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Effect of *Rhizoma gastrodiae* on glucose oxydase induced neurotoxicity in cultured mouse spinal dorsal root ganglion neurons

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Running title : Effect of *Rhizoma gastrodiae* on glucose oxydase induced neurotoxicity

SUMMARY

Effects of *Rhizoma gastrodiae* on glucose oxidase-induced neurotoxicity was investigated in cultured newborn mouse spinal dorsal root ganglion(DRG) neurons that were treated in the media with or without glucose oxidase. In addition, the protective effect of *Rhizoma gastrodiae* extract against glucose oxidase-induced neurotoxicity was examined. Cytotoxic values were expressed as a percentage of number of living cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this paper, exposure of neurons to glucose oxidase resulted in a significant call death in a dose- and time-dependent manners in DRG neuron cultures. The decrease in cell viability induced by the glucose oxidase was blocked by Rhizoma gastrodiae extract. These results indicate that the neuroprotective effect of *Rhizoma gastrodiae* extract against glucose oxidase-induced neurotoxicity may result from a prevention or attenuation of oxidative damage induced by glucose oxidase.

Key Words: Cultured DRG neuron; Oxidative stress; Rhizoma gastrodiae extract
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INTRODUCTION

Oxygen free radicals have been implicated in cerebral ischemia, Alzheimer disease and Huntington's disease (Choi, 1987; Difazio *et al.*, 1992). Several studies have suggested that an increase in oxygen radical flux lipid peroxidation are considered as important pathophysiological events in a variety of neurological diseases (Amamoto *et al.*,1983 and Lundgren *et al.*, 1991). The role of oxygen radicals lipid peroxidation is a focus of attention in the central nervous system (CNS) and peripheral nervous system(PNS) (Hall and Braughler, 1986; Rice-Evans and Diplock, 1993). Cell damage as a result of oxidant production occurs at several levels, and results from the generation of various toxic oxygen species such as superoxide or hydrogen peroxide (Chan and fishman, 1978) Many investigators have suggested that lipid peroxidation may be implicated in the

irreversible loss of neuronal function after brain or spinal cord injury (Hall and Braughler, 1989). It has been well known that many of enzymes in cytoplasm can generate oxygen radicals which have various possible targets (Dykens *et al.*, 1987 and Harken *et al.*, 1988), such as membranes and DNA. Among of them, hydrogen peroxide contributed by several enzymes causes breakdown of ATP, ultimately yielding hypoxanthine and xanthine, or glucose. A recent study has reported that a variant of familial amyotrophic lateral sclerosis (ALS) is linked to defects of the superoxide dismutase (SOD)-1 gene, which encodes cytosolic Cu, Zn-SOD (Rosen *et al.*, 1993). The SOD-1 injury would be an accumulation of highly toxic superoxide radical in the nervous system of ALS patients leading to neuronal injury. Taken together, this result strongly suggests a pathogenic role of oxygen radicals in neurological disease such as ALS. Several common neurological diseases are suspected to involve a combination of interacting excitotatory amino acids (EAAs) and oxygen radicas (Pellegrini-Giampietro *et al.*, 1988; Lundgren *et al.*, 1991).

There are evidences that antioxidant therapy has been suggested for several neural diseases (Rice-Evans and Diplock, 1993; Saunders *et al.*,1987; Liyod *et al.*, 1991). Recently, traditional oriental herbal medicinal prescription has been used for the management of various neurological diseases in Korea. However it has been left unkown how they could prevent neurological disease in studies correlated with oxygen radical-mediated neurological diseases (Dexter *et al.*, 1989).

In the present study, we examined the neuronal cell injury induced by glucose oxidase in spinal dorsal root ganglion (DRG) neurons derived from newborn mouse. Protective effects of oriental herbal medicinal extraction, Rhizoma gastrodiae against oxygen radical-induced neurotoxicity also are described.

MATERIALS AND METHODS

Cell culture

Newborn mouse spinal dorsal root ganglion (DRG) neurons were cultured in 96 multiwell plates as described previously (Michikawa *et al.*, 1994). Primary cultures of DRG neurons were prepared from newborn mice. Dissociated DRG neurons were washed three times with Dulbecco's phosphate-buffered saline (PBS), and centrifugated at 80×g. The single cells were divided in 96 multiwells coated with poly-L-lysine. A cell density was 1×10^5 cells/well, and cultures were grown in 5% CO_2/95% air atmosphere at 37°C. Cells were used for these experiments after 10-14 days in culture.

Chemicals

Glucose oxidase (GO) (type×from Aspergillus niger, G8135) were purchased from Sigma Chemicals (St. Louis, Mo).

Preparation of Rhizoma gastrodiae (RG)

An extract of RG was prepared by dissolving the dried powder of herbs with distilled water. The extract was filtered, and then stored at 4°C before use. This materials was obtained from College of Oriental Medicine, Wonkwang University.

Exposure to oxygen radicals

Spinal dorsal root ganglion (DRG) neurons derived from newborn mouse were washed three times with PBS, and incubated with the media containing various concentrations of Rhizoma gastrodiae extract for 2 hrs at 37°C, 5% CO_2/95% air. After the incubation, cells were washed and treated with 5-20 mU/ml glucose oxidase for 1-7 hrs respectively, and processed for MTT assay.

MTT cytotoxicity assay

This assay was performed by the method of Mosman et al. (1983). After appropriate incubation periods for the determination of cytotoxicity, final concentration of MTT stock solution (5 mg/ml) was added to each well, and incubated for 4 hrs at 37°C, 5% CO_2/95% air. After incubation, 96-well plates with cultures were measured on a Dynatech Microelisa reader at a wavelength of 570 nm.

Statistical analysis

Data was expressed as mean ± S.D. The Student's t-test was used and a p-value of less than 0.05 was determined to be statistically significant.

RESULTS

Cytotoxicity of glucose oxidase

To evaluate the dose-reponse relationship of glucose oxidase (GO)-induced neurotoxicity on the cultured mouse spinal dorsal root ganglion (DRG) neurons, cells in 96 multiwells were exposed to concentrations of 5, 10, 15 and 20 mU/ml GO for 5 hrs, and then processed for the MTT assay (Fig. 1). At 5 mU/ml of GO, the number of living cells was about 73% of all the unexposed cells. At 10 mU/ml of GO, 64% of total cell popuration survived of GO-induced cytotoxicity. GO at a concentration of 15 mU/ml was 48% in cell viability after 5 hrs of exposure. At 20 mU/ml of XO, cell survival was reduced to 33% of the control. The sufficient concentration of XO to cause cell death at more than 50% was found to be 15 mU/ml of XO concentration. The effects of glucose oxidase incubation time on cell survival are shown in Fig. 2. At 15 mU/ml of GO, the number of cells which were stained with MTT solutions was 67% after incubation of 1 hr of exposure. At 15 mU/ml of GO, cell viability was 59% after 3 hours of exposure, At 15 mU/ml of GO, cell survival was reduced to 52% of the control after incubation of 5 hrs of exposure. At 15 mU/ml of GO, the cell viability was 43% after incubation of 7 hrs of exposure to GO-induced oxygen radicals (Fig. 2).

The effects of Rhizoma gastrodiae (RG) extract on GO-mediated cytotoxicity

Neuroprotective effect of RG extract tested for its ability to protect against GO-mediated cytotoxicity in the cultured mouse (DRG) neuron cultures. In this study, cultures were incubated in the media containing various concentrations of RG extract for 2 hrs, and then cultures were exposed to 15 mU/ml of GO for 5 hrs. Cultures were processed for MTT assay. GO at 15 mU/ml concentration alone for 5 hrs caused cell death in 35% of cell populations (Fig. 3).

RG extract, herbal medicine extract, showed significant protection against GO-induced neurotoxicity in cultured mouse spinal DRG neurons. At 10 μg/ml of RG extract, the cell viability was 62% of the control. At 30 μg/ml of RG extract, cell survival was increased to 72% of the control, At a 50 μg/ml concentration of RG extract, the cell survival rate was 89% in spinal DRG neuron cultures (Fig. 3).

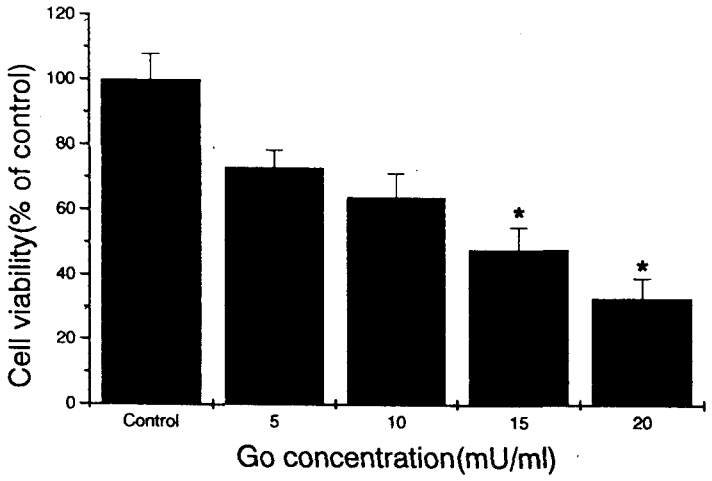

Fig 1. A Dose-dependency of glucose oxidase(GO). GO-induced neurotoxicity was measured by MTT assay in cultured mouse DRG neurons. Cultures were exposed to 5, 10, 15 and 20 mU/ml GO for 5 hours, respectively. The results indicate the mean ± SEM for 6 experiments. Significant difference from the control are marked with asterisks (*p<0.05;**p<0.01)

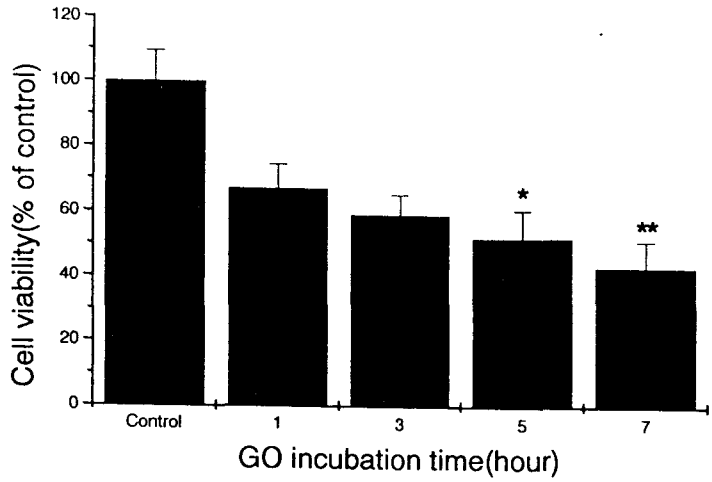

Fig 2. A dose-dependency of glucose oxidase(GO). GO-induced neurotoxicity was measured by MTT assay in cultured mouse DRG neurons. Cultures were exposed to 15mU/ml GO for 1, 3, 5 and 7 hours, respectively. The results indicate the mean ± SEM for 6 experiments. Significant difference from the control are marked with asterisks(*p<0.05;**p<0.01)

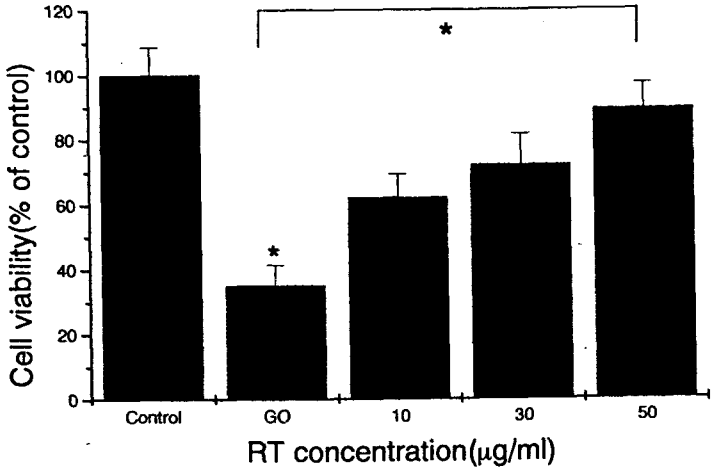

Fig 3. Dose-response relationship of Rhizoma gastrodiae(RG) for its neuroprotecitve effect on GO-induced neurotozicity by MTT assay in cultured mouse DRG neurons. Cultures were preincubated with RG for 2 hours before exposed to GO. The results indicate the mean ± SEM for 6 experiments. Asterisk indicate the significant difference(☆$p<0.05$) between groups. Other legends are same as in Fig. 1.

DISCUSSION

The purpose of this study was to determine the toxic effect of GO-induced oxygen radical neurotoxicity on cultured spinal DRG neurons derived from newborn mouse and to examine the potential protective effect of herbal extract of oriental medicine against oxygen radical-induced neurotoxicity. For this study, we used in vitro cytotoxicity assay (Francois and Lang 1886). It is known that the cell membrane is a primary site of oxygen radical mediated injury, owing to its content of peroxidizable unsaturated lipids and proteins and to its physical proximity both of intracellular and extracellular radical sources (Arslan *et al* 1985). In the present study, the cultured spinal DRG neurons treated with glucose oxidase resulted in decreasing the survival of DRG neurons. From this result, it is suggested that glucose oxidase has neurotoxic effect. For this reason, glucose oxidase produces oxygen radicals in culture medium (Rubin and Faber, 1984), and glucose oxidase- mediated oxygen radicas resulted in directly killing the cultured spinal DRG neurons (Slivka and Cohen, 1985; Kim and Kim, 1991). Therefore, oxygen radical-derived product was the actual mediator of cell damage, as has previously been demonstrated in cultured mouse spinal cord neurons (Michikawa *et al.*, 1994) and rat alveolar type II cells (Buckley *et al.*, 1987). Also, we could observe the protective effect in herbal extrct of oriental medicine on oxygen radical-induced neurotoxicity when Rhizoma gastrodiae extract were added 2 hrs before treatment of glucose oxidase in these experiments. In this study, the cell viability decreased by glucose oxidase was significantly increased by Rhizoma gastrodiae extract.

From our results, it is suggested that Rhizoma gastrodiae extract, which was taken up by neurons during the preincubation period before exposure to 15 mU/ml of glucose oxidase for 5 hours, protected neurons from the glucose oxidase-induced neurotoxicity. The mechanism of protective effect of Rhizoma gastrodiae extract against oxygen radical-induced neurotoxicity is not clear at present, but the possible mechanism of protective effect of herbal extract may be related with removal of oxygen radicals such as hydroxyl radicals or superoxide. However, it must be confirmed by further studies biochemically or physiologically. In conclusion, we demonstrated that oxygen radicals generated by the enzymatic system induced lethal toxic effect on cultured mouse spinal DRG neurons which have important function in the perception of environmental stimuli of human body, and Rhizoa gastrodiae was effective in attenuation of oxygen radical-induced neurotoxicity in these cultures. It is also important to note that culture system is a very useful tool for screening the putative agent such as oriental herbal medicine or extract of natural product for therapy of neurological disease.

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