influence of chondroitin sulfate charge density, sulfate group position, and molecular mass on Cu^{++}-mediated oxidation of human low-density lipoproteins: effect of normal human plasma-derived chondroitin sulfate", *J. Biochem.* **125**, 297–304.
[62] Palmieri, L., Conte, A., Giovannini, L., Lualdi, P. and Ronca, G. (1990) "Metabolic fate of exogenous chondroitin sulfate in the experimental animal", *Arzneimittelforschung* **40**, 319–323.
[63] Gustafson, S. and Bjorkman, T. (1997) "Circulating hyaluronan, chondroitin sulphate and dextran sulphate bind to a liver receptor that does not recognise heparin", *Glycoconjugate J.* **14**, 561–568.