The Role of *Salicornia herbacea* in Ovariectomy-Induced Oxidative Stress

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Ovarian hormone deficiency increases the generation of reactive oxygen species. Their excess induces oxidative stress, which results in the cell damage or death. It causes the aging diseases—atherosclerosis, rheumatoid arthritis, osteoporosis, etc. Ovariectomized rats are used as oxidative stress models. We verified the effects of ovariectomy-induced oxidative stress on free radical production as evaluated by DPPH elimination, lipoperoxidation evaluated by malondialdehyde levels, and antioxidant activation of superoxide dismutase, catalase, glutathione peroxidase, and estradiol in the liver and sera. Ovariectomized rats were given *Salicornia herbacea* (SH) intraperitoneally at the dose of 100 mg/kg daily for 2 months. Free radical-scavenging activity of SH was measured in comparison with that of L-ascorbic acid. The histopathology of liver tissue was also investigated. Antioxidative values in the ovariectomized group decreased, but those in the SH-treated group increased due to the free radical-scavenging activity of SH. Moreover, inflammation and cirrhosis in the liver tissue of SH-treated rats decreased significantly. These results suggest that SH may be a potential candidate for an antioxidative reagent.

Key words antioxidant; *Salicornia herbacea*; ovariectomy; oxidative stress

Reactive oxygen species (ROS), which include hydrogen peroxide and other chemical forms known as free radicals, such as superoxide anion, hydroxyl, and peroxy radicals, are produced as part of normal and essential biological processes.¹⁾ Diverse antioxidant systems, both enzymatic and nonenzymatic, operate to control excessive levels of ROS.¹⁾ A cell is generally able to maintain an appropriate balance between oxidants and antioxidants under normal conditions. When this balance is perturbed and shifts toward oxidative stress, either by an increase in oxidants or by a decrease in antioxidants, the cell becomes more susceptible to injuries, such as inflammation,²⁾ ischemia/reperfusion damage,^{2,3)} diabetes,⁴⁾ and atherosclerosis.^{5–7)} The oxidative stress established by excessive free radical generation may induce a number of alterations of cell constituents, including inactivation of enzymes, generation of reactive nitrogen species, damage of nucleic acid bases and proteins, and peroxidation of membrane lipids.

To protect against the damage caused by oxidative stress, cells have a number of antioxidant enzymes and repair activities, most of which are expressed at low levels during normal growth. In mammalian cells, ROS are normally scavenged by three major types of primary antioxidant enzymes: copper-, zinc-, and manganese-dependent superoxide dismutase (CuZn-SOD and Mn-SOD), catalase (CAT) and glutathione peroxidase (GPx). The SODs convert superoxide anion into hydrogen peroxide (H₂O₂), while CAT and GPx degrade H₂O₂ into water. Furthermore, GPx degrades H₂O₂ that originates from the oxidation of polyunsaturated fatty acids (PUFAs) in the presence of reduced glutathione (GSH) which is a key component of the antioxidant defense system.⁸⁾ In addition, ovariectomy has been shown to cause an increase in peroxide production by the liver and brain.^{9,10)} Estrogens have antioxidant properties and can inhibit lipid peroxidation *in vitro*, which might contribute directly to their neuroprotective effect,¹¹⁾ and the antioxidant metabolism of estrogen protection has been suggested to be independent of receptor binding.^{12,13)} Estrogen is an antioxidant and is capable of both reducing lipoprotein oxidation and decreasing artery wall production of superoxide anion.^{14–17)} Women undergo

natural menopause, called the primary event of the aging process, in their 40–50s. Estrogen declines precipitously in postmenopause. This ovarian hormone deficiency increases the generation of ROS. The excessive ROS induces oxidative stress that results in cell damage or death. Age-related increases in serum and tissue lipid peroxides have been demonstrated in rodents,¹⁸⁾ and increased levels of serum lipid peroxides have been found in elderly humans.¹⁹⁾

Salicornia herbacea (SH) is an annual herb growing in salt marshes and on muddy seashores. In Oriental medicine, SH has been used to treat a variety of conditions such as constipation, obesity, diabetes, asthma, arthritis, and cancer.²⁰⁾ SH contains useful organic material and many important minerals. The chemical composition of SH reported by the Korea National Fisheries Research and Development Institute²¹⁾ is as follows: moisture content 90.9%, Fe 84.8 mg, and I 70 mg per 100 g of dry weight. Amino acids such as glutamic acid, aspartic acid, tyrosine, lysine, taurine, and proline have been identified in SH.²¹⁾ This plant has previously been shown to stimulate cytokine production, nitric oxide release, and expression of surface molecules.²²⁾ There are few reports on the antioxidative function of SH, however.

The present study was performed to elucidate the production of lipid peroxides and the activity of enzymes in the oxidative stress generated by surgical menopause; the role of SH in the antioxidative effects by measuring malondialdehyde (MDA), SOD, CAT, and GPx levels in liver total homogenate and mitochondrial fraction; and by demonstrating the histopathologic effects in liver tissue.

MATERIALS AND METHODS

Animals A total of 21 Sprague-Dawley (SD) rats (8-week senescent female, 130–150 g) were supplied from the Korean Experimental Animal Center (KEAC) and acclimated for 7 d. They were maintained in separate cages with laboratory chow and tapwater *ad libitum*. During the experiment, they were housed at 22±1 °C and 60±5% relative humidity with a 12 h light/dark cycle. Body weight was measured daily. A total of 21 SD rats were divided into 3 groups: the

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nonovariectomized group (CON), ovariectomized group (OVX), ovariectomized and SH 100 mg/kg-injected group (OVX+SH). Both ovaries of the rats were removed. After 2 months, SH 100 mg/kg was injected intraperitoneally in the OVX+SH group. Physiologic saline was injected in the other groups.

Blood and Tissue Collection At week 15, all rats were anesthetized with ether and dissected. The blood and liver were used for further analysis. Blood samples were centrifuged at 800 *g* for 10 min at 4 °C to obtain serum samples and then stored at -80 °C. The livers were rinsed with saline solution and stored at -80 °C. Liver tissues were homogenized in 1:5 volumes of PBS (pH 7.4). The homogenate was centrifuged at 800 *g* for 10 min. Then, the supernatant was used as the liver total homogenate sample. The mitochondrial fraction was separated as described by Player *et al.*²³⁾

Extraction of SH SH was purchased at a medicinal store in Pujeon Dong. It was reflux-condensed by 100 g with 1 l of distilled water at 70 °C. It was concentrated with an evaporator and freeze-dried.

Analytical Procedures The free radical-scavenging test was measured as described by Blois.²⁴⁾ Superoxide anion generation was determined using modifications of the dichlorodihydrofluorescein diacetate (H₂DCFDA) staining method.²⁵⁾ DCFDA 125 μ M was added to the liver homogenate in a 96-well plate to achieve a final concentration of 25 μ M. The change in fluorescence intensity was monitored at two time points (0 and 30 min) using a microplate fluorescence reader (Bio-Tek Instruments), with excitation at 485 nm and emission at 530 nm. Protein concentrations were determined according to the method of Lowry *et al.*²⁶⁾ Lipid peroxidation was assayed by the measurement of the levels on the base of MDA reacted with thiobarbituric acid at 535 nm, according to the method of Ohkawa *et al.*²⁷⁾ Results are expressed as nmol MDA mg⁻¹ protein. The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard. SOD levels were measured using a modified assay originally described by Fridovich and Beauchamp.²⁸⁾ CAT activity was measured using the method of Aebi.²⁹⁾ GPx levels were estimated using the method of Lawrence and Burk.³⁰⁾ Estradiol levels were adapted from those described by Kwon and Ahn.³¹⁾ Collected plasma was extracted three times with 3 volumes of diethyl ether. Ether extracts were dried with a speed vacuum evaporator and reconstituted with gelatin containing PBS. Labeled 17 β -estradiol (1,2,6,7-3H-estradiol; 110 Ci/mole) was obtained from NEN. Amounts of radioactivity in the sample were quantified using a Packard Tri-Carb 2900 liquid scintillation analyzer. The 17 β -estradiol concentration was calculated with a Riasmart program (Packard) on a personal computer. For histopathologic examination of the liver tissue, it was fixed overnight in 10% formaldehyde solution (dissolved in phosphate buffer, pH 7.4), and then changed successively from 70% alcohol to 100% alcohol. Next, it was changed from 100% alcohol to xylene, and then embedded in paraffin wax, sectioned in 5 μ m slices, and stained with hematoxylin-eosin (H&E).

Statistical Analysis Statistical analysis was performed with Student's *t*-test, with **p*<0.01 and ***p*<0.05 considered statistically significant. All results are expressed as mean $\pm$ S.D. Analysis was performed using SAS version 8.2 statistical software (SAS, U.S.A.).

Fig. 1. Scavenging Effects of *Salicornia herbacea* (SH) on DPPH Free Radicals

The scavenging effects are expressed as the percentage inhibition compared with the blank control. L-Ascorbic acid was used as a positive control.

Table 1. IC₅₀ Values of SH on DPPH- and $\cdot$ O₂⁻-Scavenging Activities

Compounds	IC ₅₀ (µg/ml)	
	DPPH	$\cdot$ O ₂ ⁻
<i>Salicornia herbacea</i>	197.29	21.06
L-Ascorbic acid	79.53	—
Trolox	—	7.35

DPPH levels indicate free radical-scavenging activity. $\cdot$ O₂⁻ levels indicate inhibitory activity of superoxide anion. L-Ascorbic acid was used as a DPPH-scavenging activity positive control and Trolox was used as an $\cdot$ O₂⁻-scavenging activity positive control.

RESULTS

Free Radical-Scavenging Activity The DPPH free radical is a stable free radical and has been widely used as a tool to estimate free radical-scavenging activities of antioxidants. As shown in Fig. 1, SH exhibited significant free radical-scavenging activity in a dose-dependent manner. SH showed strong antioxidant activities at concentrations greater than 100 μ g/ml, but its effect was less than that of L-ascorbic acid. The IC₅₀ of SH for DPPH radical-scavenging activity was calculated to be approximately 197.29 μ g/ml (Table 1). To assess oxidative status, superoxide anion was measured in homogenate with the DCFDA method. Table 1 presents the IC₅₀ values of SH for $\cdot$ O₂⁻ scavenging activity. SH 21.06 μ g/ml was shown to scavenge $\cdot$ O₂⁻ effectively compared with the positive control, trolox 7.35 μ g/ml.

MDA Levels The localization of radical formation resulting in lipid peroxidation, measured as the MDA level in rat liver homogenate and mitochondrial fraction, is shown in Table 2. The MDA level in the liver total homogenate was increased in the OVX group compared with the CON group (6.19 nmol/mg protein). The MDA level in the OVX+SH group in the liver total homogenate was decreased by 1.86-fold compared with the OVX group. Based on these results, the MDA content in the OVX+SH group was inhibited by 94.12%. The MDA level in the mitochondrial fraction was also increased in the OVX group compared with the CON group. The mitochondrial MDA level in the OVX+SH group was decreased by 1.96-fold compared with the OVX group. The MDA level in the OVX+SH group was inhibited

Table 2. MDA Levels in Liver Total Homogenate and Mitochondrial Fraction of Rats

Experimental group	Liver total homogenate (nmol/mg protein)		Mitochondrial fraction (nmol/mg protein)	
	MDA contents	Inhibition (%)	MDA contents	Inhibition (%)
CON	6.19±0.17	—	4.16±1.55	—
OVX	12.19±0.32	—	9.79±3.93	—
OVX+SH	6.54±0.50*	94.12	5.00±1.83**	85.08

Ovariectomy increased the levels of MDA in both liver and mitochondria. SH treatment decreased MDA levels. CON, nonovariectomized group; OVX, ovariectomized group; OVX+SH, ovariectomized+SH 100 mg/kg body weight, i.p. group. Significantly different from the value of the OVX group at * $p<0.01$, ** $p<0.05$.

Table 3. SOD Levels in Liver Total Homogenate and Mitochondrial Fraction of Rats

Experimental group	Liver total homogenate (U/mg protein)	Mitochondrial fraction (U/mg protein)
CON	24.82±0.30	19.37±0.25
OVX	12.04±0.35	8.84±1.19
OVX+SH	18.15±0.18*	16.05±0.16*

Ovariectomy decreased the levels of SOD in both liver and mitochondria. SH treatment increased SOD. CON, nonovariectomized group; OVX, ovariectomized group; OVX+SH, ovariectomized+SH 100 mg/kg body weight, i.p. group. Significantly different from the value of the OVX group at * $p<0.01$.

markedly by 85.08%. Ovariectomy-induced oxidative stress increased the levels of MDA, a marker compound in the process of lipid peroxidation, but SH decreased MDA levels both in the liver total homogenate and mitochondrial fraction of SH-treated rats.

SOD Levels SOD levels in the liver total homogenate and mitochondrial fraction are shown in Table 3. The SOD level in the liver total homogenate was found to be lower in the OVX group than in the CON group. The SOD level in the liver total homogenate was conspicuously decreased after ovariectomy and was higher in the OVX+SH group than in the OVX group. The SOD level was markedly increased after SH treatment. The mitochondrial SOD level was also lower in the OVX group than in the CON group. The mitochondrial SOD level was also conspicuously decreased after ovariectomy and was higher in the OVX+SH group than in the OVX group. The mitochondrial SOD level was markedly increased after SH treatment. Ovariectomy-induced oxidative stress decreased the activation of SOD, but SH increased the activation of SOD in the liver total homogenate and mitochondrial fraction of SH-treated rats.

CAT Levels CAT levels in the liver total homogenate and mitochondrial fraction are shown in Table 4. The CAT level in the liver total homogenate was conspicuously lower in the OVX group than in the CON group. Ovariectomy decreased the CAT level by 58.12%. CAT levels were higher in the OVX+SH group than in the OVX group. SH treatment increased the CAT level by 54.54%. The CAT level in the mitochondrial fraction was strikingly lower in the OVX group than in the CON group. Ovariectomy decreased the CAT level by 37.27%. CAT levels were higher in the OVX+SH group than in the OVX group. SH treatment increased the CAT level by 36.02%. Based on these results, SH administration restored the decrease in CAT levels in the OVX group.

Table 4. CAT Levels in Liver Total Homogenate and Mitochondrial Fraction of Rats

Experimental group	Liver total homogenate (mU/mg protein)	Mitochondrial fraction (mU/mg protein)
CON	207.99±5.43	102.56±6.00
OVX	87.33±6.31	64.34±1.00
OVX+SH	192.12±3.94*	100.57±5.93*

Ovariectomy decreased the levels of CAT in both liver and mitochondria. SH treatment increased CAT. CON, nonovariectomized group; OVX, ovariectomized group; OVX+SH, ovariectomized+SH 100 mg/kg body weight, i.p. group. Significantly different from the value of the OVX group at * $p<0.01$.

Table 5. GPx Levels in Liver Total Homogenate and Mitochondrial Fraction of Rats

Experimental group	Liver total homogenate (mU/mg protein)	Mitochondrial fraction (mU/mg protein)
CON	233.41±3.06	185.21±6.64
OVX	181.53±4.17	136.43±4.93
OVX+SH	216.47±1.34*	161.68±5.20*

Ovariectomy decreased the levels of GPx in both liver and mitochondria. SH treatment increased GPx. CON, nonovariectomized group; OVX, ovariectomized group; OVX+SH, ovariectomized+SH 100 mg/kg body weight, i.p. group. Significantly different from the value of the OVX group at * $p<0.01$.

Ovariectomy-induced oxidative stress decreased the activation of CAT, but SH increased the activation of CAT in the liver total homogenate and mitochondrial fraction of SH-treated rats.

GPx Levels GPx levels in the liver total homogenate and mitochondrial fraction are shown in Table 5. That in the liver total homogenate was lower in the OVX group than in the CON group. Ovariectomy decreased the GPx level by 22.22%. GPx levels were higher in the OVX+SH group than in the OVX group. SH treatment increased the GPx level by 19.24%. The GPx level in the mitochondrial fraction was lower in the OVX group than in the CON group. Ovariectomy decreased the GPx level by 26.33%. GPx levels were higher in the OVX+SH group than in the OVX group. SH increased the GPx level by 18.51%. These results show that SH treatment restored the decrease in GPx levels in the OVX group. Ovariectomy-induced oxidative stress decreased the activation of GPx, one of the key enzymes to eliminate free radicals, but SH increased the GPx level in the liver total homogenate and mitochondrial fraction of SH-treated rats.

Estradiol Levels Estrogen has antioxidant properties and can inhibit lipid peroxidation *in vitro*, which might contribute directly to its neuroprotective effect,³²⁾ and the antioxidant mechanism of estrogen protection has been suggested to be independent of receptor binding.^{33,34)} The 17 β -estradiol contents in rat serum are shown in Table 6. The 17 β -estradiol level in the OVX group was decreased by 1.66-fold compared with the CON group. The 17 β -estradiol level in the OVX+SH group was increased by 1.56-fold compared with the OVX group. Therefore the 17 β -estradiol level in the OVX+SH group was inhibited by 84.70%. We found that the estrogen-like mechanism of SH had a protective effect against free radical production.

Histopathologic Findings The antioxidative effect of SH was supported by the histopathologic examination. As shown in Fig. 2, the liver tissues in OVX groups exhibited

Table 6. Effects of SH on 17 β -Estradiol Levels in the Serum of Rats

Experimental group	17 β -Estradiol (pg /50 μ l)
CON	134.12 $\pm$ 4.51
OVX	80.85 $\pm$ 7.42
OVX+SH	125.97 $\pm$ 15.58*

Ovariectomy decreased the levels of 17 β -estradiol in serum. SH treatment increased 17 β -estradiol. CON, nonovariectomized group; OVX, ovariectomized group; OVX+SH, ovariectomized+SH 100 mg/kg body weight, i.p. group. Significantly different from the value of the OVX group at * p <0.01.

Fig. 2. Histopathologic Examination of Liver Tissue of Ovariectomized Rats (H&E Staining)

Normal histology was observed in the CON (nonovariectomized (A, B), $\times 100$, $\times 200$, respectively) group. Abnormal central veins were observed in the OVX (ovariectomized (C, D), $\times 100$, $\times 200$, respectively) group. In the OVX+SH (ovariectomized+*Salicornia herbacea* 100 mg/kg body weight, i.p.) group, there were attenuated histopathologic features including inflammation and cirrhosis (E, F, $\times 100$, $\times 200$, respectively) CV, central vein; WS, wide space.

ballooning degeneration in the centrilobular zone and necrosis of hepatocytes (Figs. 2C, D). Liver tissue in the CON group (Figs. 2A, B) showed no abnormality in the central veins (CV), while that of the OVX group (Figs. 2C, D) had accumulated lipids and necrosis surrounding the CV. Conversely, the inflammation and cirrhosis in liver tissue of the OVX+SH group was significantly decreased (Figs. 2E, F). Ovariectomy-induced oxidative stress increased the accumulation of lipids and necrosis, but the inflammation and cirrhosis in the liver tissue of rats were decreased by SH.

DISCUSSION

Free radicals are known to be a major factor in biological damage and DPPH has been used to evaluate the free radical-scavenging activity of natural antioxidants.^{35,36} DPPH, which is a radical itself with a purple color, changes into a stable yellow compound by reacting with an antioxidant.³⁷ SH shows free radical-scavenging activity. The IC₅₀ of SH in DPPH radical-scavenging activity was calculated to be approximately 197.29 μ g/ml. Recently, DCFDA has been used as a probe for ROS such as $\cdot O_2^-$, H_2O_2 , etc. In the superoxide anion system, the IC₅₀ value of SH was 21.06 μ g/ml. SH was thus confirmed to exhibit antioxidant activity. Lipid peroxides are known to decompose and produce a variety of substances, the most important of which is MDA.³⁸ Therefore lipid peroxides were measured using generation of thiobarbituric acid MDA reactive species, in reference to MDA equivalents.²⁷ MDA accumulation and enzyme deactivation resulted from oxidative stress causes cell injury and cell death. Stupka and Tiidus³⁹ reported that there was a clear relation-

ship between estrogen and MDA in ovariectomized rats and that ovariectomy led to an increase in free radical production. In the present study, MDA levels in both liver tissue and the mitochondrial fraction were higher in the OVX group than in the CON group. Our findings are consistent with those of Stupka and Tiidus.³⁹ Cells have a number of mechanisms to protect themselves from the toxic effects of ROS. SOD removes superoxide anion ($\cdot O_2^-$) by converting it to H_2O_2 that can be rapidly converted to water by CAT and GPx.¹ Reduction in SOD, CAT, and GPx activity in ovariectomy may be due to a compensatory mechanism against ovariectomy-induced rapid aging. These results suggest that mitochondria are relatively deficient in the antioxidant enzymes SOD and CAT during aging and are therefore more susceptible to oxidative stress. GPx levels in the OVX group were lower than in the CON group due to the higher level of lipid peroxides in mitochondrial membranes after ovariectomy.⁴⁰⁻⁴²

SH was shown to have antioxidant effect against aging.^{43,44} The antioxidant mechanism of estrogen protection has been suggested to be independent of receptor binding.^{45,46} Other steroids have been suggested to participate in protecting brain tissue against oxidative stress induced by different injuries. For example, dehydroepiandrosterone supplementation greatly reduces oxidative damage in synaptosomes isolated from diabetic rats⁴⁷ and dehydroepiandrosterone sulfate added to the medium demonstrated a neuroprotective effect against ischemic neuronal injury *in vitro*.⁴⁸ Those results suggested a therapeutic value of ovarian hormones in diseases associated with oxidative stress. Such evidence supports the association between steroid hormones, particularly estrogens, and the oxidative state. It is known that antioxidants inhibit oxidative stress in the lipid peroxidation process by scavenging free radicals. In the present study, ovariectomy increased the accumulation of MDA, which is one end-product in the lipid peroxidative process, and inhibited the activation of antioxidant enzymes. It is thought that the oxidative stress caused by the ovariectomy-induced hormone deficiency accelerated the activity of lipid peroxidation in the PUFAs of the cell membrane and that the accumulated MDA influenced the activity of enzymes inversely. It is thought that SH increased the activities of SOD, CAT, GPx, and estradiol by scavenging the ovariectomy-derived free radicals and that the increased enzymatic activity influenced the decreased MDA level favorably.

In addition to the liver total homogenate and mitochondrial fraction, histopathologic examination was performed in the present study to confirm the role of SH in antioxidation. A comparison of liver sections of normal rats (Figs. 2A, B) with those from ovariectomized rats (Figs. 2C, D) shows that the liver cells of ovariectomized rats had marked damage, as characterized by cell vacuolation and pyknotic and degenerated nuclei. The walls of the bile capillaries were also damaged. Cell lysis was visible around the intralobular vein. Wide spaces formed at some sinusoids. The hepatic cells of SH-treated rats were concentrated and radially arranged, similar to those of normal rats.

The increase in the activity of aspartate aminotransferase (AST) in the liver (data not shown) could be expected to occur associated with pathology involving necrosis of the liver. AST is significantly increased in such cases and escapes to the plasma from the injured hepatic cells.⁴⁹ There-

fore the increment in the activities of AST and alanine aminotransferase in plasma (data not shown) may be mainly due to the leakage of those enzymes from the liver cytosol into the blood stream. This is in accordance with Greig and Pennington.⁵⁰ From those observations, it is possible that SH may considerably reduce oxidative stress in ovariectomy-induced aged rats through the scavenging of free radicals and increasing the activities of antioxidants that then detoxify free radicals. This means that SH as a modulator of antioxidants (SOD, CAT, GPx, estradiol) may play a significant role in restoration of cellular redox status and minimization of cell damage due to ROS. The results of this study suggest that SH may act to alleviate the oxidative stress associated with ovariectomy-induced aging.

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