Original Article

Effect of *Oenanthe Javanica* Extract on Antioxidant Enzyme in the Rat Liver

Choong-Hyun Lee¹, Joon-Ha Park², Jeong-Hwi Cho², In-Hye Kim², Ji-Hyeon Ahn², Jae-Chul Lee², Bai Hui Chen², Bich-Na Shin³, Hyun-Jin Tae⁴, Eun Joo Bae⁴, Il-Jun Kang⁵, Moo-Ho Won⁶, Jong-Dai Kim⁷

¹Department of Pharmacy, College of Pharmacy, Dankook University, Cheonan 330-714, South Korea
²Department of Neurobiology, School of Medicine, Kangwon National University, Chuncheon 200-701, South Korea
³Department of Physiology, College of Medicine, Hallym University, Chuncheon 200-702, South Korea
⁴Department of Biomedical Science, Research Institute for Bioscience and Biotechnology, Hallym University, Chuncheon 200-702, South Korea
⁵Department of Pediatrics, Chunchon Sacred Heart Hospital, College of Medicine, Hallym University, Chuncheon 200-701, South Korea
⁶Department of Food Science and Nutrition, Hallym University, Chuncheon 200-702, South Korea
⁷Division of Food Biotechnology, School of Biotechnology, Kangwon National University, Chuncheon 200-701, South Korea

Choong-Hyun Lee and Joon-Ha Park contributed equally to this work

Abstract

Background: *Oenanthe javanica* (*O. javanica*) has been known to have high antioxidant properties via scavenging reactive oxygen species. We examined the effect of *O. javanica* extract (OJE) on antioxidant enzymes in the rat liver.

Methods: We examined the effect of the OJE on copper, zinc-superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), catalase (CAT), and glutathione peroxidase (GPx) in the rat liver using immunohistochemistry and western blot analysis. Sprague-Dawley rats were randomly assigned to three groups; (1) normal diet fed group (normal-group), (2) diet containing ascorbic acid (AA)‑fed group (AA‑group) as a positive control, (3) diet containing OJE-fed group (OJE-group).

Results: In this study, no histopathological finding in the rat liver was found in all the experimental groups. Numbers of SOD1, SOD2, CAT, and GPx immunoreactive cells in the liver cells of the rat were significantly increased in the AA-fed group compared with those in the normal-group. On the other hand, in the OJE-group, numbers of SOD1, SOD2, CAT, and GPx immunoreactive cells in the liver were significantly increased by about 190%, 478%, 685%, and 346%, respectively, compared with those in the AA-group. Furthermore, protein levels of SOD1, SOD2, CAT, and GPx in the OJE-group were also significantly much higher than those in the AA-group.

Conclusion: OJE significantly increased expressions of SOD1 and SOD2, CAT, and GPx in the liver cells of the rat, and these suggest that significant enhancements of endogenous enzymatic antioxidants by OJE might be a legitimate strategy for decreasing oxidative stresses in the liver.

Key words: Antioxidant Enzymes; Immunohistochemistry; Liver; *Oenanthe Javanica* Extract; Western Blotting

Introduction

Reactive oxygen species (ROS), chemically reactive molecules containing oxygen, are formed by natural byproducts of the normal metabolism of oxygen and by environmental factors such as ultraviolet radiation, pollutants, and drugs. ROS has important roles in cell signaling and homeostasis. However, imbalance between ROS production and removal leads to oxidative stress. In the liver, in addition, the excessive generation of ROS, with the reduction of antioxidant defense activities, has been known to be closely related with the induction and progression of hepatic cell death. *O. javanica* displays hepatoprotective effects against tert-butyl hydroperoxide-induced damage in HepG2 cells, carbon tetrachloride-induced liver injury, alcohol intoxication, and hepatitis B virus.

Water dropwort (*Oenanthe javanica* (*O. javanica*)) is an aquatic perennial herb with a distinctive aroma and taste and is one of the medicinal plants, which has been used to treat jaundice, hypertension, fever, and abdominal pain. *O. javanica* has high antioxidant properties via scavenging ROS and has been known to protect primary cultured rat cortical cells from glutamate-induced neurotoxicity. In addition, *O. javanica* has anti-proliferative effects on HepG2 cells, not normal Chang liver cells. Furthermore, it has been well-known that *O. javanica* has protective effects against various liver injuries: *O. javanica* displays hepatoprotective effects against tert-butyl hydroperoxide-induced damage in HepG2 cells, carbon tetrachloride-induced liver injury, alcohol intoxication, and hepatitis B virus.

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Address for correspondence: Prof. Jong-Dai Kim, Division of Food Biotechnology, School of Biotechnology, Kangwon National University, Chuncheon, 200-701, South Korea.

E-Mail: Jongdai@kangwon.ac.kr
Recently, we reported that *O. javanica* extract (OJE) significantly increased immunoreactivities of superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), catalase (CAT), and glutathione peroxidase (GPx) in the rat kidney.[19] However, no studies regarding the effect of *O. javanica* on the normal liver have been reported yet, although some studies have showed hepatoprotective effects of *O. javanica* in various animal models. In the present study, therefore, we examined the effect of OJE on antioxidant enzymes, such as copper, zinc-SOD1, SOD2, CAT, and GPx, in the rat liver using immunohistochemistry and western blot analysis.

**Methods**

**Experimental animals**

Male Sprague-Dawley rats were obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, South Korea. The rats were used at 12 weeks of age (body weight, 300–320 g). They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-h light/12-h dark cycle, and free access to food and water. The procedures for animal handling and care adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th ed., 2011), and they were approved by the Institutional Animal Care and Use Committee at Kangwon National University. All efforts were also made to minimize animal suffering, as well as, the number of the animals used.

**Preparation of Oenanthe javanica extraction**

As previously described,[19] *O. javanica* was collected by Professor Jong Dai Kim in Kangwon province (South Korea), in March 2013 and kept in a deep freezer (–70°C). *O. javanica* was extracted with 10 volume (v/w) of 70% ethanol at 70°C for 4 h, and extraction was repeated three times. The extracts were filtered through Whatman filter paper (No. 2), concentrated with a vacuum evaporator, and completely dried with a freeze-drier. The extraction yield was 14.5%.

**Treatment of Oenanthe javanica extract**

The animals were divided into three groups: (1) Normal-group (*n* = 14), which is served as a negative control and received a normal composition pellets diet and tap water *ad libitum*; (2) ascorbic acid (AA)-group (*n* = 14), which is served as a positive control; AA is known as a representative antioxidant chemical, which is mixed by 0.5% of pellets weight (w/w)[20] [Table 1]; and (3) OJE-group (*n* = 14); OJE was mixed by 0.5% of pellets weight (w/w). Each group was designated to receive different amounts composition of the pellets for 28 days. Compositions of experimental diets were represented in Table 1.

**Tissue processing for histology**

For histological analysis, the animals (*n* = 7 in each group) were anesthetized with sodium pentobarbital and perfused transcardially with 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 28 days after AA or OJE diet. The liver was removed and postfixed in the 10% buffered formalin. Liver was cut sagittally, and then embedded with paraffin. The sections were cut at a 6-μm thickness with a microtome (Leica, Wetzlar, Germany).

**Hematoxylin and eosin staining**

To examine the pathological changes of the liver in each group using hematoxylin and eosin (H & E) staining, the sections were mounted on gelatin-coated microscopy slides. The sections were stained and dehydrated by immersing in serial ethanol baths, and they were then mounted with Canada Balsam (Kanto Chemical, Tokyo, Japan).

**Immunohistochemistry for antioxidant enzymes**

Immunohistochemistry was carried out with SOD1, SOD2, CAT, and GPx in order to examine changes in the antioxidants in the liver according to the method of our previous study.[21] In brief, liver sections were sequentially treated with 0.3% hydrogen peroxide (H$_2$O$_2$) in PBS for 30 min and 10% normal goat or donkey serum in 0.1 M PBS for 30 min. The sections were next incubated with goat anti-SOD1 (diluted 1:500, Calbiochem, Darmstadt, Germany), goat anti-SOD2 (diluted 1:1000, Calbiochem), rabbit anti-CAT (diluted 1:1000, Lab Frontier, Seoul, Korea) and sheep anti-GPx (diluted 1:1000, Chemicon International, Billerica, MA). Thereafter, the tissues were exposed to biotinylated goat anti-rabbit IgG, goat anti-sheep IgG, donkey anti-goat IgG, and streptavidin peroxidase complex (Vector, Burlingame, CA, USA). And they were visualized with 3,3′-diaminobenzidine in 0.1 M Tris-HCl buffer. After dehydration, the sections were mounted with Canada Balsam (Kanto, Tokyo, Japan). A negative control test was carried out using preimmune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures.

In order to quantitatively analyze SOD1, SOD2, CAT, and GPx immunoreactivity, digital images of the liver to center the central vein were captured with an AxioM1 light

| Table 1: Composition of experimental diets (%) |
|-----------------------------------------------|
| **Ingredients** | Normal-group | AA-group | OJE-group |
| Casein         | 20.0         | 20.0     | 20.0      |
| Corn oil       | 5.0          | 5.0      | 5.0       |
| Cholesterol    | 0.5          | 0.5      | 0.5       |
| Corn starch    | 15.0         | 15.0     | 15.0      |
| Cellulose      | 5.0          | 5.0      | 5.0       |
| Mineral mix (AIN-76) | 3.5 | 3.5 | 3.5 |
| Vitamin mix (AIN-76) | 1.0 | 1.0 | 1.0 |
| Methione       | 0.3          | 0.3      | 0.3       |
| Choline bitartrate | 0.2 | 0.2 | 0.2 |
| Sucrose        | 49.5         | 49       | 49        |
| Ascorbic acid  | –            | 0.5      | –         |
| 70% ethanol extracts of *oenanthe javanica* | – | – | 0.5 |
microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss, Germany) connected to a PC monitor. SOD1, SOD2, CAT, and GPx immunoreactive cells were counted at 200X magnification. Six coronal sections with 150-µm interval per animal were selected, and cell counts were obtained by averaging the counts from each animal.

**Western blot analysis for antioxidant enzymes**

To examine the protein levels of antioxidants in the liver, the animals (n = 7 in each group) were used for western blot analysis according to the method of our previous study. After sacrificing animals and removing the liver, and the tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N, N’, N” tetraacetic acid (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 20 µg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a suitable polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Corp., East Hills, NY, USA). To reduce background staining, the membranes were incubated with 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with goat anti-SOD1 (diluted 1:1000, Calbiochem, Darmstadt, Germany), goat anti-SOD2 (diluted 1:1000, Calbiochem), rabbit anti-CAT (diluted 1:1000, LabFrontier, Seoul, Korea) and sheep anti-GPx (diluted 1:1000, Chemicon International, Billerica, MA), peroxidase-conjugated donkey anti-goat IgG, goat anti-rabbit IgG or goat anti-sheep IgG (Santa Cruz, USA), and an ECL kit (Pierce Chemical, Rockford, USA).

The result of western blot analysis was scanned, and densitometric analysis for the quantification of the bands was done using Image J 1.46 (National Institutes of Health, Bethesda, MD, USA), which was used to count relative optical density (ROD): A ratio of the ROD was calibrated as %, with normal-group designated as 100%.

**Statistical analysis**

All data are presented as mean ± standard error of mean. A multiple-sample comparison was applied to test the differences between groups (ANOVA and the Tukey multiple range test as post-hoc test using the criterion of the least significant differences). Statistical significance was considered at P < 0.05.

**RESULTS**

**Histological finding**

The effect of OJE treatment on the liver histology was evaluated using H & E staining. We found that histological findings in the AA- and OJE-groups were very similar to those in the normal-group. These results show that AA and OJE did not affect normal liver histology compared with that in the normal-group [Figure 1].

**Immunoreactivity of antioxidant enzymes**

**Superoxide dismutase 1**

Superoxide dismutase 1 immunoreactive cells were hardly detected in the normal-group, although SOD1 immunoreactivity is shown in noncellular tissue [Figures 2a and 3]. SOD1 immunoreactive cells were significantly increased in the AA-group compared with those in the normal-group [Figures 2b and 3]. In the OJE-group, the mean number of SOD1 immunoreactive cells was significantly increased by about 190% compared with that in the AA-group [Figures 2c and 3].

**Manganese superoxide dismutase**

The pattern of SOD2 immunoreactivity in all the experimental groups was similar to that observed in the immunohistochemical data of SOD1 [Figure 2d–2f]. SOD2 immunoreactive cells were increased in the AA-and OJE-groups compared with those in the normal-group [Figures 2e and 3]; especially, the mean number of SOD2 immunoreactive cells in the OJE-group was significantly increased by about 478% compared with that in the AA-group [Figures 2f and 3].

**Catalase**

In the normal-group, CAT immunoreactivity was hardly detected in the liver [Figures 2g and 3]. However, a few CAT immunoreactive cells were observed in the AA-group [Figures 2h and 3]. In the OJE-group, CAT immunoreactive cells were significantly increased by about 685% compared with that in the AA-group [Figures 2i and 3].

![Figure 1: H and E staining in the rat liver of the normal-(a), ascorbic acid -(b) and Oenanthe javanica extract -(c) groups. No histopathological finding is found in all experimental groups.](image-url)
Glutathione peroxidase

Although SOD1 immunoreactivity is shown in noncellular tissue, GPx immunoreactive cells were hardly found in the liver of the normal-group [Figures 2j and 3]. However, many GPx immunoreactive cells were easily detected in the AA- and OJE-groups [Figures 2k, 2l and 3]; especially, strong GPx immunoreactive cells were significantly increased by about 346% in the OJE-group compared with that in the AA-group [Figures 2l and 3].

Protein levels of antioxidant enzymes

In this study, we examined changes in SOD1, SOD2, CAT, and GPx protein levels in the liver using western blot analysis [Figure 4]. In the AA-group, the levels of all the antioxidant enzymes were significantly increased compared with that in the normal-group, and the levels of all the antioxidant enzymes in the OJE-group were significantly higher than those in the AA-group.

Discussion

It has been widely accepted that SODs, CAT, and GPx play important roles in protection against oxidative stresses.\(^6\,\,7\,\,23\) It is well-known that SOD enzymes participate in the protection against harmful oxidative stresses by catalyzing the dismutation of superoxide anions into hydrogen peroxide and oxygen.\(^{24}\) Especially, CAT and GPx are important enzymes, which convert hydrogen peroxide into water and oxygen.\(^{24}\)

In this study, we used the AA-group as a positive control. AA has been well-known to have antioxidant property by scavenging ROS and to act as an essential coenzyme in oxidative stress pathways.\(^{25}\) It was reported that the oral...
administration of 100 mg/kg AA for 4 weeks could reduce malondialdehyde and ROS levels and increase SOD and CAT activities in the cadmium-treated rat liver.[23] We, in the present study, found that significant increases in the number of SOD1, SOD2, CAT, and GPx immunoreactive cells and their protein levels in the AA-group (positive control-group) compared with those in the normal-group. This result represents the AA treatment could increase antioxidant activity in the rat liver.

Many natural products have been known to be hepatoprotective agents through their effects on the antioxidant system.[26-28] In the present study, we first examined the effect of OJE on antioxidant enzymes in the liver, and we found that more significant increases in the number of SOD1, SOD2, CAT, and GPx immunoreactive cells and their protein levels were significantly increased in the OJE-group compared with those in the AA-group as well as the normal-group. It was reported that OJE, which has gallic acid, catechin, chlorogenic acid and caffeic acid, displayed a great potential as an antioxidant functional ingredient in food applications.[13] Furthermore, we recently showed that OJE administration in the rat markedly increased antioxidant enzymes immunoreactive cells in the kidney.[19] Therefore, it can be postulated that OJE pretreatment could provide hepatoprotection against oxidative stress-related liver cell damage via increasing antioxidant activity although we did not examine this in the present study.

In conclusion, we show that OJE treatment significantly induced augment of endogenous enzymatic antioxidants (SOD1, SOD2, CAT, and GPx) in the rat liver and the findings indicate that OJE pretreatment could provide hepatoprotection against some oxidative stresses in the liver.

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