The Neuro-Protective Effect of the Methanolic Extract of *Perilla frutescens* var. *japonica* and Rosmarinic Acid against \(H_2O_2\)-Induced Oxidative Stress in C6 Glial Cells

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Abstract

Neurodegenerative diseases are often associated with oxidative damage in neuronal cells. This study was conducted to investigate the neuro-protective effect of methanolic (MeOH) extract of *Perilla frutescens* var. *japonica* and its one of the major compounds, rosmarinic acid, under oxidative stress induced by hydrogen peroxide (\(H_2O_2\)) in C6 glial cells. Exposure of C6 glial cells to \(H_2O_2\) enhanced oxidative damage as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and thiobarbituric acid-reactive substance assays. The MeOH extract and rosmarinic acid prevented oxidative stress by increasing cell viability and inhibiting cellular lipid peroxidation. In addition, the MeOH extract and rosmarinic acid reduced \(H_2O_2\)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the transcriptional level. Moreover, iNOS and COX-2 protein expression was down-regulated in \(H_2O_2\)-induced C6 glial cells treated with the MeOH extract and rosmarinic acid. These findings suggest that *P. frutescens* var. *japonica* and rosmarinic acid could prevent the progression of neurodegenerative diseases through attenuation of neuronal oxidative stress.

Key Words: *Perilla frutescens* var. *japonica*, Rosmarinic acid, Oxidative stress, Hydrogen peroxide, C6 glial cell

INTRODUCTION

Overproduction of reactive oxygen species (ROS) damages tissue and leads to oxidative stress through lipid peroxidation, protein cross-linking, and DNA cleavage, thereby disrupting cellular function (Gorman et al., 1996). ROS are constantly produced and play a key role in the pathogenesis of a wide variety of acute and chronic neurodegenerative diseases. Hydrogen peroxide (\(H_2O_2\)) is one of the major ROS and excessive production is associated with pathological process of acute and chronic neuronal toxicity. Previous reports indicated that \(H_2O_2\) is a weak oxidant, but it can be converted to a highly reactive toxic hydroxyl radical. In addition, over-generation of nitric oxide (NO) acts as neurotoxic effector in the central nervous system, resulting in neurodegeneration (Guix et al., 2005). Activation of glial cells by \(H_2O_2\) leads to the up-regulation of inflammatory mediators, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression, and cytokine release, which cause neuronal apoptosis (Minagar et al., 2002).

COX is responsible for the conversion of arachidonic acid to prostaglandins. There are two COX isofoms, which are known to COX-1 and COX-2. COX-1 is constitutively expressed in most tissue types and is generally regarded as a "housekeeping enzyme". In contrast, COX-2 expression is induced by cytokines, growth factors, and hormones (Funk et al., 1991; Masferrer et al., 1994; Smith et al., 1996). NOS is expressed in a variety of cells under both normal and pathological conditions and produces high levels of NO (Brown, 2007). Thus, inhibiting the activity or expression of COX-2 and/or iNOS might be a promising method for prevention of degenerative diseases and inflammation. Moreover, Zhang et
al. (2006) reported that anti-oxidants can block or delay neuronal apoptosis, and thus, such substances may prevent neurodegenerative disorders.

Glial cells are believed to play a significant role in host defense and tissue repair in the central nervous system. In addition, glial cells support brain function by carrying nutrients to neurons and removing waste (Ullian et al., 2001). C6 glial cells are widely used in studies of protective mechanisms against neuronal oxidative stress, because they protect neurons from extracellular oxidants such as H$_2$O$_2$ (Dringen et al., 2000). Under pathological conditions, glial cells are activated and produce a large number of neuro-active substances, including cytokines and radicals such as NO (Minghetti and Levi, 1998; Hanisch, 2002). However, it has also been reported that pro-inflammatory mediators released by microglia inhibit the progression of neurodegenerative disorders (Eikelenboom and van Gool, 2004). Therefore, production of toxic inflammatory mediators and cytokines by activated glial cells must be regulated.

Diverse natural products, including foods, have been shown to possess biological activities that might protect neurons from oxidative injury, including anti-oxidative and anti-inflammatory effects (Aruoma et al., 2003). *Perilla frutescens var. japonica*, an annual herb with a distinctive aroma and taste, has been cultivated for centuries in Asia, especially in Korea and Japan. *P. frutescens var. japonica* has been widely used as a folk medicine and food, and it possesses several biological activities (Yang et al., 2013). In a previous study, methanolic (MeOH) extract of *Perilla* leaves was shown to produce anti-mutagenic and anti-oxidative effects (Lee et al., 1993). Kim et al. (2007) suggested that the leaves of *Perilla* have protective effects against oxidative hepatotoxicity induced by tert-butyl hydroperoxide. In addition, oral administration of a *Perilla* decoction and its constituents produced anti-allergic activity in mice (Makino et al., 2001). Rosmarinic acid (RA) is a polyphenolic phytochemical found in *Perilla* and other medicinal plants, including rosemary and mint (Makino et al., 1998; Al-Sereiti et al., 1999; Areias et al., 2000). Osakabe et al. (2002) reported that *Perilla* and RA reduced liver injury induced by lipopolysaccharides and D-galactosamine due to scavenging of superoxides or peroxynitrite.

The present study was conducted to evaluate the neuroprotective effect of *P. frutescens var. japonica* and RA against oxidative stress, as well as to explore their molecular mechanisms by investigating mRNA and protein expression related to oxidative stress.

### MATERIALS AND METHODS

#### Plant materials and chemicals

*P. frutescens var. japonica* was obtained from the Department of Functional Crop, National Institute of Crop Science, Rural Development Administration, Miryang, Korea. RA and malondialdehyde (MDA) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). All other chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich Co.

#### Preparation of sample

Freeze-dried *P. frutescens var. japonica* was extracted 3 times with 20 volumes of 100% MeOH at room temperature for 24 h. The extract was obtained by a rotary evaporator and the yield was 23.43%. The MeOH extract of *P. frutescens var. japonica* (MP) and RA were dissolved in phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO), respectively.

#### Cell culture

C6 glial cells were obtained from the KCLB (Korean Cell Line Bank, Seoul, Korea) and maintained at 37°C in a 5% CO$_2$ incubator. Cells were cultured with DMEM containing 1% fetal bovine serum, and subcultured weekly with 0.05% trypsin-EDTA in PBS.

#### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

After confluence had been reached, the cells were plated in 96-well plates at a density of 2×10$^5$ cells/well, incubated for 2 h, and treated with H$_2$O$_2$ (100 μM). After treatment of H$_2$O$_2$ for 24 h, the cells were treated with MP (5, 25, 50, and 100 μg/mL) and RA (0.5, 2.5, 5, and 10 μg/mL) for 24 h. Cell viability was determined using the MTT assay. MTT solution was added to each well of the 96-well plate, and the plate was incubated for 4 h at 37°C, after which the medium containing the MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with DMSO, and the absorbance of each well was read at 540 nm (Mosmann, 1983).

#### Thiobarbituric acid-reactive substance (TBARS) levels

Thiobarbituric acid (TBA)-reactive substance levels were determined using the Fraga assay (Fraga et al., 1988). After treatment of the cells with MP and RA, 1% TBA (1 mL) and 25% trichloroacetic acid (1 mL) were added, and the mixture was boiled at 95°C for 20 min. The mixture was cooled with ice and extracted with n-butanol (n-BuOH). After centrifugation at 4,000 rpm for 30 min, the fluorescence of the n-BuOH layer was measured at 532 nm using a fluorescence spectrophotometer (BMG LABTECH, Ortenberg, Germany).

### Table 1. Primers and conditions used in real-time quantitative PCR

| Gene     | Primer sequence | PCR conditions |
|----------|-----------------|----------------|
| iNOS     | F: CCT-CCT-CCA-CCC-TAC-CAA-GT | 53°C, cycles: 35 |
|          | R: CAC-CCA-AAG-TGC-CTC-AGT-CA | 53°C, cycles: 35 |
| COX-2    | F: AAG-ACT-TGC-CAG-GCT-GAA-CT | 53°C, cycles: 35 |
|          | R: CTT-CTG-CAG-TCC-AGG-TCG-AA | 53°C, cycles: 35 |
| GAPDH    | F: TCA-TGA-AGT-GTG-ACG-TTG-ACA-TCC-GT | 60°C, cycles: 35 |
|          | R: CCT-AGA-AGC-ATT-TGC-GGT-GCA-CGA-TG | 60°C, cycles: 35 |
RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Cells were lysed using Trizol reagent and transferred to microfuge tubes. RNA was reverse-transcribed into cDNA and used as a template for RT-PCR amplification. The primers and amplification conditions are listed in Table 1. PCR products were analyzed on 1% agarose gels, and bands were visualized with an LED slider imager (Maestrogen, Las Vegas, NV, USA).

Western blotting

C6 glial cell extracts were prepared according to the manufacturer’s instructions using RIPA buffer (Cell Signaling, Danvers, MA, USA) supplemented with 1×protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. Proteins were separated by electrophoresis in a precast 4-15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, USA), blocked with 10% skim milk solution for 1 h at 4°C and then blotted onto nitrocellulose membranes and analyzed with epitope-specific primary and secondary antibodies. Bound antibodies were visualized using enhanced chemiluminesence and an LAS 4000 imaging system (Fuji Film, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Significance level was verified by performing Duncan’s multiple range test and p-values lower than 0.05 was considered statistically significant using SAS software (version 6.0, SAS)
This result suggests that MP and RA exhibited inhibitory effects against H$_2$O$_2$-induced lipid peroxidation and cell damage.

We investigated the effects of MP and RA on cell viability after exposure to H$_2$O$_2$. As determined by the MTT assay, the viability of C6 glial cells exposed to H$_2$O$_2$ for 24 h was decreased by 45.8% (Fig. 1). However, MP significantly inhibited cell death in a dose-dependent manner, especially it was increased by 70% after treatment 100 μg/mL for 24 h. Treatment with RA also increased cell viability in a concentration-dependent manner (Fig. 2). In particular, cells treated with RA at a concentration of 10 μM showed markedly increased cell viability (78.7%) in comparison with control cells (65.56%).

### DISCUSSION

Excessive H$_2$O$_2$ can lead to neuronal cell damage by modifying cellular lipids and proteins and inducing DNA oxidation (Whitemore et al., 1994). Oxidative damage is mediated by ROS and linked to a variety of degenerative diseases such as coronary artery disease, aging, and cancer (Ames, 1998). Increased ROS formation is considered to be a crucial mediator of cell injury, and neuronal death induced by ROS is observed in patients with neurodegenerative disorders. Natural antioxidants from plant sources can inhibit excessive accumulation of free radicals and attenuate oxidative stress (Hocman, 1989). Therefore, the search for natural antioxidants in foods to replace synthetic antioxidants has attracted considerable attention. *P. frutescens var. japonica* is a traditional medicine that has been widely used in East Asian countries for centuries, and several studies have reported that extracts of *P. frutescens var. japonica* produce anti-oxidant effects (Chou et al., 2009; Meng et al., 2009). Previous study demonstrated that RA is the main phenolic compound, and other flavonoids and phenolic acids such as catechin, apigenin, luteolin, caffeic acid, ferulic acid are found in *P. frutescens* (Ishikura, 1981; Masahiro et al., 1996). Ueda et al. (2002) also reported active constituents from *Perilla* leaf extract, RA, luteolin and caffeic acid. In addition, Gu et al. (2009) isolated and identified four antioxidant compounds (RA, luteolin, apigenin, and chrysoeriol) from *P. frutescens*. Among them, RA and luteolin showed significant free radical scavenging activities. RA has four hydroxyl groups that were considered to contribute to scavenging free radicals by functioning as a proton donor (Brand-Williams et al., 1995). According to Nakamura et al. (1998), RA exhibited antioxidative activity by attenuating both intracellular superoxide and peroxide formation. In addition, RA inhibited ROS formation and lipid peroxidation against amyloid beta peptide, suggesting RA could effectively protect against oxidative stress in neuronal cell (Iuvone et al., 2006). However, the neuro-protective effects of MP and RA against oxidative stress have not been reported.

Elevated oxidative stress as a result of ROS generation and MDA formation in glial cells is a primary mediator of neuro-inflammation and an important cause of neuronal cell death in neurodegenerative diseases (Mosley et al., 2006). In this study, we found that C6 cells treated with MP and RA showed...
significantly increased cell viability after exposure to H$_2$O$_2$. This result suggests that MP and RA protect C6 glial cells from H$_2$O$_2$-induced cytotoxicity.

Determination of MDA content by measuring TBARS is an assay commonly used to assess lipid peroxidation. MDA formation is a key event in oxidative stress and an important cause of cell membrane damage (Gutteridge, 1995). H$_2$O$_2$ significantly increased MDA formation in C6 glial cells in comparison with non-stimulated cells. However, MP and RA markedly reduced MDA formation, indicating reduced oxidative stress, and thus, anti-oxidative and neuro-protective effects. Kim et al. (2008) also demonstrated that Perilla leaves protect DNA against damage and possess anti-oxidative activity. In addition, RA isolated from Perilla leaves produced anti-oxidative effects in biological systems by scavenging superoxide radicals, one of the major constituents of ROS (Nakamura et al., 1998). These results show that MP and RA possess significant protective capability against H$_2$O$_2$-induced cell damage.

Pro-inflammatory cytokines and mediators released by activated glial cells are important pathologic factors in neurodegenerative disorders including Alzheimer’s disease and central nervous system inflammation (Hull et al., 2000). Yu et al. (2012) showed that treatment with H$_2$O$_2$ induced overexpressions of iNOS and COX-2, which led to neuro-inflammation. The mechanism through which MP and RA suppress the effects of H$_2$O$_2$ in C6 glial cells was investigated by measuring iNOS and COX-2 mRNA and protein expression. Astroglial and microglial cells in a healthy brain do not express iNOS, while neurotoxic or inflammatory damage induces iNOS expression in mice, rats, and humans (Galea et al., 1992). Furthermore, there is evidence that increased COX-2 expression contributes to neurotoxicity and brain damage. In the rat brain, COX-2 is expressed in cerebral cortex and the hippocampal formation (Tocco et al., 1997). Neurotoxicity causes up-regulation of COX-2 expression, and COX-2 is known to be involved in neuronal death and survival (Ho et al., 1999). COX-2 mediates neuronal damage by producing excessive amounts of harmful prostanooids and free radicals (Nogawa et al., 1997). Our results showed that exposure of glial cells to H$_2$O$_2$ for 24 h resulted in significant induction of iNOS and COX-2 mRNA expression, whereas MP and RA treatment for 24 h after exposure of glial cells to H$_2$O$_2$ clearly reduced iNOS and COX-2 expression. These results demonstrated that MP and RA inhibit inflammatory processes by suppressing iNOS.

Fig. 4. Effect of MP (A) and RA (B) on mRNA expression of iNOS and COX-2 in C6 glial cells under H$_2$O$_2$-induced oxidative stress. Cells were pre-incubated for 24 h in the presence of 100 μM H$_2$O$_2$, followed by the addition of MP (25, 50, and 100 μg/mL) and RA (2.5, 5, and 10 μM) for 24 h. Total RNA was isolated, after which RT-PCR was performed using the indicated primers. The amplified PCR products were run in a 1% agarose gel and visualized by staining with ethidium bromide. GAPDH was used as a control gene for normalization of relative gene expression levels. Values are mean ± SD. Means with different letters are significantly different (p<0.05) as determined by Duncan’s multiple range test.
and COX-2 expression in H\textsubscript{2}O\textsubscript{2}-treated C6 glial cells.

Several natural antioxidants directly inhibit expression of iNOS and COX-2, and thus, reduce inflammation (Surh et al., 2001). To investigate the protective mechanisms of MP and RA against oxidative stress, iNOS and COX-2 expression was measured. Exposure of C6 glial cells to H\textsubscript{2}O\textsubscript{2} increased protein expression of iNOS and COX-2. However, treatment of MP or RA down-regulated iNOS and COX-2 expression. These results indicate that decreased iNOS and COX-2 protein expression by MP and RA may contribute to their attenuation of oxidative stress.

Ghaffari et al. (2014) demonstrated that RA prevents H\textsubscript{2}O\textsubscript{2}-induced oxidative stress in N2A neuroblastoma cells through mitochondrial membrane potential change and regulation of tyrosine hydroxylase and brain derived neurotropic factor gene expression. In addition, Huang et al. (2009) studied anti-inflammatory activity of RA from Prunella vulgaris using RAW 264.7 macrophage cells stimulated by lipopolysaccharide. However, the neuro-protective effect of RA on NO production against H\textsubscript{2}O\textsubscript{2}-induced stress in C6 glial cells has not been studied yet. Glial cells, the abundant cell type in mammalian brain, are support neuron by releasing antioxidant protection (Sagara et al., 1993). Glial cell lines have been widely used as a model for studies to estimate neurodegenerative condition, because these cells possess closely resembles the actual mechanisms of inflammatory and apoptosis reaction (Grobben et al., 2002; Furman et al., 2012). In addition, glial cells quickly respond to H\textsubscript{2}O\textsubscript{2} exposure, which can increases oxidative-nitrosative stress, iNOS expression and dysfunction of antioxidant defenses (Quincozes-Santos et al., 2010, 2013). Excessive NO release from glial cells mimics pathological situation which is associated with neurodegenerative disease including AD (Rampe et al., 2004). Some studies investigated that whether dietary components have possible value for inhibition of excessive production of NO by down-regulation of iNOS and COX-2 expression in C6 glial cells (Soliman and Mazzio, 1998; Kim et al., 2001; Niranjan et al., 2010). In present study, we focused on the effects of MP and RA on iNOS and COX-2 expression as the underlying mechanisms of NO production using C6 glial cells, which were activated with H\textsubscript{2}O\textsubscript{2}. The present results showed MP and RA treatment down-regulated both iNOS and COX-2 expression under NO-induced oxidative stress in C6 glial cells, suggesting neuroprotective effect of MP and RA.

![Fig. 5. Effect of MP (A) and RA (B) on protein expression of iNOS and COX-2 in C6 glial cells under H\textsubscript{2}O\textsubscript{2}-induced oxidative stress. Cells were pre-incubated for 24 h in the presence of 100 \( \mu \text{M} \) H\textsubscript{2}O\textsubscript{2}, followed by the addition of MP (25, 50, and 100 \( \mu \text{g/mL} \)) and RA (2.5, 5, and 10 \( \mu \text{M} \)) for 24 h. Cellular protein was separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was probed with anti-iNOS and anti-COX-2 antibodies. Protein was visualized using an ECL detection system. Actin was used as an internal control. Values are mean ± SD. **Means with different letters are significantly different (\( p<0.05 \)) as determined by Duncan’s multiple range test.](image-url)
This study showed that H_{2}O_{2}-induced cell damage occurred as a result of ROS over-production and lipid peroxidation. However, MP and RA reduced cell damage and lipid peroxidation in C6 glial cells through the regulation of mRNA and protein expression of iNOS and COX-2. Regulation of oxidative stress-related gene expression might be involved in the neuroprotective effects of MP and RA against oxidative stress. Although further study is necessary to determine whether P. frutescens var. japonica has protective effects against neurodegeneration in vivo, these findings show that P. frutescens var. japonica may exert protection against inflammatory brain damage induced by oxidative stress.

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