

Neuroprotective Effect of Korean Mistletoe Extract against Damage Induced by Hydrogen Peroxide in Cultured Rat Cortical Neurons

Ju Hyun Lee*, Soon Ock Cho*, Ju Yeon Ban*, Kyung-Sik Song**, and Yeon Hee Seong*†

*College of Veterinary Medicine and Research Institute of herbal Medicine, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea.

**College of Agriculture and Life-Sciences, Kyungpook National University, Daegu 702-701, Korea.

ABSTRACT : The protective effect of ethanol extract of Korean mistletoe (KM; *Viscum album coloratum*) on hydrogen peroxide (H₂O₂)-induced neurotoxicity was examined in primary cultured rat cortical neurons. H₂O₂ reduced viability of cortical neurons in a concentration-dependent manner. The addition of KM, over a concentration range of 10 to 100 µg/ml, concentration-dependently prevented the H₂O₂ (100 µM)-induced neuronal cell death, as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl-tetrazolium bromide (MTT) assay and Hoechst 33342 staining. KM significantly inhibited H₂O₂-induced elevation of the cytosolic Ca²⁺ concentration ([Ca²⁺]_c), which was measured by a fluorescent dye, fluo-4 AM. KM inhibited glutamate release into medium and generation of reactive oxygen species (ROS) induced by H₂O₂. These results suggest that KM may mitigate the H₂O₂-induced neurotoxicity by interfering with the increase of [Ca²⁺]_c, and inhibiting glutamate release and generation of ROS in cultured neurons.

Key words : Korean mistletoe, neurotoxicity, hydrogen peroxide, neuroprotection, cortical neurons

INTRODUCTION

Oxidative stress, resulting from excessive production of reactive oxygen species (ROS), has been linked causally to a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and conditions such as ischemia and excitotoxicity (Olanow, 1993). The oxidative injury causes profound cellular damage and eventual death resulting from the overactivation of glutamate receptors, and the generation of nitric oxide, superoxide and hydrogen peroxide (H₂O₂). Therefore, in vitro H₂O₂ toxicity has become an important model for studying the neuropathology of oxidative stress in the CNS disorders. Many researches demonstrated the involvement of glutamate in H₂O₂-induced neurotoxicity in cultured neurons (Gardner *et al.*, 1996). H₂O₂ and O₂⁻ inhibited the uptake of glutamate and enhanced the release of glutamate, resulting in NMDA receptor overstimulation (Volterra *et al.*, 1994; Maily *et al.*, 1999). The neurotoxic effects of H₂O₂ were strongly reduced by antagonists of NMDA receptors and enhanced in the absence of Mg²⁺ (Maily *et al.*, 1999). There are some reports on H₂O₂-induced intracellular Ca²⁺ concentration ([Ca²⁺]_i) increase (Whittemore *et al.*, 1995). NMDA receptor is a ligand-gated/voltage-sensitive cation channel, especially highly permeable to Ca²⁺. Calcium influx through NMDA receptor-coupled Ca²⁺

channel appears to be a critical role in the H₂O₂-induced neurotoxicity (Maily *et al.*, 1999). Moreover, Ca²⁺ signals activate enzymes which lead to further ROS generation (e.g. xanthine oxidase, nitric oxide synthase, phospholipase A₂); conversely, ROS generation can facilitate [Ca²⁺]_i increases by damaging the [Ca²⁺]_i regulatory mechanism and activating Ca²⁺ release from intracellular Ca²⁺ stores (Duffy & MacViar 1996). However, the clinical benefit of NMDA receptor antagonists and direct blockers of neuronal Ca²⁺ channels is debatable, since they lack convincing effectiveness or have serious side-effects (Ferber & Kriegstein, 1996; Li *et al.*, 2002). There are much efforts to develop beneficial agents from medicinal plants to achieve neuroprotection.

Semi-parasitic plants, mistletoes, belong to the families Loranthaceae and long been recognized as therapeutic herbs all over the world (Li, 1975). It has been traditionally used as a sedative, analgesic, anti-spasmodic, cardiotoxic, and anticancer agent (Li, 1975). The herb dilates the coronary vessels, reduces myocardial ischemic symptoms and decreases the oxygen demand of cardiac tissue (Huang, 1999). It can also antagonize the vasopressin-induced vasoconstriction on the coronary vessel, and has been observed to lower peripheral blood pressure (Huang, 1999). In terms of Korean mistletoe (KM; *Viscum album coloratum*), it has been reported to have cytotoxic activity against tumor cells in vitro (Khawaja *et al.*,

† Corresponding author: (Phone) +82-43-261-2968 (E-mail) vepharm@chungbuk.ac.kr

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1980). It was also reported that the water extract of KM had antitumor activity to inhibit tumor metastasis (Yoon *et al.*, 1995). Recent work suggested that KM is a potent immunoadjuvant, enhancing cellular and humoral immune responses (Yoon *et al.*, 2001). Various chemical components have been identified from the extracts of KM, such as lectin, steroid, triterpene, sesquiterpene lactone, and flavonoids. Although little research has been done on pharmacological actions of KM in the CNS, we hypothesized that KM might protect oxidative injury of neurons. In order to demonstrate the neuroprotective effect of KM, the effect of ethanol extract KM on H₂O₂-induced neuronal cell death was examined in cultured cortical neurons in the present study.

MATERIALS AND METHODS

Materials

KM was purchased from an oriental drug store in Taegu, Korea, and identified by professor K.-S. Song, Kyungpook National University. H₂O₂, 2-mercaptoethanol, trypsin (from bovine pancreas), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), *o*-phthalaldehyde (OPA), Dulbecco's modified Eagle's medium (DMEM), Joklik-modified Eagle's medium, poly-L-lysine and amino acids for HPLC standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-4 AM, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) and Hoechst 33342 dye were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from JRH Biosciences (Lenexa, KS, USA). All other chemicals used were of the highest grade available.

Preparation of ethanol extract of KM

KM (300 g) was extracted three times in a reflux condenser for 24 h each with 2 l of ethanol. The solution was combined, filtered through Whatman NO. 1 filter paper, concentrated using a rotary vacuum evaporator, and freeze-dried. The yield was about 10% (w/w).

Primary culture of cortical neurons

Primary cortical neuronal cultures were prepared using SD rat fetuses on embryonic day 15 ± 1, according to the previously described methods (Ban *et al.*, 2005). Briefly, fetuses were isolated from a dam anaesthetized with ether. Cortical hemispheres were dissected under sterile conditions and placed into Joklik-modified Eagle's medium containing trypsin (0.25 mg/ml). After slight trituration through a 5-ml pipette 5 to 6 times, the cells were incubated for 10 min at 37 °C. Dissociated cells were collected by centrifugation (1,500 rpm, 5 min) and

resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (44 mM), glucose (22.73 mM), penicillin (40 U/ml), gentamicin (50 µg/ml), KCl (5 mM) and 10% fetal bovine serum at a density of about 2 × 10⁶ cells/ml. Cells were plated onto poly-L-lysine coated 12 well-plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, and coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of cytosolic Ca²⁺ concentration ([Ca²⁺]_c), ROS and apoptosis. After 2 days' incubation, the medium was replaced with a new growth medium in which the concentrations of fetal bovine serum and KCl were changed to 5% and 20 mM, respectively. Cultures were kept at 37 °C in a 5% CO₂ atmosphere, changing the medium twice a week. Immunohistochemical staining of cultures, when grown for 5-7 days *in vitro*, with anti-microtubule associated protein-2 (MAP-2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody revealed that the culture method used in this study provided cell cultures containing about 90% neurons.

Neurotoxicity experiments

H₂O₂ was diluted freshly with the incubation buffer described below, for every experiment. KM was dissolved in ethanol with the concentration of 100 mg/ml and further diluted with experimental buffers. The final concentration of ethanol was less than 0.1%, which did not affect neuronal cell viability. Neurotoxicity experiments were performed on neurons grown for 5-7 days *in vitro*. The culture medium was removed and neurons were washed with a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM CaCl₂, at pH 7.4. They were then incubated for 30 min in the same buffer, and incubated for a further 15 min (unless otherwise indicated) in the presence of H₂O₂ at 37 °C. Exposed to H₂O₂, the neurons were washed and post-incubated in H₂O₂-free and serum-free growth medium for 15 h for the measurement of cell viability. KM was pretreated 20 min prior to the H₂O₂ treatment, and added during the H₂O₂ exposure period and the post-incubation period.

Analysis of cell viability

MTT colorimetric assay

This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductase (Ban *et al.*, 2005). Therefore, the amount of formazan produced is proportional to the number of viable cells. At the end of the incubation, the culture medium was replaced by a solution of MTT (0.5 mg/ml) in serum-free growth medium. After a 4 h incubation at 37 °C, this solution

was removed, and the resulting blue formazan was solubilized in 0.25 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm using microplate reader (Bio-Tek ELx808, VT., USA).

Measurement of apoptotic cell death

The bis-benzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. After the post-incubation as described above, neurons on coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min, then stained with Hoechst 33342 dye at the concentration of 1 $\mu\text{g}/\text{ml}$ in the incubation buffer for 15 min. The morphological change was examined under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo, Japan). The dye was excited at 340 nm and emission was filtered with a 510 nm barrier filter. To quantify the apoptotic process, neurons with fragmented or condensed DNA and normal DNA were counted. Data were shown as apoptotic cells as a percentage of total neurons.

Measurement of $[\text{Ca}^{2+}]_c$

Neurons grown on coverslips were loaded with 3 μM fluo-4 AM (dissolved in dimethylsulfoxide (DMSO)) in serum-free growth medium for 45 min at 37 °C in the CO₂ incubator, and washed with the incubation buffer. The coverslips containing fluo-4 AM labeled neurons were mounted on a perfusion chamber containing incubation buffer, subjected to a laser scanning confocal microscope (Carl Zeiss LSM 510, Oberkochen, Germany), and then scanned every 2 second with a 488 nm excitation argon laser and a 515 nm longpass emission filter. After the baseline of $[\text{Ca}^{2+}]_c$ was observed for 50 sec, 100 μM H₂O₂ was added to the perfusion chamber for the measurement of $[\text{Ca}^{2+}]_c$ change. In order to test the effect of KM on the H₂O₂-induced $[\text{Ca}^{2+}]_c$ change, neurons were pre-treated with KM (100 $\mu\text{g}/\text{ml}$) 15 min before the treatment with 100 μM H₂O₂ after being loaded with fluo-4 AM and washed. KM was also present in the perfusion chamber during the $[\text{Ca}^{2+}]_c$ measurement period. All images, about 100 images from the scanning, were processed to analyze changes of $[\text{Ca}^{2+}]_c$ in a single cell level. The results were expressed as the relative fluorescence intensity (RFI) (Lee *et al.*, 1998).

Measurement of glutamate concentration

Washed and equilibrated for 20 min with the incubation buffer, neurons were incubated with the buffer containing 100

μM H₂O₂ for 15 min and incubated with H₂O₂-free incubation buffer for a further 2 h (post-incubation) at 37 °C. KM was pre-treated 20 min prior to the H₂O₂ treatment and added during the H₂O₂ exposure period and post-incubation period. At the end of the incubation, glutamate released into the medium from the treated cells was quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (BAS MF series, IN, USA) (Ban *et al.*, 2005).

Measurement of ROS generation

The microfluorescence assay of 2',7'-dichlorofluorescein (DCF), the fluorescent product of H₂DCF-DA, was used to monitor the generation of ROS. Neurons grown on coverslips were washed with phenol red-free DMEM 3 times and incubated with the same medium for 30 min at 37 °C. Then, the medium was changed into the incubation buffer containing 100 μM H₂O₂, and cells were incubated for a further 20 min. In order to test the effect of KM on H₂O₂-induced generation of ROS, KM was added 20 min prior to the treatment with H₂O₂. The uptake of H₂DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with 100 μM H₂O₂. After being washed, coverslips containing cortical neurons loaded with H₂DCF-DA were mounted on the confocal microscope stage, and the neurons were observed by a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, UK) using 488 nm excitation and 510 nm emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. Challenge of H₂DCF-DA and measurement of fluorescence intensity was performed in the dark.

Statistical analysis

Data were expressed as mean \pm SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) with subsequent Tukey's tests. P values of < 0.05 were considered to be significant.

RESULTS

KM protects neuronal cell death induced by H₂O₂.

To assess H₂O₂-induced neuronal cell death, the MTT assay was performed. In previous experiments, we have demonstrated that H₂O₂ over the concentration range of 50-200 μM produced a concentration-dependent reduction of cell viability in cultured cortical neurons. Therefore, the concentration of 100 μM was used for the determination of the H₂O₂-induced

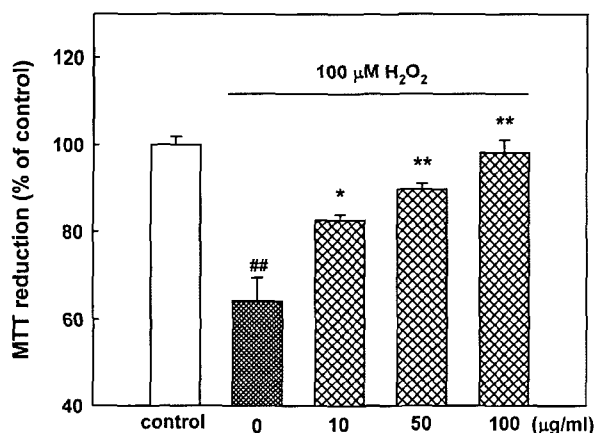


Fig. 1. Inhibitory effect of KM on H₂O₂-induced cell death in cultured cortical neurons. Cell viability was measured by the MTT assay. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean ± SEM values of the data obtained from three independent experiments performed in triplicate. ## p < 0.01 compared to control. * p < 0.05, ** p < 0.01 compared to 100 μM H₂O₂.

neuronal cell damage in the present experiments. Fig. 1 shows the effect of KM (10, 50 and 100 μg/ml) on the H₂O₂-induced decrease of MTT reduction rate. MTT reduction rate decreased to 64.2 ± 5.3% with the treatment with 100 μM H₂O₂. KM showed concentration-dependent inhibition on the H₂O₂-induced neuronal cell death, recovering cell death to 82.5 ± 1.3, 89.7 ± 1.3 and 98.1 ± 3.0% by 10, 50 and 100 μg/ml, respectively.

An additional experiment was performed with Hoechst 33342 staining to assess the neurotoxicity of H₂O₂. Cell nuclei stained by Hoechst 33342 enables the occurrence of DNA condensate to be detected, a feature of apoptosis. In neurons treated with 100 μM H₂O₂, chromatin condensation and nuclear fragmentation were observed, whereas the control culture had round blue nuclei of viable cells. The proportion of apoptotic cells was calculated and shown in Fig. 2. The treatment of neurons with 100 μM H₂O₂ induced apoptosis of 38.8 ± 1.2% of total population of cultured cerebral cortical neurons, as compared with 7.6 ± 0.7% of apoptotic neurons in control cultures. KM, 10, 50 and 100 μg/ml, decreased the H₂O₂-induced apoptotic cell death to 26.4 ± 3.5, 20.8 ± 3.1 and 19.3 ± 2.6%, respectively. KM alone did not affect cell viability (data not shown).

KM inhibits H₂O₂-induced elevation of [Ca²⁺]_c

The increase of [Ca²⁺]_c has been postulated to be associated with H₂O₂-induced cell death in many studies. In cultured cortical neurons, treatment with 100 μM H₂O₂ produced relatively slow and gradual increase of [Ca²⁺]_c. A maximal fluorescence

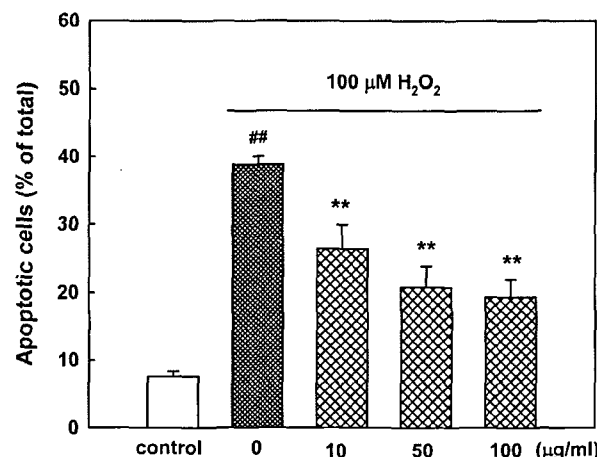


Fig. 2. Inhibitory effect of KM on H₂O₂-induced apoptosis of cultured cortical neurons. Apoptotic cell death produced by 100 μM H₂O₂ was measured by Hoechst 33342 staining and counted from five to six fields per well. Results are shown as apoptotic cells as a percentage of total number of cells and expressed as mean ± SEM values of the data obtained from three independent experiments performed in two to three wells. ## p < 0.01 compared to control. ** p < 0.01 compared to 100 μM H₂O₂.

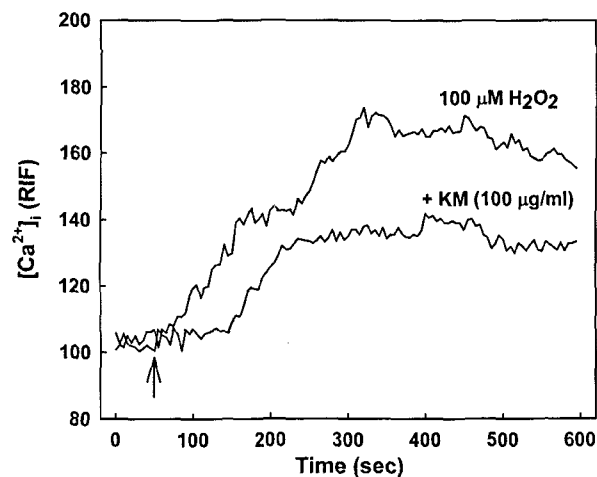


Fig. 3. Inhibitory effect of KM on H₂O₂-induced [Ca²⁺]_c elevation in cultured cortical neurons. [Ca²⁺]_c was monitored using a laser scanning confocal microscope. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative from at least three independent experiments.

intensity of about 170, compared to a base of 100, with the [Ca²⁺]_c elevation was measured about 5 min after the H₂O₂ (100 μM) application. After peaking, the fluorescence level decreased gradually. KM (100 μg/ml) produced a significant, but not complete, inhibition on the elevation of [Ca²⁺]_c induced by 100 μM H₂O₂ (Fig. 3). KM did not affect basal [Ca²⁺]_c (data not shown).

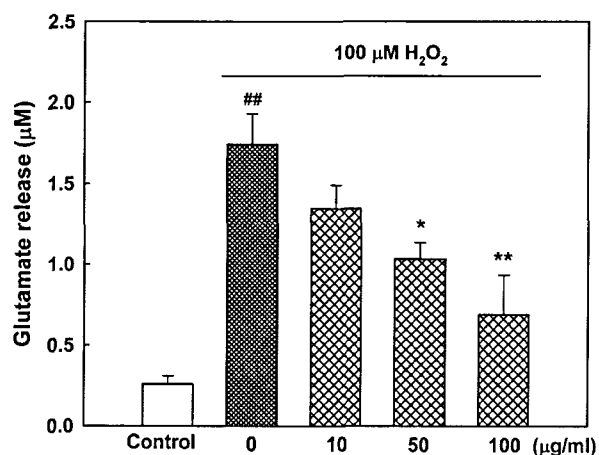


Fig. 4. Inhibitory effect of KM on H₂O₂-induced glutamate release in cultured cortical neurons. The amount of released glutamate was measured by HPLC with ECD. Results are expressed as mean ± SEM values of the data obtained in three independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 100 µM H₂O₂.

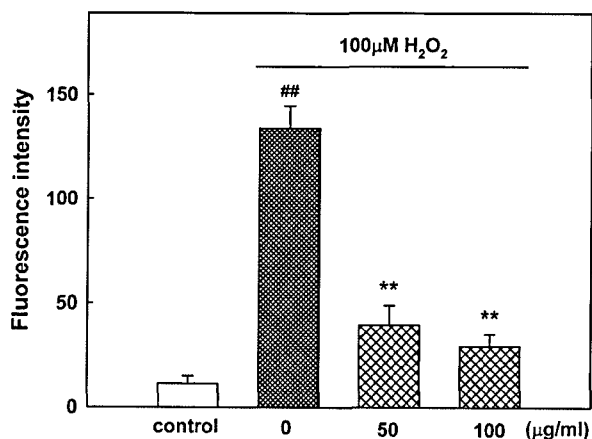


Fig. 5. Inhibitory effect of KM on H₂O₂-induced ROS generation in cultured cortical neurons. Results are expressed as mean ± SEM of relative fluorescence intensity obtained from three independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 100 µM H₂O₂.

KM inhibits H₂O₂-induced elevation of glutamate release

Glutamate released into the extracellular medium for 2 h was quantified 15 min after the incubation of cells with 100 µM H₂O₂. As shown in Fig. 4, 100 µM H₂O₂ markedly elevated the basal glutamate level from 0.26 ± 0.05 to 1.74 ± 0.19 µM. KM (100 µg/ml) strongly blocked the H₂O₂-induced elevation of glutamate release showing 0.69 ± 0.24 µM.

KM inhibits H₂O₂-induced ROS generation

H₂O₂ increased the glutamate release and the concentration of [Ca²⁺]_i. Furthermore, the pathological condition induced by H₂O₂ is associated with accelerated formation of ROS. In H₂DCF-DA-loaded cortical neurons, 100 µM H₂O₂ increased the fluorescence intensity, indicating the generation of ROS. The fluorescence intensity in H₂O₂-treated cells increased to 134.0 ± 10.5 by about 12 folds compared to control cells of 11.4 ± 3.7 . The H₂O₂-induced increase of generation was significantly inhibited by KM showing 39.6 ± 9.4 and 29.6 ± 5.7 of fluorescence intensity with 50 and 100 µg/ml, respectively (Fig. 5). KM alone did not show direct reaction with H₂DCF-DA to generate fluorescence.

DISCUSSION

The present study provides evidence that H₂O₂-induced injury to rat cortical neurons can be prevented by KM. KM was able to reduce the H₂O₂-induced [Ca²⁺]_i increase, glutamate release and ROS generation, and, in result, attenuate apoptotic neuronal death in primarily cultured rat cortical neurons.

H₂O₂ produces neuronal death by inducing a delayed accumulation of extracellular glutamate and NMDA receptor stimulation, with both effects being mediated by ·OH (Mailly *et al.*, 1999; Halliwell, 1992). Exposure to H₂O₂ causes an increase in [Ca²⁺]_i, which is blocked by Na⁺ channel antagonists as well as Ca²⁺ channel antagonists, and produces ROS concomitant with NMDA receptor stimulation in cultured neuronal cells (Mailly *et al.*, 1999; Su *et al.*, 1999; Wang & Joseph, 2000). Thus, the neurotoxic effect of H₂O₂ was strongly reduced by antagonists of NMDA receptors and Ca²⁺ channel antagonists (Mailly *et al.*, 1999). We also demonstrated the protections of MK-801, verapamil, an L-type Ca²⁺ channel blocker, and N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor against the H₂O₂-induced neuronal death in cultured rat neurons (Koh *et al.*, 2003; Lee *et al.*, 2005). Confirming these reports, the present study demonstrated that H₂O₂ stimulated glutamate release, elevation of [Ca²⁺]_i and ROS generation which in turn led to neuronal cell death in cultured cortical neurons. Many researchers have demonstrated that the neuronal loss following a transient exposure to H₂O₂ of cultured neuronal cells results from an apoptotic process (Whittemore *et al.*, 1995; Gardner *et al.*, 1996; Mailly *et al.*, 1999). Cultured cortical neurons exposed to H₂O₂ transiently followed by 15 h post-incubation with H₂O₂-free medium showed increased chromatin condensation, a typical feature of apoptotic cell death, in the present work.

Neuronal cells exposed to H₂O₂ produce membrane depolarization depending on the increased permeability to Na⁺ ions. Na⁺ ions increased by the depolarization further increase the opening of Na⁺ channels, and subsequently cause a great Ca²⁺ influx via the voltage-dependent Ca²⁺ channels (VDCC) (Halliwell, 1992; Wang & Joseph, 2000). In the present study, H₂O₂ elicited gradual and significant [Ca²⁺]_c increase, which was blocked by KM. Therefore, it is strongly suggested that the neuroprotective effect of KM is mainly due to the inhibition on the H₂O₂-induced increase of [Ca²⁺]_c through VDCC. KM also significantly inhibited the H₂O₂-induced glutamate elevation. This result indicates that the sustained inhibition on [Ca²⁺]_c elevation by KM resulted in the decrease of the H₂O₂-induced glutamate release. The elucidation of the variety of events occurring downstream of neuronal Ca²⁺ overloading is still a matter for further research. ROS generation undoubtedly takes place in glutamate neurotoxicity and is likely due to Ca²⁺ influx in the cytosol (Pereira & Oliveira, 2000). KM decreased the H₂O₂-induced increase of ROS generation. In a previous report, we demonstrated that L-NAME, an inhibitor of ROS generation, failed to show an inhibition on the H₂O₂-induced [Ca²⁺]_c increase in the earlier 20 min after the treatment, while verapamil, a Ca²⁺ channel antagonist, completely blocked ROS generation for the 20 min incubation in cultured cerebellar granule neurons (Koh *et al.*, 2003). Therefore, it is suggested that KM inhibited the H₂O₂-induced ROS generation via the blockade of [Ca²⁺]_c increase. It is thus concluded that KM may prevent the H₂O₂-induced apoptosis of neuronal cell by interfering with the increase of [Ca²⁺]_c, and then by inhibiting glutamate release and generation of ROS.

Mistletoes dilates the coronary vessels, reduces myocardial ischemic symptoms, and decreases the oxygen demand of cardiac tissue. It can antagonize the vasopressin-induced vasoconstriction action on the coronary vessel (Huang, 1999). Besides these precious somatic activities, mistletoes has also CNS inhibitory effect, antagonizing the caffeine-stimulating effect on the CNS (Huang, 1999). These results imply that KM might have a neuroprotective effect, because of very similar composition of active components between various subspecies. KM completely blocked H₂O₂-induced neuronal cell death in the present study. This KM-induced neuroprotection might be related to the prevention of oxidative damage in neurodegenerative diseases such as stroke, Alzheimer disease and Parkinson disease. It, however, must be made further studies to clarify the active components to which KM-induced neuroprotection is attributable. In conclusion, it was demonstrated a novel pharmacological action of KM and its mechanism in the present study. The protection against H₂O₂-induced neurotoxicity by KM may help to explain its inhibitory action on oxi-

dativ injury-linked various neurodegenerative pathophysiological conditions.

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