Water Extract of Samultang Reduces Apoptotic Cell Death by H₂O₂-Induced Oxidative Injury in SK-N-MC Cells

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The purpose of this study was to evaluate the effects of the water extract of Samultang (SMT), a Chinese herb, on apoptotic cell death by H_2O_2-induced oxidative stress in SK-N-MC cells. A nuclear fragmentation was observed via fluorescence imaging 12 h after exposure to 30 μM H_2O_2 and DNA laddering was detected via agarose electrophoresis gel. In addition, increases in sub-G1 phase and cleavage of the PARP protein were observed. However, treatment with SMT for 2 h prior to H_2O_2 exposure significantly reduced apoptotic cell death induced by incubation with 30 μM H_2O_2 in SK-N-MC cells. Pre-incubation with water extract of SMT for 2 h prevented the H_2O_2-induced decrease in mitochondrial transmembrane potential. SMT also attenuated the increase in caspase-3 activity and the breakdown of PARP protein caused by H_2O_2-induced oxidative stress. These results suggest that the water extract of SMT provides inhibition of apoptotic cell death against oxidative injury in SK-N-MC cells.

Key Words: Samultang, Apoptosis, Oxidative injury, SK-N-MC

INTRODUCTION

Oxidative stress is believed to be a mechanism involved in neuronal damage after cerebral ischemia (Sugawara and Chan, 2003). Namely, reactive oxygen species (ROS) can cause oxidative damage to proteins, lipids, and DNA. ROS are also mediators in signaling involving mitochondria, DNA repair enzymes, and transcription factors that may lead to apoptosis after cerebral ischemia (Chan, 2001; Sugawara and Chan, 2003). For these reasons, oxidative stress could be considered as an important target for drug development (Fujimura et al., 2005).

A composite traditional oriental medicine, Samultang (SMT) (Korean name), Si-Wu decoction (Chinese name), or Shimotsu-to (Japanese name), is a basic prescription consisting of four herbs: Radix Paeoniae Alba, Radix Angelicae Sinensis, Rhizoma Chuanxiong, and Radix Rehmanniae. For hundreds of years in Eastern Asia, SMT has been used for the treatment of gynecological diseases, chronic inflammation, and pain relief (Xie, 1997). It has also been used to treat ischemic brain and heart diseases (Kang, 1993; Shin, 1996). The main constituents of SMT are known as phenolic compounds, phthalides, alkaloids, terpene glycosides, and iridoid glycosides (Sheng et al., 2005), and some of these are known to have antioxidant or antiischemic effects (Graf, 1992; Scott et al., 1993; Yan et al., 2004).

According to a recent study, SMT improved scopolamine-impaired working memory performance in the eight-arm radial maze task (Watanabe et al., 1991). Furthermore, pretreatment with SMT prevented apoptotic cell death by oxidative stress or irradiation in C6 neuroglial cells, jejunal epithelial cells, and hematopoietic cells (Hsu et al., 1996; Lee et al., 1999; So et al., 2000).

We therefore predicted that the water extract of SMT would aid survival of neuronal cells from oxidative injury. The purpose of this study was to evaluate the neuroprotective effects of the water extract of SMT against oxidative injury in the SK-N-MC neuroblastoma cell line.

METHODS

Cell cultures

Human neuroblastoma cell line SK-N-MC was obtained from the Korean Cell Line Bank (KCLB, Korea). The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco/BRL, Canada) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cells were seeded at a density of 5×10^5 cells/well in six-well plates, 2×10^4 cells/well in 96-well plates, or 2×10^6 cells/plate in 10-cm dishes for 24 h prior to treatment.

ABBREVIATIONS: ROS, reactive oxygen species; SMT, samultang; DMEM, Dulbecco's modified eagle's medium; PARP, Poly (ADP-ribose) Polymerase; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2 ,5-diphenyl-2H-tetrazoliumbromide; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Drug exposure

The water extract of Samultang (SMT) was prepared by the College of Oriental Medicine, Wonkwang University, and dissolved with phosphate buffered saline (PBS). Various concentrations of SMT (100, 200, 300, 500 μg/ml) were added to the cultures 2 h before hydrogen peroxide (H_2O_2) treatment. H_2O_2-induced oxidative stress to cells was given under SMT treatment. Control cultures were maintained in the incubator under normal conditions.

Induction of oxidative injury

Oxidative injury was induced with H_2O_2 in SK-N-MC cells. Cells were treated with serial concentrations of H_2O_2 prepared in DMEM containing 10% FBS for up to 12 h.

MTT assay

Cell viability was determined with a modified MTT assay. This test is based on a reduction of tetrazolium salts into red formazan derivatives in intact mitochondria of viable cells. After 12 h exposure to H_2O_2, cells were washed with PBS. Tetrazolium salt solution was pipetted into each well as per the manufacturer's instructions. After a 3 h incubation period, plates were analyzed in an ELISA reader (Molecular Devices, USA) at 570 nm. MTT assay was undertaken three times in each experimental condition.

DAPI staining

Cells were washed with DAPI-methanol (1 μg/ml; Roche, USA), and then stained with DAPI-methanol for 15 min at 37°C. Stained cells were then washed once with methanol and observed under a fluorescence microscope (Olympus, Japan). Apoptotic cells were identified by features characteristic of apoptosis. Morphological analysis by DAPI staining was done three times in each experimental state.

Cell cycle study

Cells were harvested and washed once with cold PBS. Cell pellets were then suspended in 500 μl of PI solution containing 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% RNase. Cell samples were incubated at 4°C in the dark for at least 30 min, and analyzed using a flow cytometer (FACS Calibur, USA) and Cell Quest software. This flow cytometric analysis for cell cycle was performed three times in each experimental condition.

DNA fragmentation

DNA laddering in apoptotic cells was detected using an ApopLadder Ex kit (Takara Bio, Japan). Briefly, cells were suspended in lysis buffer and centrifuged. Supernatants were mixed with 10% Sodium dodecylsulfate (SDS) and Enzyme A, and then incubated at 56°C for 1 h. Enzyme B was then added, and the mixture was incubated for an additional 1 h at 37°C. To precipitate DNA fragments, the preparation was mixed with precipitant and ethanol and then stored for 15 min at -20°C. DNA pellets were washed with 80% ethanol and suspended in Tris-borate-EDTA (TBE) buffer. DNA fragmentation was visualized by electro-phoresis on a 2% agarose gel containing ethidium bromide. DNA laddering was performed three times in each experimental condition. Three times assay for DNA laddering was achieved in each experiment.

Mitochondrial membrane potentials analysis

Treated cells were stained with 25 nmol/L MitoTracker Red CMXRos, a mitochondrial selective dye (Molecular Probes, USA) at 37°C for 15 min, and washed with PBS. The mitochondrial membrane potential was measured by a flow cytometer. Assay for mitochondrial membrane potentials was undertaken three times in each experimental condition.

Reverse transcription-PCR for caspase-3

For reverse transcription (RT)-PCR experiments, total RNA was extracted using Trizol reagent (Life Technologies, USA) following the manufacturer's protocol. RT-PCR was performed according to standard protocols. For cDNA synthesis, 2.5 μg of total RNA was reversely transcribed with Superscript II (Life Technologies, USA) in a volume of 50 μl according to the manufacturer's instructions. The reaction was primed using random primers (300 ng). For the subsequent PCR reaction, 100 ng of ethanol-precipitated cDNA was used as the template. The primer sequences and cycling conditions for semiquantitative PCR were as follows: 94°C for 3 min, annealing for 30 sec, and extension for 30 sec at 72°C; caspase-3 primers, 5'-ATTCATAGTGGCACC-AAATC-3' (forward) and 5'-TAAATCAAATCCGATGTTCC-3' (reverse), annealing temperature of 64°C, 30 cycles; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, GTGATGCTGGTGCTGA (forward) and 5'-TCCACCACCC-TGTTGCTGTA-3' (reverse), annealing temperature of 64°C, 23 cycles. The expressional change in mRNA for caspase-3 was observed three times in each experimental condition.

Caspase-3 activity assay

The catalytic activity of caspase-3 was measured using a colorimetric assay according to the manufacturer's instructions (Calbiochem, USA), which was based on the spectrophotometric detection of the chromophore p-nitroanilide after cleavage from the labeled substrate of the enzyme DEVD-p-nitroanilide. The enzymatic activity for caspase-3 was analyzed three times in each experiment.

Western blotting for PARP

Cells were washed with ice-cold PBS and gently resuspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100) with freshly added 1% protease inhibitor cocktail and incubated on ice for 30 min. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Following SDS-PAGE, proteins were transferred to PVDF. The membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with anti-PARP (Santa Cruz Laboratory, USA) antibody. Immunoreactivity was detected using anti-rabbit peroxidase conjugated secondary immunoglobulin G antibody followed by chemiluminescence detection (Amersham Pharmacia Biotech, USA). The expressional change in PARP protein was observed three times in each experiment.

Statistical analysis

Data are expressed as means±S.D. for the repeated experiments. Statistical analyses were performed using the Kruskal-Wallis test followed by the Mann-Whitney U-test. Probability values of p less than 0.05 were considered significant.

RESULTS

Effects of SMT on H_2O_2-Induced cytotoxicity in SK-N-MC cells

We examined the concentration-dependent effect of H_2O_2 on cell death and determined cell viability using a MTT assay following 12 h H_2O_2 challenge. When SK-N-MC cells were treated with various concentrations of H_2O_2 (1~300 μM) for 12 h, a significant cell death in a dose-dependent manner was observed (Fig. 1A). Cell viability in SK-N-MC cells after exposure to 30 μM H_2O_2 was about 55% that of control viability. Additionally SK-N-MC cells showed time-depend-ent cytotoxicity for 48 hours after exposure to 30 μM H_2O_2 (Fig. 1B).

At concentrations less than 500 μg/ml, cells treated with various concentrations of standardized extract alone for 24 h showed no obvious changes in viability (Fig. 1C). However, SK-N-MC cells pretreated with SMT at 100, 200, 300, and 500 μg/ml 2 h before exposure to H_2O_2 (30 μM) had statistically significant increases in viability, up to 78.5±6.7 (p< 0.05), 87.2±6.8 (p<0.05), 90.1±8.7 (p<0.05), and 86.3±7.2% (p<0.05) (n=3), respectively. SMT therefore protected SK-N-MC cells from oxidative injury (Fig. 1D).

Effects of SMT on nuclear morphologic changes

SK-N-MC cells were stained with DAPI dye to investigate the nature of H_2O_2-induced cell death. Typical morphologic changes indicating apoptotic cell death, such as shrunken nuclei, chromatin condensation and appearance of apoptotic bodies, were observed in SK-N-MC cells after exposure to 30 μM H_2O_2 for 12 h (Fig 2B, D). However, pretreatment with 300 μg/ml SMT for 2 h markedly reduced the level of H_2O_2-induced apoptotic cells (n=3; p<0.05) (Fig. 2C, D).
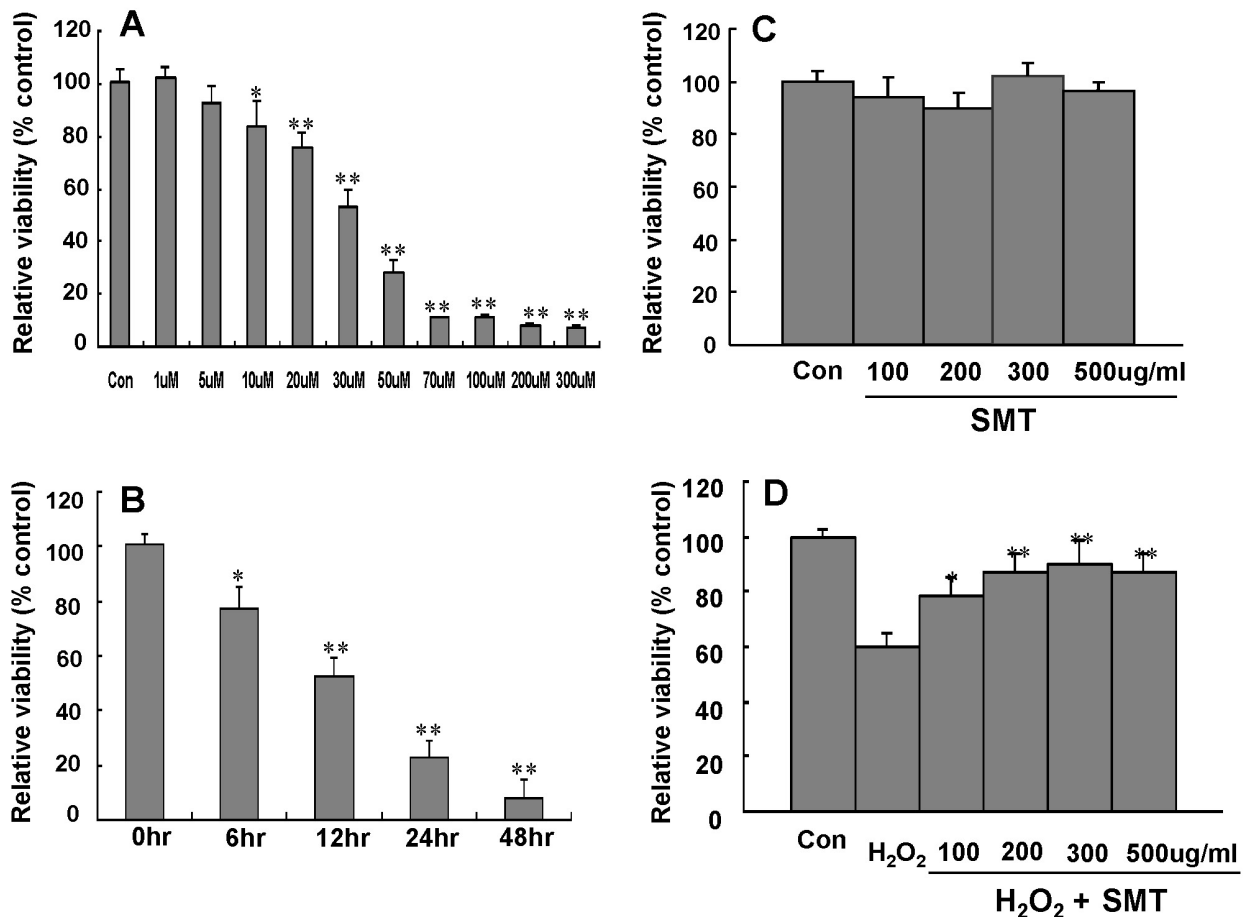

Fig. 1. Effects of H_2O_2 or SMT on cell viability in SK-N-MC cells. (A) Dose-response relationship of H_2O_2 on cell viability, (B) time-dependent relationship of H_2O_2 (30 μM) on cell viability, (C) dose-response relationship of SMT, (D) effect of SMT pretreatment on H_2O_2-induced cytotoxicity in SK-N-MC cells. The various concentrations of SMT were given 2 h before 30 μM H_2O_2 treatment. Cell viability was determined by MTT assay after 12 h H_2O_2 treatment. Values are mean±S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and H_2O_2+SMT (*p<0.05, **p<0.01).

Effects of SMT on cell cycle

To test whether treatment with H_2O_2 induced cell death via apoptosis, the percentage of apoptotic cells was measured by flow cytometry. Fig. 3B indicates that treatment with 30 μM H_2O_2 for 12 h significantly induced the sub-G1 phase, an indicator of cell apoptosis, in SK-N-MC cells com-

pared with control (Fig. 3A). Pre-incubation with 300 μg/ml SMT for 2 h significantly attenuated the accumulation of H_2O_2-induced apoptotic cells in the sub-G1 peak (n=3; p< 0.05) (Fig. 3C, D).

Effects of SMT on DNA fragmentation

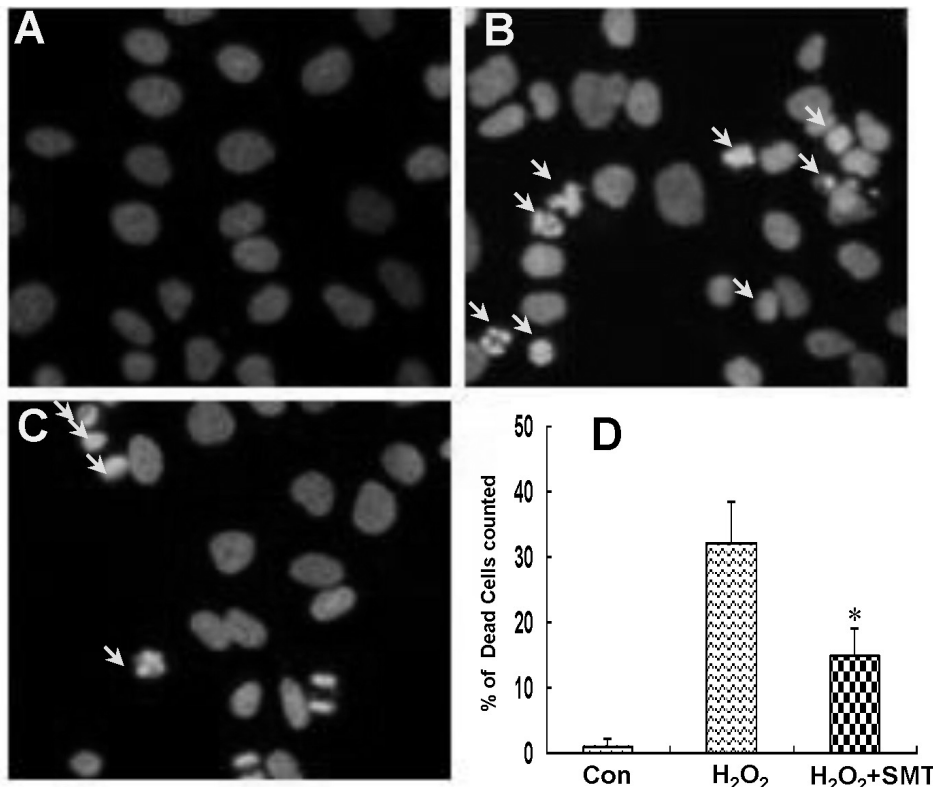Untreated SK-N-MC cells displayed no detectable DNA

Fig. 2. Photographs of DAPI staining showing changes in DNA morphology of SK-N-MC cells. (A) Control cells not treated with H_2O_2, (B) cells treated with 30 μM H_2O_2 for 12 h, (C) cells treated with SMT (300 μg/ ml) 2 h prior to 30 μM H_2O_2, (D) bar histogram showing percent of cells showing abnormal nuclear morphology counted in 3 randomly selected fields. A large amount of DNA was condensed or fragmented in cells treated only with H_2O_2 (arrows), but pretreatment with SMT significantly reduced DNA abnormalities. Values are mean±S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and $H_2O_2 +$SMT (*p<0.05).
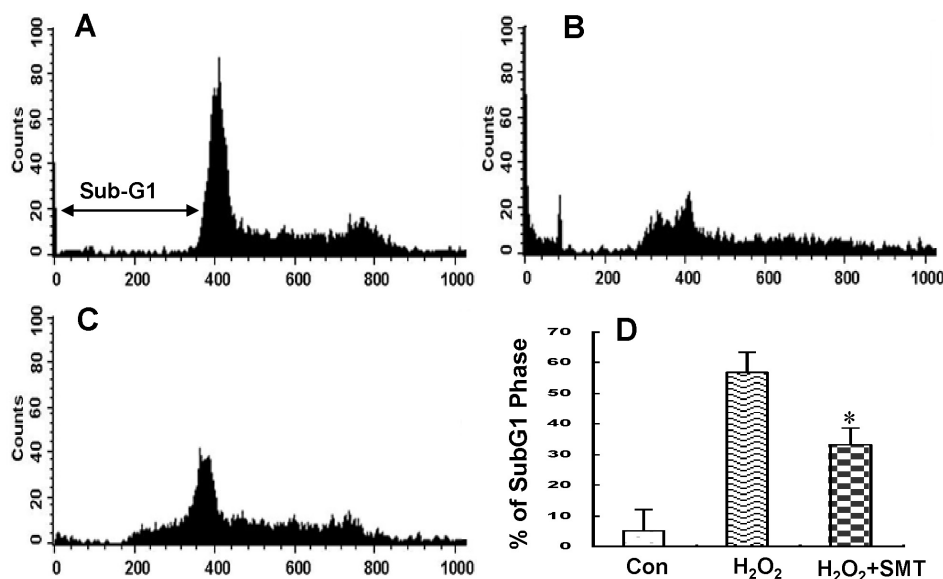

Fig. 3. Flow cytometric analysis showing changes in cell cycle of SK-N-MC cells. (A) Control, (B) treatment with 30 μM H_2O_2, (C) SMT (300 μg/ ml) treatment 2 h prior to exposure to H_2O_2 for 12 h, (D) bar histogram showing percent changes of sub-G1 phase in cell cycle. Values are mean± S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and $H_2O_2 +$SMT (*p<0.05).

fragmentation in the agarose gel electrophoresis (Fig. 4B). In contrast, SK-N-MC cells treated with 30 μM H_2O_2 displayed a marked increase in DNA laddering (Fig. 4C). Pre-incubation with 300 μg/ml SMT for 2 h, however, led to a reduction in DNA laddering caused by H_2O_2-induced apoptosis (Fig. 4D).

Effects of SMT on mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential in SK-N-MC cells treated with H_2O_2 were quantified by flow cytometry with a dye mitotracker. Treatment of SK-N-MC

cells with 30 μM H_2O_2 for 12 h produced a significant reduction in mitochondrial membrane potential (Fig. 5B, D). In contrast, pretreatment with 300 μg/ml SMT for 2 h prevented the H_2O_2-induced decrease of mitochondrial transmembrane potential (n=3; p<0.05) (Fig. 5C, D).

Effects of SMT on expression of caspase-3 mrna and caspase-3 enzyme activity

The expression of caspase-3 mRNA in SK-N-MC cells was significantly upregulated by about 450% that of the control level 3 h after H_2O_2 exposure. In contrast, a significant reduction occurred in H_2O_2-induced expression of caspase-3 mRNA by pretreatment with 300 μg/ml SMT 2 h prior to
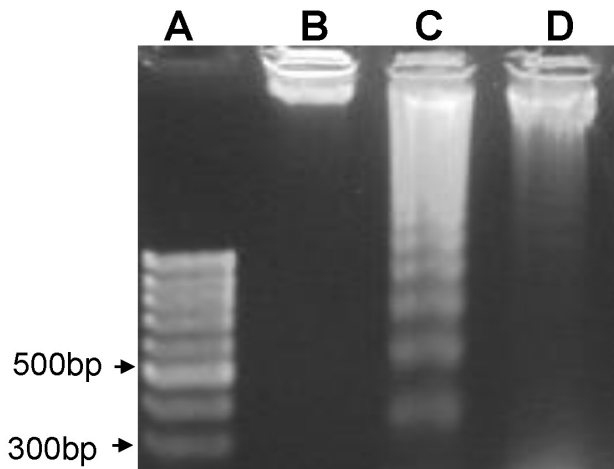

Fig. 4. Agarose gel electrophoresis of DNA extracted from SK-N-MC cells. Lane A, DNA marker (100 bp); Lane B, control; Lane C, treatment with 30 μM H_2O_2 for 12 h; Lane D, SMT (300 μg/ml) treatment 2 h prior to exposure to H_2O_2 for 12 h.
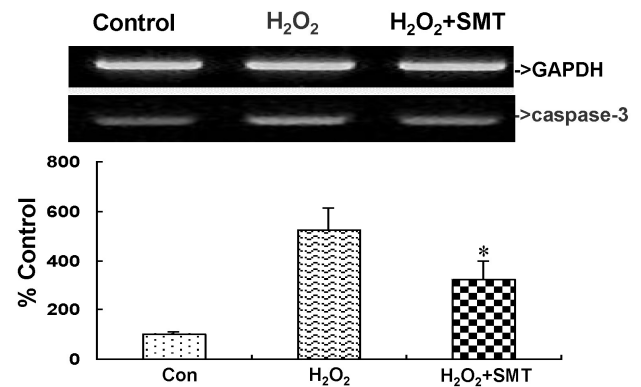

Fig. 6. RT-PCR analysis of caspase-3 mRNA expression from the cDNA of SK-N-MC cells 3 h after H_2O_2 treatment. Control, cells not treated with H_2O_2; H_2O_2, cells treated with 30 μM H_2O_2 for 3 h; SMT-H_2O_2, cells treated with 300 μg/ml SMT 2 h prior to 30 μM H_2O_2. Values are means±S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and H_2O_2+SMT (*p<0.05).

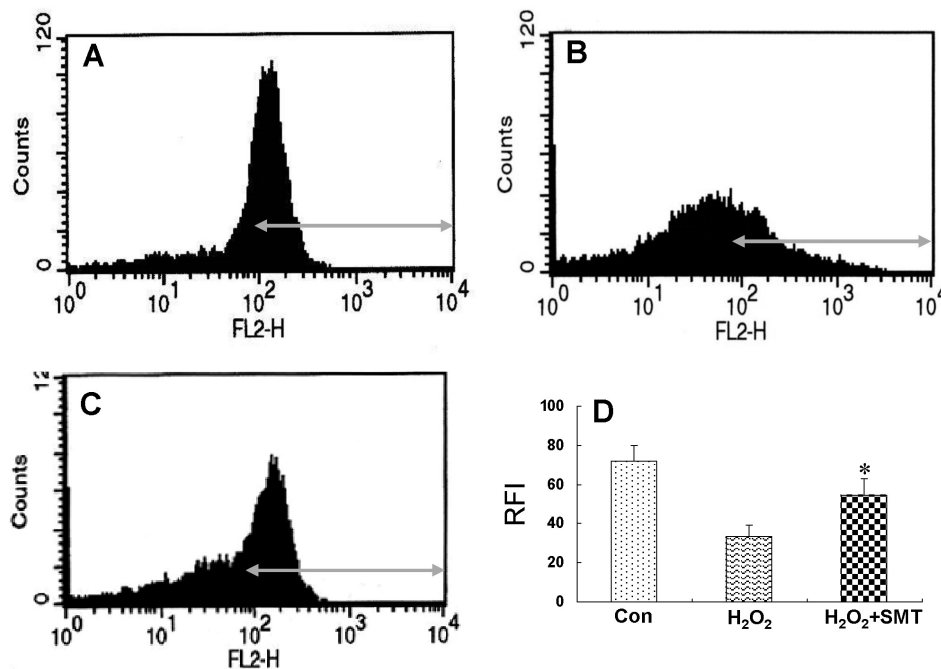**Fig. 5.** Flow cytometric analysis showing changes in the mitochondrial membrane potential of SK-N-MC cells. (A) Control, (B) treatment with 30 μM H_2O_2, (C) SMT (300 μg/ml) treatment 2 h prior to exposure to H_2O_2 for 12 h, (D) bar histogram showing the percent changes of relative fluorescence intensity (RFI) of mitochondrial membrane potential in control, H_2O_2-only treated, and SMT plus H_2O_2 treated SK-N-MC cells. Values are means±S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and H_2O_2+SMT (*p< 0.05).
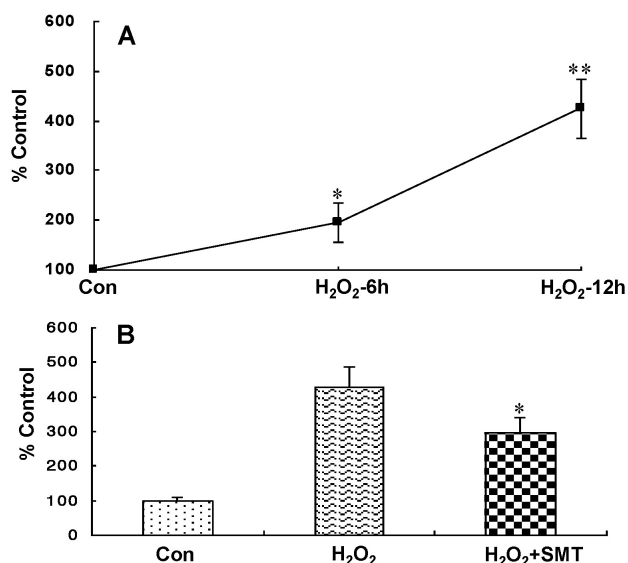
Fig. 7. Changes in caspase-3 enzyme activity from whole cell lysate of SK-N-MC cells. (A) Caspase-3 enzyme activity at 6 and 12 h after 30 μM H_2O_2 treatment, (B) effect of SMT (300 μg/ml) pretreatment on caspase-3 enzyme activity 12 h after H_2O_2 treatment. Values are means±S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and H_2O_2+SMT (*p<0.05, **p<0.01).

Fig. 8. Western blot analysis for expression of the PARP protein from whole cell lysates of SK-N-MC cells 12 h after H_2O_2 treatment. Control, cells not treated with H_2O_2; H_2O_2, cells treated with 30 μM H_2O_2 for 12 h; SMT-H_2O_2, cells treated with 300 μg/ml SMT 2 h prior to 30 μM H_2O_2. Values are means±S.D. of each triplet (n=3). *Denotes a significant difference between H_2O_2 and SMT-H_2O_2 (*p<0.05, **p<0.01).

H_2O_2 exposure (n=3; p<0.01) (Fig. 6).

The caspase-3 enzyme activity was determined by measuring relative levels of activated caspase-3 using the colorimetric caspase-3 substrate. Caspase-3 enzyme activity was significantly upregulated after 6 h of 30 μM H_2O_2 treatment, and it further increased to about four times that of the control level after 12 h of H_2O_2 treatment (n=3; p<0.01) (Fig. 7A). Enzyme activity was significantly reduced when SK-N-MC cells were treated with 300 μg/ml SMT for 2 h prior to exposure to 30 μM H_2O_2 (n=3; p<0.05) (Fig. 7B).

Effects of SMT on the expression of PARP protein

Since it is known that proteolysis of poly (ADP ribose) polymerase (PARP) by caspase-3 is an important marker for apoptosis, Western blot analysis was used to determine whether the PARP cleavage product was expressed upon H_2O_2-induced cell death in SK-N-MC cells. A marked increase in 85-kDa cleaved forms of PARP from the 116-kDa origin protein was observed after 12 h exposure to 30 μM H_2O_2 (Fig. 8A). Pre-incubation with 300 μg/ml SMT, however, significantly attenuated the breakdown of PARP protein caused by H_2O_2-induced apoptotic cell death (n=3; p<0.01) (Fig. 8B).

DISCUSSION

Oxidative stress with H_2O_2 has been widely used to assess cytoprotection, mainly in various cultured cell lines. While a high degree of oxidative stress causes necrosis, lower degrees trigger apoptosis (Dypbukt et al., 1994). A moderate concentration of H_2O_2 activates the caspase, but higher concentrations do not (Hampton and Orrenius, 1997). When SK-N-MC cell cultures were incubated with 30 μM
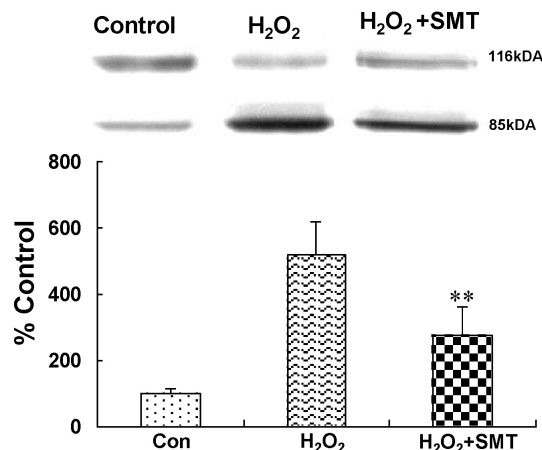
H_2O_2 for 12 h, shrunken or fragmented nuclei were observed via fluorescence imaging, as was DNA laddering via electrophoretic gel and increases in sub-G1 phase and in cleavage of the PARP protein. Additionally, a significant reduction of mitochondrial membrane potentials was observed after 6 h exposure to 30 μM H_2O_2. The expression of caspase-3 mRNA and caspase-3 enzyme activity were upregulated after 3-h and 12-h treatments of 30 μM H_2O_2, respectively. The dose (30 μM) of H_2O_2 used in this study was therefore sufficient to induce apoptotic cell death in SK-N-MC cells.

In this study, the water extract of SMT at concentrations less than 500 μg/ml yielded a minimal change in cell viability, suggesting that the concentration of SMT used for the cytoprotective tests was a sublethal dose in SK-N-MC cells. This study demonstrated that pretreatment with SMT for 2 h significantly attenuated H_2O_2-induced ctyotoxicity in a concentration-dependent manner. Moreover, when the SK-N-MC cells were treated with SMT (300 μg/ml) for 2 h prior to exposure to H_2O_2, apoptotic phenomena as described above were significantly reduced. In an earlier *in vitro* study (So et al., 2000), pretreatment with water extract of SMT (25~300 μg/ml) significantly prevented LPS/PMA-induced cell death and suppressed apoptotic morphologic changes in C6 glioma cells. Pretreatment with Radix Paeoniae Alba extract, a crude herb contained in SMT, for 3 h significantly increased cell viability in a concentration-dependent manner, demonstrating protection from oxidative damage in the HT22 hippocampal cell line (Kang et al., 2005). Accordingly, SMT or individual crude components of SMT are likely to also inhibit apoptotic cell death in glial and neuronal cell lines in the same way.

Hydrogen peroxide can induce mitochondrial permeability transition through an increase in intracellular calcium concentration, and can cause a loss in mitochondrial membrane potential in a process believed to be involved in the activation of apoptosis (Polla et al., 1996). The mitochondrial permeability transition causes the cytosolic release of

cytochrome c and apoptosis-inducing factor, which activate caspases-3 (Bouchier-Hayes et al., 2005). In this study, pre-incubation with the water extract of SMT (300 μg/ml) for 2 h prevented the H_2O_2-induced decrease in mitochondrial transmembrane potential of SK-N-MC cells. SMT also attenuated an increase in caspase-3 activity and in the breakdown of PARP protein caused by H_2O_2-induced oxidative stress.

Water extract of SMT significantly inhibited NO generation and iNOS protein expression from the LPS/PMA-treated C6 glial cells (So et al., 2000). Additionally, pretreatment with SMT completely blocked NK-κB activation induced by LPS/PMA. Radix Paeoniae Alba, a crude herb contained in SMT, exhibited potent scavenging activity in both the 1,1-diphenyl-2-picrylhydrazyl radical and the superoxide anion assays (Kang et al., 2005). Paeoniflorin, the principal bioactive component of Radix Paeoniae Alba, was also able to prevent injury from calcium overloading in cultured primary cortex neurons (Yang et al., 2001). It has been suggested that the water extract of SMT protected SK-N-MC cells from H_2O_2-induced toxicity by scavenging free radicals and preventing mitochondrial injury. However, further *in vitro* experiments are needed to elucidate the exact cellular mechanisms responsible for the neuroprotective effects of SMT.

In previous *in vivo* experiments, 7-day pretreatment with SMT extract prevented apoptosis in jejunal crypt cells and blood cells in irradiated mice with high and low doses of gamma-irradiation (Hsu et al., 1996; Lee et al., 1999). This radioprotection in jejunal crypt cells was also observed when treated only with Rhizoma Chuanxiong or Radix Paeoniae Alba. No studies have yet been undertaken, however, to observe the neuroprotective effects of SMT in brain ischemia *in vivo*. Further research is therefore needed to evaluate whether SMT suppresses neuronal death or improves behavioral deficits in an *in vivo* brain ischemic model.

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