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Antioxidant Activity of *Glycyrrhiza uralensis* Fisch Extracts on Hydrogen Peroxide-induced DNA Damage in Human Leucocytes and Cell Death in PC12 Cells

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Abstract In this study, antioxidant activity of methanol extract of *Glycyrrhiza uralensis* Fisch (GUE) against H_2O_2-induced DNA damage in human leucocytes and cell death in PC12 cells was determined. The effect of GUE on H_2O_2-induced DNA damage in human leucocytes was evaluated by the comet assay, where GUE (1-50 μg/mL) was a dose dependent inhibitor of DNA damage induced by H_2O_2. The protective effect of GUE against H_2O_2-induced damage on PC12 cells was investigated by MTT reduction assay and lactate dehydrogenase release assay. A marked reduction in cell survival induced by H_2O_2 was significantly prevented by 1-50 μg/mL of GUE. The enzyme activity of caspase-3 was elevated in H_2O_2-treated PC12 cells, while preincubation with GUE for 30 min inhibited H_2O_2-induced caspase-3 activation in a dose-dependent manner. In conclusion, GUE ameliorates H_2O_2-induced DNA damage in human leucocytes and has neuroprotective effect by preventing cell death in PC 12 cell, suggesting that GU may be a potential candidate for novel therapeutic agents for neuronal diseases associated with oxidative stress.

Keywords: *Glycyrrhiza uralensis* Fisch, antioxidant, human leucocyte, hydrogen peroxide, PC12 cell, apoptosis

Introduction

There is a growing interest in the use of natural products, as consumer awareness of their possible beneficial health effects increases (1,2). *Glycyrrhiza uralensis* Fisch. (GU) (Fam. Leguminosae), known as also licorice root, is one of the most ancient herbal medicines known (3) and extensively cultivated in China, Russia, Spain, Persia, and India (4). It has been reported to contain glycyrrhizin, a major substance and other bioactive constituents such as essential oils, alkaloids, polysacchrides, polyamines, triterpenoids, and flavonoids (5,6). The GU has long been employed in western countries as a flavoring and sweetening agent, as well as a demulcent and expectorant (7). In oriental medicine, GU has been known to possess various pharmaceutical functions, including detoxification, antiulcer, antiinflammation, antiviral, antiatherogenic, and anticarcinogenic activities (4). In addition some components of GU demonstrated significant antimicrobial activity *in vitro* (8,9), immunological (10), and antioxidant activity (1,11,12).

Reactive oxygen species (ROS) are continuously generated *in vivo* and regarded to be responsible for a variety of pathological conditions, including cardiovascular disease, cancer, aging, and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson' disease (PD) (13-15). Among a great variety of ROS, hydrogen peroxide (H_2O_2) is known to play a pivotal role because it is generated from nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues (16). Moreover, it has been shown that H_2O_2 has the ability to modulate signal transduction pathways (17,18), to provoke cell proliferation and differentiation (19,20), and finally to induce cell death either by apoptosis or necrosis (21-23), especially in organs such as the brain. The brain is believed to be particularly vulnerable to the damage affects of H_2O_2 (24). Because of its high metabolic rate and relatively reduced capacity for cellular regeneration compared with other organs (25). In addition, brain has been shown to contain low to moderate levels of enzymes such as catalase, superoxide dismutase that an important role in the metabolism of ROS (26).

Thus, a number of researchers have attempted to prevent or diminish H_2O_2-induced damage, and utilize natural products for treatments that prevent H_2O_2 generation and reduce DNA damage and cell death (27).

Until now, the protective effects of methanol extract of GU (GUE) on H_2O_2-induced DNA damage or cell death had not been assessed. In this study, therefore, we determined the antioxidant activity of GUE against H_2O_2-induced DNA damage in human leucocytes and cell death in PC12 cells.

Materials and Methods

Materials *Glycyrrhiza uralensis* Fisch. (GU) was obtained from Kumkang Pharm Co., Ltd., Masan, Korea. Hydrogen peroxide solution (H_2O_2), 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Histopaque 1077, low melting agarose, normal melting agarose, NaCl, Na_2EDTA, Tris(hydroxymethyl)amino-methane, ethidium bromide were provided by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Lactate dehydrogenase (LDH)

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release assay kit was purchased by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PC12 cells were obtained from Korean Cell Line Bank (KCLB). Dulbecco's modified Eagle's (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Preparation of methanolic extracts from GU Five g of GU were extracted with 100 mL of methanol for 2 days at room temperature and filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). Methanol solvent was then removed by evaporation, and the dried methanol extract was obtained (GUE). The methanol extract was then dissolved in dimethyl sulfoxide (DMSO) with concentration 5 mg/mL for experiments. It was designated as GUE.

Preparation of human leucocytes Blood samples were obtained from 3 healthy male volunteers. Five mL of fresh whole blood was added to 5 mL of phosphate-buffered saline (PBS) and layered onto 5 mL of Histopaque 1077. After centrifugation for 30 min at 400×g at room temperature, the leucocytes were collected from the just above the boundary with the Histopaque 1077, washed with 5 mL PBS. Finally, they were freshly used for comet assay or resuspended in freezing medium (90% fetal calf serum, 10% DMSO) at 6×10^6 cells/mL. The cells were frozen to −80°C using a Nalgene Cryo 1°C freezing container (Nalgene, Rochester, NY, USA) and stored in liquid nitrogen. The cells were thawed rapidly prior to each experiment in a water bath at 37°C.

Treatment of human leucocytes GUE dissolved in DMSO was diluted with PBS into concentrations 0, 1, 5, 10, and 50 μg/mL. Leucocytes (2×10^4 cells/mL) were incubated with each diluted extracts for 30 min at 37°C in a dark incubator. For oxidative stimulus they were then resuspended in PBS with 200 μM H_2O_2 for 5 min on ice. After each treatment, samples were centrifuged at 400×g for 5 min and washed with PBS. All the experiments were repeated twice with leucocytes from each of 3 donors on the separate day.

Determination of DNA damage The alkaline comet assay was conducted according to Singh *et al.* (28) with little modification. The cell suspension was mixed with 75 μL of 0.5% low melting agarose (LMA) and added to the slides precoated with 1.0% normal melting agarose. After solidification of the agarose, slides were covered with another 75 μL of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 hr at 4°C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4°C. The slides were washed 3 times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscope (Leica DMLB, Wetzlar, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of 2 replicate slides). Cell viability measured by trypan blue exclusion test was above 95% for all treatments.

Cell culture and treatments Rat pheochromocytoma PC12 cells were maintained in DMEM supplemented with 10% FBS, 5% horse serum (HS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 3.7 g/mL $NaHCO_3$. PC12 cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. In all experiments, cells were treated with GUE before H_2O_2-stress for the indicated times. GUE were dissolved in DMSO and added to the culture medium so that the final concentration of DMSO was less than 1%.

MTT reduction assay for cell viability Cell viability was measured with blue formazan that was metabolized from colorless MTT by mitochondrial dehydrogenases, which are active only in live cells. PC12 cells were preincubated in 96-well plates at a density of 1×10^5 cells/mL for 24 hr. Cells with various concentrations of GUE were treated with H_2O_2 for 2 hr. After incubation, MTT reagent (5 mg/mL) was added to each of the wells and the plate was incubated for an additional about 2 hr at 37°C. The intracellular formazan product was dissolved in 100 μL of DMSO. The absorbency of each well was then measured at 540 nm using the enzyme-linked immunosorbent assay (ELISA) reader (model 680; BioRad, Hercules, CA, USA) and the percentage viability was calculated.

Lactate dehydrogenase release assay Cytotoxicity was determined by measuring the release of LDH. PC12 cells with various concentrations of GUE were treated with H_2O_2 for 2 hr and the supernatant was used to assay LDH activity. The reaction was initiated by mixing 50 μL of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 μL to 96-well plate. The absorbance of sample was read at 490 nm (model 680; BioRad). Data were normalized to the activity of LDH released from H_2O_2-treated cells (obtained separate plating).

Observation of morphologic changes PC12 cells in DMEM containing 10% FSB and 5% HS were seeded into 6-well plate and incubated with various concentrations of GUE. After incubation for 30 min, cells were treated with 500 μM H_2O_2 for 2 hr. The cellular morphology was observed using phase-contrast microscope (Nikon, Tokyo, Japan). Photographs were taken at a magnification of 100×.

Nuclear staining for assessment of apoptosis Chromosomal condensation and morphological changes in the nucleus were observed using the chromatin dye Hoechst 33342 (Sigma). The PC12 cells were washed twice with PBS and then fixed in PBS containing 10% formaldehyde for 4 hr at room temperature. After twice rinsed with PBS, the cells were stained with Hoechst 33342 for 30 min at room temperature. The cells were washed twice more with PBS and the Hoechst-stained nuclei were visualized by using a fluorescence microscope (Nikon).

Measurement of caspase-3 activity Activities of caspase-3 was determined by a colorimetric assay using activation kits from R&D Systems (Wiesbaden-Nordenstadt, Germany), following the manufacturer's protocol (29). The kits used synthetic tetrapeptides labeled with *p*-nitroanilide (pNA). Briefly, 1, 5, 10, and 50 μg/mL of GUE and H_2O_2-treated cells were lysed in the supplied lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and DEVD-pNA (specific for caspase-3) as a substrate at 37°C. The reaction was measured by changes in absorbance at 405 nm using the ELISA reader (Tecan Spectra, Wetzlar, Germany). Data were expressed as a fold increase in caspase activity of apoptotic cells over that of non induced cells.

Statistical analysis The data are the means of 3 determinations and were analyzed using the SPSS package for Windows (Version 11.5). The mean values from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *p*-Value of less than 0.05 was considered significant.

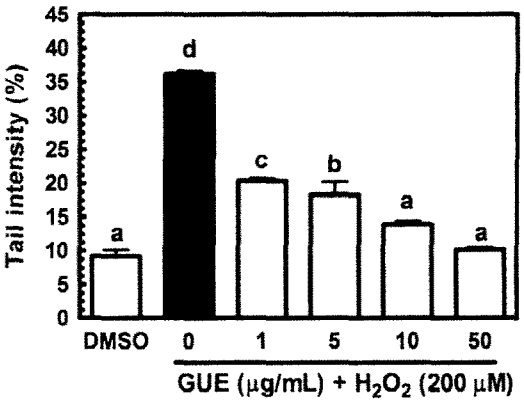

Fig. 1. Inhibition by GUE of H_2O_2-induced DNA damage in human leucocytes. Data represent the relative values±SD of 3 independent experiments and the mean values of DNA damage (tail intensity) from each treatment were compared using ANOVA followed by Duncan's test. Values not sharing the same letter are significantly different from one another ($p<0.05$).

Results and Discussion

Protective effect of GUE on H_2O_2-induced DNA damage in human leucocytes DNA damage is known to be one of the most sensitive biological markers for evaluating oxidative stress representing the imbalance between free radical generation and efficiencies of the antioxidant system (30, 31). Hydrogen peroxide is believed to cause DNA strand breakage by generation of the hydroxyl radical (OH·) close to the DNA molecule, via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe(III) + OH\cdot + OH^-$) (32). The percent fluorescence tail DNA intensity of leucocytes treated for 30 min with DMSO treated negative control was significantly different from the 200 μM of H_2O_2-preincubated positive control (tail intensity 36%) (Fig. 1). This increase of DNA damage induced by H_2O_2 was significantly inhibited in a dose dependent manner by pretreatment of the cells for 30 min with GUE at the concentrations of 1, 5, 10, and 50 μg/mL in PBS (tail intensity 20, 18, 14, and 10%, respectively). Especially, the higher concentration (10 and 50 μg/mL) of GUE indicated strong protecting effect of DNA damage, as compared to the DMSO treated normal control. The possible mechanism by which GU inhibited H_2O_2-induced DNA damage in human leucocytes might be ascribed to the antioxidant compounds contained in GU. Murcia *et al.* (33) reported that water extract of GU exhibited the good H_2O_2 scavenging effect. Polyphenols may be responsible for the antioxidant activity of GU. Licochalcones B and D strongly inhibited superoxide anion production in the xanthine/xanthine oxidase system, 1,1-diphenyl-2-picryl-hydrazyl radical system, Fe(III)-ADP/NADPH, and other lipid peroxidation systems (34-37).

GUE protection of PC12 cells against H_2O_2-induced cytotoxicity To characterize the effects of GUE on cell viability in H_2O_2-stressed PC12 cells, the cells were incubated with GUE and 500 μM of H_2O_2 and morphological alterations were verified via a phase-contrast microscope. As shown in Fig. 2, the PC12 cells had round cell bodies with clear edges and fine dendritic networks, but after 2 hr of exposure to 500 μM of H_2O_2, many cells showed cytoplasmic shrinkage, and either detached from each other, or floated in the medium. In contrast, cultures exposed to H_2O_2 in the presence of GUE appeared remarkably preserved, indicating that GUE offered protection to the H_2O_2-stressed PC12 cells.

Next, we attempted to determine the effects of GUE on neuronal protection via an MTT reduction assay and a cytoplasmic LDH release assay. As shown in Fig. 3A, PC12 cells treated for 2 hr with 500 μM of H_2O_2 had cell viability reductions of 40% compared to the control. However, after 2 hr of exposure to 500 μM of H_2O_2 and various concentrations of GUE, the cell viability recovered

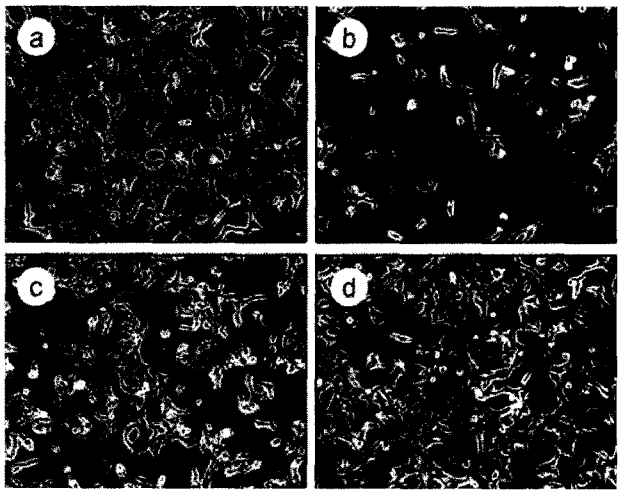

Fig. 2. Morphological characteristics of undifferentiated PC12 cells after H_2O_2 treatment in the absence or presence of GUE. (a) Control, (b) PC12 cells exposed to 500 μM of H_2O_2 for 2 hr, (c) PC12 cells treated with 10 μg/mL GUE for 30 min before exposure to 500 μM of H_2O_2 for 2 hr, (d) PC12 cells treated with 50 μg/mL GUE for 30 min before exposure to 500 μM of H_2O_2 for 2 hr. Photographs were taken with a phase-contrast microscope at 100× magnification.

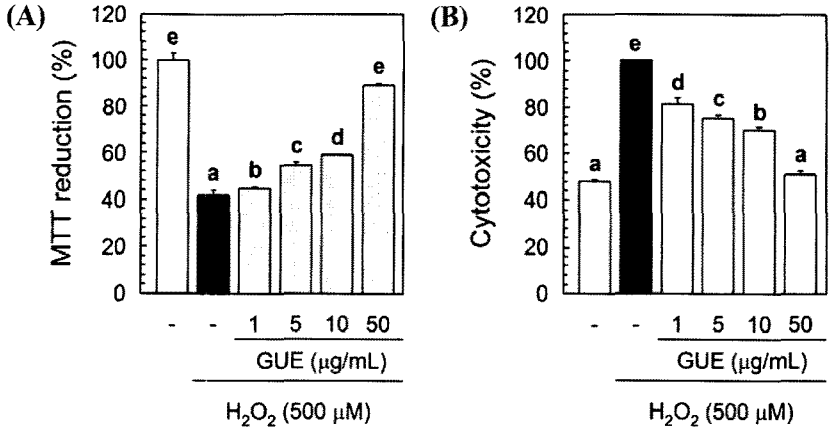

Fig. 3. Cell viability effect of GUE on H_2O_2-induced cytotoxicity in PC12 cell system (A: MTT reduction assay, B: LDH release assay). Data (means±SD) are representative of at least 3 independent experiments and the mean values from each treatment were compared using ANOVA followed by Duncan's test. Values not sharing the same letter are significantly different from one another ($p<0.05$).

from 5 to 50% when compared to the viability of the H_2O_2-stressed PC12 cells (Fig. 3A). To further investigate the protective effects of GUE, we performed the LDH assay as another indicator of cell toxicity. Here, the PC12 cells were exposed to various concentrations of GUE and H_2O_2 for 2 hr. As expected, GUE reduced cell damage in a dose-dependent manner, as was evident by a 20-50% decrease in LDH release from the H_2O_2-stressed PC12 cells (Fig. 3B).

This observation is similar to the results reported by Ahn *et al.* (38), showing that GU water extract exerts a protective effect against the cognitive impairments often observed in AD, and that in mice this effect is mediated by antioxidant actions against oxidative stress. Hence, on the basis of the above-mentioned studies, we can hypothesize that the protective effect of GUE on H_2O_2-induced cell death is related to its scavenger activity towards ROS.

Effects of GUE on apoptosis induced by H_2O_2 To determine whether cell protection by GUE was due to the inhibition of apoptosis, PC12 cells were treated with H_2O_2 and various concentrations of GUE. We found that cells stained with Hoechst 33342 revealed marked chromatin condensation and apoptotic body formation when examined by a fluorescence microscope. Hoechst 33342 staining also revealed that the H_2O_2-treated cells exhibited highly condensed and fragmented nuclei morphologies and nuclei shrinkage, which were the typical characteristics of apoptosis. In contrast, pretreatment with 10 to 50 μg/mL of GUE prevented H_2O_2-induced nuclei morphological alterations and the number of cells with nuclear condensation and fragmentation was significantly decreased (Fig. 4A).

For effective apoptosis, the activation of caspase-3 is an absolute requirement; where caspase-3 is a cysteine-dependent aspartate protease that functions as a key executor in cell death (39). Activation of caspases during H_2O_2 toxicity has frequently been seen in numerous cell types (40,41). By using a colorimetric assay, we determined that caspase-3 activity performed a central role in apoptosis within the mitochondrial pathway. As shown in Fig. 4B, the enzyme activity of caspase-3 was significantly elevated in H_2O_2-treated PC12 cells (3.5 fold). However, preincubation with GUE for 30 min inhibited H_2O_2-induced caspase-3 activation in a dose-dependent manner (2.9, 1.9, 1.1, and 1.0 fold). Thus, these results suggest that GUE may protect PC12 cells against apoptosis induced by H_2O_2. And then GUE exerts anti-apoptotic activity partly by preventing the apoptotic signaling that leads to the activation of caspase-3.

From the results it is concluded that GUE ameliorates

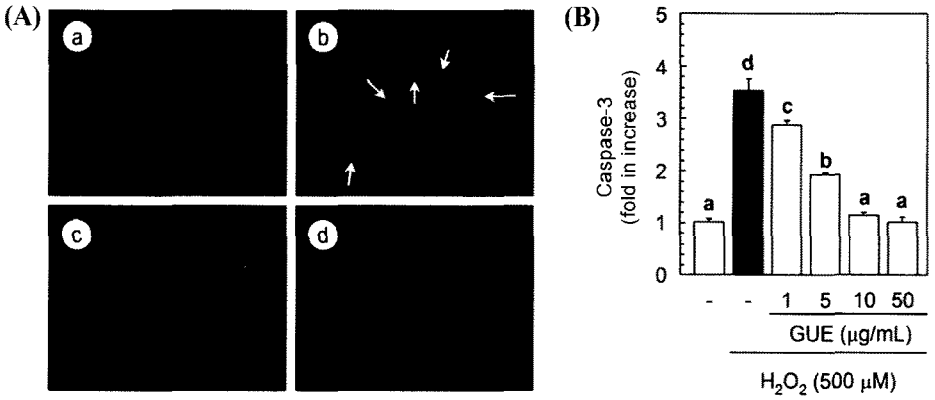

Fig. 4. Inhibition by GUE of H_2O_2-induced (A) apoptosis and (B) caspase-3 activation in PC12 cells. The arrows indicate apoptotic cells (A). The data of caspase-3 activity (B) represent the relative values±SD of 3 independent experiments and the mean values from each treatment were compared using ANOVA followed by Duncan's test. Values not sharing the same letter are significantly different from one another ($p<0.05$).

H_2O_2-induced DNA damage in human leucocytes and has neuroprotective effect by preventing cell death in PC 12 cell. Although additional research is needed to delineate the relative contribution of these pathways, our results suggest that the GU may be a potential candidate for novel therapeutic agents for neuronal diseases associated with oxidative stress.

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