

원저

Effects of *Sunghyangchungisan* (SHCS) on Oxidant-induced Cell Death in Human Neuroglioma Cells

Na-Ri Kim, Jung-Nam Kwon, Young-Kyun Kim

Dept. of Korean Medicine, Graduate school, Dong Eui University

Objectives : Reactive oxygen species (ROS) have been implicated in the pathogenesis of a wide range of acute and long-term neurodegenerative diseases. This study was undertaken to examine whether *Sunghyangchungisan* (SHCS), a well-known prescription in Korean traditional medicine, might have beneficial effects on ROS-induced brain cell injury.

Methods : Human neuroglioma cell line A172 and H₂O₂ were employed as an experimental model cell and oxidant.

Results : SHCS effectively protected the cells against both the necrotic and apoptotic cell death induced by H₂O₂. The effect of SHCS was dose-dependent at concentrations ranging from 0.2 to 5 mg/ml. SHCS significantly prevented depletion of cellular ATP and activation of poly (ADP-ribose) polymerase induced by H₂O₂. It also helped mitochondria to preserve its functional integrity estimated by MTT reduction ability. Furthermore, SHCS significantly prevented H₂O₂-induced release of cytochrome c into cytosol. Determination of intracellular ROS showed that SHCS might exert its role as a powerful scavenger of intracellular ROS.

Conclusions : The present study provides clear evidence for the beneficial effect of SHCS on ROS-induced neuroglial cell injury. The action of SHCS as an ROS-scavenger might underlie the mechanism.

Key Words: *Sunghyangchungisan* (SHCS), neuroglioma cells, reactive oxygen species (ROS)

Introduction

Ischemia- or ischemia/reperfusion- induced cell injury is associated with a variety of life threatening conditions such as myocardial infarction, cerebral stroke, and renal failure. Tissue injury resulting from ischemia or ischemia/reperfusion is mediated, in part,

by the generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen¹⁾. In the brain, they have been implicated in the pathogenesis of a wide range of acute and long-term neurodegenerative diseases including acute cerebral stroke, Parkinsons disease, Huntingtons disease, ischemic trauma, and seizures^{2,3)}.

Brain cell membranes have a high content of polyunsaturated fatty acids that are particularly susceptible to the peroxidative attack by ROS. In addition, iron, which promotes cytotoxic radical formation, is accumulated in specific brain regions, such as the globus pallidus and substantia nigra. On the other hand, antioxidative defense mechanisms are

· 접수 : 2004년 10월 27일 · 논문심사 : 2004년 12월 10일
· 채택 : 2004년 12월 30일
· 교신저자 : Young-Kyun Kim, Internal Medicine 1, Ulsan Oriental Medical Center Sinjeong-dong Nam-Gu, Ulsan, Korea
(Tel : Tel:052-226-8102, Fax:052-226-8177, E-mail: ykkim@demc.or.kr)

relatively deficient in brain cells. The brain cell contains almost no catalase and has low concentrations of glutathione, glutathione peroxidase and vitamin E ⁴⁾. The brain is, therefore, particularly vulnerable to the ROS attack.

Cytoprotection against ROS is provided by a multilevel defense system which comprises antioxidants (vitamin A, C, and E and reduced glutathione) and antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Variable constituents of natural products such as fruits, wine, tea, and green vegetables are known to be a great source of effective antioxidants ⁵⁾. Some Korean herb medicines also have been expected to exert their effects as antioxidants. However, little information is available yet.

Sunghyangchungisan (SHCS) is a well-known prescription in the Korean traditional medicine. SHCS is first mentioned in “Zheng Zhi Yao Jue(證治要訣)” written by Dai Si Gong(戴思恭) is a mixed prescription of Xing Xiang San(星香散) and Huo Xiang Zheng Qi San(藿香正氣散) ⁶⁾. It has effects of regulating vital energy(理氣), eliminating phlegm(祛痰) and is a typical treatment used for the symptoms of apoplexy(中風), zhong qi(中氣), phlegm syncope(痰厥) and a syncope with eating and drinking(食厥) etc ^{7,8)}. It is an emergency treatment for apoplexy marked by onset of fainting(中風昏倒), coma(人事不省) and abundant phlegm(痰涎壅盛) ^{9,10)}. It has been proven to be beneficial for the patients with cerebral stroke. However, the target site and mechanism responsible for the clinical efficacy are not clear yet.

The present study thus was carried out to determine whether it exerts beneficial effect against brain cell injury induced by oxidant using A172 cells, a human neuroglioma cell line. Hydrogen peroxide (H₂O₂) was employed as an oxidant model drug.

Materials and Methods

1. SHCS extract preparation

SHCS was extracted with 3000ml distilled water at 100°C for 3 hr and total extract was evaporated under reduced pressure to give 89.5g. (Table 1.)

2. Culture of A172 cells

A172 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm² culture flasks (Costar, Cambridge, MA, USA). Cells were grown in Dulbeccos modified Eagles medium (DMEM, Gibco-BRL, Grand Island, NY) containing 10% heat inactivated fetal bovine serum (Gioco-BRL, Grand Island, NY) at 37°C in humidified 95% air/5% CO₂ incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved.

Table 1. Prescription of SHCS

Herbal name	Scientific name	Weight(g)
Huo Xiang(藿香)	<i>Herba Pogostemi</i>	5.625
Su Ye(蘇葉)	<i>Folium Perillae</i>	3.750
Bai Zhi(白芷)	<i>Radix Angelicae Dahuricae</i>	1.875
Da Fu Pi(大腹皮)	<i>Pericarpium Arecae</i>	1.875
Bai Fu Ling(白茯苓)	<i>Poria</i>	1.875
Huo Po(厚朴)	<i>Cortex Magnoliae</i>	1.875
Bai Zhu(白朮)	<i>Rhizoma Atractylodis Macrocephalae</i>	1.875
Chun Pi(陳皮)	<i>Pericarpium Citri Nobilis</i>	1.875
Ban Xia(半夏)	<i>Tuber Pinelliae</i>	1.875
Jie Geng(桔梗)	<i>Radix Platycodi</i>	1.875
Gan Cao(甘草)	<i>Radix Glycyrrhizae</i>	1.875
Sheng Jiang(生薑)	<i>Rhizoma Zingiberis</i>	3.000
Da Zao(大棗)	<i>Fructus Zizyphi Jujubae</i>	3.000
Nan Xing(南星)	<i>Rhizoma Arisaematis</i>	3.750
Mu Xiang(木香)	<i>Radix Saussurea</i>	3.750
Total amount		39.750

3. Fluorescence microscopic analysis of cell injury

Cells were grown on microscopic coverglasses in 12-well culture plates and all the experiments were carried out 2-3 days postseeding when a confluent monolayer was achieved. After washout of the culture media, cells were exposed to indicated concentration of H₂O₂ in Hank's balanced salt solution (HBSS) for 3 hr at 37 °C, unless otherwise indicated. After exposure to experimental protocols, each cell group was stained with 2 μ l mixture of 270 μ M acridine orange and 254 μ M ethidium bromide (1:1), and observed by reflected fluorescence microscopy.

Injured cells fluoresced orange and intact cells fluoresced green.

4. Trypan blue exclusion assay

Measurement of cell viability by trypan blue exclusion ability is based on the fact that injured cells lose the ability to exclude the dye and is stained by it (11). After exposure to experimental protocols, cells were detached and harvested using 0.05% trypsin-0.53 mM EDTA, and incubated with 4% trypan blue solution, and were counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable.

5. Assay of LDH release

After exposure of cells on 12-well culture plates to experimental protocols, media bathing the cells were collected separately, and cells on the plates were lysed by sonication in 0.2% triton X-100.

Lactate dehydrogenase (LDH) activity in the cellular extracts and incubation media were determined using an LDH assay kit.

LDH release was calculated as the percentage of LDH activity vs. the total activity in the cellular extracts and incubation media.

6. Analysis of apoptotic cell death

Cells were exposed to 0.1 mM H₂O₂ for 3 hr and, after washout of floating dead cells by necrotic injury, returned to H₂O₂-free culture media containing 10% fetal bovine serum. After further incubation for 18 hr, cells were assayed for apoptosis by analysis of DNA fragmentation and TUNEL staining. For analysis of DNA fragmentation, low molecular weight genomic DNA was extracted as follows. Cells (2×10^6) were scraped from the culture dish, washed in phosphate-buffered saline, and lysed in 0.5ml of extraction buffer (10 mM Tris, pH 7.4, 0.5% Triton X-100 and 10 mM

EDTA) for 20 min on ice. the lysates were then centrifuged at 15,000g for 10 min at 4 °C. DNA in supernatants were extracted with phenol-chloroform, precipitated in ethanol, resuspended in Tris/EDTA, pH 8.0, containing 20 μ g/ml RNase A, and incubated for 1 hr at 37 °C to digest RNA. Recovered DNA fragments were separated by electrophoresis in 2% agarose gel and visualized by staining with ethidium bromide.

For in situ detection of apoptotic cells, TUNEL assay was performed using ApoTag peroxidase in situ apoptosis detection kit (Intergen, Purchase, NY, USA). In brief, cells cultured on coverglasses were exposed to experimental protocols and fixed in 1% paraformaldehyde for 10min. The fixed cells were incubated with digoxigenin-conjugated dUTP in a TdT-catalyzed reaction for 60min at 37 °C and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. the DNA fragments were stained using DAB as a substrate.

7. Measurement of ATP content

ATP levels were measured on cells with a luciferin-luciferase assay ¹². After an exposure to oxidant stress, the cells were solubilized with 500 μ l of 0.5% Triton X-

100 and acidified with 100 μ l of 0.6M perchloric acid and placed on ice. Cell suspension was then diluted with 10 mM potassium glutamate buffer containing 4 mM MgSO₄ (pH 7.4), and 100 μ l of 20mg/ml luciferin-luciferase was added to 10 μ l of diluted sample. Light emission was recorded at 20sec with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined on a portion of the cell sample.

8. Measurement of PARP activity

Cells were treated with H₂O₂ and preincubated for 10 min in a buffer containing 28mM NaCl, 28 mM KCl, 2 mM MgCl₂, 56 mM Hepes (pH 7.5), 0.01% digitonin, and 125mM NAD (containing 0.25 μ Ci [³H]NAD). Permeabilized cells were incubated for 5min at 37°C, and the protein ribosylated with [³H]NAD was precipitated with 200 l of 50% (w/v) trichloroacetic acid. After washing twice with trichloroacetic acid, the protein pellet was solubilized with 200 l of 2% (w/v) sodium dodecyl sulfate in 0.1M NaOH and incubated at 37°C overnight, and the radioactivity was determined by scintillation counting.

9. Assay of MTT reduction

Intact mitochondria reduce 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan¹³⁾. Therefore the ability of cells to form formazan from MTT is a good indicator of mitochondrial function. A total 5 μ l of MTT was added to each well (final concentration is 62.5 μ g/ml). After exposure to experimental protocols, the supernatant was removed and the formed formazan crystals in viable cells were solubilized in DMSO. The concentration of formazan was determined by reading the absorbance at 570 nm using a spectrophotometer.

10. Detection of cytochrome c release

After exposure to experimental protocols, cells were

washed twice with ice-cold phosphate-buffered saline at 4°C and resuspended in 1 ml of extraction buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM MgCl₂, 5mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A). Cells were lysed by five cycles of freezing in liquid nitrogen and thawing at 37°C. After verifying the more than 90% cells were lysed by microscopic examination, the lysates were centrifuged at 100,000 g for 1 hr at 4°C. The resulting supernatant, which consisted of the cytosol, was separated from the pellet containing the cellular membrane and organelles, and were analyzed for cytochrome c by Western blots using anti-cytochrome c antibody.

11. Measurement of ROS production

Intracellular generation of ROS was determined by measuring 2,7-dichlorofluorescein (DCF) fluorescence¹⁴⁾. The nonfluorescent ester dye 2,7-dichlorofluorescein diacetate (DCFH-DA) penetrates into the cells and is hydrolyzed to DCFH by the intracellular esterases. The probe is rapidly oxidized to the highly fluorescent compound DCF in the presence of cellular peroxidase and ROS such as hydrogen peroxide of fatty acid peroxides. Cells were preincubated for 10 min at 37°C in a fluorescent cuvette containing 3 ml of glucose-free buffer with 20 μ M DCFH-DA (from a stock solution of 20mM DCFH-DA in ethanol). After preincubation, cells were treated with H₂O₂ and incubated upto 60min during which fluorescent intensity was monitored on a spectrophotometer with excitation wave length at 485 nm and emission wave length at 530nm.

12. Chemicals

Acridine orange, 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR, USA). Other chemicals, otherwise indicated, were from Sigma Chemical Co. (St. Louis,

MO, USA).

13. Data analysis

Data are presented as means \pm SE. When necessary, data were analyzed by ANOVA followed by Duncans multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

Results

1. Effects of SHCS on oxidant-induced cell injury

The fluorescence microscopic analysis in Fig. 1

visualize the typical image of cells injured by 0.5mM H₂O₂. Injured cells are stained with acridine orange and fluoresce orange or red, whereas viable cells fluoresce green. Preserved contour of the nuclei indicates that the cell injury is necrotic rather than apoptotic. Protective effect of SHCS against H₂O₂-induced injury is shown in the right panel of the Fig. 1.

In Fig. 2, the protective effect of SHCS was determined as a function of its concentration. Trypan blue exclusion ability and LDH release were adopted as indexes of cell injury. Measurement of cell viability by trypan blue exclusion ability is based on the fact that injured cells lose the ability to exclude the dye and is



Fig. 1. Protection by Sunghyangchungisan (SHCS, 1mg/ml) of H₂O₂ (0.5 mM)-induced cell injury visualized by acridine orange fluorescence image. Injured cells fluoresce orange and intact cells fluoresce green.

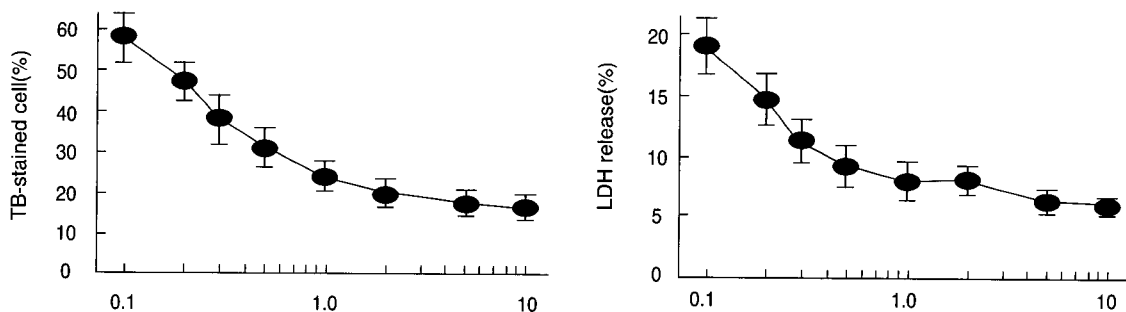


Fig. 2. Concentration-dependent protection by Sunghyangchungisan against H₂O₂-induced cell injury. Cells were treated with 0.5 mM H₂O₂ for 3 hr at 37°C in the presence of indicated concentrations of Sunghyangchungisan (SHCS), and cell viability was determined by trypan blue exclusion ability and LDH release. Each point represents mean \pm S.E. of 5 experiments.

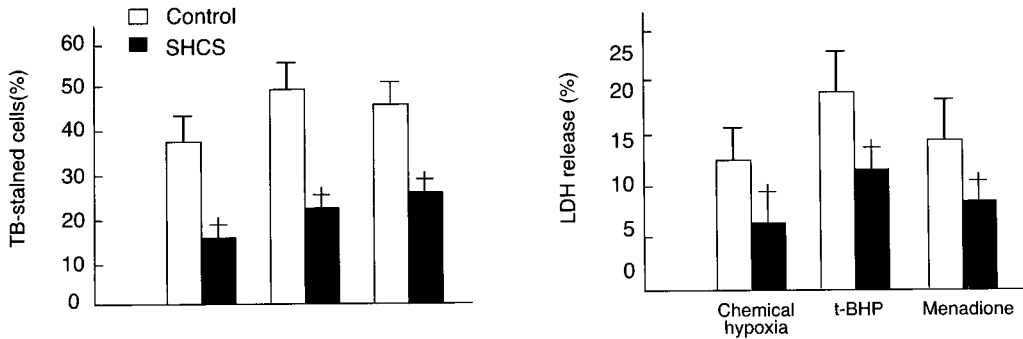


Fig. 3. Effect of Sunghyangchungisan on cell injuries induced by chemical hypoxia, t-butylhydroperoxide and menadione. Cells were exposed to chemical hypoxia or treated with t-butylhydroperoxide (t-BHP, 0.5 mM) or menadione (2 mM) for 3 hr at 37°C in the absence and presence of Sunghyangchungisan (SHCS, 1 mg/kg), and cell viability was determined by trypan blue exclusion ability and LDH release. Chemical hypoxia was induced using a combination of glucose deprivation and the mitochondrial electron transport inhibitor antimycin A (20 μM). Each point represents mean ± S.E. of 5 experiments. **p* < 0.01 vs. control.

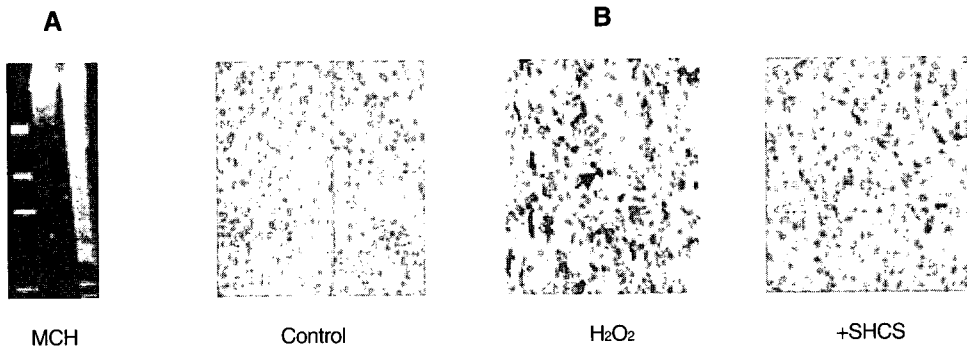


Fig. 4. Effect of SHCS on apoptotic cell death. Cells were first exposed to 0.1 mM H₂O₂ for 3 hr in the absence and presence of Sunghyangchungisan (SHCS, 1 mg/ml), and were then returned to H₂O₂-free culture media. After further incubation for 18 hr, cells were assayed for apoptosis by analysis of DNA fragmentation and TUNEL staining. Arrow indicates TUNEL-positive cells. M, molecular marker; C, control; H, H₂O₂.

stained by it ¹⁰. LDH is an intracellular enzyme and its release is a useful marker of membrane damage, a characteristic of necrotic cell death. When cells were treated with 0.5mM H₂O₂ for 3hr, 58.4% of the cells lost their ability to exclude the dye and stained by it and 19.6% of the LDH activity was found in incubation media. SHCS decreased both the indexes of cell injury in a concentration-dependent manner. At the concentration of 1 mg/ml it prevented cell injury determined by trypan blue exclusion and LDH release

by 58.2 and 63.4%, respectively.

In Fig. 3, it was examined whether SHCS can prevent other types of oxidant-induced cell injuries. Toward this end, cells were undergone chemical hypoxia, or exposed to chemical oxidants t-butyl hydroperoxide and menadione. Chemical hypoxia was induced using a combination of glucose deprivation and the mitochondrial electron transport inhibitor antimycin A (20μM). All these maneuvers induced cell injury or death determined by trypan blue exclusion ability and

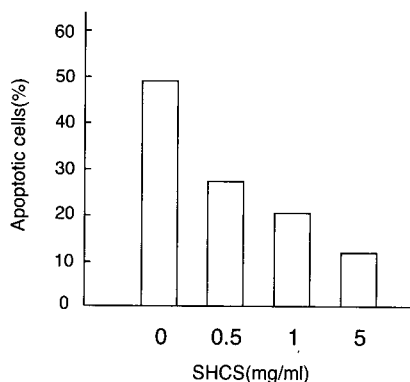


Fig. 5. Effect of SHCS on apoptotic cell death. Cells were first exposed to 0.1 mM H₂O₂ for 3 hr in the absence and presence of *Sunghyangchungisan* (SHCS, 1 mg/kg), and were then returned to H₂O₂-free culture media. After further incubation for 18 hr, cells were assayed for apoptosis by TUNEL staining. Each point represents mean \pm S.E. of 4 experiments. * $p < 0.01$ vs. the value in the absence of SHCS.

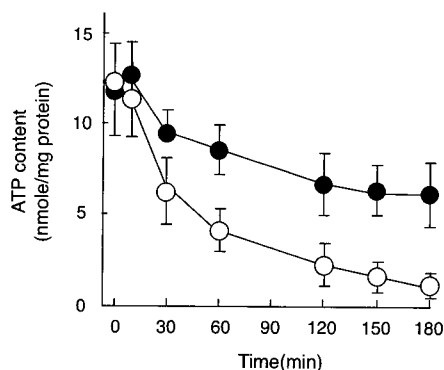


Fig. 6. Effect of *Sunghyangchungisan* on H₂O₂-induced depletion of cellular ATP. Cells were treated with 0.5 mM H₂O₂ for indicated time periods in the absence (open circle) and presence (closed circle) of *Sunghyangchungisan* (1 mg/kg). Each point represents mean \pm S.E. of 4 experiments.

LDH release assays. In the presence of SHCS (1 mg/ml), cellular injury was significantly prevented regardless of the causes of the insult. This result suggests that SHCS might have protective effect against a variety types of oxidant-induced injuries.

2. Effect of SHCS on apoptotic cell death

In the next series of experiments, it was examined whether SHCS might have a protective effect on apoptotic cell death. Toward this end A172 cells were first exposed to 0.1 mM H₂O₂ for 3hr. This maneuver resulted in usually less than 15% of necrotic cell death when analyzed by trypan blue exclusion assay. After washout of floating dead cells by necrotic injury, cells were returned to H₂O₂-free culture media. After further incubation for 18 hr, cells were assayed for apoptosis by analysis of DNA fragmentation and TUNEL staining. Treatment of cells with 0.1 mM H₂O₂ led to DNA fragmentation as shown by typical laddering pattern in the agarose gel electrophoresis (left lane of Fig. 4A). DNA fragmentation is one of the most characteristic

biochemical features of apoptosis. Apoptotic cells are visualized by TUNEL staining in the micrographs shown in Fig. 4B. Near half (47.1%) of cells in H₂O₂-treated preparations were counted as TUNEL-positive (mid panel of Fig. 4B). In the presence of SHCS, the number of TUNEL-positive cells were significantly reduced (right panel of Fig. 4B). The effects of different concentrations of SHCS on H₂O₂-induced apoptosis are summarized in Fig. 5.

3. Effect of SHCS on H₂O₂-induced ATP depletion

A decrease in intracellular ATP concentration precedes irreversible cell damage occurring upon hypoxia- or ROS-induced tissue injury¹⁾. Thus, it was examined whether SHCS might prevent H₂O₂-induced ATP depletion. In Fig. 6, time-dependent changes of intracellular ATP content in H₂O₂-treated cells in the absence and presence of SHCS (1 mg/ml) is depicted. ATP concentration in H₂O₂-treated cells decreased to lower than 10% of its control value in 3hr. However, in

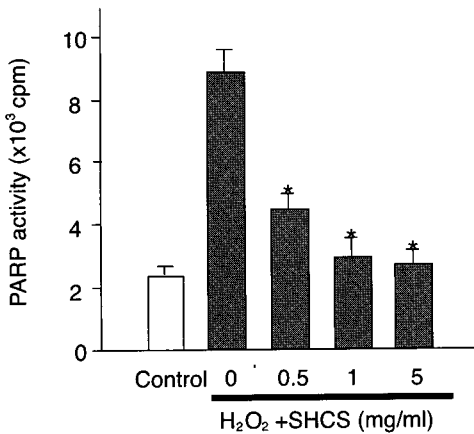


Fig. 7. Effect of Sunghyangchungisan on H₂O₂-induced activation of poly (ADP-ribose)polymerase (PARP) activity. Cells were treated for 3 hr with 0.5 mM H₂O₂ and indicated concentrations of Sunghyang chungisan (SHCS). Each point represents mean \pm S.E. of 4 experiments. * p <0.01 vs. H₂O₂ alone.

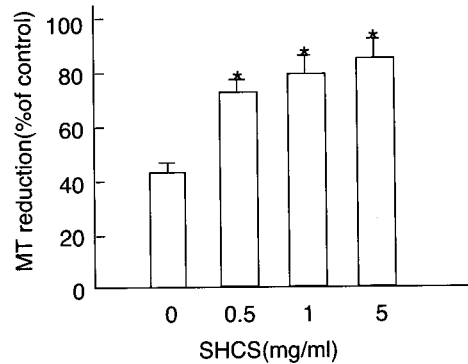


Fig. 8. Effect of Sunghyangchungisan on H₂O₂-induced deterioration of mitochondrial function. Cells were treated for 3 hr with 0.5 mM H₂O₂ and indicated concentrations of Sunghyangchungisan (SHCS), and MTT reduction ability was assayed as an index of mitochondrial function. Each point represents mean \pm S.E. of 6 experiments. * p <0.01 vs. the value in the absence of SHCS.

the presence of SHCS, H₂O₂-induced ATP depletion was significantly attenuated.

4. Effect of SHCS on H₂O₂-induced PARP activation

It has been reported that activation of poly (ADP-ribose) polymerase (PARP) is implicated in the H₂O₂-induced ATP depletion and neurotoxicity¹⁵⁾. To evaluate the possibility that the protective effect of SHCS might be related with PARP activity, H₂O₂-induced changes of PARP activity in A172 cells were examined in the presence and absence of SHCS. As shown in Fig. 7, exposure of cells to H₂O₂ resulted in a large increase in PARP activity. Moreover, this increase in PARP activity was significantly inhibited by SHCS.

5. Effect of SHCS on H₂O₂-induced mitochondrial dysfunction

In addition to PARP activation, deterioration of mitochondrial dysfunction is a major determinant to deplete intracellular ATP. In order to determine if

SHCS prevents H₂O₂-induced loss of mitochondrial function, changes in mitochondrial function was examined in cells treated with H₂O₂ in the presence or absence of SHCS using a MTT reduction assay. Mitochondria reduce MTT to formazan, and therefore, the ability of cells to form formazan from MTT is a useful indicator of intact mitochondrial function¹³⁾. Exposure of cells to 0.5mM H₂O₂ produced a significant loss of mitochondrial function as evidenced by the decrease in MTT reduction to 42.3% of control cells (Fig. 8). In the presence of SHCS, the decrease in MTT reduction was significantly prevented. These data suggest that SHCS prevents mitochondrial dysfunction induced by H₂O₂.

6. Effect of SHCS on H₂O₂-induced cytochrome release

It is well established that release of cytochrome c in mitochondrial inner membrane to cytosol is a key event to relate mitochondrial damage with apoptotic cell death¹⁶⁾. Therefore, it was examined whether SHCS could prevent

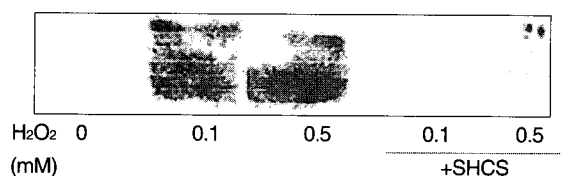


Fig. 9. Effect of *Sunghyangchungisan* on H₂O₂-induced cytochrome c release. Cells were treated for 3 hr with 0.1 or 0.5 mM H₂O₂ in the absence and presence of *Sunghyangchungisan* (SHCS, 1mg/kg). Levels of cytochrome c were analyzed by Western blot in the cytosolic fractions isolated from the treated cells.

H₂O₂-induced cytochrome c release in A172 cells. As shown in Fig. 9, treatment of cells led to release of cytochrome c and it was remarkably prevented by SHCS

7. Effect of SHCS on intracellular ROS generation

There is increasing evidence that tissue injury resulting from ischemia/reperfusion or chemical oxidant is mediated by intracellular generation of ROS¹⁾. To elucidate the effect of SHCS on ROS generation, intracellular formation of ROS was measured by monitoring the DCF fluorescence. As shown in Fig. 10, H₂O₂ induced a significant increase in the generation of intracellular ROS. In the presence of SHCS, production of ROS was remarkably reduced. The magnitude of the effect of SHCS at the concentration of 1 mg/ml to reduce intracellular ROS was comparable to the effects of well-known ROS scavengers superoxide dismutase (200U/ml), catalase (200U/ml), and dimethylthiourea (10 mM). The result suggests that SHCS might exert its effect as an effective ROS scavenger.

Discussion

All aerobic cells generate, enzymatically or nonenzymatically, superoxide, H₂O₂, and possibly hydroxyl radicals. Superoxide and H₂O₂ are generated during metabolism of a cell even under normal conditions. The mitochondrial electron transport chain

is the principal site of cellular production of ROS such as superoxide and H₂O₂ with approximately 2-5% of the O₂ consumed in state 4 respiration^{17,18)}. At the same time, the abundant antioxidant defenses of most cells, both enzymatic and nonenzymatic, prevent these species from causing cell injury. Nevertheless, there are a number of conditions in which the rate of formation of ROS is increased and/or antioxidant defenses of cells are weakened. In either case, oxidative cell injury may result. ROS can be generated during the process of phagocytosis, ischemia/reperfusion, and metabolism of many drugs and other xenobiotic chemicals¹⁾. Therefore, ROS have been implicated in the pathogenesis of a number of acute and chronic diseases. In this regard, the targeted development of new antioxidant drugs has been required.

There appear to be at least three distinct modes of cell death that participate in cell death, namely, apoptosis autophagocytotic cell death, and necrotic cell death^{19,20,21)}. Necrosis or necrotic cell death is fundamentally different from the other types of cell death in that it only occurs after an exogenous insult. It is usually accompanied by cellular swelling and membrane damage²⁰⁾. It does not appear to be a programmed, or a physiological process. Apoptosis is a form of cell death with morphological features quite distinct from necrosis^{20,22)}. In contrast to necrosis, it is characterized by both cytosolic and nuclear shrinkage. The nuclear chromatin in apoptotic cells undergoes condensation, whereas

plasma membrane and mitochondria remain morphologically intact. The condensed nuclei ultimately disintegrate into small membrane-bound fragmentation of DNA called 'apoptotic body'.

In the present study, necrotic cell death or apoptosis could be induced by varying concentrations of H₂O₂. Exposure of cells for 3hr to 0.5mM H₂O₂ induced cell death dominated by necrosis. Whereas, exposure of cells for 3hr to 0.1 mM H₂O₂ and further incubation in H₂O₂-free culture media overnight could lead to apoptotic cell death. Exposure of cells for 3hr only to 0.1 mM H₂O₂ induced usually less than 15% of necrotic cell death when analyzed by trypan blue exclusion assay. The characteristic features of apoptosis was shown clearly by the analysis of DNA fragmentation and TUNEL staining (Fig. 4).

Let,s survey the pharmacological working of SHCS. Huo Xiang(藿香) regulates vital energy(理氣), harmonizes middle-jiao(和中)and treats exterior syndrome and interior syndrome(兼治表裏). Huo Xiang(藿香), Bai Zhi(白芷) and Su Ye(蘇葉) dissipates cold pathogem(寒邪) by warming, benefits dysphagia(利膈), removes pathogenic factors attacking the exterior of the body, and eliminates wetness-evil(濁氣) with drugs of fragrant flavour(芳香). Jie Geng(桔梗), Huo Po(厚朴) and Da Fu Pi(大腹皮) regulates qi(調氣), removes mass of the abdomen(消脹) and transports water(行水). Ban Xia(半夏), Chun Pi(陳皮) and Sheng Jiang(生薑) keeps the adverse energy(降逆), removes dampness(除濕), eliminates the phlegm(化痰), and resolves interior substance. Bai Fu Ling(白茯苓), Bai Zhu(白朮) and Da Zao(大棗) reinforces the spleen(健脾), dries damp(祛濕) and invigorates normal resistance of the body(正氣)^{8,23)}. Nan Xing(南星) removes wind phlegm syndrome(風痰) and damp phlegm syndrome(濕痰) from the effect of drying damp(燥濕), eliminating the phlegm(化痰), dispelling wind-evil and spasmolysis(祛風解癇). Mu Xiang(木

香) resolves the stagnation of intestine,s vital energy from the effect of regulating qi(行氣), arresting pain(止痛), reinforcing the spleen(健脾), removing food retention and promoting digestion(消食)^{8,23,24)}. SHCS is a prescription which adds Nan Xing(南星) and Mu Xiang(木香) to Huo Xiang Zheng Qi San(藿香正氣散), and used for apoplexy(中風), zhong qi(中氣), phlegm syncope(痰厥) and a syncope with eating and drinking(食厥) as an emergency treatment^{7,8,10)}.

The data in the present study clearly suggested that SHCS might act as an effective cytoprotectant against neuroglial cell injury induced by ROS. It protected A172 cells against cell death induced by various types of oxidants as well as H₂O₂. It was effective to protect apoptotic as well as necrotic cell death. These results suggest that the protective effect of SHCS should be explained in relation with the common mechanism responsible for both the necrotic and apoptotic cell death.

Several lines of evidence suggest that the beneficial effect of SHCS to preserve mitochondrial function during oxidative insult might be a key event responsible for its protective effect against cell death. It helped mitochondria to preserve the ability to reduce MTT against H₂O₂-induced oxidative insult. Furthermore it significantly attenuated H₂O₂-induced ATP depletion. However, the beneficial effect of SHCS to preserve intracellular ATP during oxidative insult could not be ascribed entirely to the effect on mitochondrial function. The effect of SHCS to prevent PARP activation as shown in Fig. 7 could provide another clue to explain the mechanism of ATP preservation.

Oxidants have been known to cause DNA damage²⁵⁾ and PARP activation^{26,27)}. Since PARP catalyzes the transfer of ADP-ribose from NAD to protein with the concomitant release of nicotinamide, the activation of this enzyme results in depletion of NAD and a consequent reduction in ATP, which may be involved

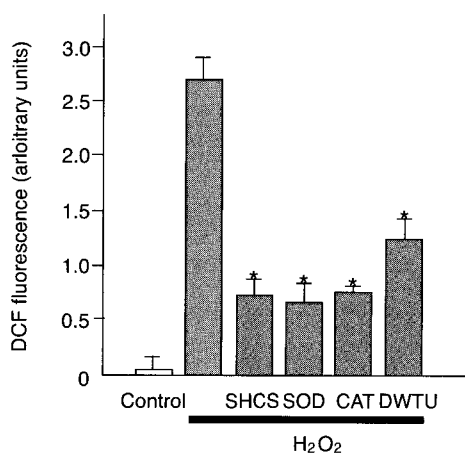


Fig. 10. Effect of *Sunghyangchungisan* and various ROS scavengers on H₂O₂-induced formation of ROS in A172 cells. H₂O₂ (0.5 mM)-induced increase in ROS formation for 60min was determined by DCF fluorescence assay in the absence and presence of *Sunghyangchungisan* (SHCS, 1mg/kg), superoxide dismutase (SOD, 200 U/ml), catalase (CAT, 200 U/ml), and dimethylthiourea (DMTU, 10mM). Each point represents mean \pm S.E. of 4 experiments. * $p < 0.01$ vs. H₂O₂ alone.

in the pathogenesis of oxidant-induced injury. PARP has been demonstrated to be involved in the cell death caused by H₂O₂ in neuronal cells^{15,28}) and various nonneuronal cell systems^{29,30}). However, the role of PARP in oxidant-induced death to glial cells has not been established. The present study demonstrated clearly that PARP is activated as a result of oxidant stress by H₂O₂ and that the SHCS prevents the stimulation of PARP caused by H₂O₂ in A172 cells. However, it is unclear whether the ATP depletion resulting from PARP activation is a cause of H₂O₂-induced cell death. While ATP depletion has been reported to induce the oxidant-induced cell death³¹), other studies have shown that the oxidant-induced injury is dissociated with ATP depletion^{32,33}).

It has been suggested that ROS-induced cell injury is associated with mitochondrial permeability transition (MPT) and subsequent release of cytochrome c through this MPT pore¹⁶). Released cytochrome c activates caspase cascades, which are critical for the execution phase of apoptosis. It is interesting to note that SHCS

prevents H₂O₂-induced cytochrome c release (Fig. 9). It might be closely related with the protective effect of SHCS against apoptotic cell death. However, as noted above, it is not likely that SHCS might act on a specific intracellular pathway to induce MPT and apoptosis. With this point of view it is noteworthy that SHCS itself might exert as an effective ROS scavenger as evidenced in Fig. 10.

In the present study, the fluorescent dye DCFDA was used as a quantitative mean to assess the intracellular ROS generation. DCFDA has been used as a sensitive probe for cellular, mitochondrial, cytosolic, and microsomal production of ROS such as superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and etc. in many tissues¹⁴). The results clearly showed that SHCS is an effective scavenger of ROS. It suggests that the overall protective effects observed in this study might be associated with the role of SHCS as an ROS-scavenger. It was not deciphered in this study which component of SHCS is responsible for the ROS-scavenging effect. It may be a natural component(s)

included in SHCS or a newly formed compound(s) through a chemical reaction among the natural components of SHCS. Further analytical study should be needed to elucidate this.

In conclusion, the present study provides clear evidence for the beneficial effect of SHCS on neuroglial cell injury. The action of SHCS as an ROS-scavenger might underlie the mechanism. The exact component and mechanism responsible for the cytoprotection re

Conclusion

This study was carried out to determine whether SHCS exerts beneficial effect against brain cell injury induced by oxidant using A172 cells, a human neuroglioma cell line.

The results are as follows;

1. SHCS significantly prevented cell injury induced by various types of oxidants.
2. SHCS significantly reduced apoptotic cell death.
3. SHCS significantly attenuated H₂O₂-induced ATP depletion.
4. SHCS significantly prevented increase in PARP activity.
5. SHCS significantly prevented mitochondrial dysfunction induced by H₂O₂.
6. SHCS remarkably prevented H₂O₂-induced cytochrome c release in A172 cells.
7. SHCS remarkably reduced intracellular ROS generation.

In conclusion, the present study provides clear evidences for the beneficial effect of SHCS on neuroglial cell injury.

References

1. Farber JL, Kyle ME, Coleman JB. Mechanisms of cell injury by activated oxygen species. *Lab*

*Invest.*1990;62(6):670-9.

2. Bondy SC. The relation of oxidative stress and hyperexcitation to neurological disease. *Proc Soc Exp Biol Med.* 1995;208(4):337-45.
3. Sendtner M, Thoenen H. Neurodegenerative disease. Oxidative stress and motoneuron disease. *Curr Biol,* 1994;4(11):1036-9.
4. Olanow CW. A radical hypothesis for neurodegeneration. *TINS.* 1993;16:439-44
5. Kanter M. Free radicals, exercise and antioxidant supplementation. *Proc Nutr Soc.* 1998;57:9-13.
6. Dai Si Gong, "Zheng Zhi Yao Jue", Bei Jing : The People,s Medical Publishing House. 1983:35-6.
7. Hwang Do Yeon, "Zeung Mak Bang Yak Hap Pyun", Seou : Nam San Dang. 1978:138-40.
8. Yun Gil Young, "Catagraphology of Oriental Medicine", Seoul : Ko Mun Company. 1980:40-1,108,144,155,173.
9. Ku Bon Hong, "Cardiology of Oriental Medicine" Seoul : Seo Won Dang. 1985:.305-11.
10. Kim Jung Jae, "Summary of Treatment" Seoul : Laboratory of Oriental Medicine. 1974; vol. 1: 448-9, 453, vol. 2:431.
11. Bjorkerud S, Bondjers G. Endothelial integrity and viability in the aorta of the normal rabbit and rat as evaluated with dye exclusion tests and interference contrast microscopy. *Atherosclerosis.* 1972;15:285-300.
12. Lyman GE, DeVincenzo JP. Determination of picogram amounts of ATP using the luciferin-luciferase enzyme system. *Anal Biochem.* 1967;21:435-43.
13. Morgan DM. Tetrazolium (MTT) assay for cellular viability and activity. *Methods Mol Biol.* 1998;79:179-83.
14. LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species

- formation and oxidative stress. *Chem Res Toxicol.* 1992;5:227-31.
15. Cookson MR, Ince PG, Shaw PJ. Peroxynitrite and hydrogen peroxide induced cell death in the NSC34 neuroblastoma x spinal cord cell line: role of poly (ADP-ribose) polymerase. *J Neurochem.* 1998;70(2):501-8.
 16. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol.* 1998;60:619-42.
 17. Boveris A, Oshino N and Chance B. The cellular production of hydrogen peroxide. *Biochem J.* 1972;128:617-30.
 18. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J.* 1980;191:421-7.
 19. Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol.* 1990;181(3):195-213.
 20. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol.* 1995;146(1):3-15.
 21. Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev.* 1993;14(2):133-51.
 22. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* 1972;26:239-57.
 23. Shin Jae Yong, "Explanation of Bang Yak Hap Pyun". Seoul :Sung Bo Compony. 1989:98-9.
 24. Shin Min Gyo, "Colorful Clinical Phytology". Seoul : Nam San Dang. 1986 : 172~81, 294~5, 329~31, 460~1, 464~5, 469~70, 590~3, 598~600, 649~52, 724~6, 744~7, 749~50, 819-21.
 25. Jackson JH, Schraufstatter IU, Hyslop PA, Vosbeck K, Sauerheber R, Weitzman SA, Cochrane CG. Role of oxidants in DNA damage. Hydroxyl radical mediates the synergistic DNA damaging effects of asbestos and cigarette smoke. *J Clin Invest.* 1987;80:1090-5.
 26. Szabo C, Virag L, Cuzzocrea S, Scott GS, Hake P, O'Connor MP, Zingarelli B, Salzman A, Kun E. Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly(ADP-ribose)synthase. *Proc Natl Acad Sci USA,* 1998;95:3867-72.
 27. Yamamoto K, Farber JL. Metabolism of pyridine nucleotides in cultured rat hepatocytes intoxicated with tert-butyl hydroperoxide. *Biochem Pharmacol.* 1992;43:1119-26.
 28. Mailly F, Marin P, Israel M, Glowinski J, Premont J. Increase in external glutamate and NMDA receptor activation contribute to H₂O₂-induced neuronal apoptosis. *J Neurochem.* 1999;73:1181-8.
 29. Chatterjee PP, Cuzzocrea S, Thiernemann C. Inhibitors of poly (ADP-ribose) synthetase protect rat proximal tubular cells against oxidant stress. *Kidney Int.* 1999;56:973-84.
 30. Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG. Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA.* 1986; 83:4908-12.
 31. Thies RL, Autor AP. Reactive oxygen injury to cultured pulmonary artery endothelial cells: mediation by poly(ADP-ribose)polymerase activation causing NAD depletion and altered energy balance. *Arch Biochem Biophys.* 1991; 286:353-63.
 32. Andreoli SP, Mallett CP. Disassociation of oxidant-induced ATP depletion and DNA damage from early cytotoxicity in LLC-PK1 cells. *Am J Physiol.* 1997;272:F729-35.

(282) 대한한의학회지 제26권 제2호(2005년 6월)

33. Frenkel K, Gleichauf C. Hydrogen peroxide formation by cells treated with a tumor promoter.

Free Radic Res Commun. 1991;12:783-94.