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Effect of *Rhizoma gastrodiae* on oxidative stress in cultured mouse spinal motor neurons

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SUMMARY

To clarify the toxic effect of oxidative stress, hydrogen peroxide (H_2O_2)-induced neurotoxicity was examined in cultured newborn mouse spinal motor neurons after spinal motor neurons were grown in the media containing various concentrations of glucose oxidase (GO). And also, the protective effect of *Rhizoma gastrodiae* extract against GO-induced neurotoxicity was evaluated. Cytotoxicity was expressed as a cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this study, exposure of motor neurons to GO-induced cell death significantly, in a dose- and time-dependent manners in spinal motor neuron cultures. The decrease in cell viability of motor neurons damaged by GO was proventioned by *Rhizoma gastrodiae* extract. These results suggest that the neuroprotective effect of *Rhizoma gastrodiae* extract on GO-induced neurotoxicity may result from a attenuation of H_2O_2-induced oxidative stress.

INTRODUCTION

It is well known that Parkinsonism, Alzheimer disease as well as amyotrophic lateral sclerosis (ALS) are induced by oxidative stress of oxygen free radicals (Choi, 1987; Difazio *et al.*, 1992). Recent studies have suggested that both of accumulation of oxygen radicals and an injury of antioxidant system bring about considered important pathophysiological events in a variety of neurological diseases(Lundgren *et al.*, 1991). Therfore,

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The oxidative stress of oxygen radicals play a key role in neurological diseases of the central nervous system and peripheral nervous system (Hall and Braughler, 1986; Rice-Evans and Diplock, 1993). Oxygen free radicals such as superoxide or hydrogen peroxide damage neuronal cells by oxidant production at several levels, and results from the cell degeneration and cell death (Chan and fishman, 1978). Many studies have suggested that oxygen free radicals iniciate lipid peroxidation chains of cells and they may be implicated in the serious loss of neuronal function after neuronal cells are injured (Hall and Braughler, 1989). It has been suggested that various kind of oxygen radicals generated from enzymes have active targets (Dykens et al.,1987; Harken et al.,1988), such as antioxidants, cell membranes and DNA. Among of them, glucose oxidase (GO) causes breakdown of ATP following to contribution of xanthine, or glucose. A recent study reported that acumulation of a lots of oxygen free radicals induce variant of familial amyotrophic lateral sclerosis (ALS) by the mutation of the superoxide dismutase (SOD)-1 gene. The injury of this gene lead to an accumulation of highly toxic superoxide radical in the nervous system of ALS patients leading to neuronal injury. This result implys that oxygen radicals are a causative factor in neurological disease such as ALS. Several studies have been demonstrated that oxygen free radicals secrete excitotatory amino acids (EAAs) which activate N-methyl-D-aspartate (NMDA) leading to increase intracellular calcium level (Pellegrini-Giampietro et al., 1988; Lundgren *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 GO in cultured mouse spinal motor neurons. In addition, protective effects of oriental herbal medicinal extraction, *Rhizoma gastrodiae* (RG) against GO-induced neurotoxicity is assessd.

MATERIALS AND METHODS

Cell culture

Spinal motor neurons derived from mouse were grown in 96 multiwell plates as described previously (Michikawa *et al.*, 1994). Spinal motor neurons were prepared by enzymatical dissociation from newborn mice. Dissociated spinal motor neurons were washed three times with Dulbecco's phosphate-buffered saline (PBS), and centrifugated at 80 × g. The single cells were harvsted and divided in 96 multiwells coated with poly-L-lysine. Cells were plated as a density of 1×10^5 cells/well, and cells were incubated in 5% CO_2/95% air atmosphere at 37℃. Cells were used for these experiments after 5-10 days in culture.

Chemicals

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

Preparation of 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℃ before use. This materials was obtained from College of Oriental Medicine, Wonkwang University.

Exposure to oxygen radicals

Spinal motor 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 hours at 37℃, 5% CO_2/95% air. After the incubation, cells were washed and treated with 5~20 mU/ml glucose oxidase for 1-7 hours respectively, and processed for MTT assay.

MTT cytotoxicity assay

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

2.6. Statistical analysis

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

RESULTS

Cytotoxicity of glucose oxidase

To measure the dose-reponse relationship of glucose oxidase (GO)-induced neurotoxicity on the cultured mouse spinal motor neurons, cells in 96 multiwells were exposed to concentrations of 1, 5, 10 and 15mU/㎖ GO for 5 hours, and then processed for the MTT assay. At 1mU/ml of GO, the number of living cells was about 67% of all the unexposed cells. At 5mU/ml of GO, 62% of total cell population survived of GO-induced cytotoxicity. GO at a concentration of 10mU/㎖ was 51% in cell viability after 5 hours of exposure. At 15mU/ml of GO, cell survival was reduced to 48% of the control. The midcytotoxic value (MCV) of GO to cause cell death at more than 50% was found to be 10mU/ml of GO concentration (Fig. 1).

The effects of GO incubation time on cell survival are shown in Fig. 2. At 10mU/㎖ of GO, the number of cells which were stained with MTT solutions was 78% after incubation of 1 hour of exposure. At 10mU/㎖ of GO, cell viability was 62% after 2 hours of exposure, At 10mU/ml of GO, cell survival was reduced to 48% of the control after incubation of 3 hours of exposure. At 10mU/㎖ of GO, the cell viability was 23% after incubation of 4 hours of exposure to GO-induced oxygen radicals (Fig. 2).

The effects of RG extract on GO mediated cytotoxicity

Protective effect of RG extract tested for its ability to protect against GO-mediated cytotoxicity in the cultured mouse spinal motor neuron cultures. In this study, cultures were incubated in the media containing various concentrations of RG extract for 2 hours, and then cultures were exposed to 10mU/㎖ of GO for 5 hours. Cultures were processed for MTT assay. GO at 10mU/㎖ concentration alone for 5 hours caused cell death in 47% of cell populations (Fig. 3).

RG extract, herbal medicine extract, showed significant protection against GO-induced neurotoxicity in cultured mouse spinal motor neurons. At 25 ㎍/㎖ of RG extract, the cell viability was 73% of the control. At 50 ㎍/㎖ of RG extract, cell survival was increased to 84% of the control, At a 100 ㎍/㎖ concentration of RG extract, the cell survival rate was 89% in spinal motor neuron cultures (Fig. 3).

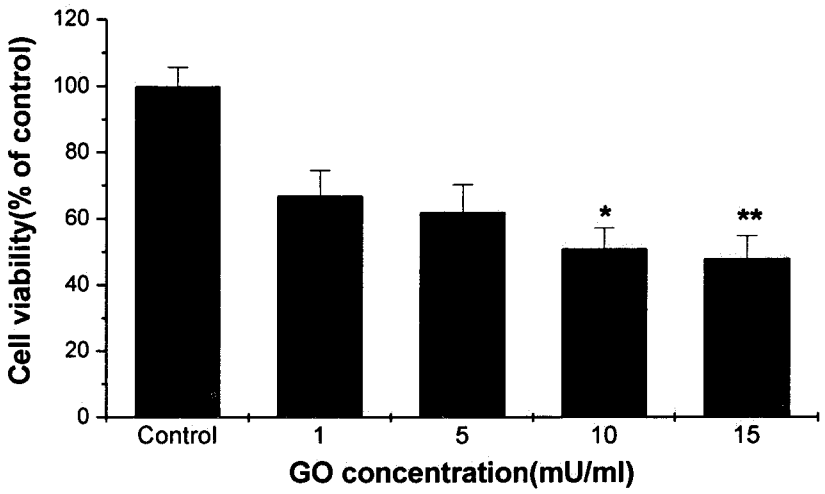

Fig. 1. A dose-dependency of GO. GO-induced neurotoxicity was measured by MTT assay in cultured mouse spinal motor neurons. Cultures were exposed to 1, 5, 10 and 15 mU/㎖ GO for 5 hours, respectively. The results indicate the mean ± SEM for 6 experiments. Significant difference from the control are marked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

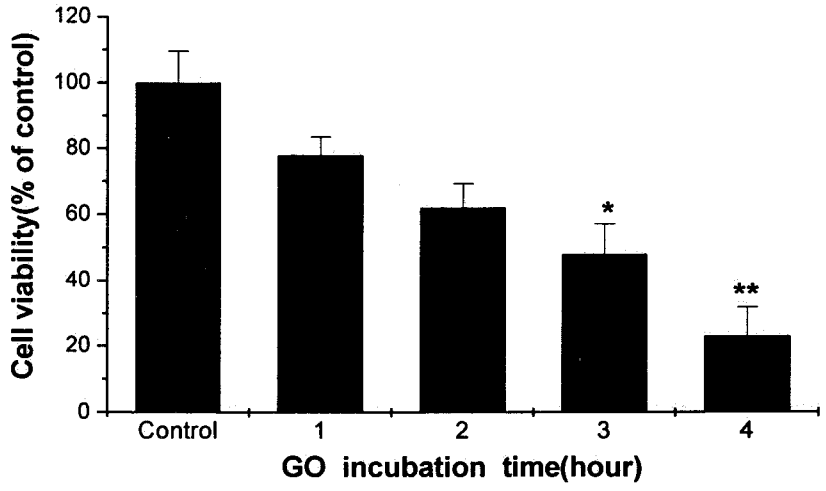

Fig. 2. A dose-dependency of GO. GO-induced neurotoxicity was measured by MTT assay in cultured mouse spinal motor neurons. Cultures were exposed to 10mU/㎖ GO for 1, 2, 3 and 4 hours, respectively. The results indicate the mean ± SEM for 6 experiments. Significant difference from the control are marked with asterisks (*p<0.05, **p<0.01)

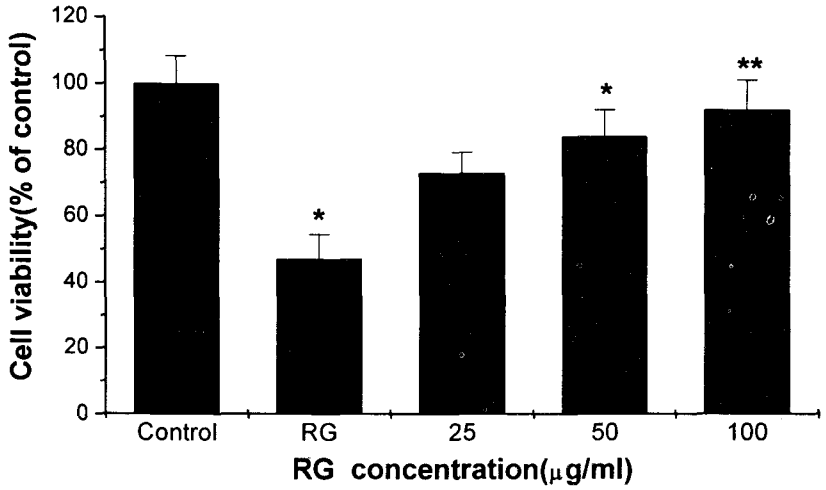

Fig. 3. Dose-response relationship of RG for its neuroprotective effect on GO-induced neurotoxicity by MTT assay in cultured mouse spinal motor neurons. Cultures were preincubated with RG for 2 hours before exposed to GO. The results indicate the mean ± SEM for 6 experiments. Significant difference from the control are marked with asterisks (*$p<0.05$, **$p<0.01$)

DISCUSSION

We examined the toxic effect of GO-induced oxygen radical neurotoxicity on cultured spinal motor neurons derived from newborn mouse and to evaluate the potential protective effect of herbal extract of oriental medicine against glucose oxidase (GO)-induced neurotoxicity. For this study, we applied MTT assay (Mosmann, 1983). 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 (Francois and Lang 1886). In the present study, the cultured spinal motor neurons treated with GO resulted in decreasing the survival of spinal motor neurons. From this result, it is suggested that GO has neurotoxic effect on cultured mouse spinal motor neurons. For this reason, GO produces oxygen radicals in culture medium (Kim and Kim, 1991), and glucose oxidase-mediated oxygen radicas resulted in directly killing the cultured spinal motor neurons (Slivka and Cohen, 1985; Rubin and Faber, 1993). Therefore, oxygen radical-derived product was the actual mediator of cell damage, as has previously been demonstrated in cultured mouse spinal cord neurons (Kim and Kim, 1991; Michikawa *et al.*, 1994) and rat alveolar type II cells (Buckley *et al.*, 1987). Also, we could observed the protective effect in herbal extrct of oriental

medicine on oxygen radical-induced neurotoxicity when Rhizoma gastrodiae extract were added 2 hours before treatment of GO in these experiments. In this study, the cell viability decreased by glucose oxidase was significantly increased by Rhizoma gastrodiae extract. From the our results, it suggests that RG extract, which was taken up by neurons during the preincubation period before exposure to 10mU/ml of glucose oxidase for 5 hours, protected neurons from the GO-induced neurotoxicity. The mechanism of protective effect of RG extract against GO-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. From these results, we conclued that oxygen radicals generated by the enzymatic system induced lethal toxic effect on cultured mouse spinal motor neurons playing a key role in the perception of environmental stimuli of human body, and also, it is implyed that herbal extract such as RG is effective in attenuation of oxygen radicalinduced neurotoxicity in these cultures.

REFERENCES

Burkley BA, Tanswell AK, Freeman BA(1987) Liposome-mediated augmentation of catalase in alveolar type II cells protects against H_2O_2 injury. *J. Appl Physiol.* **63**, 359-367.

Chan, P.H. and Fishman, R.A. (1978) Transient formation of superoxide radicals in polyunsaturated fatty acid induced brain swelling, *J. Neur. Chem.* **35**, 1004-1007.

Choi, D.W. (1987) Ionic dependence of glutamate neurotoxicity, *J. Neurosci.* **7**, 369-379.

Difazio MC, Hollingsworth Z, Young AB, Penny JB(1992) Glutamate receptor im the substentia nigra of Parkinson's disease brains. *Neurology* **42**, 402-406.

Dexter, D.T., Wells, F.R., Lees, A.J., Agid, F., Agid, Y., Jenner, P. and Marsden C.D. (1989) Increased nigral iron content and alteration in other metal ions occuring in brain in Parkinson's disease, *J. Neur. Chem.* **52**, 1830-1836.

Dykens, J., Stern, A. and Trenkner, E. (1987) Mechanism of kainate toxicity to cerebellar neurons in vitro is analogous to reperfusion tissue injury, *J. Neurochem.* **49**, 1222-1228.

Francois, D. and Lang, L. (1986) Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, *J. Immmuno. Meth.* **89**, 271-277.

Hall, E.D. and Braughler, J.M. (1986) Role of lipid peroxidation in post-traumatic spinal cord degeneration, *CNS Trauma* **3**, 281-294.

Hall, E.D. and Braughler, J.M. (1989) Central nervous system trauma and stroke. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation, *Free Rad. Biol. Med.* **6**, 303-313.

Harken, P.A., Burton, L.S., Rodell, T.C., Piermattei, D., Schorr, W.J., Parker, N.B., Berger, E.M., Horesh, I.R., Terada, L.S., Linas, S.L., Cheronis, J.C. and Repine, J.E. (1988) Xanthine oxidase-derived hydrogen peroxide contributes to ischemia reperfusion-induced edema in gerbil brain, *J. Clin. Invest.* **81**, 1556-1562.

Kim, Y.S. and Kim, S.U. (1991) Oligodendroglial cell death induced by oxygen radicals and its protection by catalase, *J. Neurosci. Res.* **29**, 100-106.

Lioyd, J.B., Cable, H. and Rice-Evans, C.A.

(1991) Evidence that deferoxamine cannot enter cells by passive diffusion. *Biochem. Pharmacol.* **41**, 1361-1366.

Lundgren, J., Zhang, H., Agardh, C.D., Smith, M.L., Evans, P.J., Haliwell, B. and Siesjoe, B.K. (1991) Acidosis-induced ishemic brain damage: Are free radicals involved? *J. Cereb. Blood Flow Meta.* **11**, 587-596.

Michikawa, M., Lim, K.T., McLarnon, J.G. and Kim, S.U. (1994) Oxygen radical-induced neurotoxicity in spinal cord neuron cultures. *J. Neurosci Res.* **37**, 62-70.

Mosmann, T. (1983) Rapid colorimetric assay for growth and survival: Application to proliferation and cytotoxicity assays. *J. Immuno. Meth.* **65**, 55-63.

Pellegrini-Giampietro, D.E., Cherici, G., Alesiani, M., Carla, V. and Moroni, F. (1988) Excitatory amino acids release from rat hippocampal slices as a consequence of free radical formation. *J. Neurochem.* **51**, 1960-1963.

Rice-Evans, C.A. and Diplock, A.T. (1993) Current status of antioxidant therapy. *Free Rad. Biol. & Med.* **15**, 77-96.

Rosen D, Siddique T, Patterson D, Figlewicz D, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan J, Deng H, Rahmani Z, Krizus A, (1993) Mutatuon in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature(London).* **362**, 59-62.

Rubin, R. and Faber, J.L. (1984) Mechanism of killing of cultured hepatocytes by hydrogen peroxide. *Arch. Biochem. Biophys.* **228**, 450-459.

Saunders, R.D., Dugan, L.I., Demediuk, P., Means, E.C., Harrocks, L.A. and Anderson, D.K. (1987) Effects of methylprednisolone and the combination of alphatocopherol and selenium on arachidonic acid metabolism and lipid peroxidation in traumatized spinal cord tissue. *J Neurochem.* **49**, 24-31.

Slivka, A. and Cohen, G. (1985) Hydroxyl radical attack on dopamine, *J. Biol. Chem.* **260**, 15466-15472.