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The Preventive Inhibition of Chondroitin Sulfate Against the CCl_4-Induced Oxidative Stress of Subcellular Level

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Our work in this study was made in the microsomal fraction to evaluate the lipid peroxidation by measuring superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) and to elucidate the preventive role of CS in the CCl_4-induced oxidative stress. The excessive lipid peroxidation by free radicals derived from CCl_4 leads to the condition of oxidative stress which results in the accumulation of MDA. MDA is one of the end-products in the lipid peroxidation process and oxidative stress. MDA, lipid peroxide, produced in this oxidative stress causes various diseases related to aging and hepatotoxicity, etc. Normal cells have a number of enzymatic and nonenzymatic endogenous defense systems to protect themselves from reactive species. The enzymes in the defense systems, for example, are SOD, CAT, and GPx. They quickly eliminate reactive oxygen species (ROS) such as superoxide anion free radical $\cdot O_2^-$, hydrogen peroxide H_2O_2 and hydroxyl free radical ·OH. CS inhibited the accumulation of MDA and the deactivation of SOD, CAT and GPx in the dose-dependent and preventive manner. Our study suggests that CS might be a potential scavenger of free radicals in the oxidative stress originated from the lipid peroxidation of the liver cells of CCl_4-treated rats.

Key words: Chondroitin sulfate, Carbone tetrachloride, Malondialdehyde, Antioxidative enzyme, Radical scavenger

INTRODUCTION

Normal cells have a number of enzymatic and nonenzymatic endogenous defense systems to protect themselves from reactive species (RS) as shown in Table I, II (Yu *et al.*, 1994). The enzymes in the defense systems, for example, are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). They quickly eliminate reactive oxygen species (ROS) such as superoxide anion free radical $\cdot O_2^-$, hydrogen peroxide H_2O_2 and hydroxyl free radical ·OH (Reilly *et al.*, 1991). SOD removes superoxide $\cdot O_2^-$ by converting it into hydrogen peroxide H_2O_2. This H_2O_2 is converted into molecular oxygen and water by CAT and GPx (Halliwell *et al.*, 1992). Furthermore, GPx degrades hydroperoxide which originates from the oxidation of polyunsaturated fatty acids (PUFA) in the presense of reduced glutathione (GSH) which is a key component of the antioxidant defense system (Puglia and Powell, 1984; Salminen *et al.*, 19984). These enzymes prevent the formation of highly reactive hydroxyl radicals. But in the process of these mechanisms, free radicals uneliminated by enzymes generate strong free radical, hydroxyl radical ·OH which attacks PUFA in the phospholipids of various membranes in the cells and generates highly reactive peroxyl free radical of hydroperoxide, initiating the lipid peroxidation. The initial event of carbon tetrachloride (CCl_4) is carbon-halogen bond cleavage, probably by one electron reduction of CCl_4 made by a particular ferrous cytochrome p-450 (Lai *et al.*, 1979). Chloride ion Cl^- and trichloromethyl free radical $\cdot CCl_3$ are the major initial products. $\cdot CCl_3$ is converted into $\cdot CCl_3O_2$ by the reaction with molecular oxygen. Like above other free radicals, lipid peroxidation

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Table I. Major defense system

Intracellular compartment	Cellular membranes	Extracellular space
Superoxide dismutase	Vitamin E	Ceruloplasmin
Catalase and peroxidase	β-carotene	Transferrin
DT-diaphorase (GSH)	Phospholipase	Albumin
Ascorbic acid		Lactoferrin
Metal-binding proteins		Vitamin E
Proteolytic enzymes		Ascorbic acid
Repair system		

Table II. Reactive species

Radicals		Non-radicals	
Reactive oxygen species (ROS)			
Superoxide	$\cdot O_2^-$	Hydrogen peroxide	H_2O_2
Hydroxyl	·OH	Hydrochlorous acid	HOCl
Peroxyl	LOO·	Ozone	O_3
alkoxyl	LO·	Singlet oxygen	$^1O_2$
Hydroperoxyl	HOO·	Hydroxy alkenals	
Reactive nitrogen species(RNS)			
Nitric oxide	NO·	Nitrous acid	HNO_2
Nitrogen dioxide	$NO_2^·$	Dinitrogen trioxide	N_2O_3
		Peroxynitrite	ONOO·
		Alkyl peroxynitrites	LOONO

is initiated by the interaction of this reactive free radical $\cdot CCl_3O_2$ with PUFA of membrane lipids. The excessive lipid peroxidation by these free radicals leads to the condition of oxidative stress which results in the accumulation of malondialdehyde (MDA). MDA is one of the end-products in the lipid peroxidation process and oxidative stress (Kurata *et al.*, 1993; Hagihara *et al.*, 1984). MDA, lipid peroxide, produced in this oxidative stress causes various diseases related to aging and hepatotoxicity, etc. Extracellular matrix (ECM) is essential to many tissues of the body (Lin and Bissel, 1993). Among other components, ECM contains proteoglycans. Proteoglycans are complex macromolecules containing a core protein with one or more covalently bound glycosaminoglycan (GAG) chains. GAG is complex polysaccharides and include heparan sulfate, keratan sulfate, and chondroitin sulfate. Chondroitin sulfate (CS) consists of an alternating polymer of sulfated *N*-acetylgalactosamine and uronic acid residues linked by glycosidic bonds. It has been suggested that heparin, one of GAGs acts as a potential antioxidant in the metal ion-induced lipid peroxidation (Ross *et al.*, 1992; Albertini *et al.*, 1996). In the previous study, we have reported that CS, one of GAGs, plays a role as a potential antioxidant in the lipid peroxidation of mitochondria (Ha and Lee, 2003). Like mitochondria, microsome is susceptible to lipid peroxidation because it contains high levels of phospholipids in the membrane that harbor PUFA such as linoleic acid and arachidonic acid. Also microsome has much cytochrome p-450 which catalyzes CCl_4, leading to the formation of free radical $\cdot CCl_3$ and the lipid peroxidation. Therefore our work in this study was made in the microsomal fraction to evaluate the lipid peroxidation by measuring SOD, CAT, GPx and MDA and to elucidate the preventive role of CS in the CCl_4-induced oxidative stress.

MATERIALS and METHODS

Animals

Sparague-Dowley (SD) rats (Female, 130 g~150 g) were supplied from Korean Experimental Animal Center (KEAC) and acclimated for 7 days. All animals were maintained in separated cages with laboratory chow and tap water *ad libitum*. They were housed at 22±1°C and 60±5% relative humidity and kept on a 12-h light/dark cycle throughout the period of experiment. Body weight was measured daily. A total of 28 SD rats were divided into 4 groups : non CCl_4-treated [NCT] group, CCl_4-treated [CCT] group, CS (100 mg/kg)-injected and CCl_4-treated [CS100] group, and CS (200 mg/kg)-injected and CCl_4-treated [CS200] group. CS was injected intraperitoneally. NCT and CCT groups were injected saline (0.9% physiological saline). CS C type (sigma no. C4384) was used. Because of the viscosity of CS, it was prepared by sonication. After two weeks from the acclimation, CCl_4 (3.3 mL/kg body weight) was treated. CCl_4 was dissolved in the same volume of olive oil. After the 12 h from CCl_4 treatment, animals were anesthesized with ether and dissected. Finally, liver was collected.

Microsomal fraction of liver tissue

Fresh liver tissues were homogenized in 1:10 volumes of solution A (10 mM Tris, 0.2 M Mannitol, 0.07 M sucrose, 0.1 mM EDTA). The homogenate was centrifuged at 600 g for 10 min. The supernatant was centrifuged 8000 g for 10 min. The obtained pellet was used for mitochondrial fraction. The obtained supernatant was centrifuged at 10000 g for 10 min. The pellet was discarded and the supernatant was centrifuged at 105000 g for 60 min. The obtained pellet was dissolved 0.1 M phosphate buffer. It was used for microsomal fraction.

Analytical procedures

Protein concentrations were determined according to the method of Lowry *et al.* (1951). Lipid peroxidation was

assayed by the measurement of the levels on the base of MDA reacted with thiobarbituric acid at 535nm, according to Ohkawa *et al.* (1979). Results were expressed as nmol MDA mg^{-1} protein. The breakdown product of 1,1,3,3-tetraethoxypropane was used as standard. SOD activity was measured by using a modified assay originally described by Beauchamp *et al.* (1971). CAT activity was measured by using the method of Aebi (1984). GPx activities were estimated by the method of Lawrence and Burk (1976).

Statistical analysis

Statistical analysis was performed by applying the Student's *t*-test, with $P<0.01$ and $P<0.05$ considered statistically significant. All results are expressed as mean ± S.D.

RESULTS

MDA levels

The pattern of MDA values in the microsomal fractions of NCT, CCT, CS 100 and CS 200 groups was expressed in Fig. 1. MDA contents in the microsomal fraction of NCT group which was non CCl_4-treated group were 4.91 nmol/mg protein. It was regarded as the value of MDA in the microsome of the normal liver cells of rats. Those of CCT group which was CCl_4-treated group were 11.34 nmol/mg protein. Free radicals originating from CCl_4 increased the value of MDA in CCT group by 130.95% compared to the value of MDA in NCT group. The strong oxidative stress occurred in the process of lipid peroxidation due to the treatment of CCl_4. This coincides with the fact that CCl_4^- induced toxicity is severe in the microsomal vesicles derived from the endoplasmic reticulum (ER) of the centrilobular liver cells. This condition can lead to the cell injury and finally to the cell death if it is not controlled by any anti-oxidant. In addition, MDA reactivity towards amino groups can result in inhibition of DNA and RNA protein synthesis (Bird and Draper, 1980). The MDA value of CS 100 group which was CS (100 mg/kg)-preinjected and CCl_4-posttreated group was 9.54 nmol/mg protein ($P<0.01$). It was inhibited by 36.65% compared to the MDA value of NCT group. The MDA value of CS 200 group which was CS (200 mg/kg)-preinjected and CCl_4-posttreated group was 6.90 nmol/mg protein ($P<0.01$). It was inhibited by 90.42% compared to the MDA value of NCT group. CS inhibited the increase of MDA value. These inhibitions in NCT and CCT groups are preventive because CS was injected in advance before the CCl_4 treatment. CS acted as an inhibitior of free radical $\cdot CCl_3O_2$ generated by the CCl_4-treatment. Furthermore, CS in the CS 200 group inhibited MDA value by 54.06% more than CS in the CS 100 group. This means the dose-dependent inhibition pattern of CS. Although CS didn't restore the level of MDA value to the normal state of NCT group, it played a significant role dose-dependently in the preventive inhibition of lipid peroxides in the CCl_4-induced lipid oxidative stress in the subcellular microsomal fraction of the liver cell.

SOD levels

The pattern of SOD values in the microsomal fractions of NCT, CCT, CS 100 and CS 200 groups was expressed in Fig. 2.

$$2\cdot O_2^- + 2H^+ \xrightarrow{SOD} {}^3O_2 + H_2O_2$$

SOD converts $\cdot O_2^-$ into H_2O_2. SOD prevents the attack of PUFA by ·OH which is produced in the process of the

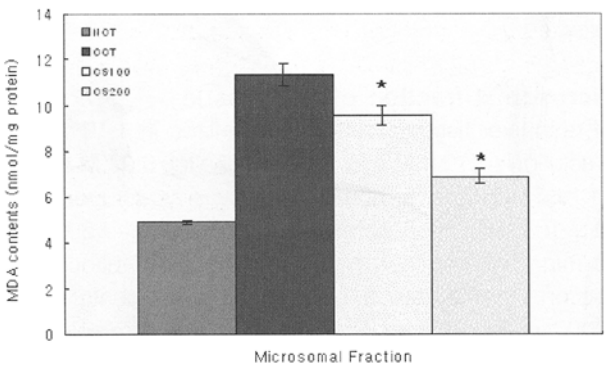

Fig. 1. MDA value in the microsomal fractions of CCl_4-induced oxidative stress rat liver. NCT: Non CCl_4-treated; CCT: CCl_4-treated (dissolved in equal vol. of olive oil, 3.3 mL/kg body weight, i.p.); CS100: Chondroitin sulfate (100 mg/kg body weight, i.p.) + CCl_4; CS200: Chondroitin sulfate (200 mg/kg body weight, i.p.) + CCl_4. Results are presented as the the mean±S.D. (n=7). Significantly different from the value of CCT group at *p<0.01.

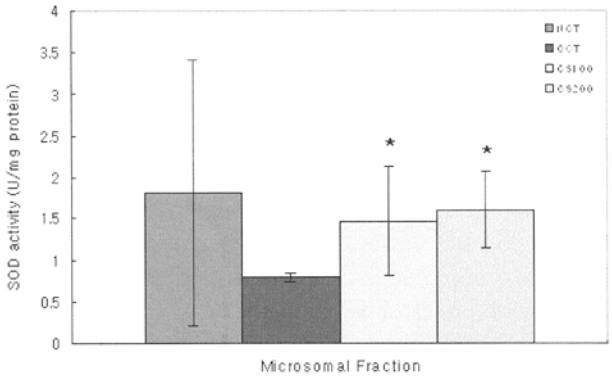

Fig. 2. SOD value in the microsomal fractions of CCl_4-induced oxidative stress rat liver. NCT: Non CCl_4-treated; CCT: CCl_4-treated (dissolved in equal vol. of olive oil, 3.3 mL/kg body weight, i.p.); CS100: Chondroitin sulfate (100 mg/kg body weight, i.p.) + CCl_4; CS200: Chondroitin sulfate (200 mg/kg body weight, i.p.) + CCl_4. Results are presented as the the mean±S.D. (n=7). Significantly different from the value of CCT group at *p<0.01.

reaction of $\cdot O_2^-$ with H_2O_2. SOD activity in the microsomal fraction of NCT group which was non CCl_4-treated group was 1.81 U/mg protein. It was regarded as the activity of SOD in the microsome of the normal liver cells of rats. SOD activity of CCT group which was CCl_4-treated group was 0.79 U/mg protein. CCl_4 in CCT group deactivated the activity of SOD by 56.63%, compared to the activity of SOD in NCT group. This coincides with the fact that the reaction of MDA with the primary amino group proteins forms schiff base compounds which lead to the deactivation of enzymes. This deactivation is correlated with the elevation of MDA value of CCT group described in the above MDA levels section. The SOD value of CS 100 group which was CS (100 mg/kg)-preinjected and CCl_4-posttreated group was 1.47 U/mg protein ($P<0.01$). It was deactivated by 18.78% compared to the SOD value of NCT group. CS inhibited the deactivation of SOD by 37.57%. The SOD value of CS 200 group which was CS (200 mg/kg)-preinjected and CCl_4-posttreated group was 1.60 U/mg protein ($P<0.01$). It was deactivated by 11.60% compared to the SOD value of NCT group. CS inhibited the deactivation of SOD by 44.75%. These inhibitions in NCT and CCT groups are preventive because CS was injected prior to the CCl_4-treatment. CS is thought to have acted as an inhibitor of the deactivation of SOD enzyme. Furthermore, CS in the CS 200 group inhibited the value of SOD deactivation by 7.18% more than CS in the CS 100 group. This means the dose-dependent inhibition pattern of CS. Although CS didn't prevent the deactivation of SOD to the level of NCT group, CS played a remarkable role dose-dependently in the preventive inhibition of the deactivation of SOD resulted from the oxidative stress of free radical $\cdot CCl_3O_2$ of CCl_4.

CAT levels

The pattern of CAT values in the microsomal fractions of NCT, CCT, CS 100 and CS 200 groups was expressed in Fig. 3.

$$2H_2O_2 \xrightarrow{CAT} 2H_1O + {}^3O_2$$

CAT converts H_2O_2 into H_2O following SOD. CAT prevents the attack of PUFA by OH which is generated in the process of the reaction of $\cdot O_2^-$ with H_2O_2. CAT activity in the microsomal fraction of NCT group which was non CCl_4-treated group was 353.11 mU/mg protein. It was regarded as the activity of SOD in the microsome of the normal liver cells of rats. CAT activity of CCT group which was CCl_4-treated group was 235.65 mU/mg protein. CCl_4 in CCT group deactivated the activity of CAT by 33.26% compared to the activity of CAT in NCT group. This deactivation is also correlated with the elevation of MDA value of CCT group described in the above MDA levels

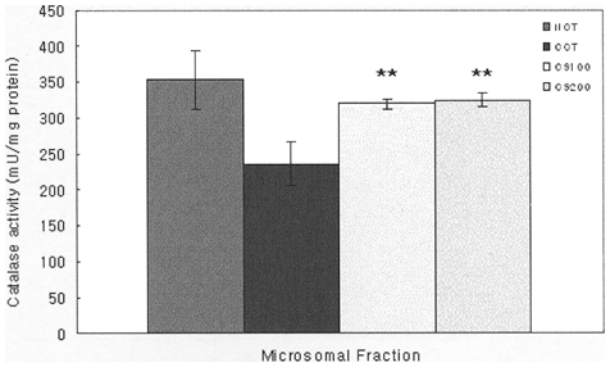

Fig. 3. CAT value in the microsomal fractions of CCl_4-induced oxidative stress rat liver. NCT: Non CCl_4-treated; CCT: CCl_4-treated (dissolved in equal vol. of olive oil, 3.3 mL/kg body weight, i.p.); CS100: Chondroitin sulfate (100 mg/kg body weight, i.p.) + CCl_4; CS200: Chondroitin sulfate (200 mg/kg body weight, i.p.) + CCl_4. Results are presented as the the mean±S.D. (n=7). Significantly different from the value of CCT group at *$p<0.01$, **$p<0.05$, respectively.

section. The CAT value of CS 100 group which was CS (100 mg/kg)-preinjected and CCl_4-posttreated group was 319.34 mU/mg protein ($P<0.05$). It was deactivated by 9.56% compared to the CAT value of NCT group. CS inhibited the deactivation of CAT by 23.70%. The CAT value of CS 200 group which was CS (200 mg/kg)-preinjected and CCl_4-posttreated group was 324.64 mU/mg protein ($P<0.05$). It was deactivated by 8.06% compared to the CAT value of NCT group. CS inhibited the deactivation of CAT by 25.20%. These inhibitions in NCT and CCT groups are preventive because CS was injected in advance before the CCl_4-treatment. Furthermore, CS in the CS 200 group inhibited the value of CAT deactivation by 1.50% more than CS in the CS 100 group. This means the dose-dependent inhibition pattern of CS. Although CS didn't prevent the deactivation of CAT to the level of NCT group, CS played an important role dose-dependently in the preventive inhibition of the deactivation of CAT resulted from the oxidative stress of free radical $\cdot CCl_3O_2$ of CCl_4.

GPx levels

The pattern of GPx values in the microsomal fractions of NCT, CCT, CS 100 and CS 200 groups was expressed in Fig. 4.

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

GPx converts hydroperoxide (ROOH) into GSSG. GPx prevents the attack of PUFA by RO· and ·OH which are derived from the unstable ROOH. GPx activity in the microsomal fraction of NCT group which was non CCl_4-treated group was 126.89 mU/mg protein. It was regarded as the activity of GPx in the microsome of the normal liver cells of rats. GPx activity of CCT group which was CCl_4-

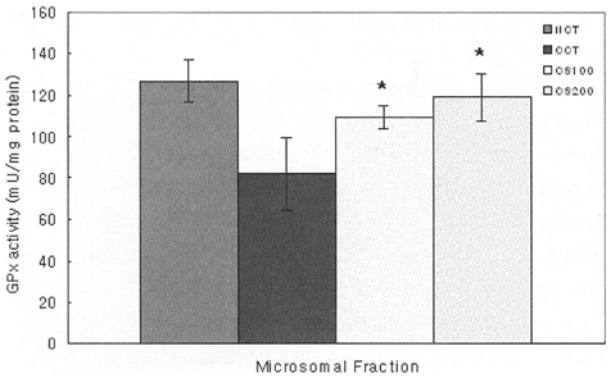

Fig. 4. GPx value in the microsomal fractions of CCl_4-induced oxidative stress rat liver. NCT: Non CCl_4-treated; CCT: CCl_4-treated (dissolved in equal vol. of olive oil, 3.3 mL/kg body weight, i.p.); CS100: Chondroitin sulfate (100 mg/kg body weight, i.p.) + CCl_4; CS200: Chondroitin sulfate (200 mg/kg body weight, i.p.) + CCl_4. Results are presented as the the mean±S.D. (n=7). Significantly different from the value of CCT group at *p<0.01.

treated group was 81.89 mU/mg protein. CCl_4 in CCT group deactivated the activity of GPx by 35.46% compared to the activity of GPx in NCT group. This deactivation is also correlated with the elevation of MDA value of CCT group described in the above MDA levels section. The GPx value of CS 100 group which was CS (100 mg/kg)-preinjected and CCl_4-posttreated group was 109.12 mU/mg protein (P<0.01). It was deactivated by 14.00% compared to the GPx value of NCT group. CS inhibited the deactivation of GPx by 21.46%. The GPx value of CS 200 group which was CS (200 mg/kg)-preinjected and CCl_4-posttreated group was 118.92 mU/mg protein (P<0.01). It was deactivated by 6.28% compared to the GPx value of NCT group. CS inhibited the deactivation of GPx by 29.18%. These inhibitions in NCT and CCT groups are preventive because CS was injected in advance before the CCl_4-treatment. Furthermore, CS in the CS 200 group inhibited the value of GPx deactivation by 7.72% more than CS in the CS 100 group. This means the dose-dependent inhibition pattern of CS. Although CS didn't prevent the deactivation of GPx to the level of NCT group, CS played an important role dose-dependently in the preventive inhibition of the deactivation of GPx resulted from the oxidative stress of free radical $\cdot CCl_3O_2$ of CCl_4.

DISCUSSIONS

It has been reported that free radicals derived from CCl_4 lead to the accumulation of MDA as one of end-products of lipid peroxidation and that the accumulation of MDA results in the deactivation of antioxidative enzymes SOD, CAT and GPx (Ha and Lee, 2003). In our present study, MDA level was elevated and SOD, CAT, GPx were deactivated. The results of our study were in parallel with the reports. The oxidative stress such as MDA accumulation and enzyme deactivation causes the cell injury and

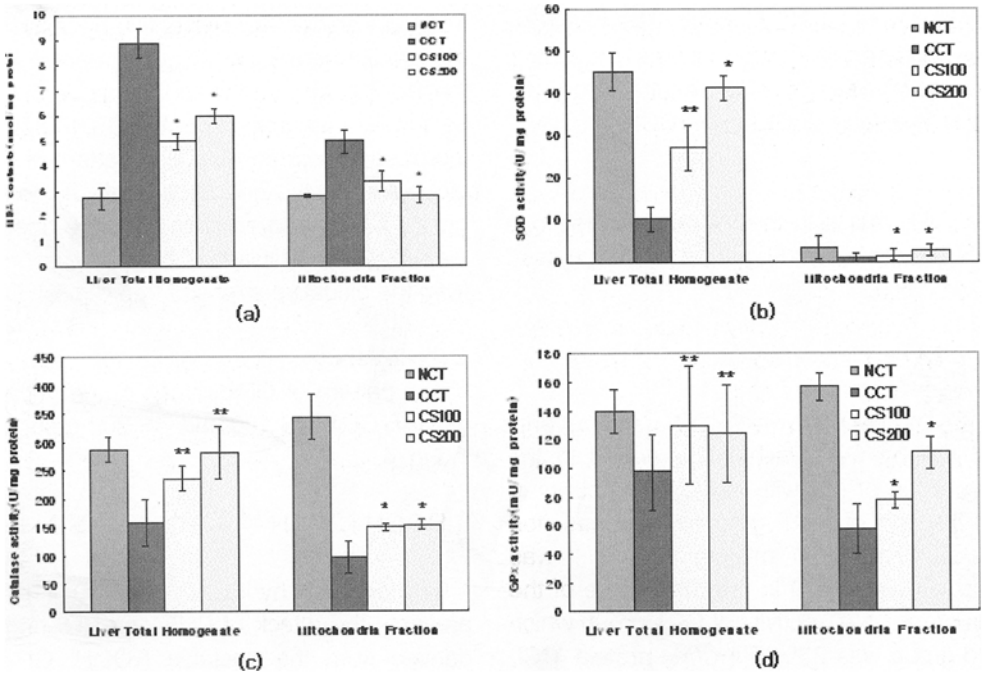

Fig. 5. MDA, SOD, CAT, GPx values in the total homogenate and mitochondrial fraction of CCl_4-induced oxidative stress rat liver. NCT: Non CCl_4-treated; CCT: CCl_4-treated (dissolved in equal vol. of olive oil, 3.3 mL/kg body weight, i.p.); CS100: Chondroitin sulfate (100 mg/kg body weight, i.p.) + CCl_4; CS200: Chondroitin sulfate (200 mg/kg body weight, i.p.) + CCl_4. Results are presented as the the mean±S.D. (n=7). Significantly different from the value of CCT group at *p<0.01, **p<0.05, respectively.

the cell death. It is known that antioxidants such as vitamin C and vitamin E inhibit the oxidative stress in the lipid peroxidation process by playing a scavenging role of free radicals. In our present study, CS inhibited the accumulation of MDA and the deactivation of SOD, CAT and GPx in the dose-dependent and preventive manner. It is thought that CS decreased MDA level by scavenging the CCl_4-derived free radicals and that the decrease of MDA level reduced the deactivity of SOD, CAT and GPx. In our previous study as shown in Fig. 5, CS showed the same pattern as CS in our present study. In addition to the liver total homogenate and mitochondrial fraction of our pervious study, our present study was made in the microsomal fraction. Furthermore, in the histopathological examination of the previous study CS improved the state of inflammation and cirrhosis in the liver tissue of rats. Also our other study (not published) showed that CS decreased AST and ALT in the serum of CCl_4-treated rats. The consistency in the role of CS against the oxidative stress was found in a series of our studies. Therefore, our study suggests that CS might be a potential scavenger of free radicals in the oxidative stress originated from the lipid peroxidation of the liver cells of CCl_4-treated rats.

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