Short communication

Protective effects of *Orostachys japonicus* A. Berger (Crassulaceae) on H$_2$O$_2$-induced apoptosis in GT1-1 mouse hypothalamic neuronal cell line

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Abstract

Oxidative stress is a major cause of neurodegenerative diseases, so the protection of neuronal cells from reactive oxygen species can be beneficial for the prevention and treatment of these diseases. Methanol extract of *Orostachys japonicus* A. Berger (Crassulaceae), a traditional oriental medical herb, was shown to have a protective effect on H$_2$O$_2$-induced apoptosis in GT1-1 mouse hypothalamic neuronal cell line which was detected by flow cytometry after propidium iodide staining. Among fractions of *O. japonicus*, chloroform fraction had the highest protective effect, and water fraction had no protective effect suggesting that the active ingredients might be hydrophobic compounds. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords**: *Orostachys japonicus*; Hydrogen peroxide; Neurodegenerative disease; Apoptosis; GT1-1

1. Introduction

Traditional oriental herbs contain many useful compounds, which can be used for the treatment of chronic disease. Many reports suggested that traditional herbs have potentials for preventing pathological outcome of neurodegenerative disease. Kim et al. (1998) reported that ginsenoside Rbl and Rg3 isolated from *Panax ginseng* protected cultured rat cortical cells from glutamate-induced neurodegeneration. Watanabe et al. (1995) reported that Chinese herbal medicine, toki-shakuyaku-san protected cultured cerebellar granule cells from glutamate-induced neuronal damage. Zhao et al. (1996) reported that H$_2$O$_2$-induced neuronal apoptosis can be reduced by *Ginkgo biloba* extract.

Reactive oxygen species has been suggested as a major cause of neurodegenerative disorders such
as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease (Simonian and Coyle, 1996). Hydrogen peroxide (H$_2$O$_2$), one of the major reactive oxygen species, exerts its toxic effects mainly through the ferrous iron-dependent formation of the highly reactive hydroxyl radical (Fenton, 1984). Recent studies have shown a close association between H$_2$O$_2$ and neurodegenerative disease, and it was suggested that the level of H$_2$O$_2$ was increased in pathological conditions including ischemia and neurodegenerative disease. Hyslop et al. (1995) measured H$_2$O$_2$ concentration in rat brain using microdialysis system during ischemia and reperfusion, and found that a significant rise in H$_2$O$_2$ level was observed for about 1 h during reperfusion. Amyloid $\beta$ which is known to be the cause of Alzheimer’s disease also induces the increase of H$_2$O$_2$ level (Behl et al., 1994). Because of its high membrane permeability, H$_2$O$_2$ can be cytotoxic not only for the producing cell but also for neighboring cells (Halliwell, 1992). Recently, it was reported that H$_2$O$_2$ destroys neurons by the process of apoptosis, spontaneous cellular suicide. H$_2$O$_2$-induced apoptosis was observed in cultured cortical neurons and PC 12 sympathetic neuronal cell line (Whittemore et al., 1994; Sato, et al., 1996). The prevention of H$_2$O$_2$-induced neuronal apoptosis may be beneficial for the prevention and treatment of neurodegenerative diseases.

In our laboratory, out of many traditional oriental medical herbs and prescriptions studied, *Orostachys japonicus* A. Berger (Crassulaceae) was found to possess good protective effect on H$_2$O$_2$-induced neuronal apoptosis.

### 2. Materials and methods

#### 2.1. Preparation of methanol extract of *O. japonicus*

Dried whole plant of *O. japonicus* A. Berger (Crassulaceae) was collected at Youngwol, Kangwon-do, Korea. Voucher specimen No. CA-1998-05-33 is preserved in Korea Institute of Oriental Medicine, Seoul, Korea. Twenty grams of *O. japonicus* was extracted by overnight incubation at 60°C in 500 ml of 80% methanol. The solution was filtered with Whatman No. 1 filter paper and concentrated using rotary evaporator. The concentrated solution was freeze-dried to get 1.8 g of powdered extract. The powdered extract was dissolved in culture medium and sterilized using a 0.25-μm pore size filter before treatment.

#### 2.2. Cell culture

GT1-1 is a immortalized neuronal cell line developed by genetically targeting the expression of SV40 T-antigen in hypothalamic neurons of transgenic mice (Wetsel et al., 1991), and it was kindly provided by Dr K. Kim, Department of Molecular Biology, Seoul National University, Seoul, Korea. It was cultured using DMEM medium containing 10% heat-inactivated fetal calf serum at 37°C in humidified atmosphere of 95% air and 5% CO$_2$, and was successively subcultured every 2–3 days.

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Fig. 1. Internucleosomal DNA fragmentation in GT1-1 mouse hypothalamic neuronal cells treated with H$_2$O$_2$ for 16 h. SM is DNA size marker.
2.3. Internucleosomal DNA fragmentation

Cells were harvested and suspended in 500 µl of lysis buffer containing 20 mM Tris–HCl (pH 7.4), 4 mM EDTA, 0.4% (v/v) Triton X-100 and 10 µg/ml digitonin, and incubated on ice for 30 min. After centrifugation for 5 min at 13 000 rpm, supernatants were collected. Each supernatant was extracted with phenol three times and once with chloroform. Then, DNA was precipitated by incubating at −80°C for 30 min after the addition of 1 µg of glycogen, 100 µl of 5 M NaCl, and 700 µl of isopropanol. DNA was collected by centrifuging at 13 000 rpm for 5 min, and washed once with 70% ethanol. DNA pellets were dissolved in 30 µl of TE buffer containing 10 µg/ml RNase A, and incubated at 37°C for 30 min. Ten milliliters of each DNA samples were loaded on a 1.8% agarose gel.

2.4. Flow cytometer analysis

Cells were harvested and washed once with cold phosphate buffered saline. Then cell pellets were suspended in 500 µl of staining solution containing 50 µg/ml of propidium iodide, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40. Cell samples were incubated at 4°C in the dark for at least 15 min, and analyzed using flow cytometer (FACSCalibur, Beckton Dickinson, USA). The percentage of apoptotic cell population was measured using Cell Quest Software (Beckton Dickinson, USA).

2.5. Fractionation of O. japonicus extract

Two hundred grams of dry O. japonicus (whole plant) was extracted in 2 l of 80% methanol and filtered using Whatman No. 1 filter paper. After
removing all methanol using a rotary evaporator, the remaining solution was extracted with 500 ml of chloroform three times. The chloroform fractions were combined, and the remaining solution was extracted with 500 ml of ethyl acetate three times to get ethyl acetate fraction. The remaining water-soluble fraction, ethyl acetate fraction and chloroform fraction were evaporated using rotary evaporator to get powdered extracts. Finally, 3.0 g of chloroform fraction, 0.5 g of ethyl acetate fraction, and 2.3 g of water fraction were obtained.

Fig. 3. Protective effect of *O. japonicus* methanol extract (OJEx) on H$_2$O$_2$-induced morphological change in GT1-1 neuronal cells. Cellular morphological changes were observed using a phase contrast microscope at the magnitude of 100.

Fig. 4. Flow cytometer analysis of the protective effect of *O. japonicus* extract on H$_2$O$_2$-induced apoptosis. (A) Control cells; (B) cells treated with 750 μM of H$_2$O$_2$ for 16 h; (C) cells treated with 750 μM of H$_2$O$_2$ and 500 μg/ml of *O. japonicus* methanol extract for 16 h. The description of each peak is same as that of Fig. 2.

3. Results

H$_2$O$_2$-induced cell death in GT1-1 mouse hypothalamic neuronal cell line was accompanied by internucleosomal DNA fragmentation which is characteristic of apoptosis (Fig. 1). H$_2$O$_2$ treatment induced the cellular morphological changes
including degeneration of cellular processes and detachment from the culture dishes (data not shown). Flow cytometer analysis of propidium iodide-stained GT1-1 neuronal cell line treated with various concentration of H$_2$O$_2$ is shown in Fig. 2. The percentage of apoptotic cell population was 8.5% after 500 μM of H$_2$O$_2$ treatment, and increased to 91% after 750 μM of H$_2$O$_2$ treatment. After 1 mM H$_2$O$_2$ treatment, 100% of cells became apoptotic. Fig. 3 shows protective effect of O. japonicus extract on H$_2$O$_2$-induced apoptosis in GT1-1 cells. Cells treated with 750 μM of H$_2$O$_2$ showed morphological changes of cellular process degeneration, rounding up, and detachment from the culture dish, but co-treatment of 500 μg/ml of O. japonicus methanol extract inhibited morphological changes induced by H$_2$O$_2$. O. japonicus extract alone has no effect on cell morphology (data not shown). Protective effect of O. japonicus extract was confirmed by flow cytometer analysis (Fig. 4). The treatment of 750 μM H$_2$O$_2$ induced apoptosis in 85% of cell populations, but the co-treatment of 500 μg/ml of O. japonicus methanol extract reduced it to 15%.

To fractionate active ingredients, O. japonicus methanol extract was serially extracted with chloroform and ethyl acetate as described in Section 2, and each fraction was tested for neuro-protective effects (Fig. 5). Each fraction of 50 μg/ml concentration was co-treated with H$_2$O$_2$ for 16 h and the effects were analyzed using flow cytometer. Chloroform fraction markedly reduced apoptotic cell population from 42 to 4%, Ethyl acetate fraction showed weak protective effect reducing apoptosis from 42 to 22%, but water fraction showed no protective effect at all. These results suggest that the active ingredients of O. japonicus might be hydrophobic compounds and concentrated in chloroform fraction.

Fig. 5. Flow cytometric analysis of the protective effects of O. japonicus fractions on H$_2$O$_2$-induced apoptosis. GT1-1 cells were treated with 750 μM of H$_2$O$_2$ alone or with 50 μg/ml of each fraction (chloroform Fr., EtOAc Fr., and water Fr.) for 16 h. Apoptotic cell populations were marked as M1. Abbreviation: Fr, fraction.
4. Discussion

Oxygen-derived free radical generation has been implicated in neuronal cell death after acute injury such as ischemia-reperfusion or trauma (Traystman et al., 1991). In particular, superoxide anion (O$_2^-$), which has limited toxic effect in itself, can be converted into H$_2$O$_2$ by the action of superoxide dismutase. In turn, H$_2$O$_2$ exerts its toxic effects mainly through the formation of the highly reactive hydroxy radical (OH$^-$), which leads to alterations of lipids, proteins, and DNA (Halliwell, 1992). Probably less important, the modification of the redox thiol status of the cytosol could also contribute to H$_2$O$_2$ toxicity. Under pathological situations such as ischemia-reperfusion, various cell types including neurons produce large amounts of H$_2$O$_2$. In this study, O. japonicus extract showed neuro-protective effect on H$_2$O$_2$-induced apoptosis in GT1-1 mouse hypothalamic neuronal cell line. Although, it should be confirmed by primary neuronal culture and in vivo study, O. japonicus has a potential to be used for prevention and treatment of neurodegenerative disease.

O. japonicus A. Berger is a perenial herbaceous plant which has been traditionally used as an anti-inflammatory agent to treat hepatitis, boils, and piles, and also as a hemostatic agent for the treatment of vomiting blood, nose bleeding, and blood excrement. The traditional use of O. japonicus A. Berger suggests that it has effects on reducing inflammation and improving platelet function. Recent studies suggest the role of H$_2$O$_2$ in inflammation and platelet function. It has been reported that H$_2$O$_2$ is produced by leukocytes and induces tissue damage during inflammation (Schalkwijk et al., 1986; Dannenberg et al., 1994). Naseem et al. (1996) proposed that H$_2$O$_2$ plays a role in the inhibition of platelet aggregation. The anti-inflammatory and hemostatic usage in traditional medicine and neuro-protective effects shown in this study imply that the effects of O. japonicus are related to the modulation of H$_2$O$_2$.

References