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, Huntington’s 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. 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. 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(霍香正氣散). It has effects of regulating vital energy, eliminating phlegm and a syncope with eating and drinking etc. It is an emergency treatment for apoplexy, coma and abundant phlegm. 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 Dulbecco 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>
<thead>
<tr>
<th>Herbal name</th>
<th>Scientific name</th>
<th>Weight(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huo Xiang(藿香)</td>
<td>Herba Pogostemi</td>
<td>5.625</td>
</tr>
<tr>
<td>Su Ye(蘇葉)</td>
<td>Folium Perillae</td>
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<tr>
<td>Bai Zhi(白芷)</td>
<td>Radix Angelicae Dahuricae</td>
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<tr>
<td>Da Fu Pi(大腹皮)</td>
<td>Pericarpium Arecae</td>
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<tr>
<td>Bai Fu Ling(白茯苓)</td>
<td>Poria</td>
<td>1.875</td>
</tr>
<tr>
<td>Huo Po(厚朴)</td>
<td>Cortex Magnoliae</td>
<td>1.875</td>
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<tr>
<td>Bai Zhu(白朮)</td>
<td>Rhizoma Atractylodis Macrocephalae</td>
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<tr>
<td>Chun Pi(陳皮)</td>
<td>Pericarpium Citri Nobilis</td>
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<tr>
<td>Ban Xia(半夏)</td>
<td>Tuber Pinelliae</td>
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<td>Gan Cao(甘草)</td>
<td>Radix Glycyrrhizae</td>
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<td>Sheng Jiang(生薑)</td>
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<td>3.000</td>
</tr>
<tr>
<td>Da Zao(大棗)</td>
<td>Fructus Zizyphi Jujubae</td>
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<tr>
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<td>Rhizoma Arisaematis</td>
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<tr>
<td>Mu Xiang(木香)</td>
<td>Radix Saussurea</td>
<td>3.750</td>
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**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 2µl mixture of 270µM acridine orange and 254µM 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. 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 × 10⁶) 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 20µg/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 antidigoxigenin 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. After an exposure to oxidant stress, the cells were solubilized with 500µl of 0.5% Triton X-
and acidified with 100 µl 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 µl of 20 µg/ml luciferin-luciferase was added to 10 µl 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 28 mM NaCl, 28 mM KCl, 2 mM MgCl2, 56 mM Hepes (pH 7.5), 0.01% digitonin, and 125mM NAD (containing 0.25µCi [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 µl of MTT was added to each well (final concentration is 62.5 µg/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, 10µg/ml leupeptin and 10µg/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 20µM 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,
13. Data analysis
Data are presented as means ± SE. When necessary, data were analyzed by ANOVA followed by Duncan's 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 0.5 mM H\(_2\)O\(_2\). 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\(_2\)O\(_2\)-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
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 0.5 mM H\(_2\)O\(_2\) for 3 hr, 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 (20 \(\mu\)M). All these maneuvers induced cell injury or death determined by trypan blue exclusion ability and

**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 \(\mu\)M). 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 H\(_2\)O\(_2\) for 3 hr in the absence and presence of Sunghyangchungisan (SHCS, 1 mg/ml), and were then returned to H\(_2\)O\(_2\)-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, H\(_2\)O\(_2\).
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 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. *

![Graph showing effect of SHCS on apoptotic cell death](image)

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.

![Graph showing effect of SHCS on ATP depletion](image)

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.

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 injury 1). 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 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.
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. 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 function. 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 c 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 death. Therefore, it was examined whether SHCS could prevent
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. 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 mg/ml to reduce intracellular ROS was comparable to the effects of well-known ROS scavengers superoxide dismutase (200U/ml), catalase (200U/ml), and dimethyldithiorea (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. 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. 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. 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. 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. 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\textsubscript{2}O\textsubscript{2}. Exposure of cells for 3 hr to 0.5 mM H\textsubscript{2}O\textsubscript{2} induced cell death dominated by necrosis. Whereas, exposure of cells for 3 hr to 0.1 mM H\textsubscript{2}O\textsubscript{2} and further incubation in H\textsubscript{2}O\textsubscript{2}-free culture media overnight could lead to apoptotic cell death. Exposure of cells for 3 hr only to 0.1 mM H\textsubscript{2}O\textsubscript{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 (濕氣) 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 (消腫) 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 (祛湿) and invigorates normal resistance of the body (正氣) \(^{4,23}\). 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 spasmylosis (祛風解痺). 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\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2}-induced oxidative insult. Furthermore it significantly attenuated H\textsubscript{2}O\textsubscript{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
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 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 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 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$_2$O$_2$-induced ATP depletion.
4. SHCS significantly prevented increase in PARP activity.
5. SHCS significantly prevented mitochondrial dysfunction induced by H$_2$O$_2$.
6. SHCS remarkably prevented H$_2$O$_2$-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.

**References**

14. LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species