Chondroitin Sulphate: Antioxidant Properties and Beneficial Effects

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Abstract: Most biological molecules exhibit more than one function. In particular, many molecules have the ability to directly/indirectly scavenge free radicals and thus act in living organisms as antioxidant. During oxidative stress, the increase of these molecules levels seems to be a biological response that in synergism with the other antioxidant defence systems may protect cells from oxidation. Among these structures, chondroitin sulphate is a biomolecule which has increasingly focused the interest of many research groups due to its antioxidant activity. This review briefly summarises the action of chondroitin sulphate in reducing molecular damage caused by free radicals and associated oxygen reactants.

Key Words: Glycosaminoglycans, antioxidants, lipid peroxidation, oxidative stress, reactive oxygen species, hydroxyl radical, transition metals, free radical scavenger, chondroitin sulphate, metalloproteinases.

INTRODUCTION

Cellular exposure to exogenously or endogenously generated oxidants causes macromolecular damage including protein oxidation, lipid peroxidation, and nucleic acid instability and mutation [1-2]. Oxidative damage of cellular constituents has been associated with increased incidence of a number of diseases, and is likely to be an important contributor to inflammation [1], carcinogenesis [3], rheumatoid arthritis [4] diabetes [5] ischaemia [6] ulcerative colitis [7] liver disease [8], atherosclerosis [9], etc. Oxidative injury occurs to some extent not only through the direct action of superoxide anions (O₂⁻) and other reactive oxygen species (ROS), but it is also partly secondarily derived from peroxide radicals, lipid hydroperoxides and several lipid fragmentation products that behave as active oxidising agents in various tissues such as the kidney, heart, liver, brain, lung, gut, skin etc. [10]. A number of endogenous antioxidant defence mechanisms that limit the levels of potentially dangerous reactive oxygen species have been identified. Endogenous defences are enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and nonenzymatic systems that are able, in normal conditions, to neutralise free radicals.

The MMPs (Matrix MetalloProteinases) are a family of calcium-dependent zinc-containing endopeptidases, which are capable of degrading a wide variety of extracellular matrix components [11]. MMPs play important roles in tissue remodelling and repair. The activity of MMPs is regulated by several types of inhibitors, of which the TIMPs (Tissue Inhibitors of MetalloProteinases) are the most important [12]. The balance between MMPs and TIMPs is important for physiological tissue remodelling. A deregulation of this balance is a characteristic feature of pathological conditions involving extensive tissue degradation and destruction, such as arthritis, diabetes, liver injury and atherosclerosis. ROS are known to react with thiol groups, such as those involved in preserving MMP latency, so they could modulate the activity of MMPs [13].

Although oxidative stress is an unavoidable consequence of aerobic metabolism, the majority of four electron reduction of the O₂ molecule is of a rather low reactivity. However, trace amounts of unprotected transition metal ions like Fe³⁺ and Cu⁺ can catalyse the Haber-Weiss reaction of the low reactive O₂⁻ and H₂O₂ that gives rise to the highly toxic hydroxyl radical. O₂⁻‘s role in the iron or copper catalysed Haber-Weiss reaction is the superoxide-assisted Fenton reaction [14].

The catalytic effect of transition metal ions-induced OH⁻ generation can be reduced by using molecules possessing chelating activity against these metal ions [15]. For instance, the reactivity of iron varies greatly dependent upon its ligand-based environment. In general, iron is coordinated by O, N and S atoms. Oxygen ligands prefer Fe³⁺, thus decreasing the reduction potential of the iron [16]. Therefore chelators with oxygen ligands, such as citrate, promote the oxidation of Fe²⁺ to Fe³⁺, while chelators that contain nitrogen ligands, such as phenanthrolines, inhibit the oxidation of Fe³⁺. Many chelators, such as ethylenediaminetetraacetic acid (EDTA) and Desferal, are able to bind both Fe³⁺ and Fe²⁺; however, the stability constants are much greater for the Fe³⁺-chelator complexes.

Glycosaminoglycans (GAGs) have recently been suggested to show antioxidant properties mainly for hyaluronan (HA) and chondroitin-4-sulphate (C4S), in both in vitro and in vivo experimental models [17-21]. This antioxidant activity is probably due to their capacity to chelate transition metals like Cu⁺ or Fe³⁺ that are in turn responsible for the initiation of Haber-Weiss and Fenton’s reaction [18, 21-22].

Chondroitin sulphate (CS) is the main GAG representative in blood and significant increases with respect to normal values of plasma glycosaminoglycan concentration were
observed in patients with different types of diseases such as systemic lupus erythematosus [23], rheumatoid arthritis [24], and liver disease [25]. The obvious explanation is that glycosaminoglycans originate from the metabolism of inflamed tissues. Nevertheless, the exact meaning of their rise is at the moment unclear.

**MOLECULAR CHARACTERISTICS OF CHONDROITIN SULPHATES**

CS is the most abundant glycosaminoglycan in the body. GAGs are long unbranched polysaccharides containing a repeating disaccharide unit [26-27]. The disaccharide units contain either of the two modified sugars N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) and a uronic acid such as glucuronic acid (GlcUA) or iduronic acid (IdUA). With the exception of hyaluronan, being composed of alternate units of GlcNAc and GlcUA, the GAGs bear sulphate groups, either ester or amido groups, and are build up as copolymer of different disaccharide units. In CS molecules, the disaccharide unit contains GlcUA and GalNAc and usually has one sulphate group per disaccharide, which is predominantly either on the 4 or 6 carbon of the GalNAc residue: the chondroitin sulphate chains are copolymers of segments of one to several 6-sulphated disaccharides interrupted by segments of one to several 4-sulphated disaccharides. They are named chondroitin-4-sulphate (Fig. 1A) or chondroitin-6-sulphate (C6S) (Fig. 1B) on the basis of the prevalence of 4-sulphated or 6-sulphated segments, respectively. The number of repeat disaccharides within a preparation of chains varies from 20 to 60, with an average of about 40, corresponding to about 20 thousand molecular weight. Although cartilage and intervertebral disc are tissues having the highest content of chondroitin sulphate, these molecules are largely distributed in tissues, and are the main glycosaminoglycan components in blood and urine. Again except hyaluronan, glycosaminoglycans in the body are linked to core proteins, forming protein-GAG covalent complexes called proteoglycans (PGs). In water solution, the highly polar glycosaminoglycan chains extend perpendicularly from the core in a brush-like structure. The linkage of GAGs to the protein core involves a specific trisaccharide composed of two galactosyl residues and a xylose residue (GAG-GalGalXyl-O-CH2-protein). The trisaccharide linker is coupled to the protein core through an O-glycosidic bond to a serine residue in the protein. Chondroitin sulphate containing proteoglycans (CS-PGs) are a large family of heterogeneous structures according to the type, length and charge of the chondroitin sulphate chains, their number and distribution, and the nature of the protein moiety. There have emerged amongst them, however, some distinctive sub-families that contain protein motifs of related sequence, such as the aggrecan, the leucine-rich and the serglycin proteoglycan families. The aggrecan family PGs are modular structures composed of structural motifs, such as epidermal growth factor-like domains, lectin-like domains, complement regulatory protein-like domain, immunoglobulin folds and proteoglycans tandem repeats [28]. The family includes aggrecan, which is expressed in cartilage and contains a high number of chondroitin sulphate chains, neurocan and brevican, expressed in brain tissues, versican and the cell surface receptor CD44. PGs of the leucine-rich family contain in their proteins repeating leucine-rich motifs, and only one or two glycosaminoglycan chains. They include decorin, having a role in regulating collagen fibrillogenesis and biglycan. Serglycin are serine and glycine rich PGs, which are present in myeloid cells. A further CS-PG is thrombomodulin of the endothelial cell membrane, interacting with thrombin. The biosynthesis of CS-PGs starts from the protein core, which is synthesised first and serves as an acceptor for the monosaccharide transferases involved in chondroitin sulphate chain biosynthesis, which occurs in the endoplasmic reticulum: the first enzyme involved is xylosyltransferase, followed by two distinct galactosyltransferases, and one specific glucuronyltransferase. The subsequent steps in chain elongation are the alternating additions of GalNAc and GlcUA, by specific transferases. The process is terminated by sulphation, by transfer of the sulphate group from phosphoadenosine phosphosulphate (PAPS), catalysed by two enzymes specific for positions 4 and 6, respectively. Sulphation starts before chain elongation is stopped.

**CS AS METAL ION CHELATOR**

The GAGs capacity to bind metal ions and other charged molecules is widely known from long time. The oldest findings related to the chondroitin sulphate and its interaction with metal ions were reported by Einbinder and Schubert [29]. At that time, the metal cation widely studied was the calcium ion and also Bowness [30], Bettelheim [31], and Urist et al. [32] reported interesting studies on calcium binding by chondroitin sulphate alone or associated with collagen.

Calcium ion environment in chondroitin-4-sulphate has been elucidated by Cael et al. [33]: the ion bridges carboxylate groups in separate chains, and carboxylate and sulphate ester groups within a single chain. Previous studies [34] by equilibrium dialysis showed that chondroitin sulphate in nasal septa cartilage has more affinity for copper (II) ion than

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**Fig. (1).** Chemical structures of chondroitin-4-sulphate (A) and chondroitin-6-sulphate (B).
for calcium ion. Copper (II) interaction with heparin was investigated by Stivala [35-36] and interactions with both carboxylic acid and sulphate groups were suggested.

An accurate analysis of spectra of chondroitin-4-sulphate and chondroitin-6-sulphate structures in the presence of ytterbium(III) by nuclear magnetic resonance (NMR) was carried out by Balt et al. [37]. In this study, it has been found that the structure of the ytterbium-polysaccharide complex in solution is similar to that of calcium-C4S, in which Ca^{++} coordinates to the carboxylate group. The same workers [38] reported a study in which the binding of metal ions, such as Cu^{++} and Na⁺, to polysaccharides was investigated. In this report, Authors elucidated that the site binding of chondroitin sulphate is localised into the carboxylate group and the sulphate group binds the ions with electrostatic interaction only. The nitrogen atom of the N-acetyl group appeared not to be involved in the bonds of cations to chondroitin sulphate. Yang et al. [39] performed a study which showed the high ordered state of the iron into the chondroitin sulphate complex with respect to other iron complexes. The diverse chondroitin sulphate structures and conformations are very important to well define the CS-metal ion interaction. An extended analysis of the different types of chondroitin sulphate structures and the physicochemical consequence of epimerisation evaluated in terms of intrinsic flexibility were investigated by Scott et al. [40].

It is clear that polysaccharides may interact with cations to very different degrees, depending upon the incorporation of anionic side chains. Very low density of such side chains, as in celluloses, rules out the role of cations in aqueous solution. Increased anionic density due to sulphation favours chain repulsion and water binding rather than interaction with cations. Increased anionic density due to carboxylate or ester phosphate certainly favours the interaction of cations. The interaction is selective: calcium ion shows the ability to interact with neutral oxygen donors as carbonyls and alcohols, and immediate binding of a large number of centres. Copper (II) ion prefers N or S donors [41].

Acid glycosaminoglycans contain both carboxylic acid and ester sulphate groups. As already mentioned, calcium ion may bind both groups in chondroitin-4-sulphate. It is clear that the position of sulphate ester group may affects the GAG-cation interaction. In carrageenans, the sulphation at O-2 on the 3. 6-anhydro-D-galactose and O-4 on the 1,3-substituted galactose residue does not interfere with double helix formation, while sulphation at O-6 of the 1,4-substituted galactose residues inhibits double helix formation [42]. In chondroitin-4-sulphate, all the major substituents of the disaccharide sugar rings are equatorial, except for the axial sulphate ester group on position 4 of N-acetylgalactosamine. The position of the sulphate groups along the center line of the polymer backbone gives high negative charge density on the long axis of the molecule, and restricts the chondroitin-4-sulphate from self-aggregation. Chondroitin-6-sulphate, in which the sulphate groups are at the periphery of the molecule, can self-aggregate [43]. C4S exerts antioxidant effects while C6S does not. Cations may bind carboxylic groups. The antioxidant activity of chondroitin-4-sulphate higher than that of HA suggests that the sulphate groups are synergistically involved in cation binding, as shown for calcium ion.

**IN VITRO EVIDENCES**

Many in vitro evidences that GAGs may protect endothelial cells by reactive oxygen species were provided. In an experimental model of glutamate-induced neuronal toxicity, Okamoto et al. [44] investigated the neuroprotective activity of chondroitin sulphate proteoglycans (CSPGs) on excitotoxic cell death and long-term survival of neurons in primary cultured neurons of rat cortex. In another study, CSPGs protected neuronal death induced by 200 μM N-methyl-D-aspartate (NMDA), kainate or 100 μM alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) [45].

Albertini et al. [46] showed that the antioxidant effect of proteoglycans obtained from bovine cornea protected liposome from peroxidation induced by Fe^{++} and from fragmentation. Papain digestion of core protein reduced the protective effect of chondroitin sulphate, dermanatan sulphate-proteoglycan, whereas it abolished completely that of keratan sulphate-proteoglycan.

Oxidation of low density lipoprotein (LDL) may play a crucial role in the initiation and progression of atherosclerosis. The oxidation is mediated by transition metals that in turn catalyse ROS production. Oxidised LDL are removed by macrophages via scavenger receptors, leading to foamcell formation that is the basal compound of atheromatous plaque [47]. Albertini et al. [48] showed that C4S (and not C6S) inhibited copper-induced LDL oxidation by prolonging the lag time and reducing the rate of propagation. A possible initial key reaction in LDL oxidation, the reduction of copper(II) to copper(I) by LDL, was rather inhibited in the presence of C4S, probably by masking copper binding sites.

The effect of chondroitin-4-sulphate and chondroitin-6-sulphate on the oxidation of human high density lipoprotein (HDL) has been investigated by kinetic analysis [18]. Chondroitin-4-sulphate increased the lag time and reduced the maximum rate of HDL oxidation induced by Cu^{++}. On the contrary, chondroitin-6-sulphate was ineffective. Since chondroitin-4-sulphate was able to bind Cu^{++}, Authors suggest that this resulted in less Cu^{++} available for HDL oxidation and likely represented the mechanism of the protective effect.

Volpi and Tarugi [49] investigated the antioxidant activity of chondroitin sulphate, obtained from different sources, on Cu^{++}-induced LDL oxidation, and the influence of CS charge (decreasing charge densities proved effective) and size (low molecular masses were ineffective). Furthermore, Volpi and Tarugi [50] evaluated the antioxidant effect of various glycosaminoglycans of different origin both on Cu^{++}- and 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)-induced human LDL oxidation. Hyaluronan had no effect. Chondroitin sulphate from beef trachea produced a very strong protective antioxidant effect.

Arai et al. [17] studied the antioxidant activity of glycosaminoglycans on Cu^{++} and α,α-diphenyl-beta-picrylhydrazyl radical-induced oxidative modification of apolipoprotein E (apoE) in human very low-density lipoprotein (VLDL).
The VLDL oxidation catalysed by Cu\(^{++}\) led to the lipid peroxidation, the formation of aggregates and covalent modification of apoE. These effects were suppressed by heparan sulphate, heparin and chondroitin-4-sulphate, even though GAGs demonstrated no ability to scavenge α,α-diphenyl-β-picrylhydrazyl radical and no charge influence relationship between inhibitory activity and their number of sulphate groups. An interaction between GAG and VLDL, preserving the biological functions of apoE from oxidative stress is suggested.

On the contrary, Camejo et al. [51] showed a pro-oxidant effect of glycosaminoglycans on Cu\(^{++}\)-induced LDL oxidation. Authors have assumed that chondroitin-sulphate-glycosaminoglycans and GAGs could induce apparent increase in Cu\(^{++}\) affinity and enhanced access to internal regions of the apoB-100. Abuja [52] also described a pro-oxidant action of chondroitin-4-sulphate on LDL oxidation: aggregation of LDL in the presence of chondroitin-4-sulphate, and not chondroitin-6-sulphate, gives rise to a complex, which can oxidise in the presence of ascorbate and urate and suggests the entrapment of Cu\(^{++}\) within.

Phacoemulsification is the most common and advanced surgery technique widely used to eliminate the cataract [53]. However, since ultrasonic medical devices were used, some biological and cellular effects on the production of large amounts of reactive oxygen species and ultraviolet radiation within tissues have been documented [54]. A mixture of hyaluronic and chondroitin sulphate (Viscoat) is commonly used to limit eye tissues damage from oxidative attack. Takahashi et al. [55] showed in vitro the protective effect of these agents. The Authors detected free radicals in phacoemulsification and aspiration procedures by using electron-spin resonance. A characteristic signal corresponding to hydroxyl radicals was detected and similar inhibition by Healon and Viscoat was observed. The inhibition by Healon ceased at 20 s, whereas Viscoat suppressed the signal throughout the time course. Authors concluded that phacoemulsification produces hydroxyl radicals in the anterior chamber even with irrigation and aspiration and the effect of ophthalmic visco-surgical devices on free radicals depends on the retention of the materials within the anterior chamber.

Morawski et al. [56] reported that perineuronal nets, mainly consisting of large chondroitin-sulphate-proteoglycans of the aggreca family (brevican), interacting with hyaluronic and tenascin, which surround subpopulations of neurons, protect neurons against oxidative stress.

Chondroitin-4-sulphate showed antioxidant properties in reducing oxidative injury induced by different oxidising agents (CuSO\(_4\) plus ascorbate, FeSO\(_4\) plus ascorbate or H\(_2\)O\(_2\)) in human skin fibroblast cultures [57]. Three different methods were utilised to induce oxidative stress in human skin fibroblast cultures, with inhibition of cell growth, cell death, increase of lipid peroxidation evaluated by the analysis of malonylaldehyde, decrease of GSH and SOD levels, and rise of lactate dehydrogenase (LDH) activity. The treatment with commercial glycosaminoglycans at different doses showed beneficial effects on cell growth, lipid peroxidation, GSH, SOD and LDH levels in all oxidative models, although less in system H\(_2\)O\(_2\). Hyaluronic and C4S exhibited the highest protection.

In a further study, chondroitin-4-sulphate reduced DNA fragmentation and OH production in fibroblast cultures exposed to FeSO\(_4\) plus ascorbate [58]. The data obtained showed that C4S and HA were able to limit cell death, they reduced DNA strand breaks and protein oxidation, decreased OH generation, inhibited lipid peroxidation and improved antioxidant defences. A similar investigation was also performed by the same Authors by using purified chondroitin-4-sulphate obtained from human plasma [59]. In this study, purified human plasma GAGs were added to the fibroblast cultures in which oxidative stress was induced by the oxidising system employing Fe\(^{2+}\) plus ascorbate. Purified human glycosaminoglycans at three different doses reduced cell death, limited DNA fragmentation and protein oxidation, decreased OH generation and LDH activity, inhibited lipid peroxidation and improved endogenous antioxidant defences. Authors suggested that these results further support the hypothesis that these molecules may function as antioxidants.

Imbalance between metallo proteinase and their tissue inhibitors is an important control point in tissue remodelling [12]. Several findings have reported a marked MMPs/TIMPs imbalance in a variety of in vitro models, in which oxidative stress was induced [60, 13].

Purified human plasma chondroitin-4-sulphate, by reducing reactive oxygen species generation, was able to improve MMPs/TIMPs imbalance in fibroblast cultures that underwent oxidative stress [61]. Authors concluded that these results further support the hypothesis that these biomolecules possess antioxidant activity and they suggest that by reducing ROS production, C4S may limit cell injury produced by MMPs/TIMPs imbalance.

Chan et al., [62] studied the effect of physiologically relevant concentrations of glucosamine (GLN) and chondroitin sulphate on gene expression and synthesis of nitric oxide (NO) and prostaglandin E\(_2\) in cytokine-stimulated articular cartilage explants. Results showed that CS and the GLN and CS combination at concentrations attainable in the blood down regulated IL-1 induced mRNA expression of inducible nitric oxide synthase (iNOS). Authors concluded that these data indicate that physiologically relevant concentrations of GLN and CS can regulate gene expression and synthesis of NO and prostaglandin E\(_2\), providing a plausible explanation for their purported anti-inflammatory properties.

**IN VIVO EXPERIMENTS**

**Animal Studies**

Many in vivo laboratory works on antioxidant effect of glycosaminoglycans were carried out which supported the findings of the in vitro researches.

Due to its ability to bind iron, chondroitin sulphate was used, complexed with this metal, in order to act as antianemic. In a dated study, the complex CS-Fe (Condrofer) and iron (Proteoferrina) or ferritin were given orally for 4 weeks to rats in which severe experimental anaemia had previously been induced. The results showed a more complete reversal of anaemia in the rats that received Condrofer rather than iron and this was most probably due to the higher bioavailability of iron administered under this complex formulation [63].
Supplementation in rats of the infused saline with CS reduced peroxidation of the peritoneum and prevented loss of ultrafiltration during peritoneal dialysis [64].

The contents of chondroitin sulphate and hyaluronan in the surroundings of the bronchi were significantly increased after exposure to Diesel exhaust particles (DEP), which were shown to generate ROS in the same areas in which cell damage and proliferating cell nuclear antigen-positive cells also increased [65], suggesting that CS and HA in the lung contribute to cell process of recovery from injury caused by exposure to DEP.

A number of reports, although of controversial results, described an antioxidant activity exerted by chondroitin sulphate in rheumatoid arthritis (RA), the most common disease of connective tissue in which reactive oxygen species are thought to play an important role. Ronca et al. [66] studied the pharmacokinetics and tested the anti-inflammatory activity of chondroitin sulphate in rats, by using tritiated chondroitin sulphate at the reducing end and chondroitin sulphate labelled with 131I. Chondroitin sulphate and its fractions inhibited the directional chemotaxis induced by zymosan-activated serum, were able to decrease the phagocytosis and the release of lysozyme induced by zymosan and protected the plasma membrane from ROS. The results showed that compared with nonsteroidal anti-inflammatory drugs (indomethacin, ibuprofen), CS appears to be more effective on cellular events of inflammation than on oedema formation.

Beren et al. [67] described an anti-inflammatory effect of a nutritional supplement consisting of a combination of glucosamine hydrochloride, purified sodium chondroitin sulphate and manganese ascorbate in a rat model of collagen-induced autoimmune arthritis (CIA).

Campo et al. [19] evaluated the antioxidant activity of HA and C4S in a rat model of CIA. Treatment with the two compounds, starting at the onset of arthritis for 10 days, limited the erosive action of the disease in the articular joints of knee and paw, reduced lipid peroxidation, restored the endogenous antioxidants GSH and SOD, decreased plasma tumour necrosis factor-α (TNF-α) levels and limited synovial neutrophil infiltration, suggesting that erosive destruction of the joint cartilage in CIA is due at least in part to free radicals released by activated neutrophils and produced by other biochemical pathways and that hyaluronan and chondroitin-4-sulphate could be considered natural endogenous macromolecules to limit erosive damage in CIA. In Lewis rats subjected to CIA, the treatment of rats with HA and C4S starting at the onset of arthritis for 20 days again limited inflammation and the clinical signs in the knee and paw, reduced OH· production, decreased conjugated dienes levels, partially restored the endogenous antioxidants vitamin E and catalase, reduced macrophage inflammatory protein-2 serum levels and limited polymorphonuclear cells (PMNs) infiltration. [20]. These data give further support to the possible role of endogenous glycosaminoglycans to limit/control the progression of this detrimental disease, probably by working as metal chelators.

Ha and Lee [68], with the aim to develop a new biomaterial to be used as an antioxidant drug, studied the hepatoprotective effect of chondroitin sulphate on the antioxidant enzyme activity in total homogenate liver and mitochondria fraction, by using CCl4-induced liver injury in rats. MDA levels, SOD and CAT activities, GSH, oxidised-glutathione (GSSG) and glutathione peroxidase concentrations in rat livers were determined. CS treatment restored liver antioxidant enzyme activities and decreased lipid peroxidation, in a dose dependent manner, acting as a radical scavenger. The same workers evaluated the same endogenous antioxidants superoxide dismutase, catalase and glutathione peroxidase, and malonylaldehyde in the microsomal fraction of rats treated with CCl4-induced oxidative damage, following chondroitin sulphate treatment [69]. Results showed that chondroitin sulphate limited the injury induced by CCl4 treatment as a potential scavenger of reactive oxygen species.

Campo et al. [22] studied the effects of hyaluronan and chondroitin-4-sulphate in a model of CCl4-induced acute rat liver damage. In this paper, liver damage was induced in rats by an intraperitoneal injection of CCl4, Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatic MDA, plasma TNF-α, hepatic GSH and CAT, and myeloperoxidase (MPO), an index of PMNs infiltration in the jeopardised hepatic tissue, were evaluated 24 h after CCl4 administration. Intraperitoneal treatment of rats with HA or C4S failed to exert any effect on the considered parameters, while the combination treatment with both GAGs decreased the serum levels of aminotransferases, inhibited lipid peroxidation by reducing hepatic MDA, reduced plasma TNF-α, restored the endogenous antioxidants and decreased myeloperoxidase activity. Authors concluded that hyaluronan and chondroitin-4-sulphate could possess a different antioxidant mechanism and consequently, the combined administration of both GAGs exerts a synergistic effect with respect to the single treatment.

The chronic treatment with CCl4 generates a cascade of events that result in hepatic fibrosis. Campo et al. [70] recently evaluated the antioxidant effects of HA and C4S treatment in a rat model of liver fibrosis. Liver fibrosis was induced in rats by several intraperitoneal injections of CCl4, for 6 weeks. Hyaluronan or chondroitin-4-sulphate alone or in combination were administered daily by the same route during the 6 weeks. Treatment with the two molecules, especially when in combination, successfully reduced the aminotransferase rise, lipid peroxidation, TIMPs activation and mRNA expression, partially restored SOD and GPx activities, and limited collagen deposition in the hepatic tissue. In this way, they limited the hepatic injury induced by chronic CCl4 intoxication and specifically limited the liver fibrosis.

A number of reports described a loss of endogenous antioxidants and molecular oxidative damage during acute pancreatitis [71]. Campo et al. [72] investigated the effect of the administration of C4S and HA in a cerulein-induced acute pancreatitis in rats. The results obtained showed that intraperitoneal pretreatment of rats with chondroitin-4-sulphate, hyaluronan or with both compounds ameliorated pancreatic cell conditions, restored the endogenous antioxidants, limited cell membrane peroxidation and reduced neutrophil activation.

Finally, the treatment with chondroitin sulphate decreased MDA concentration and restored antioxidant activi-
ties, in a dose-dependent manner, in an experimental post-menopausal model in rats [73].

**Human Studies**

There are a multitude of positive clinical studies involving different pathologies, in which chondroitin sulphate acutely or chronically was administered, although the bioavailability of orally administered CS is controversial [74].

Koch et al. [75] showed that Viscoat (containing CS in addition to HA) provided greater corneal endothelial protection than Healon (containing only HA) during iris-plane phacoemulsification. Kim and Joo [76] concluded by using the soft-shell technique that Viscoat and Hyal-2000 (containing only HA) protected corneal endothelial cells during cataract surgery.

Shimamatsu [77] reported that iron supplementation as i. v. iron-CS colloid may be a safely feasible ultimate way to rule out iron deficiency in haemodialysis patients with anaemia resistant to recombinant human erythropoietin i. v. therapy. A plausible explanation of these results could be that the iron chelating activity of chondroitin sulphate allows the iron to be gradually released, so preventing ROS formation due to free iron-catalysed Fenton and Haber-Weiss reactions.

From many years it is widely known that the treatment with chondroitin sulphate ameliorates symptoms and progression of osteoarthritis (OA). A large number of human studies have been performed with positive outcomes (for review, see 78). Although several possible mechanisms have been proposed, the exact role played by CS in limiting the effects of OA has not yet been elucidated. Some of these investigations compared the effects of chondroitin sulphate with nonsteroidal anti-inflammatory drugs (NSAIDs). A human study has been carried out by Fioravanti et al. [79] by comparing the efficacy and the tolerance of galactosaminogluuronoglycan sulphates (Matrix) with those of ibuprofen lysine in patients affected by OA. At the end of the study, an improvement in all clinical considered variables was found, with no significant differences between the oral and the intramuscular administrations. By the achieved results, Authors concluded that this study confirms the efficacy and above all, the good tolerance of Matrix in OA.

Rovetta [80] also evaluated the efficacy and tolerance of Matrix in the therapy of tibiofibular arthritis of the knee. In this study, forty patients suffering from this illness undergoing concomitant therapy with NSAIDS were randomised into two groups of twenty. The treatment group received the drug under study and the control group received placebo. Treatment was carried out in double blind trial. The therapy protocol comprised two intramuscular injection a week for 6 months. Analysis of the results showed a statistically significant higher therapeutic effect by treatment with Matrix for all the symptoms taken into consideration. The author concluded that the good clinical results obtained, together with the excellent tolerance shown by the drug, suggest that Matrix could be the drug of choice in the basic therapy of OA.

Morreale et al. [81] assessed the clinical efficacy of chondroitin sulphate by intramuscular injection in comparison with sodium diclofenac (SD), in a medium/long-term clinical study in patients with knee osteoarthritis. Authors concluded that chondroitin sulphate seems to have slow but gradually increasing clinical activity in osteoarthritis and these benefits last for a long period after the end of treatment.

Coaccioli et al. [82] evaluated the clinical efficacy and the tolerance of galactosaminogluuronoglycan sulphate, administered both orally and intra-articularly, for the treatment of generalised and localised OA. Again a significant improvement of the articular function and excellent tolerance were observed.

Several other controlled clinical studies have been performed in osteoarthritic patients in order to evaluate the efficacy and tolerability against placebo only. Uebelhart et al. [83] assessed the clinical, radiological and biological efficacy and tolerability of the CS in patients suffering from knee osteoarthritis. Authors concluded that oral chondroitin sulphate is an effective and safe symptomatic slow-acting drug for the treatment of knee OA and, in addition, CS might be able to stabilise the joint space width and can modulate bone and joint metabolism. Again Uebelhart et al. [84] investigated the efficacy and tolerability of a 3-month duration, twice a year, intermittent treatment with oral chondroitin sulphate in knee osteoarthritis patients with support to previous results.

In a randomised, double-blind, placebo-controlled trial, Verbruggen et al. [85] recruited 119 patients in order to test the beneficial properties of chondroitin sulphate in OA. Authors observed a significant decrease in the number of patients with new ‘erosive’ osteoarthritis finger joints in the CS group. This result is particularly important since OA of the finger joints become a clinical problem. In light of these results, Authors were able to assert that treated patients were protected against erosive evolution. The progression of erosion at 24 months resulted lower in patients treated with chondroitin sulphate and naproxen than in patients taking naproxen only in a more recent observation [86].

In an other study, Bourgeois et al., [87] performed a multicenter randomised, double-blind, controlled study to compare the oral efficacy and tolerability of CS vs placebo, in patients with mono or bilateral knee OA. The results showed that the treatment carried out with different formulations was very well tolerated. Finally, the Authors concluded that chondroitin sulphate favours the improvement of the subjective symptoms, improving the joint mobility.

Bucsi and Poor [88] carried out in two different centres, a randomised, double-blind, placebo-controlled study by treating with chondroitin sulphate, patients with osteoarthrisis of the knee. The efficacy and tolerability of oral CS vs placebo were assessed for 6-month study period. The results showed that both treatments were very well tolerated, but efficacy was significant in favour of the CS group. The Authors concluded that these results strongly suggest that chondroitin sulphate acts as a symptomatic slow-acting drug in knee osteoarthritis. Although not investigated, also for these reports it is plausible to suppose that in part, the limitation in cartilage damage and resolution of OA symptoms could be due to the antioxidant effect of chondroitin sulphate.
There are also several deepened reviews that well described the rationale for use and efficacy of chondroitin sulphate in OA [78, 89-96].

ANTIOXIDANT ACTIVITY

Several hypotheses about the antioxidant mechanism of chondroitin sulphate and generally for all GAGs have been proposed. Karlsson et al. [97] found that sulphated glycosaminoglycans were responsible for binding extracellular superoxide dismutase (E-SOD) and they suggest that in this way, the complex may protect mammalian cells from free-radical damage. Nevertheless, the most plausible explanation about the antioxidant mechanism of CS as well as for HA is due to their particular chemical structure. In fact, as other glycosaminoglycans, chondroitin sulphate and hyaluronan are linear polymer formed by alternating hexuronic acid and hexosamine units, although these units are characteristic of hyaluronan and of the chondroitin sulphate family, respectively. The explanation of the antioxidant activity for CS and HA is the presence, in their structure, of a carboxylic group always in the same spatial position on the glucuronic acid residue that is not or occasionally present in the other GAGs; and, for C4S or C6S chains, the presence of sulphated group at position 4 or 6 of the aminosugar moiety, in the opposite side of carboxylic group. As shown by Cael et al. [33] for chondroitin-4-sulphate and calcium ions, both types of these carboxylate and sulphate charged groups may interact with the transition metals ions like Cu^{++} or Fe^{++} that are in turn responsible for the initiation of Fenton and Haber-Weiss reactions. It was already mentioned that Cu^{++} ions bind CS more strongly than Ca^{++} ions [34, 98]. The ability of these polysaccharides to chelate different ions and transition metals was extensively reported by several authors [18, 21, 48, 99-101]. Although the C4S seems to be more effective than C6S, since the sulphated group in position 4 may better bind positive metal ions, the C6S may also positively reduce free radicals activity [57]. Albertini et al. [18], suggested that a reasonable explanation for the different Cu^{++} binding ability of C4S and C6S might be the distance between carboxylic and sulphate groups, which is shorter in C4S than in C6S. In this way, the interaction between Cu^{++} and carboxylic group could be stabilised more efficiently by the sulphate in C4 of the N-acetylgalactosaminyl residue, as shown by Cael et al. [33] for calcium ion, than by sulphate in C6 of the same residue. In addition, another important explanation may be that the sulphate group in C4S is placed along the centre line of the polymer so creating a high negative density charge. In C6S, the sulphate groups are, instead, at the periphery of the polymer. All these data strongly suggest that CS is able to bind iron and copper cations in solution. This metal entrapment could certainly decrease their availability for oxidation processes.

About the protective effect of several glycosaminoglycans on Cu^{++}-induced LDL oxidation, Albertini et al. [48] suggested that since part of the copper binding sites involved in the initiation of LDL oxidation might be located on Apo-B-100, and an ionic interaction between the positively charged amino acids of Apo-B-100 and negatively charged glycosaminoglycans, dependent on their sulphation pattern, has been reported [102], C4S may interfere with LDL oxidation by covering or masking some copper binding sites on Apo-B-100, as a result of its interaction with the particle. While C6S may be unable to realise a similar interaction, owing to the different position of the sulphate group, outside the sugar ring, Volpi and Tarugi [50], instead, stated that the mechanism of protection in this case does not seem to be related to the strong capacity of glycosaminoglycans to bind the Cu^{++} ion. They suggested that since an increased access of Cu^{+} ions in hydrophobic regions of the LDL molecule, after forming reversible complexes between LDL and GAGs or chondroitin sulphate-proteoglycans, was previously reported [51], it is possible to assume that the protective effect of glycosaminoglycans on Cu^{++}-mediated LDL oxidation could be due to their capacity to interact, especially by their hydrophobic groups, with hydrophobic regions of LDL protein, so introducing structural modifications that mask some copper binding sites and decrease LDL susceptibility to copper-catalysed oxidation by the formation of radicals from the fatty acids.

Mostly, Albertini et al. [103] suggested that since chondroitin sulphate exposed to reactive oxygen species resulted in marginal desulphation, the presence of sulphate on the GAG chain may protect the molecule against ROS attack. In this way, reactive oxygen species would come drastically reduced. Arai et al. [17], instead, have supposed that the decomposition of glycosaminoglycans by reactive oxygen species produced neutralising molecules that in turn may act as radical scavengers with consequent reduction in free radical activity.

Presti and Scott [104] showed a direct scavenger action of hyaluronan on OH^{-} generated by various oxidative systems. This mechanism could be an additional or alternative antioxidant activity by which chondroitin sulphate may directly scavenge free radical molecules and especially the detrimental OH^{-} or other Fenton’s reaction intermediates like O_{2}^{-}.

Free radical attack to biological membranes leads to lipid peroxidation of polyunsaturated fatty acids (PUFA) and the formation of chemotactic peroxide intermediates that in turn amplify the inflammatory mechanism by attracting neutrophils at the site of damage [105]. In fact, neutrophils may increase cellular injury by releasing superoxide radicals, proteolytic enzymes and cytokines. A secondary activity of chondroitin sulphate could be an anti-inflammatory effect exerted by chelating transition metals or by scavenging ROS. The inhibition of lipid peroxidation may decrease the formation of the chemotactic intermediates thus reducing PMNs recruitment [19-20, 22]. While the neutralisation of the reactive oxygen species released by activated PMNs may directly reduce inflammation.

FUTURE STRATEGIES

Chondroitin sulphate administration orally or intravenously seems to be an interesting prospective of drug therapy to reduce the severity of some diseases, in particular, as widely reported, to limit the detrimental effects of osteoarthritis [106-107]. Since free radical generation is involved in several pathologies and the direct or indirect cellular damage plays a central role in the progression and evolution of the morbid state, the reduction in free radical activity could be an important step in the therapy of the disease. In
The antioxidants may exert effect on different functions; such as by suppressing the formation of active species by reducing hydroperoxides and \( \text{H}_2\text{O}_2 \) and by sequestering metal ions, scavenging active free radicals, and repairing and/or clearing damage; and besides, some antioxidants may induce the biosynthesis of other antioxidants or defence enzymes.

The characteristics of CS molecules suggest that the mechanism of antioxidant action should be limited to chelation activity and influence on antioxidants biosynthesis, or both. In order to exert its antioxidant effect, the compound must be able to reach the target sites at sufficient concentration. The intravenous administration is the way in which a drug effectively reaches target cells. Nevertheless, the oral route is more preferable because it is less invasive and easier for the patients, although only a small amount of the active drug can reach the blood. Hence the key property for an orally active molecule is its ability to be efficiently absorbed from the gastrointestinal tract and to cross biological membranes thereby gaining access to the desired target sites such as the liver.

The absorption of CS compound administered by oral route has always been a controversial question. Ingested chondroitin sulphate in man remains intact in the stomach and small intestine, so to be used as a carrier for colon-specific drug delivery [109]. The polar character of the polysaccharide, its mass and charge would strongly suggest that no absorption is possible by the mammalian gastrointestinal tract. However, a number of experimental findings are consistent with an increase of plasma CS concentration following oral administration in man [110]. Furthermore, the usual localisation of polysaccharide structures are outside the cell: chondroitin sulphate-proteoglycans are components of the extracellular matrix. Among their different function, the emerging one is the regulatory function on cell activity, by binding with most molecules, as hormones and cytokines are able to interact with cell receptors. The function would reinforce the suggestion of antioxidant activity mediated by antioxidants biosynthesis control. However, a component of the external cell space is also to sequester, by chelation, the metal ions. The molecular size may be critical for this function. Since the molecular size is another critical factor which influences the rate of drug absorption, a study may be developed to identify and isolate the shortest CS chain able to chelate metal ions. In addition, CS could be chemically modified in order to insert into the molecular structure, active chelating groups such as additional sulphated groups. However, the metabolic properties of chelating agents play a critical role in determining both their efficacy and toxicity. It is also important to ensure that the agent is not degraded to metabolites, which lack the ability to further bind the metal ion. The toxicity associated with metal chelators, mainly for iron chelators, originates from a number of factors, including inhibition of metal-containing enzymes.

In general, iron chelators do not directly inhibit haem-containing enzymes due to the inaccessibility of porphyrin-bound iron to chelating agents. In contrast, many nonhæm iron-containing enzymes such as the lipoxygenase and aromatic hydroxylase families and ribonucleotide reductase are susceptible to chelator-induced inhibition [111]. Lipoygenases are generally inhibited by hydrophobic chelators, therefore the introduction of hydrophilic characteristics into a chelator tend to minimise such inhibitor potential. The modified chondroitin sulphate should be deeply tested in order to avoid this toxic effect.

Chondroitin sulphate and glycosaminoglycans have also showed to possess a direct free radical scavenger effect. Although this property is low, it may be enhanced by chemical insertion into the molecular CS structure, some reactive groups able to react against free radicals. For example, the phenolic compounds possess high reactivity toward lipid peroxyl radical, a chain carrying species in lipid peroxidation. The activity of phenolic antioxidant toward peroxyl radicals is determined primarily by the bond dissociation energy of the phenolic O-H bond, its redox potential and the steric hindrance to the abstraction of the phenolic hydrogen by peroxyl radicals. A chemical insertion of phenolic groups into the chondroitin sulphate structure could enhance the antioxidant activity of this natural compound.

CONCLUDING REMARKS

Several basic science evidences, such as cell culture, or in vitro biochemical studies, suggest an antioxidant activity for chondroitin sulphate that is able to reduce cell and tissue damage, due to free radical attack, mainly by sequestering transition metals that in turn catalyse reactive oxygen species production. CS seems also to possess a slight radical scavenger activity. In a number of in vitro studies, chondroitin sulphate clearly showed the capacity to chelate iron, copper and other metal cations, as well as beneficial antioxidant effect in cultured cells from different tissue sources, such as endothelial cells, chondrocytes, neurons, and fibroblast. Although some outcomes are in contrast, CS molecules have been reported to be beneficial in the prevention of the \( \text{Cu}^{2+} \)-induced LDL, HDL and VLDL oxidation. The protective effect exerted by this natural compound was evaluated by biochemical and morphometric analysis. Chondroitin sulphate inhibited lipid peroxidation, the main mechanism capable of both destroying cellular membranes and generating new free radical species; CS limited the failure of the natural antioxidant defences by restoring the endogenous antioxidants such as SOD, CAT, GSH, GPx, vitamin E, etc.; and increased cell survival. By reducing the oxidative burst, CS limited DNA damage and protein degradation and MMPs/TIMPs imbalance.

Equally beneficial results were obtained by using experimental animal models of diseases in which free radicals play an important role. Chondroitin sulphate was effective in the reduction of cartilage degradation and biochemical parameters amelioration in rats with arthritis induced experimentally by CIA. Chondroitin sulphate improved the general conditions and decreased hepatic damage in rats that underwent liver injury induced by \( \text{CCl}_4 \) treatment. The treatment with CS showed positive outcomes in experimental models of lung intoxication, pancreatitis and liver fibrosis.

Some beneficial effects of chondroitin sulphate in humans were also described, although mainly in the treatment of OA. In this cases there are not clear evidences that CS
Chondroitin Sulphate
does improve conditions of the patient suffering from os-
teoarthritis by exerting an antioxidant mechanism. In the
light of these data, the antioxidant activity of chondroitin
sulphate could be chemically enhanced and improved with
the aim to upgrade its therapeutic effect in OA or other pa-
thologies in which the damage induced by free radical gen-
eration is of great interest.

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