원 저

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 oxygen [1]. 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

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 symtoms 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 (H_2O_2) was employed as an oxidant model drug.

Materials and Methods

1. SHCS extract preparation

SHCS was extracted with 3000ml distilled water at 100℃ 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-cm^2 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℃ in humidified 95% air/5% CO_2 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.

Tabel 1. Prescription of SHCS

Herbal name	Scientific name	Weight(g)
Huo Xiang(藿香)	*Herba Pogostemi*	5.625
Su Ye(蘇葉)	*Folium Perillae*	3.750
Bai Zhi(白芷)	*Radix Angelicae Dahuricae*	1.875
Da Fu Pi(大腹皮)	*Pericarpium Arecae*	1.875
Bai Fu Ling(白茯笭)	*Poria*	1.875
Huo Po(厚朴)	*Cortex Magnoliae*	1.875
Bai Zhu(白朮)	*Rhizoma Atractylodis Macrocephalae*	1.875
Chun Pi(陳皮)	*Pericarpium Citri Nobilis*	1.875
Ban Xia(半夏)	*Tuber Pinelliae*	1.875
Jie Geng(桔梗)	*Radix Platycodi*	1.875
Gan Cao(甘草)	*Radix Glycyrrhizae*	1.875
Sheng Jiang(生薑)	*Rhizoma Zingiberis*	3.000
Da Zao(大棗)	*Fructus Zizyphi Jujubae*	3.000
Nan Xing(南星)	*Rhizoma Arisaematis*	3.750
Mu Xiang(木香)	*Radix Saussurea*	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 H_2O_2 in Hank's balanced salt solution (HBSS) for 3 hr at 37℃, unless otherwise indicated. After exposure to experimental protocols, each cell group was stained with 2μl mixture of 270μM acridine orange and 254 μM 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 H_2O_2 for 3 hr and, after washout of floating dead cells by necrotic injury, returned to H_2O_2-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 mM on ice. the lysates were then centrifuged at 15,000g for 10 min at 4℃. DNA in supernatants were extracted with phenol-chloroform, precipitated in ethanol, resuspended in Tris/EDTA, pH 8.0, containing 20μg/ml RNAse A, and incubated for 1 hr at 37℃ 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 coverglsses 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℃ 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 500μl of 0.5% Triton X-

100 and acidified with $100\mu l$ 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 $100\mu l$ of 20㎎/㎖ luciferin-luciferase was added to $10\mu l$ 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 H_2O_2 and preincubated for 10 min in a buffer containing 28mM NaCl, 28 mM KCl, 2 mM $MgCl_2$, 56 mM Hepes (pH 7.5), 0.01％ digitonin, and 125mM NAD (containing $0.25\mu C_i$ [^{3}H]NAD). Permeabilized cells were incubated for 5min at 37℃, and the protein ribosylated with [^{3}H]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℃ 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 formazan [13]. Therefore the ability of cells to form formazan from MTT is a good indicator of mitochondrial function. A total $5\mu l$ of MTT was added to each well (final concentration is $62.5\mu g/ml$). 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℃ and resuspended in 1 *ml* of extraction buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 5mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10µg/*ml* leupeptin and 10µg/*ml* pepstatin A). Cells were lysed by five cycles of freezing in liquid nitrogen and thawing at 37℃. 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℃. 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) fluorescence[14]. 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℃ in a fluorescent cuvette containing 3 ml of glucose-free buffer with 20µM DCFH-DA (from a stock solution of 20mM DCFH-DA in ethanol). After preincubation, cells were treated with H_2O_2 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±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 H_2O_2. 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 H_2O_2-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
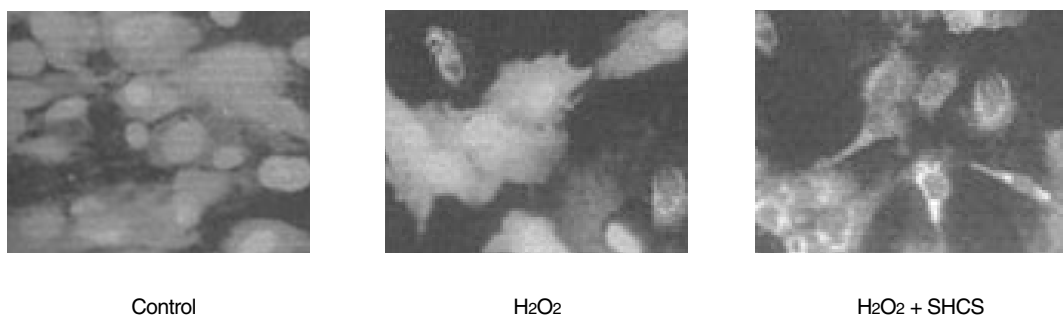

Control H_2O_2 H_2O_2 + SHCS

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.
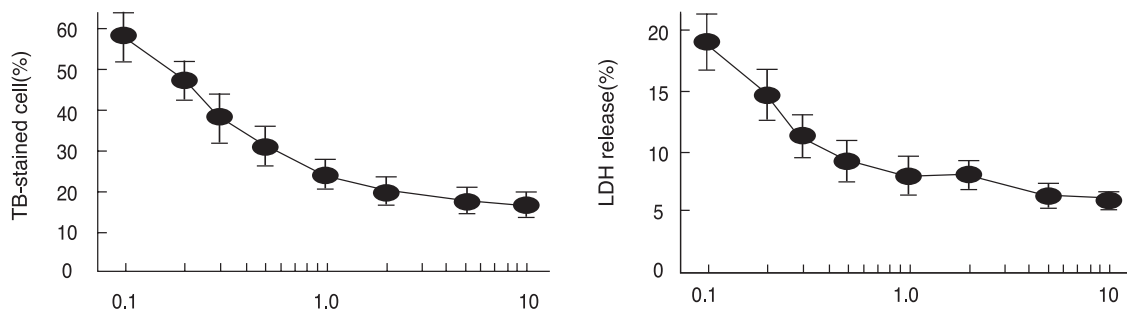

Fig. 2. Concentration-dependent protection by Sunghyangchungisan against H2O2-induced cell injury. Cells were treated with 0.5 mM H2O2 for 3 hr at 37oC in the presence of indicated concentrations of Sunghyangchungisan (SHCS), and cell viability was determined by trypan blue exclusion ability and LDH release. Each point represents mean±S.E. of 5 experiments.
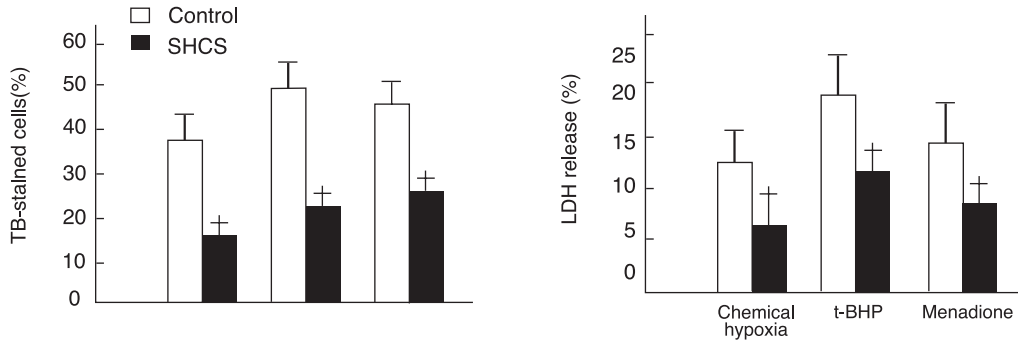
Fig. 3. Effect of Sunghyangchungisan on cell injuries induced by chemical hypoxia, t-butylhydroperoxide and menadione. Cells were exposed to chemical hypoxia or treated with t-butylhydroperoxide (t-BHP, 0.5 mM) or menadione (2 mM) for 3 hr at 37oC in the absence and presence of Sunghyangchungisan(SHCS, 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 depriva-tion and the mitochondrial electron transport inhibitor antimycin A (20 µM). Each point represents mean±S.E. of 5 experiments. *p<0.01 vs. control.

A   **B**

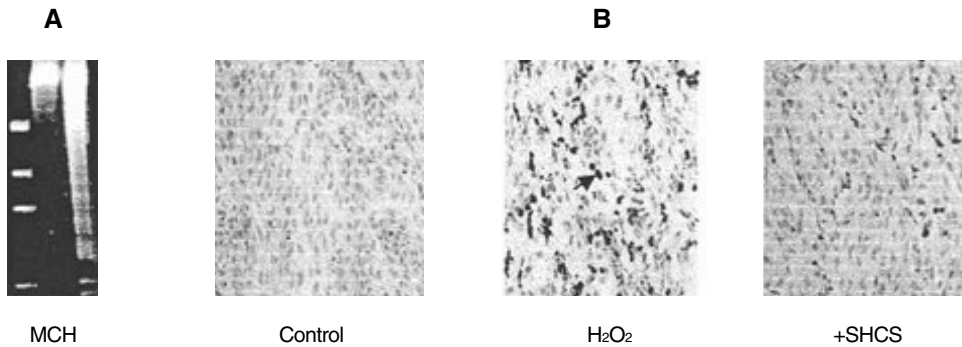MCH   Control   H₂O₂   +SHCS

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 [11]. 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 H_2O_2 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
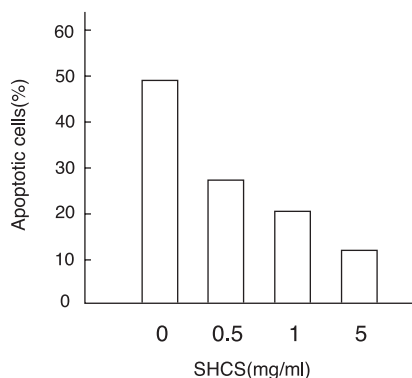
Fig. 5. Effect of SHCS on apoptotic cell death. Cells were first exposed to 0.1 mM H2O2 for 3 hr in the absence and presence of Sunghyangchungisan (SHCS, 1 mg/kg), and were then returned to H2O2-free culture media. After further incubation for 18 hr, cells were assayed for apoptosis by TUNEL staining. Each point represents mean±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±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 H_2O_2 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 H_2O_2-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 H_2O_2 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
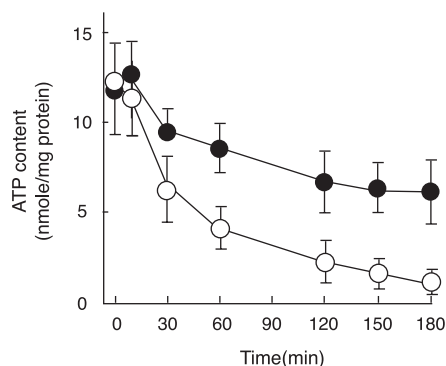
biochemical features of apoptosis. Apoptotic cells are visualized by TUNEL staining in the micrographs shown in Fig. 4B. Near half (47.1%) of cells in H_2O_2-treated preparations were counted as TUNEL-positive (mid panel of Fig. 4B). In the presence of SHCS, the number of TUNEL-positive cells were significantly reduced (right panel of Fig. 4B). The effects of different concentrations of SHCS on H_2O_2-induced apoptosis are summarized in Fig. 5.

3. Effect of SHCS on H_2O_2-induced ATP depletion

A decrease in intracellular ATP concentration precedes irreversible cell damage occurring upon hypoxia- or ROS-induced tissue injury [1]. Thus, it was examined whether SHCS might prevent H_2O_2-induced ATP depletion. In Fig. 6, time-dependent changes of intracellular ATP content in H_2O_2-treated cells in the absence and presence of SHCS (1 mg/ml) is depicted. ATP concentration in H_2O_2-treated cells decreased to lower than 10% of its control value in 3hr. However, in
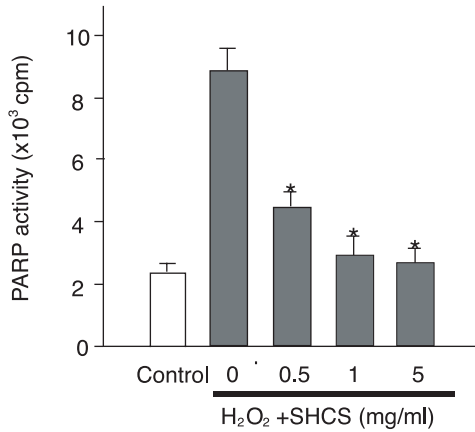
Fig. 7. Effect of Sunghyangchungisan on H2O2-induced activation of poly (ADP-ribose)polymerase (PARP) activity. Cells were treated for 3 hr with 0.5 mM H2O2 and indicated concentrations of Sunghyang - chungisan (SHCS). Each point represents mean$\pm$ S.E. of 4 experiments. *p<0.01 vs. H2O2 alone.
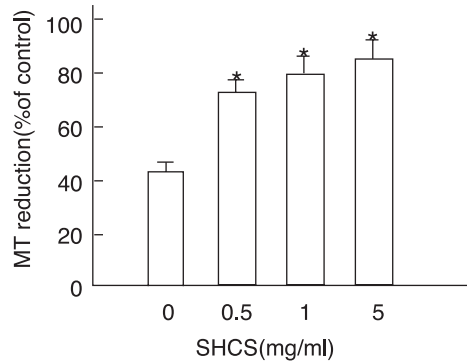

Fig. 8. Effect of Sunghyangchungisan on H2O2-induced deterioation of mitochondrial function. Cells were treated for 3 hr with 0.5 mM H2O2 and indicated concentrations of Sunghyangchungisan (SHCS), and MTT reduction ability was assayed as an index of mitochondrial function. Each point represents mean$\pm$S.E. 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 neurotoxicity [15]. 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 mitocho - ndrial 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 function [13]. Exposure of cells to 0.5mM H2O2 produced a significant loss of mitochondrial function as evidenced by the decease in MTT reduction to 42.3% of control cells (Fig. 8). In the presence of SHCS, the decease in MTT reduction was significantly prevented. These data suggest that SHCS prevents mitochondrial dysfunction induced by H2O2.

6. Effect of SHCS on H2O2-induced cytochr- ome crelease

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 death[16]. Therefore, it was examined whether SHCS could prevent
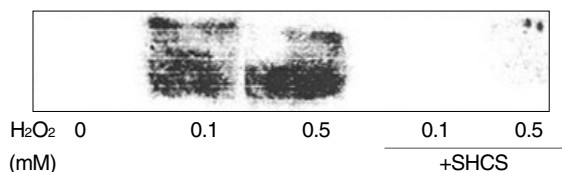
H$_2$O$_2$	0	0.1	0.5	0.1	0.5
(mM)				+SHCS	

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, $1\mathrm{mg/kg}$). Levels of cytochrome c were analyzed by Western blot in the cytosolic fractions isolated from the treated cells.

H$_2$O$_2$-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 ROS [1]. 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, H$_2$O$_2$ 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 $1\mathrm{mg}/ml$ 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, H$_2$O$_2$, and possibly hydroxyl radicals. Superoxide and H$_2$O$_2$ 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 H$_2$O$_2$ with approximately 2-5% of the O$_2$ 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 chemicals [1]. 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 damage [20]. 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 H_2O_2. Exposure of cells for 3hr to 0.5mM H_2O_2 induced cell death dominated by necrosis. Whereas, exposure of cells for 3hr to 0.1 mM H_2O_2 and further incubation in H_2O_2-free culture media overnight could lead to apoptotic cell death. Exposure of cells for 3hr only to 0.1 mM H_2O_2 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 dempness(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(祛風解痙). 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 H_2O_2. 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 H_2O_2-induced oxidative insult. Furthermore it significantly attenuated H_2O_2-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 damage[25] 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
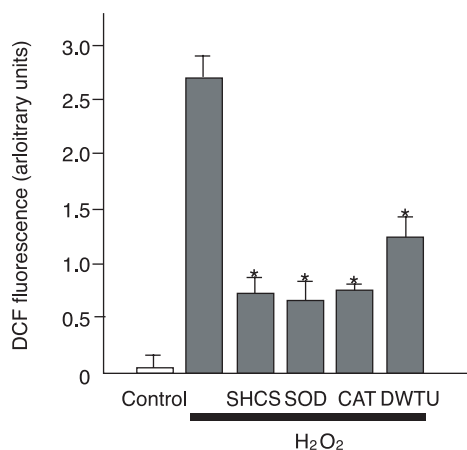
Fig. 10. Effect of Sunghyangchungisan and various ROS scavengers on H2O2-induced formation of ROS in A172 cells. H2O2 (0.5 mM)-induced increase in ROS formation for 60min was determined by DCF fluorescence assay in the absence and presence of Sunghyangchungisan (SHCS, $1mg/kg$), superoxide dismutase (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 H_2O_2 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 H_2O_2 and that the SHCS prevents the stimulation of PARP caused by H_2O_2 in A172 cells. However, it is unclear whether the ATP depletion resulting from PARP activation is a cause of H_2O_2-induced cell death. While ATP depletion has been reported to induce the oxidant-induced cell death [31], 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 pore [16]. Released cytochrome c activates caspase cascades, which are critical for the execution phase of apoptosis. It is interesting to note that SHCS

prevents H_2O_2-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 exerts 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 tissues [14]. 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 H_2O_2-induced ATP depletion.
4. SHCS significantly prevented increase in PARP activity.
5. SHCS significantly prevented mitochondrial dysfunction induced by H_2O_2.
6. SHCS remarkably prevented H_2O_2-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.

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