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과산화수소수로 유도된 배양신경세포손상에 대한 참죽나무잎 추출물의 보호효과

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Extract of *Cedrela sinensis* Leaves Protects Neuronal Cell Damage Induced by Hydrogen Peroxide in Cultured Rat Neurons

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ABSTRACT : Dried leaves from *Cedrela sinensis* A. Juss. (CS), have been observed to possess various pharmacological activity and contain various antioxidant constituents. The protective effect of ethanol extract of CS on hydrogen peroxide (H_2O_2)-induced neurotoxicity was examined using primary cultured rat cortical neurons in the present study. Exposure of cultured neurons to 100 μM H_2O_2 caused a significant neuronal death as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and Hoechst 33342 staining. The addition of CS, over a concentration range of 10 to 50 ㎍/㎖, concentration-dependently prevented the H_2O_2-induced neuronal apoptotic death. CS (50 ㎍/㎖) significantly inhibited H_2O_2-induced elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which was measured by a fluorescent dye, Fluo-4 AM. CS (30 and 50 ㎍/㎖) inhibited glutamate release and generation of reactive oxygen species (ROS) induced by 100 μM H_2O_2. These results suggest that CS may mitigate the H_2O_2-induced neurotoxiciy by interfering with the increase of $[Ca^{2+}]_c$, and then inhibiting glutamate release and generation of ROS in cultured neurons.

Key words : *Cedrela sinensis*, 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, amyotropic 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 (H_2O_2). Therefore, in vitro H_2O_2 toxicity has become an important model for studying the neuropathology of oxidative stress in the central nervous system (CNS) disorders. Many researches demonstrated the involvement of glutamate in H_2O_2-induced neurotoxicity in cultured neurons (Gardner *et al.*, 1996). H_2O_2 and O_2^- inhibited the uptake of glutamate and enhanced the release of glutamate, resulting in N-methyl-D-aspartic acid (NMDA) receptor overstimulation (Volterra *et al.*, 1994; Mailly *et al.*, 1999). The neurotoxic effects of H_2O_2 were strongly reduced by antagonists of NMDA receptors and enhanced in the absence of Mg^{2+} (Mailly *et al.*, 1999). NMDA receptor is a ligand-gated/voltage-sensitive cation channel, especially highly permeable to Ca^{2+}. Calcium influx through NMDA receptor-coupled Ca^{2+} channel appears to be a critical role in the H_2O_2-induced neurotoxicity (Mailly *et al.*, 1999). Moreover, Ca^{2+} signals activate enzymes which lead to further ROS generation (e.g. xanthine oxidase, nitric oxide synthase, phospholipase A_2); conversely, ROS generation can facilitate $[Ca^{2+}]_i$ increases by damaging the $[Ca^{2+}]_i$ regulatory mechanism and activating Ca^{2+} release from intracellular Ca^{2+} stores (Duffy & MacViar 1996).

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Cedrela sinensis A. Juss (Meliaceae) is a medicinal plant, which is widely distributed in China and cultivated in Korea. All most every part of C. *sinensis*, including seeds, root bark, peptioles, and leaves, has a medicinal effect (Edmonds & Staniforth, 1998). The leaves and stems of C. *sinensis* have been used for the treatment itch, dysentery, and enteritis in oriental medicine (Lee, 1985). The powdered root has been used as a corrective, and the fruits have been used as an astringent and for the treatment of eye infection (Stuart, 1911; Perry, 1980). There have been potent evidences that C. *sinensis* has antioxidant activity (Cho *et al.*, 2003). C. *sinensis* has shown scavenging effects on peroxynitrite, and inhibitory effects on human immunodeficiency virus (HIV) type 1 protease (Kang *et al.*, 2003; Park *et al.*, 2000). As regards its phytochemical investigation, isolation of limonoids, phytol derivatives, flavonoids, and phenolic compounds was previously reported (Luo *et al.*, 2000; Mitsui *et al.*, 2004). Furthermore, a recent phytochemical study of C. *sinensis* leaves has isolated several antioxidant constituents such as catechin, quercetin, and their derivatives, which showed significant DPPH and superoxide radical scavenging effects and exhibited inhibitory effect on LDL peroxidation (Lee *et al.*, 2006). Although little research has been done on pharmacological actions of C. *sinensis* leaves (CS) in the CNS, we hypothesized that CS might protect oxidative injury of neurons because of its possession of various antioxidants. In the current study, we investigated the protective effect of ethanol extract of CS on H_2O_2-induced neuronal damage using cultured rat cortical neurons.

MATERIALS AND METHODS

1. Chemicals

H_2O_2, 2-mercaptoethanol, trypsin (from bovine pancreas), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), *o*-phthaldialdehyde, Dulbecco's modified Eagle's medium, Joklik-modified Eagle's medium, and poly-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-4 AM, 2',7'-dichlorodihydrofluorescin diacetate (H_2DCF-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.

2. Plant materials and preparation of ethanol extract

The leaves of CS were collected in Daejeon, Korea, in October 2005, and identified by Prof. KiHwan Bae. A voucher specimen (CNU 1269) has been deposited in the herbarium at the College of Pharmacy, Chungnam National University, Daejeon, Korea. The dried CS (6.0 kg) was extracted three times with ethanol at room temperature for 7 days, filtered and concentrated to afford an ethanol extract. The yield was approximately 10% (w/w).

3. Animals

Sprague-Dawley (SD) rats (Daehan Biolink Co. Ltd., Chungbuk, Korea) were housed in an environmentally controlled room at 22±2℃, with a relative humidity of 55±5%, a 12-h light/dark cycle, and food and water ad libitum. The procedures involving experimental animals complied with the regulations for the care and use of laboratory animals of the animal ethics committee of Chungbuk National University.

4. Induction of oxidative toxicity in primary cultures of rat cerebral cortical neurons

Primary cortical neuron cultures were prepared using embryonic day 15 to 16 (E15-E16) SD rat fetuses, as described previously (Lee *et al.*, 2007). Neurotoxicity experiments were performed on neurons after 5-7 days in culture. Cultured neurons were treated with 100 μM H_2O_2 in a HEPES-buffered solution containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM $CaCl_2$, pH 7.4, for 15 min (unless otherwise indicated) at 37℃. Exposed to H_2O_2, the neurons were washed and further incubated in H_2O_2-free and serum-free growth medium for 15 h (post-incubaion), and cell viability was measured. H_2O_2 was diluted freshly with the HEPES-buffer. CS was dissolved in ethanol with the concentration of 50 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. For each experiment, CS was added 20 min prior to treatment with H_2O_2, and was also present in the buffer during the H_2O_2 incubation and post-incubation.

5. Analysis of neuronal viability

Neuronal viability was monitored using the colorimetric MTT assay, as previously described (Park *et al*, 2006; Lee *et al.*, 2007). After the H_2O_2 incubation and post-incubation,

the culture medium was replaced by a solution of MTT (0.5 ㎎/㎖) in serum-free growth medium. After a 4 h incubation at 37℃, this solution was removed, the resulting blue formazan was solubilized in acidic isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 ㎚ using a microplate reader (Bio-Tek ELX808, Vermont, USA). Results are expressed as the percentage of MTT reduction, with the absorbance of control cells normalized to 100%. Apoptotic cell death was characterized using Hoechst 33342 staining (Park *et al*, 2006; Lee *et al*., 2007). Neurons on coverslips exposed to 100 μM H_2O_2 and post-incubated were fixed in 4% paraformaldehyde at room temperature for 20 min, and then stained with 1 ㎍/㎖ Hoechst 33342 in HEPES buffer for 15 min. The dye was excited at 340 ㎚, and emission was filtered with a 510 ㎚ barrier filter under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo). Neurons with fragmented or condensed DNA and normal DNA were counted and the data are presented as apoptotic neurons as a percentage of total neurons.

6. Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)

Neurons grown on coverslips were loaded with 3 μM Fluo-4 AM (dissolved in DMSO) in serum-free growth medium for 45 min at 37℃ in a CO_2 incubator, and washed with the HEPES buffer. Coverslips with Fluo-4 AM labeled neurons were mounted in a perfusion chamber containing HEPES buffer, and examined by laser scanning confocal microscopy (Carl Zeiss LSM 510, Oberkochen, Germany). Samples were scanned every 5 s using a 488 ㎚ excitation argon laser and a 515 ㎚ longpass emission filter. After the baseline of $[Ca^{2+}]_c$ was observed for 30 s, 100 μM H_2O_2 was added to the perfusion chamber and changes in $[Ca^{2+}]_c$ were measured. All images (~120 images per cell) were processed to analyze changes in $[Ca^{2+}]_c$ at the single cell level. The results are expressed as relative fluorescence intensity (RFI) (Lee *et al*., 1998).

7. Measurement of glutamate concentration

Washed and equilibrated for 20 min with the HEPES buffer, neurons were incubated with the buffer containing 100 μM H_2O_2 for 15 min and incubated with H_2O_2-free HEPES buffer for a further 2.5 h (post-incubation) at 37℃. 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).

8. Measurement of ROS generation

A microfluorescence assay to detect 2',7'-dichlorofluorescin (DCF), the fluorescent product of H_2DCF-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 100 μM H_2O_2 in HEPES buffer at 37℃ for 20 min. H_2DCF-DA (final concentration, 5 μM) dissolved in DMSO was loaded immediately after the treatment with H_2O_2. After washing, coverslips containing cortical neurons loaded with H_2DCF-DA were mounted and the neurons were observed using a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, UK) with 488 ㎚ excitation and 510 ㎚ emission filters. The average pixel intensity of the fluorescence was measured in each cell in the field and expressed in relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer.

9. Statistical analysis

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

RESULTS

1. Protective effect of CS on H_2O_2-induced neuronal cell death

The MTT assay was used to quantify cell death in response to H_2O_2 treatment. When cerebral cortical neurons were exposed to 100 μM H_2O_2, MTT absorbance was 56.3 ± 1.8% of that of untreated controls, indicating that H_2O_2 resulted in neuronal cell death. In cultures treated with CS (30 and 50 ㎍/㎖), H_2O_2-induced cell death was significantly reduced (MTT, 86.1 ± 4.8 and 87.4 ± 1.4% with 30 and 50 ㎍/㎖ SC, respectively) (Fig. 1). Hoechst 33342 staining was used to detect condensed or fragmented DNA, which was indicative of H_2O_2-induced neuronal apoptotic cell death. As shown in Fig. 2A, in treated cells with 100 μM H_2O_2, chromatin condensation and nuclear fragmentation

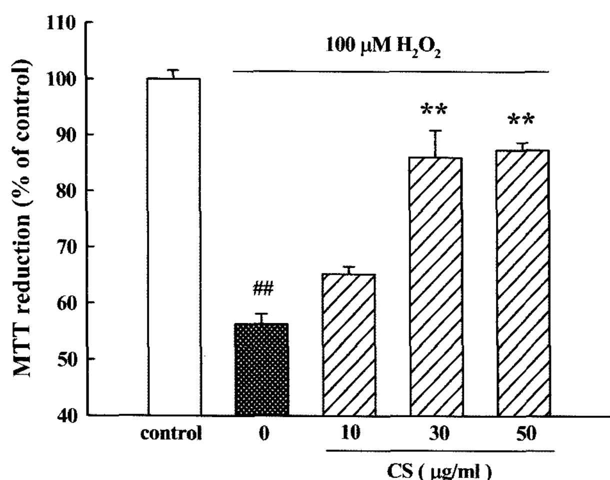
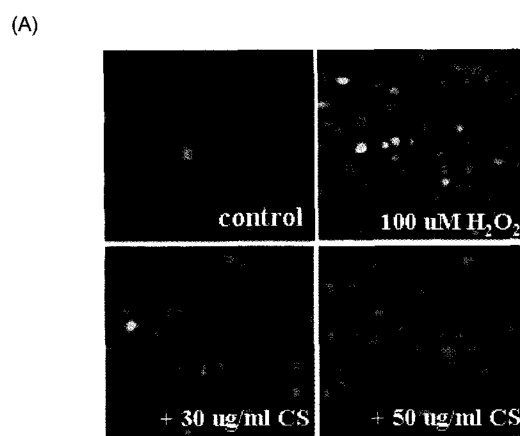


Fig. 1. Inhibitory effect of *Cedrela sinensis* (CS) on H_2O_2-induced cell death in cultured cortical neurons. Neuronal death was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl-tetrazolium bromide (MTT) assay. The MTT absorbance from non-treated cells was normalized to 100%. Results are expressed as mean ± SEM of the data obtained from three independent experiments performed in three to five wells. ## $P < 0.01$ compared to control. ** $P < 0.01$ compared to 100 μM H_2O_2.

(A)



(B)

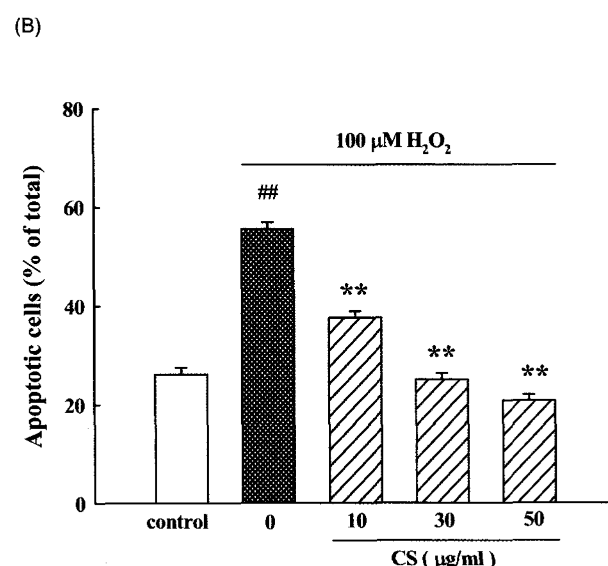


Fig. 2. Inhibitory effect of *Cedrela sinensis* (CS) on H_2O_2-induced apoptosis of cultured cortical neurons. Apoptotic cell death produced by 100 μM H_2O_2 was measured by Hoechst 33342 staining and counted from five to six fields per well. (A) Representative photomicrographs of cultured neurons showing H_2O_2-induced apoptosis. (B) Results shown are apoptotic cells as a percentage of the total number of cell and expressed as mean ± SEM of the data obtained from three independent experiments performed in three to five wells. ## $P < 0.01$ compared to control. ** $P < 0.01$ compared to 100 μM H_2O_2.

were observed, whereas the control culture had round blue nuclei of viable cells. Treatment of neurons with 100 μM H_2O_2 induced apoptosis in 55.5 ± 1.4% of cultured cortical neurons, compared to 26.2 ± 1.4% apoptotic neurons in control cultures. The addition of SC (10, 30 and 50 ㎍/㎖) significantly decreased 100 μM H_2O_2-induced apoptotic cell death, to 37.6 ± 1.3, 25.1 ± 1.3, and 20.8 ± 1.2% apoptotic cells in 10, 30, and 50 ㎍/㎖ SC, respectively (Fig. 2B).

2. Inhibitory effect of CS on H_2O_2-induced elevation of $[Ca^{2+}]_c$

Accumulation of $[Ca^{2+}]_c$ may play a critical role in the early stages of H_2O_2-induced neurotoxicity. As shown in Fig. 3, $[Ca^{2+}]_c$ relatively slowly and gradually increased in response to treatment with 100 μM H_2O_2 throughout the measurement time. In contrast, pretreatment with CS (50 ㎍/㎖) completely inhibited the H_2O_2 induced increase in $[Ca^{2+}]_c$. CS did not affect basal $[Ca^{2+}]_c$ (data not shown).

3. Inhibitory effect of CS on H_2O_2-induced elevation of glutamate release

Glutamate released into the extracellular medium for 2.5 h after the treatment with 100 μM H_2O_2 was quantified. As shown in Fig. 4, 100 μM H_2O_2 markedly elevated the basal glutamate level from 0.44 ± 0.16 to 3.85 ± 0.41 μM. CS (30 and 50 ㎍/㎖) significantly blocked the H_2O_2-induced elevation of glutamate release showing 1.63 ± 0.16 and 1.43 ± 0.17 μM, respectively (Fig. 4).

4. Inhibitory effect of CS on H_2O_2-induced ROS generation

H_2O_2 treatment increased the cytosolic concentration of

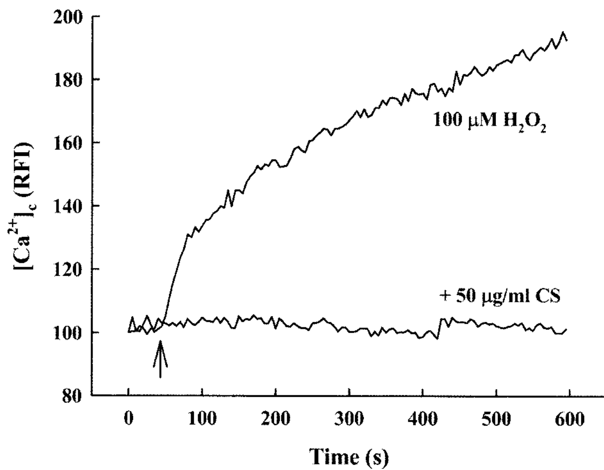


Fig. 3. Inhibitory effect of *Cedrela sinensis* (CS) on H_2O_2-induced $[Ca^{2+}]_c$ elevation in cultured cortical neurons. $[Ca^{2+}]_c$ was monitored using a laser scanning confocal microscope. All images were processed to analyze changes in $[Ca^{2+}]_c$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative of at least three independent experiments.

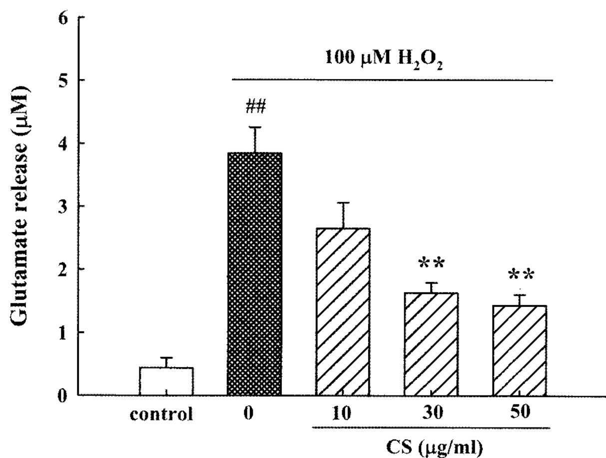


Fig. 4. Inhibitory effect of *Cedrela sinensis* (CS) on H_2O_2-induced glutamate release in cultured cortical neurons. The amount of released glutamate was measured by HPLC with ECD. Results are expressed as mean ± SEM of the data obtained in three independent experiments performed in two to three wells. ## $P < 0.01$ compared to control. ** $P < 0.01$ compared to 100 μM H_2O_2.

free Ca^{2+}. Furthermore, the pathological conditions induced by H_2O_2 are associated with accelerated formation of ROS. In H_2DCF-DA-loaded cerebral cortical neurons, 100 μM H_2O_2 increased the fluorescence intensity, indicating the generation of ROS. When treated with 100 μM H_2O_2 for 20 min, the fluorescence intensity increased approximately 3 fold to 228.9 ± 12.8 compared to control neurons (69.6 ± 6.7). CS

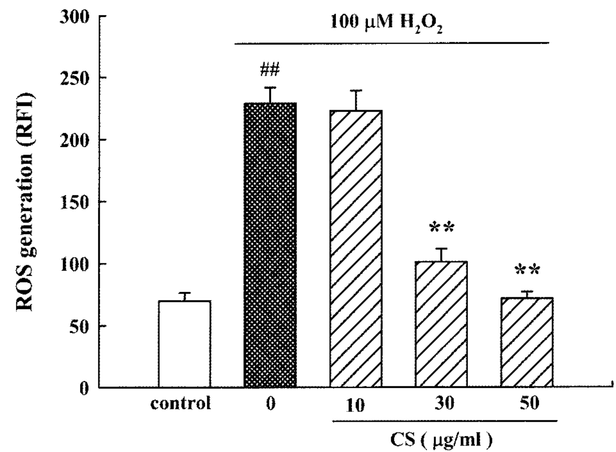


Fig. 5. Inhibitory effect of *Cedrela sinensis* (CS) on H_2O_2-induced ROS generation in cultured cortical neurons. Values represent mean ± SEM of RFI obtained from three independent experiments performed in two to three wells. ## $P < 0.01$ compared to control. ** $P < 0.01$ compared to 100 μM H_2O_2.

(30 and 50 μg/mℓ), significantly blocked the H_2O_2-induced increase in fluorescence intensity to 100.8 ± 10.7 and 71.5 ± 5.5, respectively (Fig. 5).

DISCUSSION

The present study provides evidence that H_2O_2-induced injury to rat cortical neurons can be prevented by CS. CS was able to reduce the H_2O_2-induced $[Ca^{2+}]_c$ increase, glutamate release and ROS generation, and, in result, attenuate apoptotic neuronal death in primarily cultured rat cortical neurons.

H_2O_2 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 H_2O_2 causes an increase in $[Ca^{2+}]_i$, which is blocked by Na^+ channel antagonists as well as Ca^{2+} 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 H_2O_2 was strongly reduced by antagonists of NMDA receptors and Ca^{2+} channel antagonists (Mailly *et al.*, 1999). In previous reports, H_2O_2 caused the increase in $[Ca^{2+}]_c$, glutamate release and ROS generation (Koh *et al.*, 2003; Lee *et al.*, 2005; Park *et al.*, 2006; Lee *et al.*, 2007). We also demonstrated the protections of MK-801, verapamil, an

L-type Ca^{2+} channel blocker, and N^G-nitro-L-arginine methyl ester ($_L$-NAME), a nitric oxide synthase inhibitor against the H_2O_2-induced elevation of $[Ca^{2+}]_c$, glutamate release, ROS generation, and neuronal death in cultured rat neurons (Koh *et al.*, 2003; Lee *et al.*, 2005). Confirming these reports, the present study demonstrated that H_2O_2 stimulated elevation of $[Ca^{2+}]_c$, glutamate release 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 H_2O_2 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 H_2O_2 transiently followed by 15 h post-incubation with H_2O_2-free medium showed increased chromatin condensation, a typical feature of apoptotic cell death, in the present study.

Neuronal cells exposed to H_2O_2 produce membrane depolarization depending on the increased permeability to Na^+. Na^+ increased by the depolarization further increase the opening of Na^+ channels, and subsequently cause a great Ca^{2+} influx via the voltage-dependent Ca^{2+} channels (VDCC) (Halliwell, 1992; Wang & Joseph, 2000). In the present study, H_2O_2 elicited gradual and significant $[Ca^{2+}]_c$ increase, which was blocked by CS. Therefore, it is strongly suggested that the neuroprotective effect of CS is mainly due to the inhibition on the H_2O_2-induced increase of $[Ca^{2+}]_c$ through VDCC. CS also significantly blocked H_2O_2-induced glutamate release. This result indicates that the sustained inhibition on $[Ca^{2+}]_c$ elevation by CS resulted in the decrease of the H_2O_2-induced glutamate release. Elucidation of the events occurring downstream of neuronal Ca^{2+} overloading requires further research. ROS generation undoubtedly takes place in glutamate neurotoxicity and is likely due to Ca^{2+} influx in the cytosol (Pereira & Oliveira, 2000). CS decreased the H_2O_2-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 H_2O_2-induced $[Ca^{2+}]_c$ increase in the earlier 20 min after the treatment, while verapamil, a Ca^{2+} 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 CS inhibited the H_2O_2-induced ROS generation via the blockade of $[Ca^{2+}]_c$ increase. It is thus concluded that CS may prevent the H_2O_2-induced apoptosis of neuronal cell by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release and generation of ROS.

CS has been widely used for the various medicinal effect of anti-allergy, anti-inflammation and anti-infection (Lee, 1985; Stuart, 1911; Perry, 1980). Antioxidant constituents such as phenolic compounds including catechin and quercetin have been identified from CS (Lou *et al.*, 2000; Mitsui *et al.*, 2004; Lee *et al.*, 2006). A significant DPPH, peroxynitrite and superoxide radical scavenging effect and inhibitory effect on LDL peroxidation of CS have been demonstrated (Kang *et al.*, 2003; Lee *et al.*, 2006; Park *et al.*, 2000). We could hypothetize that CS would have neuroprotective effect against oxidative stress-induced neuronal death, because of its potent anti-oxidative effect corresponding to its abundant anti-oxidant constituents. Moreover, natural catechin and quercetin, which is also contained in CS, have been demonstrated as good candidates for neuroprotection against ischemic insult *in vivo* as well as *in vitro* (Pu *et al.*, 2007; Bureau *et al.*, 2007). The present study firstly investigated that CS could block oxidative stress-induced cultured neuronal death. This CS-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 CS-induced neuroprotection is attributable.

In conclusion, it was demonstrated a novel pharmacological action of CS in the CNS and its mechanism in the present study. The protection against H_2O_2-induced neurotoxicity by CS may prove its inhibitory action on oxidative injury-linked various neurodegenerative pathophysiological conditions.

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