

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 (H₂O₂)-induced neurotoxicity were investigated in cultured newborn rat spinal dorsal root ganglion (DRG) neurons after DRG neurons were treated in the media containing various concentrations of H₂O₂. In addition, the protective effect of Herba Epimedii (HE) extract against H₂O₂-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 H₂O₂ resulted in a significant cell death in a dose- and time-dependent manners in cultured DRG neurons. The decrement of cell viability by H₂O₂ was blocked by HE. These results suggest that the neuroprotective effect of HE against H₂O₂-induced cytotoxicity may result from the prevention of injury induced by H₂O₂.

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

INTRODUCTION

Hydrogen peroxide (H₂O₂) has been known as a reactive oxygen intermediate (ROI). ROI such as H₂O₂ 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 excitatory 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 unknown 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 H₂O₂ in cultured rat spinal dorsal root ganglion (DRG) neurons. In addition, protective effect of oriental herbal medicinal extraction, Herba Epimedii (HE) over H₂O₂-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 1×10⁵ cells/well, and cultures were grown in 5% CO₂/95% air atmosphere at 37°C. Cells were used for these experiments after 7 days in culture.

Chemicals

Hydrogen peroxide (H₂O₂) 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% CO₂/95% 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% CO₂/95% 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 H₂O₂-induced neurotoxicity on the cultured rat DRG neurons, cells in 96 multiwells were exposed to concentrations of 2, 4, 6, and 8 μM H₂O₂ for 24 hours, and then processed for the MTT assay (Fig. 1). At 2 μM of H₂O₂, the number of living cells was about 72% of all the unexposed cells. At 4 μM of H₂O₂, 66% of total cell population survived of H₂O₂-induced cytotoxicity. H₂O₂ at a concentration of 6 μM was 46% (*P*<0.05) in cell viability after 24 hours of exposure. At 8 μM of H₂O₂, cell survival



Fig. 1. Dose-response relationship of hydrogen peroxide (H₂O₂) 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.

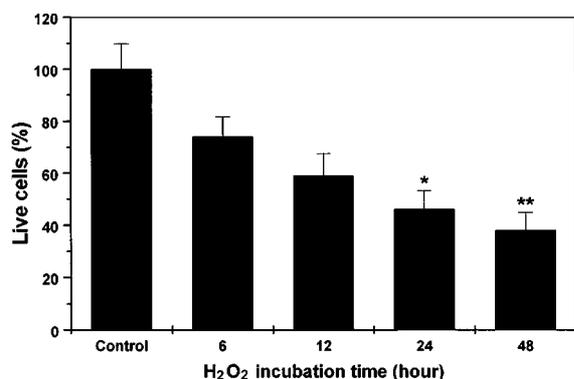


Fig. 2. Time-response relationship of hydrogen peroxide (H₂O₂) on cultured rat spinal DRG neurons. Cytotoxicity was measured by MTT assay. The results indicate the mean±SE for 6 experiments. **P*<0.05; ***P*<0.01.

was reduced to 31% (*P*<0.01) of the control. The midcytotoxicity value (MCV) of H₂O₂ to cause cell death at more than 50% was found to be 6 μM of H₂O₂ concentration.

The effects of H₂O₂ incubation time on cell survival are shown in Fig. 2. At 6 μM of H₂O₂, 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 μM H₂O₂. At 6 μM of H₂O₂, cell survival was reduced to 46% (*P*<0.05) of the control after incubation of 24 hours of exposure. At 6 μM of H₂O₂, the cell viability was 36% (*P*<0.01) after incubation of 48 hours of exposure to H₂O₂-induced oxygen radicals (Fig. 2).

The effects of Herba Epimedii(HE) extract on H₂O₂ mediated cytotoxicity

Neuroprotective effect of HE extract was tested for its ability to protect against H₂O₂-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 μM of H₂O₂ for 24 hours. Cultures were processed for MTT assay. H₂O₂ at 6 μM concentration alone for 24 hours caused cell death in 46% of cell populations (Fig. 3).

HE, herbal medicine extract showed significant protection against H₂O₂-induced neurotoxicity in cultured mouse spinal DRG neurons. At 40 g/ml of HE extract, the cell viability was 56% of the

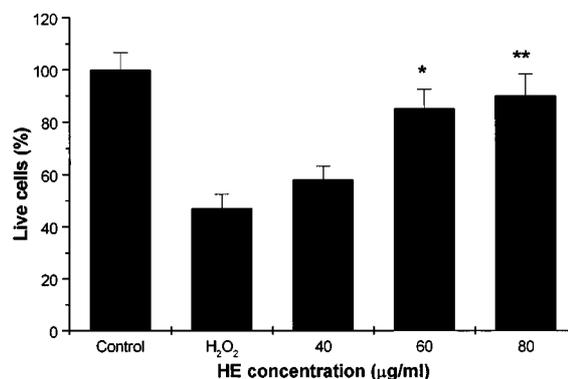


Fig. 3. Dose-response relationship of Herba Epimedii (HE) for its neuroprotective effect on hydrogen peroxide (H₂O₂)-induced cytotoxicity by MTT assay in cultured rat spinal DRG neurons. Cultured were preincubated with HE for 2 hours before the exposure of H₂O₂. The results indicate the mean±SE for 6 experiments. **P*<0.05.

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 H₂O₂-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 H₂O₂ showed the decrease of the survival of DRG neurons. These results support the previous studies demonstrating that H₂O₂ had neurotoxic effect (Yamamoto *et al.*, 1983; Rubin and Faber, 1984), and also H₂O₂-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 H₂O₂ 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 H₂O₂ decreased catalase activity, and they results in the decrease of cell viability by glucose oxidase-mediated H₂O₂ means the decrease of catalase activity. Also we could observe examined the protective effect in herbal extract of oriental medicine on H₂O₂-induced oxidative stress after HE extract were pretreated for 2 hours before treatment of H₂O₂ in these experiments. In this study, the cell viability decreased by H₂O₂ 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 H₂O₂ protected neurons from the H₂O₂-induced oxidative stress. The mechanism of protective effect of HE extract against H₂O₂-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 H₂O₂ 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 H₂O₂-induced neurotoxicity by the increase of cell viability. Therefore, HE may be partially responsible for the treatment of brain disease such as AD or ALS. In conclusion, we demonstrated that H₂O₂ induced lethal toxic effect on cultured rat spinal DRG neurons, and HE was effective in attenuation of H₂O₂-induced oxidative stress in these cultures.

ACKNOWLEDGEMENTS

This research was supported by the grants of Wonkwang University and partially by BK-21 project.

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