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Effect of Herba Epimedii on hydrogen peroxide induced neurotoxicity in cultured rat dorsal root ganglion neurons

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SUMMARY

Effects of hydrogen peroxide (H_2O_2)-induced neurotoxicity were investigated in cultured newborn rat spinal dorsal root ganglion (DRG) neurons after DRG neurons were treated in the media containning various concentrations of H_2O_2. In addition, the protective effect of Herba Epimedii (HE) extract against H_2O_2-induced neurotoxicity was examined. Cytotoxic values were determined by the cell viability of living cells using 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay. In the present study, exposure of neurons to H_2O_2 resulted in a significant cell death in a dose- and time-dependent manners in cultured DRG neurons. The decrement of cell viability by H_2O_2 was blocked by HE. These results suggest that the neuroprotective effect of HE against H_2O_2-induced cytotoxicity may result from the prevention of injury induced by H_2O_2.

Key words: Hydrogen peroxide; Cultured DRG neuron; Herba Epimedii

INTRODUCTION

Hydrogen peroxide (H_2O_2) has beeen known as a reactive oxygen intermediate (ROI). ROI such as H_2O_2 play a key role in multiple sclerosis, brain ischemia and Huntington's disease (Choi, 1987; Difazio *et al.*, 1992). Many of study have suggested that ROI induces intracellular calcium flux and lipid peroxidation which are considered as important pathophysiological events in a variety of neurological diseases (Agranoff, 1984 and Lundgren *et al.*, 1991). ROI cascade the lipid peroxidation and cell injury in the central nervous system (CNS) or peripheral nervous system (PNS) (Hall and Braughler, 1986; Rice-Evans and Diplock, 1993). Metabolic products of ROI result in the generation of various toxic oxygen species such as superoxide or hydrogen peroxide (Chan and fishman, 1978). Many researches have demonstrated that oxidative stress of ROI induces the neuronal function and cell damage (Hall and Braughler, 1989). While, anti-oxidative enzymes such as superoxide dismutase (SOD) and catalase have been well known that they convert harmful oxygen species to water (Dykens *et al.*, 1987 and Harken *et al.*, 1988). Of them, hydrogen peroxide is contributed to breakdown of ATP, or the increase of protein kinase C (PKC). A study reported that amyotrophic lateral sclerosis (ALS) is due to the point mutation of the superoxide dismutase (SOD)-1 gene, which encodes cytosolic Cu, Zn-SOD (Rosen *et al.*, 1993). In addition, ROI have been strongly suggested that they are involved in the release of excitotatory amino acids (EAAs) (Pellegrini-Giampietro *et al.*, 1988; Lundgren *et al.*, 1991).

Therefore, there is evidence that N-methyl-D-aspartate (NMDA) receptor antagonist or antioxidant

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therapy has been suggested for the treatment of several neural diseases (Rice-Evans and Diplock, 1993, Saunders *et al.*, 1987, Liyod *et al.*, 1991). Recently, traditional oriental herbal medicinal prescription has been demonstrated to be effective in the management of various neurological diseases in Korea., but it has been left unkown how they could prevent neurological disease correlated with ROI - mediated neurological diseases (Dexter *et al.*, 1989).

In the present study, we examined the neuronal cell injury induced by H_2O_2 in cultured rat spinal dosal root ganglion (DRG) neurons. In addition, protective effect of oriental herbal medicinal extraction, Herba Epimedii (HE) over H_2O_2-induced neurotoxicity is described.

MATERIALS AND METHODS

Cell culture

Newborn rat spinal dorsal root ganglion (DRG) neurons were cultured in 96 multiwell plates as described previously (Michikawa *et al.*, 1994). 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. 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 7 days in culture.

Chemicals

Hydrogen peroxide (H_2O_2) was purchased from Sigma Chemicals (St. Louis, Mo).

Preparation of Herba Epimedii (HE)

An extract of HE 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 material was obtained from College of Oriental Medicine, Wonkwang University.

Exposure to oxygen radicals

Cultured rat spinal dorsal root ganglion (DRG) neurons were washed three times with PBS, and incubated with the media containing various concentrations of HE extract for 2 hours at 37°C, 5% CO_2/95% air. After the incubation, cells were washed and incubated, and processed for MTT assay.

MTT assay

This assay was performed by the method of Mosmann *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 hours 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 to significant with a *P*-value of less than 0.05.

RESULTS

Cytotoxicity of hydrogen peroxide

To evaluate the dose-reponse relationship of H_2O_2-induced neurotoxicity on the cultured rat DRG neurons, cells in 96 multiwells were exposed to concentrations of 2, 4, 6, and 8 uM H_2O_2 for 24 hours, and then processed for the MTT assay (Fig. 1). At 2 uM of H_2O_2, the number of living cells was about 72% of all the unexposed cells. At 4 uM of H_2O_2, 66% of total cell population survived of H_2O_2-induced cytotoxicity. H_2O_2 at a concentration of 6 uM was 46% ($P<0.05$) in cell viability after 24 hours of exposure. At 8 uM of H_2O_2, cell survival

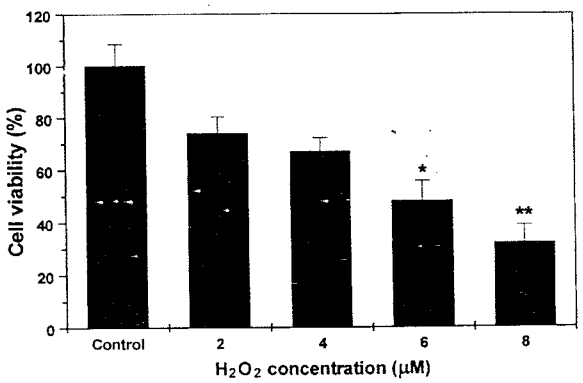

Fig. 1. Dose-response relationship of hydrogen peroxide (H_2O_2) on cultured rat spinal DRG neurons. Cytotoxicity was measured by MTT assay. The results indicate the mean±SE for 6 experiments. *$P<0.05$; **$P<0.01$.

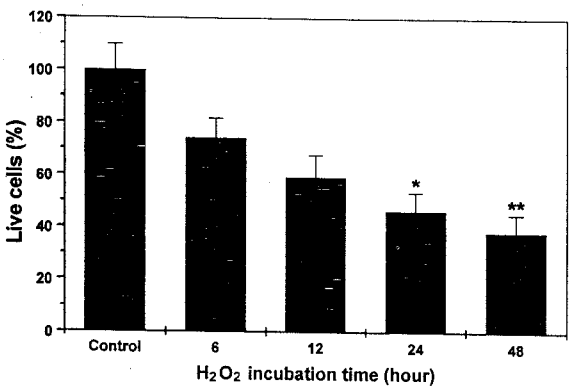

Fig. 2. Time-response relationship of hydrogen peroxide (H_2O_2) on cultured rat spinal DRG neurons. Cytotoxicity was measured by MTT assay. The results indicate the mean±SE for 6 experiments. *P<0.05; **P<0.01.

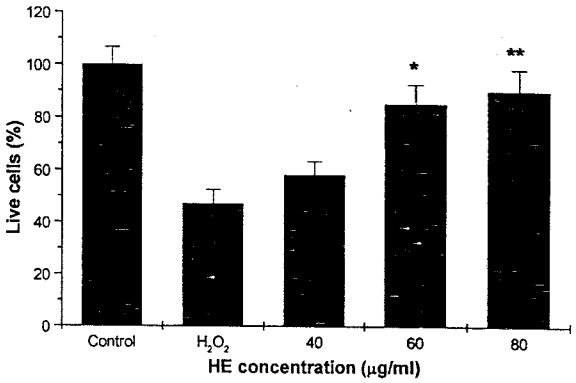

Fig. 3. Dose-response relationship of Herba Epimedii (HE) for its neuroprotective effect on hydrogen peroxide (H_2O_2)-induced cytotoxicity by MTT assay in cultured rat spinal DRG neurons. Cultured were preincubated with HE for 2 hours before the exposure of H_2O_2. The results indicate the mean±SE for 6 experiments. *P<0.05.

was reduced to 31% (P<0.01) of the control. The midcytotoxicity value (MCV) of H_2O_2 to cause cell death at more than 50% was found to be 6 uM of H_2O_2 concentration.

The effects of H_2O_2 incubation time on cell survival are shown in Fig. 2. At 6 uM of H_2O_2, the number of cells which were stained with MTT solutions was 72% after incubation of 1 hour of exposure. The cell viability was 57% after 12 hours of exposure of 6 uM H_2O_2, At 6 uM of H_2O_2, cell survival was reduced to 46% (P<0.05) of the control after incubation of 24 hours of exposure. At 6 uM of H_2O_2, the cell viability was 36% (P<0.01) after incubation of 48 hours of exposure to H_2O_2-induced oxygen radicals (Fig. 2).

The effects of Herba Epimedii(HE) extract on H_2O_2 mediated cytotoxicity

Neuroprotective effect of HE extract was tested for its ability to protect against H_2O_2-mediated cytotoxicity in DRG neuron cultures. In this study, cultures were incubated in the media containing various concentrations of HE extract for 2 hours, and then cultures were exposed to 6 uM of H_2O_2 for 24 hours. Cultures were processed for MTT assay. H_2O_2 at 6 uM concentration alone for 24 hours caused cell death in 46% of cell populations (Fig. 3).

HE, herbal medicine extract showed significant protection against H_2O_2-induced neurotoxicity in cultured mouse spinal DRG neurons. At 40 g/ml of HE extract, the cell viability was 56% of the control. At 60 g/ml of HE extract, cell survival was increased to 84% (P<0.05) of the control, At a 80 g/ml concentration of HE extract, the cell survival rate was 90% (P<0.01) in spinal DRG neuron cultures (Fig. 3).

DISCUSSION

The toxic effect of H_2O_2-induced oxidative stress on cultured rat spinal DRG neurons was determined and to the potential protective effect of herbal extract of oriental medicine was examined. For this study, we used colorimetric assay (Arslan *et al.*, 1985; Francois and Lang 1886). In the present study, the cultured spinal DRG neurons treated with H_2O_2 showed the decrease of the survival of DRG neurons. These results support the previous studies demonstrating that H_2O_2 had neurotoxic effect (Yamamoto *et al.*, 1983; Rubin and Faber, 1984), and also H_2O_2-mediated oxidative stress resulted in directly killing the cultured spinal DRG neurons (Brooksbank and Balazs, 1984; Slivka and Cohen, 1985; Kim and Kim, 1991). Kim and Kim (1993) also reported that H_2O_2 induced by glucose oxidase decreased cell viability of cultured bovine oligodendrocytes, but glucose oxidase plus catalase significantly increased cell viability against glucose oxidase-induced cytotoxicity. These results imply

that glucose oxidase-mediated H_2O_2 decreased catalase activity, and they results in the decrease of cell viability by glucose oxidase-mediated H_2O_2 means the decrease of catalase activity. Also we could observe examined the protective effect in herbal extract of oriental medicine on H_2O_2-induced oxidative stress after HE extract were pretreated for 2 hours before treatment of H_2O_2 in these experiments. In this study, the cell viability decreased by H_2O_2 was significantly increased by addition of HE extract. Our results suggests that HE extract taken up by neurons during the preincubation period before exposure to H_2O_2 protected neurons from the H_2O_2-induced oxidative stress. The mechanism of protective effect of HE extract against H_2O_2-induced neurotoxicity is not clear at present, but the possible mechanism of protective effect of herbal extract may be suggested to relate with removal of oxygen radicals such as H_2O_2 or superoxide. On the other hand, Alzheimer disease (AD) or amyolaterltrophic sclerosis (ALS) is closely related with the cell damages by oxygen free radicals(Pellegrini-Giampietro *et al.*, 1988; Rosen *et al.*, 1993). Especially, AD is based on the damage of acetylcholinergic neurons (Jackisch *et al.*, 1986). In the present study, HE effectively prevented from H_2O_2-induced neurotoxicity by the increase of cell viability. Therfore, HE may be partially responsible for the treatment of brain disease such as AD or ALS. In conclusion, we demonstrated that H_2O_2 induced lethal toxic effect on cultured rat spinal DRG neurons, and HE was effective in attenuation of H_2O_2-induced oxidative stress in these cultures.

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REFERENCES

Agranoff BW. (1984) Lipid peroxidation and membrane aging. *Neurobiol. Aging* **5**, 337-338 977-982.

Arslan P, Virgilio F, Beltrane M, Tsien RY, Pozzan T. (1985) Cytosolic Ca^{2+} homeostasis in Ehrrich and Yoshida carcinomas: A new, membrane-permeable chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca^{2+}. *J. Biol. Chem.* **260**, 2719-2727.

Brooksbank BWL, Balazs R. (1984) Superoxide dismutase. glutathione peroxidase, and lipoperoxidation in Down'syndrome fetal brain. *Dev. Brain Res.* **16**, 37-44.

Chan PH, Fishman RA. (1978) Transient formation of superoxide radicals in polyunsaturated fatty acid induced brain swelling. *J. Neur. Chem.* **35**, 1004-1007.

Choi DW. (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 DT, Wells FR, Lees AJ, Agid F, Agid Y, Jenner P, Marsden CD. (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, 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, 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 ED, Braughler JM. (1986) Role of lipid peroxidation in post-traumatic spinal cord degeneration. *CNS Trauma.* **3**, 281-294.

Hall ED, Braughler JM. (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 PA, Burton LS, Rodell TC, Piermattei D, Schorr WJ, Parker NB, Berger EM, Horesh IR, Terada LS, Linas SL, Cheronis JC, Repine JE. (1988) Xanthine oxidase-derived hydrogen perooxid contributes to ischemia reperfusion-induced edema in gerbil brain. *J. Clin. Invest.* **81**, 1556-1562.

Jackisch R, Geppert M, Brenner AS, Illes P. (1986) Presynaptic opioid receptors modulating acetylcholline release in the hippocampus of the rabbit. *Naunyn Schmiedehergs Acta Pharmacol.* **332**, 56-162.

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

Lioyd JB, Cable H, Rice-Evans CA. (1991) Evidence that deferoxamine cannot enter cells by passive diffusion. *Biochem. Pharmacol.* **41**, 1361-1366.

Lundgren J, Zhang H, Agardh CD, Smith ML, Evans PJ, Haliwell B, Siesjoe BK. (1991) Acidosis-induced ishemic brain damage: Are free radicals involved?.

J. Cereb. Blood Flow Meta. **11**, 587-596.

Michikawa M, Lim KT, McLarnon JG, Kim SU. (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 DE, Cherici G, Alesiani M, Carla V, 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 CA, Diplock AT. (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, *et al.*, (1993) Mutatuon in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* (London) **362**, 59-62.

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

Saunders RD, Dugan LI, Demediuk P, Means EC, Harrocks LA, Anderson DK. (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, Cohen G. (1985) Hydroxyl radical attack on dopamine. *J. Biol. Chem.* **260**, 15466-15472.

Yamamoto M, Shima T, Uozumi T, Sogabe T, Yamada K, Kawasaki T. (1983) A possible role of lipid peroxidation in cellular damages caused by cerebral ischemia and protective effect of alpa-tocopherol administration. *Stroke* **14**, 977-982.