Leonurus sibiricus Herb Extract Suppresses Oxidative Stress and Ameliorates Hypercholesterolemia in C57BL/6 Mice and TNF-α Induced Expression of Adhesion Molecules and Lectin-Like Oxidized LDL Receptor-1 in Human Umbilical Vein Endothelial Cells

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In Leonurus sibiricus herb extract (LHE)-supplemented animals, plasma cholesterol decreased and high-density lipoprotein-cholesterol increased, resulting in a lowered atherogenic index. The plasma trolox equivalent antioxidant capacity, levels of hepatic thiobarbituric acid-reactive substances, and protein carbonyl values decreased significantly in LHE-supplemented mice \((p < 0.05)\), whereas the hepatic antioxidant indicators were all significantly elevated \((p < 0.05)\). In human umbilical vein endothelial cells stimulated with tumor necrosis factor alpha, LHE significantly suppressed intracellular reactive oxygen species, LOX-1, and adhesion molecules. LHE supplementation may modulate the lipoprotein composition and attenuate oxidative stress by elevated antioxidant processes, thus suppressing the activation of inflammatory mediators. This is a possible mechanism of the anti-atherogenic effect.

Key words: Leonurus sibiricus herb; LOX-1; adhesion molecules; C57BL/6 mice; human umbilical vein endothelial cells

Since atherosclerosis is a slowly progressing disease, early detection allows for more efficient treatment or even reversal.¹ Therefore, the ability to detect the early stages of atherosclerosis is of clinical interest in order to select patients who will best benefit from treatment. The first stage of atherosclerosis is considered to be endothelial dysfunction.²³ Oxidative stress induced by reactive oxygen species (ROS), reactive nitrogen species (RNS), and related inflammation probably plays an important role in the etiology of atherosclerosis.²⁹ Oxidized low-density lipoprotein (ox-LDL) is recognized as a major risk factor in the initiation and progression of atherosclerotic lesions, which may promote endothelial dysfunction by increasing the production of endothelial-derived ROS and enhancing the expression of adhesion molecules on the endothelium via lectin-like ox-LDL receptor-1 (LOX-1).⁴¹ LOX-1 expression is upregulated in the vessel tissues of animals and humans suffering from atherosclerosis and related cardiovascular diseases.⁵³ The expression of LOX-1 in endothelial cells may provide a molecular link for the incorporation of ox-LDL into cells, thus inducing the generation of ROS; this, in turn, activates oxidative stress-sensitive factors including nuclear factor kappa B (NFκB) and p38 mitogen-activated protein kinase (p38 MAPK).⁶⁰ Subsequently, overexpression of endothelin-1 (ET-1) and adhesion molecules such as P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) is induced or the activation of endothelial nitric oxide synthase (eNOS) is impaired.³⁷ Up- or downregulated expression is all involved in the pathomechanisms of endothelial dysfunction. LOX-1 probably functions as a mediator and biomarker of endothelial or vascular dysfunction.⁸³ As a marker of oxidative stress, ox-LDL and other molecules related to oxidative stress can induce LOX-1 expression,⁹⁶ whereas antioxidant treatment suppresses its expression; this suggest redox-sensitive regulation of LOX-1 expression.¹⁰ The best explanation of these collective findings is that elevated oxidative stress contributes to the development of atherosclerosis through inflammatory processes. The biological effects of Leonurus sibiricus herb (LH) may reflect the presence of phytochemicals and their antioxidant capacities. The antioxidant capacities of LH extract (LHE) are well known, as are those of LH phytochemicals with antioxidant activity.¹¹ However, much less is known about the in vitro and in vivo health benefits of LH. Our previous in vitro study revealed that LH possesses potent antioxidant capacity and can scavenge ROS and RNS. These observations led us to investigate whether supplementation with LHE can ameliorate the risk factors of cardiovascular diseases, hypercholesterolemia and atherosclerosis.

Materials and Methods

Preparation of plant extracts. LH (Leonurus sibiricus H.) was obtained from Omniherb (Yeongcheon, Gyeongbuk, Republic of Korea) and was stored at 2–4°C until use. When required, an LH

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sample was milled (maximum particle size, 0.4 mm). In preparing the LHE, the powdered material was diluted 20-fold, refluxed 3 times (12, 6, and 3 h) with 70% ethanol, and then filtered through a G4 glass filter funnel. The extract was gathered and the ethanol was evaporated under reduced pressure at 45 °C in a Buchi RII rotary vacuum evaporator (Buchi, Flawil, Switzerland). The extract was then lyophilized. The 70% ethanol extract yielded 21.3 g/500 g of LH. The total phenolic content of the 70% ethanol extract, as estimated by the Folin-Ciocalteu reagent method, contain to 723.67 μg GA eq/mg.

Animals and diets. This study was conducted in accordance with the Guidelines for Animal Experiments approved by Dongguk University. The 30 female C57BL/6 mice obtained from Orient-Bio (Seongnam, Korea) were approximately 5 weeks old at the start of the experiment. They were kept in cages in pairs in a temperature-controlled (25 °C) vivarium under a 12 h light/dark cycle (lights off at 12:00 PM). They were kept in cages in pairs in a temperature-controlled (25 °C) vivarium under a 12 h light/dark cycle (lights off at 12:00 PM). They were anesthetized with dry ice following a 12 h fast. Blood samples were obtained from the abdominal aortas, and the livers were removed, rinsed with phosphate-buffered saline (PBS, pH 7.4), and stored at −70 °C until analysis.

Lipid assay. The plasma concentrations of cholesterol, triglyceride, and high-density lipoprotein (HDL)-cholesterol were determined using colorimetric kits (Sigma-Aldrich, St. Louis, MO).

Oxidative stress status. The total antioxidant capacity of the plasma was determined using a modified plasma trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich) equivalent antioxidant capacity (TEAC) assay.125 The oxidative stress in the liver was also quantified by measuring the levels of thiobarbituric acid-reactive substances (TBARS).13 Hepatic protein carbonyl values were determined by a previously described method,14 which is based on spectrophotometric detection of the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyl to form protein hydrazones.

Antioxidant enzyme activities. The protein concentration of each fraction was measured as described previously15 with bovine serum albumin (BSA) as the standard. Cu/Zn-superoxide dismutase (SOD) and Mn-SOD activities were determined as described previously.16 Previously described methods were used to determine the activities of catalase,17 glutathione peroxidase (GPX),18 and glutathione reductase (GR).19

Cell culture and vitality. Human umbilical vein endothelial cells (HUVECs) purchased from Lonza Walkersville Inc. (Walkersville, MD) were cultured in endothelial cell basam medium-2 (EGM-2) (Clonetics). Cells from passages 4–7 were used; they were maintained in an incubator at 37 °C in an atmosphere of 5% CO2. For all experiments, HUVECs were grown to 80–90% confluence and made quiescent by starvation for at least 18 h. Cell vitality was ascertained by Cell Titer 96 colorimetric assay (Promega, Madison, WI) using 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxy methoxyphenyl)-2(4-sulfenyl)-2H-tetrazolium (MTS) according to the manufacturer’s instructions. HUVECs (5 × 104) were dispersed into the wells of a 96-well plate and supplemented with various concentrations of LHE 1 h before treatment with 10 ng/ml tumor necrosis factor alpha (TNF-α). After 10 h of incubation, 20 μl MTS reagent was added to each well. The cells were incubated for another 2 h at 37 °C in 5% CO2 and the absorbance was measured at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

ROS generation in TNF-α-stimulated HUVECs. The intracellular ROS levels were determined using the ROS-sensitive fluorescent dye 2,7'-dichlorofluorescin diacetate (DCF-DA) as previously described.20

Immunoblot analysis. HUVECs (5 × 104) were supplemented with various concentrations of LHE 1 h before the addition of 10 ng/ml TNF-α, which was maintained in an incubation at 37 °C in an atmosphere of 5% CO2 for 12 h. The cells were washed twice using ice-cold PBS and harvested by scraping in 1 ml of ice-cold PBS. After centrifugation at 13,000 rpm, the cell pellets were lysed in 400 μl protein extraction solution (Intron Biotechnology, Gyeonggi-do, Korea) for 20 min at −20 °C. The lysates were centrifuged at 13,000 rpm for 5 min and the supernatants were immediately aliquoted and stored at −70 °C until use. The protein concentration was determined with a commercial protein assay kit (Bio-Rad, Hercules, CA) using BSA as the standard. Protein samples of equal concentrations were electrophoresed in a 7.5–12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking with PBS containing 5% non-fat dry milk for 1 h at room temperature, each membrane was incubated with anti-LOX-1 (R&D System, Madison, WI), anti-VCAM-1, and anti-ICAM-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After washing twice with PBS containing 0.1% Tween-20 (PBST), each membrane was immunoblotted with horseradish peroxidase-conjugated anti-mouse or anti-goat IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and then washed thrice in PBST; the proteins on the membranes were visualized by enhanced chemiluminescence western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were determined by densitometry.

Statistical analysis. All experiments were performed at least three times by conducting each assay in triplicate. Data were analyzed with SPSS version 17.0 for Windows (SPSS, Chicago, IL) and by expressed as the mean ± standard deviation. Statistical analyses were conducted using analysis of variance (ANOVA, Tukey’s) test between groups and statistical significance was considered at p < 0.05.

Results

Weight gain, food intake, food efficiency, and liver weight

The data for weight gain, food intake, and food efficiency are summarized in Table 1. The weight gain of the atherogenic diet-control group (AC) mice was lower than that of the normal-control group (NC) mice because of decreased food intake. However, the food efficiency did not differ among these experimental groups. The liver weight of the AC mice was higher.

Table 1. Effects of LHE Supplementation on Weight Gain, Food Intake, Food Efficiency, Liver Weight and Plasma Lipid Profile in C57BL/6 Mice Fed a Atherogenic Diet

<table>
<thead>
<tr>
<th>Groups</th>
<th>NC</th>
<th>AC</th>
<th>AC + LHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (g/d)</td>
<td>0.06 ± 0.01a</td>
<td>0.05 ± 0.01b</td>
<td>0.06 ± 0.01b</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>2.94 ± 0.11a</td>
<td>2.51 ± 0.09b</td>
<td>2.79 ± 0.07b</td>
</tr>
<tr>
<td>Food efficiency (%)</td>
<td>1.95 ± 0.17a</td>
<td>1.96 ± 0.07b</td>
<td>2.02 ± 0.15b</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>2.77 ± 0.05b</td>
<td>3.01 ± 0.03c</td>
<td>2.76 ± 0.08b</td>
</tr>
<tr>
<td>Lipid profiles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>96.6 ± 3.96c</td>
<td>213.9 ± 7.99a</td>
<td>155.5 ± 3.99b</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>34.0 ± 0.83a</td>
<td>35.5 ± 0.89a</td>
<td>32.6 ± 0.79a</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dl)</td>
<td>43.2 ± 1.21a</td>
<td>38.8 ± 0.59a</td>
<td>50.1 ± 1.10a</td>
</tr>
<tr>
<td>Atherogenic Index (AI)</td>
<td>1.23 ± 0.10a</td>
<td>4.52 ± 0.22a</td>
<td>2.11 ± 0.12a</td>
</tr>
</tbody>
</table>

**Statistical significance:** *p* < 0.05 by the Tukey test. NS: not significant. NC: Normal-Control group; AC: Atherogenic diet-Control group; AC + LHE: Atherogenic diet + 0.01 g LHE/20 g body weight. Values are mean ± S.D. (n = 10).
than that of the mice fed the normal diet, which may be due to the fatty liver; however, in the AC mice fed the LHE-supplemented diet, the liver weight recovered, and it was comparable to that of the NC mice.

**Plasma lipid profile**

The plasma lipid levels in the C57BL/6 mice after 14 weeks are shown in Table 1. Plasma total cholesterol levels were considerably higher in the AC group than in the NC group, but they decreased significantly due to LHE supplementation. The final plasma triglyceride level was slightly elevated in the AC group as compared to that in the NC group, but was reduced in the AC + LHE group. The HDL-cholesterol concentration slightly decreased in the mice receiving the atherogenic diet, but was significantly higher in the LHE-supplemented groups than in the NC and AC groups ($p < 0.05$). The atherogenic index was significantly elevated in the AC group relative to that in the NC group. Supplementation of the atherogenic diet with LHE significantly suppressed the atherogenic index as compared to the NC and AC groups ($p < 0.05$).

**Oxidative stress status and antioxidant enzyme activities**

The level of oxidative stress was determined by the status of plasma TEAC, hepatic TBARS, and protein carbonyls. The plasma TEAC value was suppressed by the atherogenic diet, and it improved upon LHE supplementation ($p < 0.05$) (Fig. 1). The levels of hepatic TBARS and protein carbonyls were higher in the AC group than in the NC group. However, LHE supplementation significantly suppressed these levels in liver homogenates ($p < 0.05$) (Fig. 1). The activities of hepatic antioxidant enzymes are shown in Fig. 1. The activities of all the examined antioxidant enzymes (Cu,Zn-SOD, Mn-SOD, catalase, GPX, and GR) were significantly lower in the AC group than in the NC group; however, LHE supplementation significantly increased the activities of these enzymes ($p < 0.05$).

**Effects of LHE on viability and ROS generation in TNF-α-stimulated HUVECs**

In order to assess the effect of LHE on the growth of HUVECs, viable cell numbers were estimated by MTS.
assay. Incubation of LHE alone or TNF-α-stimulated HUVECs with 10–400 μg LHE for 12 h did not have a discernable cytotoxic effect (Fig. 2). The effect of LHE on ROS generation in the TNF-α-stimulated HUVECs was evaluated using the ROS-sensitive fluorescent dye DCF-DA. The level of ROS in the TNF-α-stimulated HUVECs was markedly higher than that in the control. LHE treatment reduced ROS production in the TNF-α-stimulated HUVECs in a dose-dependent manner; the levels of ROS suppression were 26%, 49%, 55%, 67%, and 73% at LHE concentrations of 10, 50, 100, 200, and 400 μg/ml, respectively (Fig. 2).

**Effects of LHE on LOX-1 expression in TNF-α-stimulated HUVECs**

Western blot analysis of cell lysates for the LOX-1 protein indicated that stimulation of cells with TNF-α sharply increased LOX-1 expression. Although LOX-1 expression was slightly induced at low concentrations of LHE (<100 μg/ml) in the TNF-α-stimulated HUVECs, no statistical variation was found. Incubation of cells with both TNF-α and LHE significantly inhibited such an increase in receptor expression in a concentration-dependent manner at >200 μg/ml of LHE (Fig. 3). In particular, treatment with high levels of LHE (400 μg/ml) resulted in a significant reduction (27%) in LOX-1 expression as compared to treatment solely with TNF-α.

**Effects of LHE on VCAM-1 and ICAM-1 expression in TNF-α-stimulated HUVECs**

Expression of VCAM-1 and ICAM-1 proteins in the TNF-α-stimulated HUVECs was measured using β-actin as an internal standard. Expression of VCAM-1 and ICAM-1 was significantly lower in LHE-treated HUVECs than in the controls. In particular, the expression of VCAM-1 was reduced by 83% and 94% at LHE concentrations of 200 μg/ml and 400 μg/ml, respectively (Fig. 3).

**Discussion**

In this study, we examined the suppressive effects of LHE supplementation on atherogenic diet-induced hypercholesterolemia and oxidative stress in plasma and livers of C57BL/6 mice. Our study demonstrated that LHE supplementation in C57BL/6 mice for 14 weeks significantly lowered plasma cholesterol and elevated HDL cholesterol, decreasing the atherogenic index. Although it is still unclear how LH affects the cholesterol level, present evidence supports the notion that LHE supplementation can modulate unfavorable lipoprotein composition in hypercholesterolemic patients. Oxidative stress induced by ROS, RNS, and inflammation are considered to be influential in the development of atherosclerosis. In this study, we found that elevated antioxidant capacity suppressed lipid peroxidation and protein oxidation in mice fed an atherogenic diet supplemented with LHE. Furthermore,
we observed that generation of intracellular ROS and expression of adhesion molecules such as VCAM-1, ICAM-1, and LOX-1 were significantly inhibited by LHE treatment in TNF-α stimulated HUVECs. *In vitro*, LOX-1 expression is induced by many inflammatory cytokines, oxidative stress, hemodynamic stimuli, and ox-LDL; *in vivo*, expression is enhanced in proatherogenic settings including hypertension, hyperlipidemia, and diabetes, and LOX-1 accumulates in the atherosclerotic and glomerulosclerotic lesions. These researchers found that the LOX-1 level is significantly increased in the plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits and that LOX-1 rapidly increases postprandially in the plasma of humans consuming a westernized diet. Additionally, they reported that this increase can be attenuated by supplementing the diet with antioxidants. These observations suggest that LHE may also suppress the expression of VCAM-1 and ICAM-1 in HUVECs by the same mechanism. Furthermore, the attenuated LOX-1 expression observed presently might be due to a decrease in the oxidative stress in the AC + LHE group. Indeed, hepatic TBARS and protein carbonyl values were lower in the mice fed the LHE-supplemented diet, which corresponds to decreased lipid peroxidation and protein oxidation. According to these findings, LHE supplementation contribute to the amelioration of oxidative stress and the suppression of adhesion molecules and LOX-1 expression induced by an atherogenic diet in mice; this effect might be attribute in part to the antioxidant properties of LHE. Several other lines of evidence indicate that LHE has antioxidant activity and pharmaceutical effects *in vitro*, perhaps due to the presence of phenolic compounds. Our *in vitro* study also confirmed that a 70% ethanol extract of LH possesses stronger antioxidant capacity than the reference data, and that the total phenol content of LHE, as estimated by the Folin-Ciocalteu method, was high; however, the compounds with the antioxidant activity have not yet been identified. Furthermore, the activities of hepatic antioxidant enzymes such as Cu,Zn-SOD, GPX, GR, and catalase were observed to be higher in the mice fed the LHE-supplemented diet than in mice fed the AC diet. Intracellular antioxidant enzymes such as SOD and catalase protect cells from free radical-mediated disturbances by scavenging ROS and the product of lipid peroxidation. These results suggest that LHE supple-

![Fig. 3.](image)

Inhibition of TNF-α-Induced LOX-1 (A), VCAM-1 (B), and ICAM-1 (C) Expression by LHE.

HUVECs were pretreated with different concentrations of LHE for 1 h before being incubated with 10 ng/ml TNF-α for 12 h and subjected to Western blotting using antibody specific for LOX-1, VCAM-1, and ICAM-1. A representative figure is calculated and compared with the vehicle by densitometry quantification of bands. Bars with different letters are significantly different at \( p < 0.05 \) by the Tukey test. Values are mean ± S.D. (n = 3).
mentation attenuates the hepatic oxidative stress induced by an atherogenic diet through direct antioxidant action, the free radical-scavenging action of LHE, and/or by enhanced antioxidant enzyme activity to ameliorate ROS toxicity, which subsequently results in the suppression of adhesion molecule and LOX-1 expression.

In summary, our demonstration of the hypocholesterolemic effect, improved redox status, and suppression of adhesion molecule and LOX-1 expression suggests that LHE supplementation is beneficial in preventing cardiovascular diseases, especially atherosclerosis, in humans due to attenuated intracellular oxidative stress and inflammation.

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**References**