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Neuroprotective Effects of Methanol Extracts of Jeju Native Plants on Hydrogen Peroxide-induced Cytotoxicity in SH-SY5Y Human Neuroblastoma Cells

Pil-Jae Kong[1], Yu-Mi Kim[1], Hee Jae Lee[1], Sung-Soo Kim[1], Eun Sook Yoo[2], and Wanjoo Chun[1]

[1]Department of Pharmacology, College of Medicine, Kangwon National University, Chunchon 200-701, [2]Department of Pharmacology, College of Medicine, Cheju National University, Jeju 690-756, Korea

Neuronal death is a common characteristic hallmark of a variety of neurodegenerative disorders including Alzheimer's disease and Parkinson's disease. However, there have been no effective drugs to successfully prevent neuronal death in those diseases, whereas oriental medicinal plants have to possess valuable therapeutic potentials to treat neurodegenerative diseases. In the present study, in an attempt to provide neuroprotective agents from natural plants, 80% methanol extracts of a wide range of medicinal plants, which are native to Jeju Island in Korea, were prepared and their protective effects on hydrogen peroxide-induced apoptotic cell death were examined. Among those tested, extracts from *Smilax china* and *Saururus chinesis* significantly decreased hydrogen peroxide-induced apoptotic cell death. The extracts attenuated hydrogen peroxide (H_2O_2)-induced caspase-3 activation in a dose-dependent manner. Further, plant extracts restored H_2O_2-induced depletion of intracellular glutathione, a major endogenous antioxidant. The data suggest that Jeju native medicinal plants could potentially be used as therapeutic agents for treating or preventing neurodegenerative diseases in which oxidative stress is implicated.

Key Words: Neuroprotection, Jeju native plants, Hydrogen peroxide, Caspase-3

INTRODUCTION

Oxidative stress has long been implicated in a number of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and ischemia (Simonian and Coyle, 1996; Murphy, 1999; Liu et al, 2002). Cell death from oxidative stress has also been associated with general decline of the central nervous system function with senescence (Serrano and Blasco, 2001). Many physiological and environmental toxicants exert oxidative stress to cells by overproducing reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals. Although hydrogen peroxide (H_2O_2) is not a free radical *per se* and has a limited reactivity, it is thought to be the major precursor of highly reactive free radicals. Furthermore, it has been reported that hydrogen peroxide induces apoptosis in neurons of the central nervous system (CNS) (Chandra et al, 2000). An increasing body of evidence suggest that neuronal apoptosis is involved in the pathological processes of neurodegenerative disorders such AD, PD, and HD (Burke and Kholodilov, 1998; Friedlander, 2003).

Corresponding to: Wanjoo Chun, Department of Pharmacology, College of Medicine, Kangwon National University, Hyoja 2-dong, Chunchon 200-701, Korea. (Tel) 82-33-250-8853, (Fax) 82-33-242-7571, (E-mail) wchun@kangwon.ac.kr

It has been suggested that traditional oriental medicinal plants exhibit a wide range of biological properties and have potentials for preventing pathological progresses of various neurodegenerative diseases (Yoon et al, 2000; Leem and Park, 2007). In the present study, to provide useful protective agents to cope with oxidative stress, medicinal plants which are native to Jeju Island, Korea, were examined for their possible protective properties in an oxidative stress cell model. Given their previously reported beneficial biological properties, methanol extracts of *Smilax china* (Lee and Lin, 1988; Song et al, 1998; Lee et al, 2001), *Machilus thunbergii* (Ma et al, 2004), *Saururus chinesis* (Cho et al, 2005; Kang et al, 2005), Crinum asiaticum (Samud et al, 1999), and *Achyranthes japonica* (Kim et al, 2004a), were prepared and their protective effects were examined under oxidative condition.

METHODS

Preparation of plant extracts

Root, fruit, leaf, stem, or seed of following plants were used in the present study; root of *Smilax china* (1), fruit of *Machilus thunbergii* (2), leaf of *Machilus thunbergii* (3), stem of *Saururus chinesis* (4), root of *Saururus chinesis* (5), seed of *Crinum asiaticum* (6), and roots of *Achyranthes japonica* (7). Dried plant parts were extracted with 80% methanol at 80°C, and the extracts were concentrated with

a vacuum rotary evaporator (Eyela, Japan) under reduced pressure. These extracts were dissolved in ethanol and diluted with phosphate buffered saline (PBS, pH 7.4) to give final concentration in the range of 1.0 to 100 μg/ml.

Cell culture and extract treatment

Human neuroblastoma SH-SY5Y cells obtained from the American Type Culture Collection (Rockville, USA) were cultured in DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 20 mM glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. Hydrogen peroxide (Sigma Chemical Co, MO, USA) was used for 2 hr to induce apoptotic cell death in SH-SY5Y cells. The cell cultures were preincubated with the extracts 12 hr prior to the addition of H_2O_2. All experiments were carried out on sub-confluent cultures.

Determination of cell viability

Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. MTT dissolved in PBS was added to a final concentration of 0.5 mg/ml at the end of incubation, and then incubated further at 37°C for 2 hr. The resultant formazan product was extracted for 4 h with extraction solution (20% SDS, 50% DMF and 2% acetic acid) and detected by a UV-VIS spectrometer (Perkin Elmer Co.) at 570 nm.

Measurement of in situ caspase-3 activity

In situ caspase-3 activity was measured using a protocol previously described (Bijur et al, 2000). In brief, 200 μl of assay buffer (20 mM Hepes, pH 7.5, 10% glycerol, and 2 mM dithiothreitol) containing the peptide substrate for caspase-3 (AC-DEVD-AMC) was added to each well (final concentration of 25 ng/μl) of a 96-well clear bottom plate (Corning). Cell lysate (20 μg of protein) was added to start the reaction. Triplicate measurements were done for each sample. Background fluorescence was measured in wells containing assay buffer, substrate, and lysis buffer without the cell lysate. Assay plates were incubated at 37°C for 1 h, and fluorescence was measured on a fluorescence plate reader (Bio-Tek, Winooski, VT) set at 360 nm excitation and 460 nm emission.

Measurement of glutathione levels

Cells (60-mm plate, 0.2~0.4 mg of protein) were washed twice with ice-cold PBS and deproteinized with 200 μl of ice-cold 5% (w/v) 5-sulfosalicylic acid (SSA). Forty microliters of 1 M HEPES/1 M KOH solution was added to protein-free SSA, and the pH was adjusted to 7.0 with 1 M KOH. Total amount of glutathione (GSH±GSSG) was measured by an enzymatic recycling procedure in which it was sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase (Tietze, 1969). The rate of 2-nitro-5-thiobenzoic formation was monitored at 412 nm, and the concentration of glutathione determined by comparing the result with a standard curve generated with known amounts of GSH. All GSH assays were performed in quadruplicate.

Statistics

All values are expressed as mean±SD from at least three independent experiments. Data were analyzed using Student's *t*-test and considered significantly different when the two-tailed p value was <0.05.

RESULTS

In the present study, neuroprotective effects of the methanol extracts of Jeju native plants were examined with H_2O_2-induced cytotoxicity model using SH-SY5Y human neuroblastoma cells. To obtain appropriate concentration of H_2O_2, which results in approximately 50% of cell death, SH-SY5Y cells were treated with different concentrations of H_2O_2 for 2 hr, and cell viability was determined using MTT assay (Fig. 1). Concentration dependent cell death was observed with up to 400 μM concentration of H_2O_2, and 100 μM H_2O_2, which exhibited approximately 50% cell death, was chosen in the following experiments.

To examine possible protective effects of plant extracts on cell viability, cells were pretreated with plant extracts for 12 hr prior to H_2O_2 treatment, and then cell viability was examined using MTT assay. As shown in Fig. 2, the extracts 1, 4, and 5 among 7 xtracts tested significantly decreased H_2O_2-induced cell death, albeit not completely decreased (Fig. 2). Although a dose-dependent trend seemed to exist with increasing concentration of plant extracts, no statistically significant dose-dependency was observed. Other plant extracts did not show any significant protection against H_2O_2-induced cell death in SH-SY5Y cells. Protective effects of the extracts 1, 4, and 5 reached maximum at 100 μg/ml and then decreased with higher concentrations (data not shown). This phenomenon was most likely due to the cytotoxic effect of these compounds at higher concentration.

To examine whether H_2O_2-induced cell death in SH-SY5Y human neuroblastoma cells was mediated through apopto-

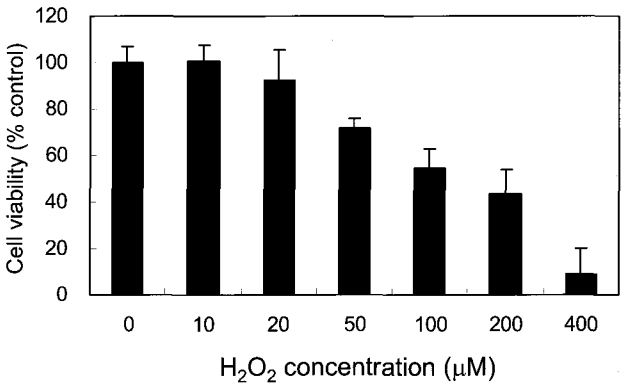

Fig. 1. Concentration-dependent cytotoxicity by hydrogen peroxide in SH-SY5Y human neuroblastoma cells. After two hours of incubation with hydrogen peroxide, neuronal cell viability at indicated concentration of hydrogen peroxide was measured using MTT assay. Concentration-dependent cell death was observed, and 100 μM concentration, which exhibited approximately 50% cell death, was chosen in the following experiments.

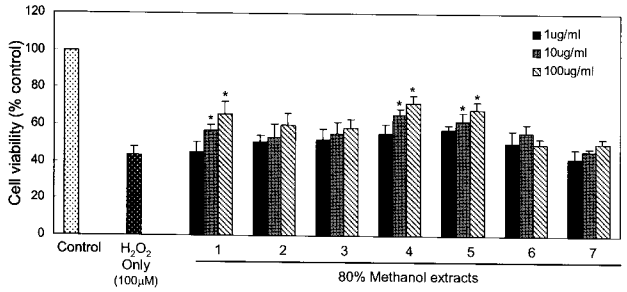

Fig. 2. Effects of methanol extracts of Jeju native natural plants on hydrogen peroxide-induced neuronal cell viability. Effect of 80% methanol extracts of Jeju natural plants on hydrogen peroxide-induced cell death was examined using MTT assay. Twelve hours of pre-treatment of the cells with the extracts 1, 4, and 5 at 10 μg/ml and 100 μg/ml concentrations significantly attenuated hydrogen peroxide-induced cell death. Data are expressed as mean(SD from three independent experiments. *p <0.05 indicates statistically significant difference from the cells exposed to hydrogen peroxide alone.

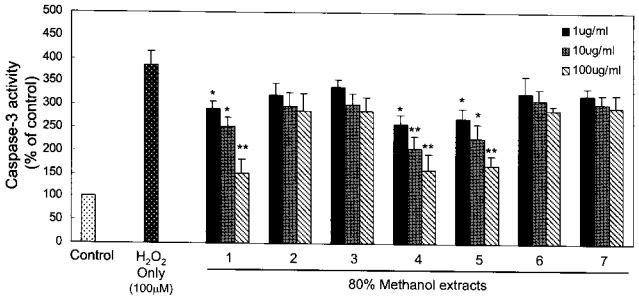

Fig. 3. Effects of methanol extracts of Jeju native natural plants on hydrogen peroxide-induced caspase-3 activation in SH-SY5Y cells. Effect of 80% methanol extracts of Jeju natural plants on hydrogen peroxide-induced caspase-3 activation was examined using fluometric substrate (AC-DEVD-AMC). Twelve hours of pre-treatment of the cells with the extracts 1, 4, and 5 significantly attenuated hydrogen peroxide-induced cell death in a dose-dependent manner. Data are expressed as mean±SD from three independent experiments. *$p<0.05$, **$p<0.01$ indicate statistically significant difference from the cells exposed to hydrogen peroxide alone.

sis, activation of caspase-3, which is the effector caspase in the apoptotic cascade, was examined using a fluometric substrate. Treatment of the cells with H_2O_2 resulted in approximately 4-fold activation of casapse-3 compared to control (Fig. 3). In accordance with the data on cell viability, the extracts 1, 4, and 5 significantly attenuated H_2O_2-induced caspase-3 activation (Fig. 3). Furthermore, attenuation of H_2O_2-induced caspase-3 activation showed a dose-dependent pattern in the concentration range tested. These data strongly suggest that caspase-3 activation is responsible for the H_2O_2-induced cell death in SH-SY5Y cells.

In an effort to examine a possible underlying mechanism by which these plant extracts provide neuroprotection, the level of glutathione, a major intracellular antioxidant, was measured. Treatment of SH-SY5Y cells with H_2O_2 resulted in depletion of intracellular glutathione in concentration- and incubation time-dependent manners (data not shown). Pretreatment of the cells with the extracts 1, 4, and 5 significantly restored H_2O_2-induced glutathione depletion, although not completely (Fig. 4). However, no dose-dependent pattern was observed in the concentration range tested.

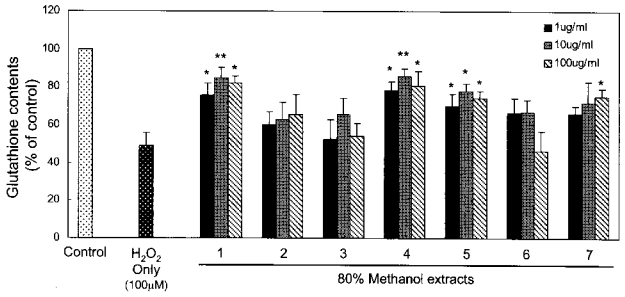

Fig. 4. Effects of methanol extracts of Jeju native natural plants on hydrogen peroxide-induced glutathione depletion. Effect of 80% methanol extracts of Jeju natural plants on hydrogen peroxide-induced glutathione depletion was examined by determining total glutathione levels in cells. In accordance with the data from cell viability and caspase-3 activity levels, 12 hr pre-treatment of the cells with the extracts 1, 4, and 5 significantly restored hydrogen peroxide-induced glutathione depletion. However, dose-dependency was not observed. Data are expressed as mean±SD from three independent experiments. *$p<0.05$, **$p<0.01$ indicate statistically significant differences from the cells exposed to hydrogen peroxide alone.

DISCUSSION

Reactive oxygen species (ROS) may disturb the inherent cellular antioxidant defense system, resulting in damage to biological macromolecules such as nucleic acids, proteins, carbohydrates, and lipids. Such forms of damage have been implicated in several neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and ischemia (Hyslop et al, 1995; Chandra et al, 2000). Therefore, the protection of neuronal cells from reactive oxygen species can be beneficial for the prevention and treatment of these diseases. In the present study, Jeju Island (Korea) native medicinal plants were examined for their possible neuroprotective properties against oxidative stress in a human neuroblastoma cell model.

Among plant extracts tested in the present study, *Smilax china* has been reported to exhibit biological activities such as antimicrobial(Song et al, 1998), antimutagenic (Lee and Lin, 1988), and antioxidant properties (Lee et al, 2001). However, previous studies examined its effects mostly *in vitro* and on non neuronal cells (Lee and Lin, 1988). Furthermore, neuroprotective properties of *Smilax china* have not been observed. *Saururus chinensis,* another plant tested in the present study, has been reported to exert a wide range of biological properties such as antioxidant, anti-inflammatory, and neuroprotective properties (Kim et al, 2004b; Cho et al, 2005). Therefore, the present study was carried out to determine whether extracts of Jeju native plants affect neuronal cell viability, apoptotic cell death, and intracellular protective environment such as intracellular level of glutathione, which is a major endogenous antioxidant in cells. Among plant extracts tested, extracts from *Smilax china* and *Saururus chinensis* showed anti-apoptotic neuroprotective effects against hydrogen per-

oxide-induced cytotoxicity. These extracts significantly attenuated hydrogen peroxide-induced cell death and caspase-3 activation in a dose-dependent manner. It has also been observed that these extracts restored hydrogen peroxide-induced depletion of intracellular glutathione. However, the degree of protection was not high enough to completely abolish H_2O_2-induced cellular injury in the present experimental model. This is most likely to relatively low amount of active ingredients in the extracts. Therefore, identification of active ingredients by further fractionation may enhance their biological activities. The antimicrobial property of *Smilax china* has been attributed to its phenolic compounds, since that the potency of antimicrobial activity was proportional to the amount of phenolic compounds in each fraction (Song et al, 1998). In addition to phenolic compounds, *Smilax china* has been shown to contain a wide range of biologically active compounds including steroidal saponins (Kim et al, 1989; Song et al, 1998). Furthermore, *Saururus chinensis* has also been reported to exert various biological properties due to the active compounds (Cho et al, 2005; Lee et al, 2006; Park et al, 2007): For example, lignan compounds have been reported to exhibit immunosuppressive property (Park et al, 2007) and flavonol glycosides exhibit free radical scavenging activity (Kang et al, 2005). Since *Saururus chinensis* possesses various biological activities, opposing biological activities appear to depend on the presence of different active compounds. For example, Saucernetin-7 has been reported to exhibit apoptosis-inducing activity in a leukemia cell model (Choi et al, 2007), whereas aristolactam BII attenuates glutamate-induced neurotoxicity in cortical neuronal culture (Kim et al, 2004b). Therefore, identification of active components appears to be absolutely necessary for the treatment purpose.

In conclusion, the present data indicate that methanol extracts of Jeju native medicinal plants possesses neuroprotective properties against H_2O_2-induced neuronal cell death by suppressing caspase-3 activation and restoring GSH levels. These properties of *Smilax china* and *Saururus chinensis* would be useful in development of therapeutic protection as well as treatment of neurodegenerative diseases caused by oxidative stress. However, further studies are necessary to determine which compounds exert these neuroprotective properties and the mechanism by which these compounds exert neuroprotective action.

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