Antioxidant Activities of *Perilla frutescens* against Low-Density Lipoprotein Oxidation *in Vitro* and in Human Subjects

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Abstract: Perilla (*Perilla frutescens* (L.) Britt.) is a popular food as well as a traditional medicine in Japan, China, and other Asian countries. The aim of this study was to investigate the inhibitory effects of perilla on low-density lipoprotein (LDL) oxidation *in vitro* and in human subjects. We compared the antioxidant activities of red perilla and green perilla. Both green and red perilla had high 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities and were abundant in polyphenol compounds. In addition, the radical scavenging activity and polyphenol content of red perilla were higher than those of green perilla. Perilla dramatically inhibited azo-radical-induced LDL oxidation and endothelial-cell-mediated LDL oxidation *in vitro*. Moreover, red perilla significantly increased mRNA and protein expression levels of antioxidant enzymes in endothelial cells. We further examined the antioxidant effects against LDL in human subjects after the consumption of perilla extracts. After oral intake of red perilla, the subjects’ LDL oxidation lag times were significantly longer than those before the intake. Furthermore, lipid peroxide formation and the electrophoretic mobility of LDL decreased markedly. These results suggested that perilla, especially the red variety, had high antioxidant activity and prevented the oxidation of LDL, which is a process strongly related to the development of atherosclerosis.

Key words: Perilla, LDL oxidation, antioxidant enzyme, atherosclerosis, endothelial cell

1 INTRODUCTION

Recent reports have shown that leaves contain high quantities of polyphenols and could therefore play a positive role in preventing low-density lipoprotein (LDL) oxidation. *Perilla (Perilla frutescens (L.) Britt.)* is a widely cultivated leafy vegetable that is commonly consumed as well as used in traditional medicine in Japan, China, and other Asian countries. Perilla can be categorized into two types based on their morphology and use: *Perilla frutescens var. frutescens* is mostly used as an oil crop, whereas *Perilla frutescens var. crispa* is eaten raw and used for medicinal and nutritional purposes. *Perilla frutescens var. crispa* can be further categorized into two types: a red variety (red perilla, “Aka-jiso” in Japanese) and a green variety (green perilla, “Ao-jiso”); these types differ in anthocyanin content. Chemical analysis has indicated that only the red variety produces anthocyanins, with malonylshisonin being the main pigment. Many studies have been conducted on the compounds contained in perilla that have anti-inflammatory, anti-HIV, anti-allergic, and anti-tumor properties. Nonetheless, the antioxidant activity of perilla remains poorly understood.

The oxidative modification of LDL may play a critical role in the development of atherosclerosis. Oxidized LDL (oxLDL) is internalized by endothelial cells and macrophages, leading to endothelial dysfunction and foam cell formation, respectively. Therefore, reducing LDL oxidation is assumed to be a useful strategy to prevent atherogenic disease.

Reactive oxygen species (ROS) are free radicals produced during metabolism and the aging process. ROS include free radicals such as superoxide (O$_2^{-}$), hydroxyl (OH), peroxyl (RO$_2^{-}$), and hydroperoxy (HRO$_2^{-}$), as well as nonradical species such as hydrogen peroxide (H$_2$O$_2$) and hypochlorous acid (HOCl). ROS can cause lipid peroxidation, result-
ing in oxidative stress. A variety of non-enzymatic antioxidants (e.g., glutathione and uric acid) and enzymatic antioxidants (e.g., catalase (CAT) and Cu-Zn-superoxide dismutase (SOD)) have been known to play an active role against oxidative stress\(^{16,17}\). The mechanism by which perilla protects against oxidative stress still remains largely unknown. In this study, we investigated the effects of perilla on LDL oxidation and antioxidant enzyme expression both in vitro and in human subjects.

2 EXPERIMENTAL PROCEDURES
2.1 Antioxidative activities of perilla
2.1.1 Perilla preparation

The leaves of a green- and red-type \textit{P. frutescens} were purchased from a market (Tokyo, Japan). The dried perilla leaves (1 g) were extracted with 10 mL distilled water for 1 h at room temperature under gentle stirring. The extracted solution was then filtered and stored at \(-20^\circ\text{C}\) for use in the following in vitro studies.

2.1.2 Free radical-scavenging activity

Free radical-scavenging activity was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Wako Pure Chemical Industries, Osaka, Japan). An aliquot of each extract was mixed with 2 mL of 0.1 mM DPPH in ethanol. Following incubation for 20 min at 37°C, the absorbance of each solution was measured at 516 nm using a Beckman Model DU 640 spectrophotometer. The volume of each perilla extract that was required to cause a 50% decrease in the absorbance at 516 nm relative to the control was then calculated.

2.1.3 Determination of total polyphenol content

The total polyphenol content was determined by the Folin-Ciocalteu assay as described previously\(^{18}\). The content was expressed as a (+)-catechin equivalent.

2.1.4 Isolation of LDL from human subjects

Blood samples were collected in sodium EDTA-containing tubes from fasting normolipidemic volunteers after obtaining their informed consent. Plasma samples were immediately prepared by centrifugation at 3,000 rpm for 15 min at 4°C. The LDL was separated by single-spin density ultracentrifugation (100,000 rpm, 40 min, 4°C). LDL protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Laboratories, Rockford, IL).

2.1.5 Determination of LDL oxidizability (lag time assay)

LDL oxidizability was measured according to the method described in our previous report\(^{19}\). The prepared LDL samples (final concentration of protein: 70 μg/mL) were oxidized with or without 5 μL of perilla extract by 200 μM 2,2-azobis-2,4-dimethylvaleronitrile (V70; AMVN-CH\(_{3}\)O) (Wako Pure Chemical Industries, Ltd.), which is an oxidative inducer. The kinetics of LDL oxidation were determined by monitoring the absorbance of conjugated dienes at 234 nm using a Beckman Model DU 800 spectrophotometer at 4 min intervals at 37°C.

2.1.6 Endothelial cell-mediated LDL oxidation

Human umbilical vein endothelial cells (HUVECs) (Sanko Junyaku Co., Tokyo, Japan) were cultured in EGM-2 (Lonza Walkersville, Inc., Walkersville, USA). The Cells were grown to confluence at 37°C in 5% CO\(_2\) and used for experiments at passage 4. HUVECs were pre-incubated for 6 h in the presence or absence of perilla extract in M-199 medium. The medium was subsequently removed, and the cells were washed twice with PBS. LDL (100 μg protein/mL) was incubated with the cells for 18 h in Ham’s F10 medium (Lonza Walkersville) containing 3 μM FeSO\(_4\) and CuSO\(_4\). After this incubation, the medium was analyzed for LDL oxidation as described below.

2.1.7 Thiobarbituric acid reactive substances assay

Malondialdehyde (MDA) generated in a medium containing LDL was measured by using the thiobarbituric acid reactive substances (TBARS) assay as described by Buege and Aust\(^{20}\). Sample absorbance was measured at 535 nm. Results were expressed in terms of relative MDA content (nmol/mg-LDL protein) calculated using the extinction coefficient for MDA as previously described.

2.1.8 Lipid peroxide assay

Lipid peroxide (LPO) in LDL was measured by using a Determiner LPO (Kyowa Medex, Tokyo, Japan). Samples were measured spectrophotometrically at 675 nm.

2.1.9 Agarose gel electrophoresis

The media containing LDL were collected and subjected to agarose gel electrophoresis. Electrophoresis was performed at 400 V for 25 min by using a rapid electrophoresis system (Helena Laboratories, Saitama, Japan). After electrophoresis, the gels were stained with CHO/Trig CONBO CH (Helena Laboratories, Saitama, Japan)\(^{21}\).

2.2 Effect of perilla on antioxidant enzyme
2.2.1 Real-time PCR analysis

Total cellular RNA was extracted using TRIZOL Reagent (Invitrogen, Tokyo, Japan), and first-strand cDNA was synthesized from the total RNA (0.2 μg) by using TaqMan\(^{\text{®}}\) Reverse Transcription Reagents (Applied Biosystems, CA, USA). Real-time PCR was performed on an ABI 7300 cycler (Applied Biosystems, CA, USA) using SYBR green PCR mix. The results were expressed as the copy number ratio of the target mRNA to GAPDH mRNA. The primer sequences were as follows: for catalase, forward 5’-TGAC-CAGGGCATCAAAAAACC-3’; reverse 5’-CGGATTGC-CATAGTCAGGATCTT-3’; for SOD, forward 5’-CATCATCATTCTTGCAGCAGA-3’; reverse 5’-GCCACACCATTTTGTTCGACG-3’; and for GPDH, forward 5’-TGCACCCAACACTGTTAGC-3’, reverse 5’-GCCATGGAAGTGGTCAGAG-3’.

2.2.2 Western blot analysis

After treatment, cells were washed with ice-cold PBS
and lysed with a protein extraction reagent (Pierce Biotechnology, IL, USA) containing 10 μg/mL leupeptin, 10 μg/mL aproatin, and 1 mM phenylmethylsulfonyl fluoride. Aliquots of cellular proteins were electrophoresed in a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore, MA, USA). The membrane was allowed to react with catalase and SOD (Santa Cruz, CA, USA) and specific proteins were detected by enhanced chemiluminescence. Loading differences were normalized using polyclonal actin antibodies. All signals were detected by LAS-4000 (Fujifilm, Tokyo, Japan). Densitometric analysis was performed by using Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan) to scan the signals.

2.3 Clinical study
The study group consisted of eight healthy female volunteers ranging from 20 to 25 yrs of age; none took any medications or special dietary supplements. All were nonsmokers and in good states of health. This study was approved by the Ethics Committee of Ochanomizu University and established in Helsinki (established in 1964 and revised in 2004). All subjects gave their informed consent to participate in the study. After over 12 h of fasting, blood samples were collected between 8:00 and 9:00 a.m. The subjects then ingested 120 mL of perilla extract. The perilla extract contained about 1,000 mg of polyphenols. Plasma samples were taken at baseline, then 0.5, 1, 2, and 4 h after consumption of perilla extract; these samples were then subjected to the isolation and preparation of LDL described above. Next, we measured the lag time. After diluting the samples with PBS to a final concentration of 140 μg/mL LDL protein, the LDL was oxidized by 400 μM AMVN-CH$_3$O. Lag time, TBARS, LPO products, and LDL mobility were determined as described above.

2.4 Statistical analysis
Results were expressed in the form, mean ± SD. Human study results were expressed in the form, mean ± SEM. Differences between groups were analyzed by ANOVA with Fisher’s PLSD using StatView-J5.0 (SAS Institute Inc., NC, USA). The minimum significance level was set at a P value of 0.05 for all analyses.

3 RESULTS
3.1 Antioxidant activities of perilla
The antioxidant activities of green and red perilla are listed in Table 1. The DPPH solutions required 7.9 and 29 μg/mL of red perilla and green perilla, respectively, to scavenge 50% of DPPH radicals. The polyphenol concentration of green perilla was 1.7 mg/mL, and that of red perilla was 4.6 mg/mL.

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<th>Table 1</th>
<th>Antioxidant activity of perilla.</th>
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<td>Polyphenol (mg/leaf)</td>
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<tr>
<td>Green perilla</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Red perilla</td>
<td>4.6 ± 0.5***</td>
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Data are mean ± S.D. n = 6. ***p<0.001 vs. green perilla

3.2 Inhibitory effects of perilla on LDL oxidation in vitro
We first analyzed the cytotoxic effects of perilla in HUVECs with an MTT assay. Neither green nor red perilla affected cell viability under our experimental conditions (data not shown).

To evaluate the antioxidant effects of perilla extracts on LDL oxidation, we carried out an LDL lag time assay. As shown in Fig. 1, both green and red perilla extracts significantly prolonged LDL oxidation lag time compared with the control (control 24 ± 3 min; green perilla 51 ± 5 min; red perilla 87 ± 7 min (p<0.001)).

We also examined the inhibitory effects of perilla on endothelial-cell-mediated LDL oxidation. The pretreatment of HUVECs with red perilla significantly reduced the TBARS response and LPO formation from oxidation (p<0.001) (Fig 2A, B). Furthermore, we evaluated the change in the surface charge of LDL by agarose gel electrophoresis. The oxidation of LDL could be monitored by comparing the relative electrophoresis mobility of unmodified and modified LDLs. Red perilla significantly suppressed LDL oxidation lag time.

![Fig 1](image-url) Effects of perilla extracts on LDL oxidation lag time.
Perilla extract was incubated with 70 μg protein/mL of LDL in PBS (1 mL in total) with V-70 at 37°C for 400 min, and conjugated diene formation was monitored by the changes in 234 nm wavelength absorbance. Results are expressed in the form, mean ± S.D., n = 3. ***p<0.001, **p<0.01 vs. control.
3.3 Effect of perilla on antioxidant enzymes

To investigate the mechanism by which endothelial cells mediated LDL oxidation, we tested the effect of perilla on antioxidant enzyme expression in HUVECs. Treatment of HUVECs with red perilla led to a significant increase in SOD and catalase mRNA expression (Table 2). In addition, red perilla increased protein expression of SOD and catalase (Fig. 3), indicating that perilla increased antioxidant enzyme expression by direct modulation of gene transcription.

Table 2  Effects of perilla extracts on mRNA expression of the antioxidant enzymes in HUVECs.

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<tr>
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<th>Catalase</th>
<th>SOD</th>
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<tr>
<td>Green perilla</td>
<td>2.3 ± 1.3</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>Red perilla</td>
<td>3.2 ± 1.4***</td>
<td>2.1 ± 0.5***</td>
</tr>
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Values were expressed as the ratio of catalase or SOD to GAPDH mRNA expressions. (mean ± S.D. n = 4) ***p<0.001 vs. control

3.4 Inhibitory effects of perilla on LDL oxidation in human subjects

Eight subjects consumed 120 mL of red perilla extract, which contained about 1,000 mg of polyphenols. As shown in Fig. 4A, the LDL oxidation lag time was prolonged to 2 h and 4 h after the ingestion of red perilla extract compared to that before intake (p<0.001, p<0.05, respectively). The consumption of red perilla extract decreased the amount of LPO products at each time point (Fig. 4B). TBARS products also tended to decrease (Fig. 4C). LDL mobility was markedly decreased at 4 h (p<0.05) (Fig. 4D). These results showed that red perilla could suppress LDL oxidation in human subjects as well as in vitro.

4 DISCUSSION

The oxidative modification of LDL is thought to play a central role in the pathogenesis of atherosclerosis. In this study, we demonstrated that perilla reduced LDL oxidizability both in vitro and in human subjects.

First, we checked the antioxidant activity of green perilla and red perilla. Both green and red perilla had DPPH radical-scavenging activities and contained high polyphenol concentrations. Moreover, red perilla showed stronger antioxidant activity than green perilla. Perilla leaves are known to contain several kinds of polyphenols, such as cin...
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**Fig. 3** Effects of perilla extracts on antioxidant enzyme expression in HUVECs. HUVECs were incubated with or without perilla extract for 6 h. We measured the expression of antioxidant enzyme (catalase, SOD) levels by Western blot analysis. Representative data from four independent experiments are shown.

next, we demonstrated the protective effect of perilla against pro-oxidant-initiated oxidative modification or endothelial-cell-mediated oxidation in vitro. Perilla significantly prolonged the azo-radical-induced LDL oxidation lag time, indicating that it could prevent free-radical-induced lipid peroxidation of LDL. To examine the effect of perilla on cell-mediated LDL oxidation, we used Ham’s F10 medium containing metal ions to induce the oxidation of LDL in HUVECs. As shown in Fig. 2A, B and C, the interaction of LDL and HUVECs significantly increased TBARS and LPO production, but these were dramatically inhibited by incubation with red perilla. Red perilla also reduced the negative charge of LDL particles that resulted from HUVEC incubation. This indicated that perilla could play a role in preventing apolipoprotein B100 modification in LDL. Based on these past studies, we could postulate that the consumption of red perilla extract containing 1,000 mg of polyphenols significantly extended the lag time of LDL oxidation and reduced TBARS production, LPO production and LDL mobility.

A past study of the antioxidant abilities of grapes and balsamic vinegar, both containing anthocyanin, reported that the prolongation of the lag time was related to an increase in plasma polyphenol levels. Plasma lipopolysomes have been suggested as potential carriers of polyphenols. In the previous in vitro study, maximum plasma concentrations of anthocyanins were observed 120-240 min after intake of anthocyanin rich foods. These data suggest that polyphenols may be absorbed into the bloodstream and incorporated into LDL after perilla consumption. Moreover, it must be taken into account that perilla has many different antioxidants, not only polyphenols but also vitamins E and carotenoids. Previous studies have suggested that some hydrophilic antioxidants bind to phospholipids or proteins on the LDL surface, whereas hydrophobic antioxidants bind closer to the LDL core. Based on these past studies, we could postulate that the consumption of red perilla may have inhibited LDL oxidation due to the combination of hydrophilic and hydrophobic antioxidants.

In recent years, it has been theorized that phytochemicals may not only act as “primary antioxidants” by scavenging ROS but also as “secondary antioxidants” by interacting with several signaling pathways to induce production of cytoprotective enzymes. There are reports that antioxidant-rich food may be able to enhance the activity of antioxidant enzymes. Moreover, previous studies have shown that phenolic acids significantly induce antioxidant enzyme production and increase the antioxidizing potential of the liver. These phenolic acids seem to selectively induce transcription of hepatic mRNAs for CuZnSOD, GPx, and catalase, likely through upregulation of gene transcription as well as the NF-E2-related factor 2 (Nrf2) transcription factor.

Since the in vitro study found that perilla upregulated both SOD and catalase, we may conclude that the consumption of red perilla inhibited LDL oxidation by increasing the level of the antioxidant enzymes. The reason for the increase might be related to up-regulation of hepatic...
multidrug resistance-associated protein and transcription factor Nrf2, although we do not have data to support this claim.

In conclusion, our results showed that perilla exhibits antioxidant abilities and inhibits LDL oxidation. Furthermore, the consumption of perilla may reduce the risk factors associated with atherosclerosis.

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Reference


