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Original Article

Ethanol extract of *Prunus mume* fruit attenuates hydrogen peroxide-induced oxidative stress and apoptosis involving Nrf2/HO-1 activation in C2C12 myoblasts

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ABSTRACT

The fruit of the *Prunus mume* (Siebold) Siebold & Zucc., Rosaceae (Korean name: Maesil) has long been used as a health food or valuable medicinal material in traditional herb medicine in Southeast Asian countries. In this study, we determined the potential therapeutic efficacy of the ethanol extract of *P. mume* fruits (EEPM) against H_2O_2-induced oxidative stress and apoptosis in the murine skeletal muscle myoblast cell line C2C12, and sought to understand the associated molecular mechanisms. The results indicated that exposure of C2C12 cells to H_2O_2 caused a reduction in cell viability by increasing the generation of intracellular reactive oxygen species and by disrupting mitochondrial membrane permeability, leading to DNA damage and apoptosis. However, pretreatment of the cells with EEPM before H_2O_2 exposure effectively attenuated these changes, suggesting that EEPM prevented H_2O_2-induced mitochondria-dependent apoptosis. Furthermore, the increased ex-pression and phosphorylation of nuclear factor erythroid 2-related factor 2 (Nrf2) and up-regulation of heme oxygenase-1 (HO-1), a phase II antioxidant enzyme, were detected in EEPM-treated C2C12 cells. We also found that zinc protoporphyrin IX, an HO-1 inhibitor, attenuated the protective effects of EEPM against H_2O_2-induced reactive oxygen species accumulation and cytotoxicity. Therefore, these results indicate that the activation of the Nrf2/HO-1 pathway might be involved in the protection of EEPM against H_2O_2-induced cellular oxidative damage. In conclusion, these results show that EEPM contributes to the prevention of oxidative damage and could be used as a nutritional agent for oxidative stress-related diseases.

Introduction

Oxidative stress is implicated in numerous diseases caused by the overproduction of reactive oxygen species (ROS). ROS, derivatives of cellular metabolic reactions, modulate the fundamental physiological functions of aerobic life. Excess amounts of ROS damage various cellular molecules, such as proteins, lipids, nucleic acids, and other macromolecular substances, resulting in cellular dysfunction and apoptosis (Wang et al., 2013; Wu et al., 2010). Therefore, supplementation with antioxidants, including synthetic and natural antioxidants, could reduce oxidative stress and ameliorate oxidative stress-related diseases through induction of phase II antioxidant enzymes as well as superoxide dismutase (SOD) and catalase (Wojcik et al., 2010; Guerra-Araiza et al., 2013).

Among phase II antioxidant enzymes, which are regulated by antioxidant responsive elements (AREs) at the transcription level,

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heme oxygenase-1 (HO-1) is a rate-limiting enzyme involved in the conversion of heme to biliverdin and carbon monoxide, among other cellular reactions (Martin et al., 2004; Chen et al., 2003). Bilirubin functions as a potent antioxidant and carbon monoxide has been reported to mediate the anti-apoptotic effects of HO-1 in response to inflammatory cytokine stimulation (Surh et al., 2008; Son et al., 2013). Nuclear factor erythroid 2-related factor 2 (Nrf2), a leucine zipper redox-sensitive transcription factor, is a key regulator of antioxidant and detoxification gene expression. Under normal conditions, Nrf2 binds to kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and is later subjected to proteasomal degradation. However, Nrf2 translocates from the cytoplasm to the nucleus following a variety of stimuli, and subsequently binds to ARE present within the promoter region of genes encoding for phase II enzymes, resulting in the up-regulation of their transcription (Terazawa et al., 2013; Niture et al., 2014). Moreover, recent evidence indicates that Nrf2 promotes cell survival by preventing an increase in ROS in various conditions of oxidative stress (Hybertson et al., 2011; Niture et al., 2014). Therefore, the Nrf2-ARE pathway is currently the most important endogenous antioxidant signaling pathway.

Prunus mume (Siebold) Siebold & Zucc. (Korean name: Maesil) is a deciduous tree of the Family Rosaceae, which is now widely cultivated in Southeast Asia countries, including Korea, China, and Japan (Matsuda et al., 2003; Wen and Shi, 2012). The various parts of this plant have been used as health foods or medicinal materials in traditional medicine for generations (Wen and Shi, 2012; Yan et al., 2014). In particular, its fruit has been eaten since ancient times in Asian countries as a traditional herbal medicine for relief of fatigue, diarrhea, fever, dyspepsia, and intestinal and skin disorders for thousands of years (Yan et al., 2014; Wen and Shi, 2012; Zhang et al., 2011; Jeong et al., 2006). The fruit of the *P. mume* contains abundant phenolic compounds, such as phenolic acids and flavonoids (Jeong et al., 2006; Kita et al., 2007; Mitani et al., 2013), which may be involved in the biological effects of anti-viral, anti-inflammatory, immunoenhancing, and antineoplastic activities (Zhang et al., 2011; Yingsakmongkon et al., 2008; Park et al., 2011; Enomoto et al., 2010; Tsuji et al., 2011; Jung et al., 2010; Tada et al., 2012; Lee et al., 2013; Jeong et al., 2006). Although there are several reports on the antioxidant activity and free radical scavenging activities of *P. mume* (Yan et al., 2014; Sang et al., 2002; Lee et al., 2013), the exact molecular mechanism(s) of actions of *P. mume* extract against oxidative stress involved in the Nrf2/HO-1 signaling pathway are yet to be described. Therefore, in the present study, we examined the ability of the ethanol extract of *P. mume* fruits (EEPM) to protect cells from hydrogen peroxide (H_2O_2)-induced cell damage and elucidated the mechanism underlying the protective action in a mouse-derived C2C12 myoblast model.

Materials and methods

Preparation of EEPM

Dried fruits of *Prunus mume* (Siebold) Siebold & Zucc., Rosaceae, were obtained from Institute of Hukyong Food (Busan, Republic of Korea), which were authenticated by Professor Su Hyun Hong, Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Republic of Korea). For the preparation of EEPM, freeze-dried fruits of *P. mume* were extracted with ethanol (100 g per 1 l) at 60 °C for three days using a blender. The extract was centrifuged at 10,000 × *g* for 20 min, and the supernatants were then collected and immediately filtered through a Whatman filter (pore size, 0.22 μm). The filtrate was lyophilized and stored at −70 °C. The yield (w/w) of the extract was ~5.0%. The powder was dissolved to a 100 mg/ml concentration with dimethylsulfoxide (DMSO, Sigma–Aldrich Chemical Co., St. Paul, MN, USA). The voucher specimens (accession number DEU-36) have been deposited at a publicly available Natural Resource Bank of Dongeui University College of Oriental Medicine.

Cell culture and EEPM treatment

The C2C12 myoblast cell line was purchased from the American Type Culture Collection (Manassa, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, WelGENE Inc.) and 100 μg/ml penicillin/streptomycin antibiotics (WelGENE Inc.) in a humidified 5% CO_2 atmosphere at 37 °C. The stock solution of EEPM was diluted with DMEM before every experiment.

Measurement of cell viability

To investigate the cytotoxicity, cells were seeded into 6-well plates and exposed to various concentrations of EEPM in the absence or presence of H_2O_2 and/or zinc protoporphyrin IX, a HO-1 inhibitor (ZnPP, Sigma–Aldrich) for the indicated times. After completion of the treatments, 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich) was added to each well continuously at 37 °C. After 3 h incubation, the culture supernatant was removed from the wells and the formazan complex was dissolved in DMSO. The absorbance of each well was detected at 540 nm with a microplate reader (Molecular Devices, Palo Alto, CA, USA). Cell viability is expressed as a percentage of untreated cells.

Measurement of intracellular ROS levels

The production of intracellular ROS was quantified using the oxidation-sensitive fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes, Eugene, OR, USA). After treatment, the cells were harvested, suspended in phosphate-buffered saline (PBS), and then incubated with 10 μM H2DCFDA for 20 min at room temperature in the dark. The fluorescence intensity was measured by a flow cytometer (Becton Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm (Kim et al., 2014).

Measurement of mitochondrial membrane potential (MMP)

The mitochondrial transmembrane electrochemical gradient was measured using the mitochondrial potential sensor 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma–Aldrich), a cell permeable, cationic, and lipophilic dye, which is internalized and concentrated by respiring mitochondria, reflecting changes in MMP in live cells. Briefly, cells were collected, resuspended in PBS, and then incubated with 10 μM JC-1 under dark conditions for 30 min at 37 °C. After the JC-1 was removed, the cells were washed with PBS to remove unbound dye. The cellular fluorescence intensity was quantified using a flow cytometry at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Seo et al., 2014).

DAPI nuclear staining

For the assessment of apoptosis, morphological changes of nuclei were visualized following DNA staining by t4,6-diamidino-2-phenyllindile (DAPI, Sigma–Aldrich), a fluorescent dye. In brief, the cells were fixed with 3.7% paraformaldehyde (Sigma–Aldrich) in PBS for 10 min at room temperature, and then stained with