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Oxidative Stress in Ovariectomy Menopause and Role of Chondroitin Sulfate

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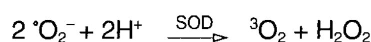
Oxidative stress due to reactive oxygen species (ROS) can cause oxidative damage to cells. Cells have a number of defense mechanisms to protect themselves from the toxicity of ROS. Mitochondria are especially important in the oxidative stress as ROS have been found to be constantly generated as an endogen threat. Mitochondrial defense depends mainly on superoxide dismutase (SOD) and glutathione peroxidase (GPx), whereas microsomal defense depends on catalase (CAT), which is an enzyme abundant in microsomes. SOD removes superoxide anions by converting them to H_2O_2, which can be rapidly converted to water by CAT and GPx. Also, GPx converts hydroperoxide (ROOH) into oxidized-glutathione (GSSG). Ovariectomized (OVX) rats are used as an oxidative stress model. An ovariectomy increased the levels of MDA, one of the end-products in the lipid peroxidative process, and decreased levels of the antioxidative enzymes; SOD, CAT and GPx. However, Chondroitin sulfate (CS) decreased the levels of MDA, but increased the levels of SOD, CAT and GPx in a dose-dependent manner. Moreover, inflammation and cirrhosis of liver tissue in CS- treated rats were significantly decreased. These results suggest that CS might be a potential candidate as an antioxidative reagent.

Key words: Chondroitin sulfate, Oxidative stress, Ovariectomy, Antioxidative enzyme

INTRODUCTION

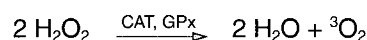
Cells have a number of defense mechanisms to protect themselves against the toxic effects of reactive oxygen species (ROS). One prominent biological target of ROS is polyunsaturated fatty acid (PUFA). Mitochondria are especially important in oxidative stress since ROS have been found to be constantly generated as an endogen threat. Mitochondrial defense depends mainly on superoxide dismutase (SOD) and glutathione peroxidase (GPx), while microsomal defense depends on catalase (CAT), which is an enzyme abundant in microsomes.

SOD removes superoxide anions by converting them to H_2O_2, which can be rapidly converted to water by CAT and GPx (Halliwell *et al.*, 1992).



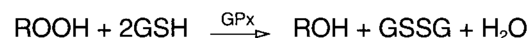
$$2\ {}^{\bullet}O_2^- + 2H^+ \xrightarrow{SOD} {}^3O_2 + H_2O_2$$

SOD converts ${}^{\bullet}O_2^-$ into H_2O_2 and prevents the attack of PUFA by the ${}^{\bullet}OH$ produced in the reaction of ${}^{\bullet}O_2^-$ with H_2O_2.



$$2\ H_2O_2 \xrightarrow{CAT,\ GPx} 2\ H_2O + {}^3O_2$$

CAT and GPx convert H_2O_2 to H_2O following the SOD reaction and prevent the attack of PUFA by the ${}^{\bullet}OH$ generated in the reaction of ${}^{\bullet}O_2^-$ with H_2O_2. Mitochondria, which do not have CAT, rely only on GPx to detoxify H_2O_2.



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

GPx converts hydroperoxide (ROOH) into oxidized-glutathione (GSSG), prevents attack of PUFA by $RO^{\bullet}$ and ${}^{\bullet}OH$, which are derived from the unstable ROOH, and is one of the main enzymes involved in the glutathione redox cycle.

Although various enzymatic systems have been developed by cells to cope with ROS, it is generally accepted, with increasing age, particularly in mitochondria, a condition of oxidative stress is established in cells (Tomalsoff, 1980; Cutler, 1985; Cand and Verdetti, 1989), when the

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rate of ROS generation exceeds the cell capacity for their removal. Oxidative stress refers to a biological redox condition where excessive oxidative modifications of cellular constituents occur due to increased oxidizing power. A disturbance in the cellular redox balance is assumed to interfere with the proper maintenance of cellular homeostasis (Yu, 1994).

The oxidative stress established by excessive free radical generation may induce a number of alterations of cell constituents, including inactivation of enzymes, generations of reactive nitrogen species, damages of nucleic acid bases and proteins and peroxidation of membrane lipid.

Numerous marker compounds of lipid peroxidation (LPO) processes have been detected, including pentane (Zarling *et al.*, 1993; Matsuo *et al.*, 1993), malondialdehyde (MDA) (Esterbauer and Cheeseman, 1990; Esterbauer *et al.*, 1991), 4-hydroxynonenal (4-HNE) (Esterbauer *et al.*, 1991; Waeg *et al.*, 1996; Mattson *et al.*, 1997) and hydroxyoctadecadienoic acids (HODEs) (Jira *et al.*, 1996), etc.

The extracellular matrix (ECM) is essential to many tissues of the body (Lin and Bissel, 1993). Among other components, the ECM contains proteoglycans, which are complex macromolecules of a core protein with one or more covalently bound glycosaminoglycan (GAG) chains. GAG is a complex polysaccharide and includes 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 the GAGs acts as a potential antioxidant in metal ion-induced lipid peroxidation (Ross *et al.*, 1992; Albertini *et al.*, 1996). In a previous study, CS, one of GAGs, was reported to play a role as a potential antioxidant in the lipid peroxidation of mitochondria (Ha and Lee, 2003) and microsome (Lee *et al.*, 2004) in CCl_4-treated rat liver.

Generally, MDA, SOD, CAT and GPx have been studied in the blood, but study focused on MDA, SOD, CAT and GPx in liver tissue, not the blood from the view of the four following points. It is known that the levels of MDA in the blood were increased in postmenopausal elderly people. Our previous studies showed that the levels of MDA were increased in the liver tissue of CCl_4-treated rat. It was reported that total cholesterol (TC) and triglyceride (TG) were measured in the liver tissue of ovariectomized rats. Also, a large reserve of reduced glutathione (GSH) is present in hepatocytes, as well as erythrocytes, for detoxification of free radicals.

The present work was performed to elucidate the role of CS in ovariectomy-induced oxidative stress by measuring the levels of MDA, SOD and GPx in liver total homogenate and the mitochondrial fraction, as well as the levels of CAT in liver total homogenate and the microsomal fraction, and demonstrate its histopathological effect in the liver tissue.

MATERIALS AND METHODS

Animal

Sparague-Dowley (SD) rats (female, 130 g~150 g) were supplied from the Korean Experimental Animal Center (KEAC), and acclimatized for 7 days. All the animals were maintained in separated cages, with laboratory chow and tap water *ad libitum*. During the experiment, the animals were housed at 22±1°C and 60±5% relative humidity, with a 12 h light/dark cycle. The body weight was measured daily. A total of 28 SD rats were divided into 4 groups: the non-ovariectomized [CON], ovariectomized [OVX], ovariectomized + CS 100 mg/kg -injected [OVX+CS100] and ovariectomized + CS 200 mg/kg-injected [OVX+CS200] groups. Both the ovaries of the animals were ectomized. After 2 days, CS C type (sigma no. 4384) was used, and 100 and 200 mg/kg were intraperitoneally injected into the OVX+CS100 and OVX+CS200 groups, respectively. Physiological saline was injected into the OVX group.

Tissue collection

On the fifteenth week, the animals were anesthetized with ether, dissected and the livers collected. They were rinsed with saline solution, and stored at -80°C. The liver tissues were homogenized in 1:5 volumes of PBS (pH 7.4) and the homogenate centrifuged at 600×g for 10 min. The supernatant was used as the liver total homogenate sample. Mitochondrial and microsomal fractions were separated as described by Player *et al.* (Player, 1997).

Analytical procedures

The Protein concentrations were determined according to the method of Lowry *et al.* (Lowry *et al.*, 1951). **Lipid peroxidation** was assayed by measuring the levels of MDA reacted with thiobarbituric acid at 535 nm, according to the method of Ohkawa *et al.* (Ohkawa *et al.*, 1979). The results were expressed as nmol MDA mg^{-1} protein. The breakdown product of 1, 1, 3, 3-tetraethoxypropane was used as a standard. The **SOD levels** were measured using a modified assay originally described by Fridovich *et al.* (Fridovich *et al.*, 1971). The **CAT levels** were measured using the method of Aebi (Aebi, 1984). The **GPx levels** were estimated by the method of Lawrence and Burk (Lawrence and Burk, 1976). In order to make **the histopathological examination,** the liver tissue was fixed overnight in 10% formaldehyde solution (dissolved in phosphate buffer (pH 7.4)), which was then consecutively changed within the range from 70 to 100% alcohol, from 100% alcohol to xylene and then *embedded in paraffin*

wax, sectioned in 5 sizes, followed by staining with heamatoxylin-eosin (H&E).

Statistical analysis

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

RESULT

MDA levels

The levels of MDA in the liver total homogenate and mitochondrial fraction are shown in Fig. 1, and that in the liver total homogenate of the OVX group (9.10 nmol/mg protein) was increased compared to the CON group (6.32 nmol/mg protein). The MDA level in the liver total homogenate of the OVX+CS100 group (7.19 nmol/mg protein) was inhibited by 21% compared to the OVX group. Moreover, the MDA level in liver total homogenate of the OVX+CS200 group (6.72 nmol/mg protein) was inhibited by 26%. Also, the MDA level in mitochondrial fraction was increased in the OVX (12.12 nmol/mg protein) compared to the CON group (8.84 nmol/mg protein). The mitochondrial MDA level of the OVX+CS100 (9.41 nmol/mg protein) and OVX+CS200 (9.12 nmol/mg protein) groups were inhibited by 23 and 25%, respectively. Ovariectomy-induced oxidative stress increased the levels of MDA, one of the marker compounds in the process of lipid peroxidation, but CS decreased the MDA levels in both the liver total homogenate and mitochondrial fraction of the CS-treated rats in a dose-dependent manner.

SOD levels

The SOD levels in the liver total homogenate and mitochondrial fraction are shown in Fig. 2, and that in the liver total homogenate was found to be lower in the OVX (0.16 mU/mg protein) than in the CON group (35.41 mU/mg protein) and decreased by 99% after an ovariectomy. The SOD level in the liver total homogenate was higher in the OVX+CS100 (2.95 mU/mg protein) and OVX+CS200 (3.17 mU/mg protein) groups than in the OVX group and was markedly increased after CS treatment. The mitochondrial SOD level was also lower in the OVX (0.31 mU/mg protein) than the CON group (29.11 mU/mg protein) and was markedly decreased after an ovariectomy. The mitochondrial SOD levels were observed to be higher in the OVX + CS100 (6.17 mU/mg protein) and OVX+CS200 (13.84 mU/mg protein) groups than in the OVX group and was markedly increased after CS treatment. The ovariectomy-induced oxidative stress decreased the levels of SOD, an enzyme that eliminates free radicals, but CS increased the SOD activity in the liver total homogenate and mitochondrial fraction of the CS-treated rats in a dose-dependent manner.

CAT levels

The CAT levels in the liver total homogenate and microsomal fraction are shown in Fig. 3, and that in the liver total homogenate was found to be lower in the OVX (317.03 mU/mg protein) than the CON group (415.61 mU/mg protein). An ovariectomy decreased the level of CAT by 24%. The CAT levels were found to be higher in the OVX+CS100 (376.31 mU/mg protein) and OVX+CS200 (392.17 mU/mg protein) groups than the OVX group. CS in the OVX+CS100 and OVX+CS200 groups increased the level of CAT by 19 and 24%, respectively. The level of

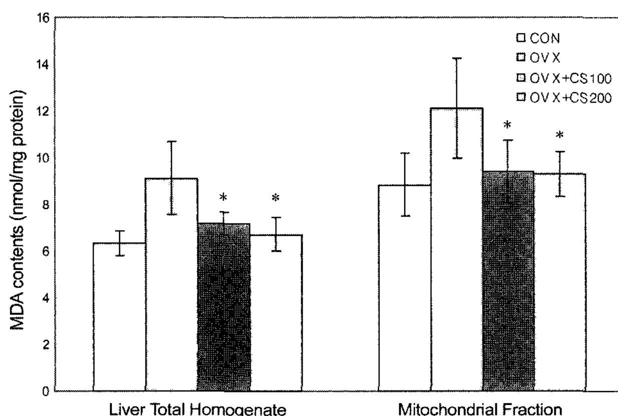

Fig. 1. MDA levels in liver total homogenate and the mitochondrial fraction of ovariectomized rat. CON: Non-ovariectomized group; OVX: Ovariectomized group; OVX+CS100: Ovariectomized + Chondroitin sulfate (100 mg/kg body weight, i.p.) group; OVX+CS200: Ovariectomized + Chondroitin sulfate (200 mg/kg body weight, i.p.) group. Significantly different from the OVX group at *$p<0.01$.

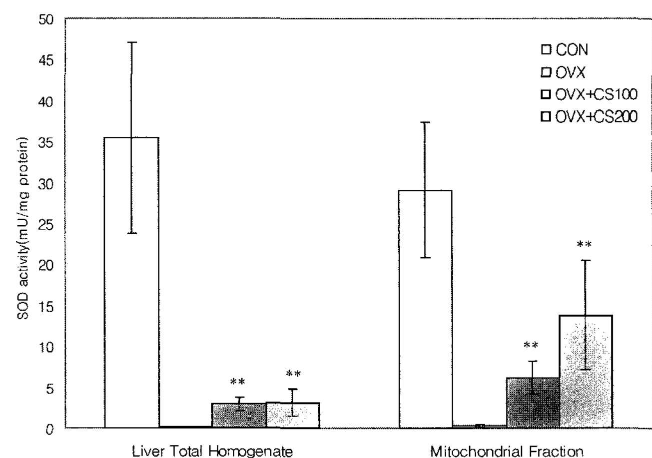

Fig. 2. SOD levels in liver total homogenate and the mitochondrial fraction of ovariectomized rat. CON: Non-ovariectomized group; OVX: Ovariectomized group; OVX+CS100: Ovariectomized + Chondroitin sulfate (100 mg/kg body weight, i.p.) group; OVX+CS200: Ovariectomized + Chondroitin sulfate (200 mg/kg body weight, i.p.) group. Significantly different from the OVX group at **$p<0.05$.

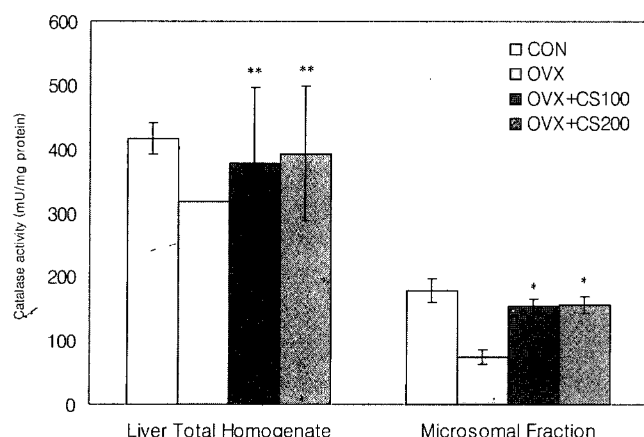

Fig. 3. CAT levels in liver total homogenate and the microsomal fraction of ovariectomized rat. CON : Non-ovariectomized group; OVX : Ovariectomized group; OVX+CS100 : Ovariectomized + Chondroitin sulfate (100 mg/kg body weight, i.p.) group; OVX+CS200 : Ovariectomized + Chondroitin sulfate (200 mg/kg body weight, i.p.) group. Significantly different from the OVX group at *p<0.01 and **p<0.05, respectively.

CAT in the microsomal fraction was lower in the OVX (74.94 mU/mg protein) than the CON group (177.80 mU/mg protein). An ovariectomy decreased the level of CAT by 58%. The measured levels of CAT in the OVX+CS100 (154.38 mU/mg protein) and OVX+CS200 (155.80 mU/mg protein) groups were found to be higher than in the OVX group. CS in the OVX+CS100 and OVX+CS200 groups increased the levels of CAT by 106 and 108%, respectively. The ovariectomy-induced oxidative stress decreased the levels of CAT, an enzyme that eliminates free radicals, but CS increased the levels of CAT in the liver total homogenate and microsomal fraction of CS-treated rats in a dose-dependent manner.

GPx levels

The levels of GPx in the liver total homogenate and mitochondrial fraction are shown in Fig. 4, and that in the liver total homogenate was found to be lower in the OVX (136.09 mU/mg protein) than the CON group (162.32 mU/mg protein). An ovariectomy decreased the level of GPx by 16%. The levels of GPx were found to be higher in the OVX+CS100 (143.74 mU/mg protein) and OVX+CS200 (159.39 mU/mg protein) groups than in the OVX group. CS in the OVX+CS100 and OVX+CS200 groups increased the levels of GPx by 6 and 17%, respectively. The level of GPx in the mitochondrial fraction was lower in the OVX (64.26 mU/mg protein) than the CON group (78.69 mU/mg protein). An ovariectomy decreased the level of GPx by 18%. The levels of GPx were found to be higher in the OVX+CS100 (68.40 mU/mg protein) and OVX+CS200 (70.41 mU/mg protein) groups than in the OVX group. CS in the OVX+CS100 and OVX+CS200 groups increased the levels of GPx by 6 and 10%, respectively. The ovariectomy-induced oxidative stress decreased the levels of GPx, an enzyme that eliminates free radicals, but CS increased the levels of GPx in the liver total homogenate and mitochondrial fraction of CS-treated rats in a dose-dependent manner.

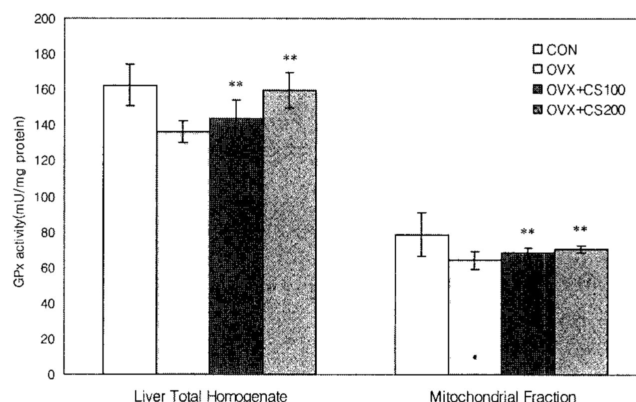

Fig. 4. GPx levels in liver total homogenate and the mitochondrial fraction of ovariectomized rat. CON: Non-ovariectomized group; OVX: Ovariectomized group; OVX+CS100: Ovariectomized + Chondroitin sulfate (100 mg/kg body weight, i.p.) group; OVX+CS200: Ovariectomized + Chondroitin sulfate (200 mg/kg body weight, i.p.) group. Significantly different from the OVX group at **p<0.05.

Histopathological findings

The antioxidative effect of CS was supported by the histopathological examination. As shown in Fig. 5, the liver tissue of the CON group (Fig. 5-A) showed no abnormal state in the central and portal veins (CV and PV), while

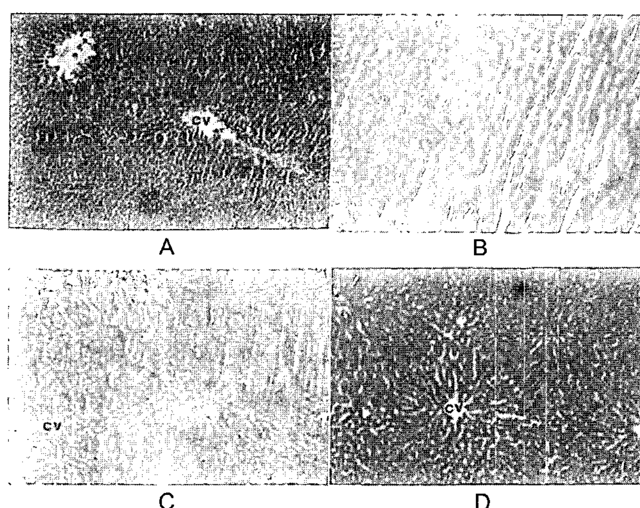

Fig. 5. Histopathological examination of the liver tissue of ovariectomized rat (H&E, ×200). (A) CON: Non-ovariectomized group; (B) OVX: Ovariectomized group; (C) OVX+CS100: Ovariectomized + Chondroitin sulfate (100 mg/kg body weight, i.p.) group; (D) OVX+CS200: Ovariectomy+ Chondroitin sulfate (200 mg/kg body weight, i.p.) group.

that of the OVX group (Fig. 5-B) showed accumulated lipid and necrosis surrounding the CV. Conversely, the inflammation and cirrhosis in liver tissue of the OVX+CS groups were significantly decreased (Fig. 5-C, D). The ovariectomy-induced oxidative stress increased the accumulation of lipid and necrosis, but CS decreased the inflammation and cirrhosis in the liver tissue of CS-treated rats.

DISCUSSION

Oxidative stress can lead to cell injury and finally to cell death if it is not controlled by an antioxidant. An ovariectomy increased the levels of MDA, one of the end-products in the lipid peroxidative process, and decreased the levels of the antioxidative enzymes, SOD, CAT and GPx. It is thought that the ovariectomy-induced ROS accelerated the activity of lipid peroxidation in the polyunsaturated fatty acids of the cell membrane, and that the increased level of MDA inversely influenced the activity of the enzymes. This coincides with two facts; the MDA reactivity towards amino groups can result in inhibition of DNA and RNA protein syntheses (Bird and Draper, 1980), and the reaction of MDA with the primary amino group proteins forms Schiff base compounds that lead to the deactivation of the enzymes. In our next study, it will be necessary to investigate the mechanism of the relation between MDA, the inhibition of DNA and RNA protein syntheses and the deactivation of the enzymes. CS decreased the levels of MDA and increased the levels of SOD, CAT and GPx in a dose-dependent manner. It is thought that CS increased the levels of SOD, CAT and GPx by scavenging the ovariectomy-derived free radicals, and that these increased enzymatic levels favorably influenced the decrease in the MDA level. In two of our previous studies (Ha and Lee, 2003; Lee *et al.*, 2004), CS showed the same pattern as in this study. In addition to the liver total homogenate, mitochondrial fraction and microsomal fraction, a histopathological examination was performed to support the role of CS in the antioxidation. CS improved the states of inflammation and cirrhosis in the liver tissue of rats. Also, another study (not published) showed that CS decreased the AST and ALT in the serum of CCl_4-treated rats. As the consistency of the role of CS against oxidative stress was found in a series of other studies, this study suggests that CS might be a potential candidate as an antioxidative reagent in oxidative stress originating from lipid peroxidation of liver cells of ovariectomized rats.

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