Schisandrae semen essential oil attenuates oxidative stress-induced cell damage in C2C12 murine skeletal muscle cells through Nrf2-mediated upregulation of *HO-1*

JI SOOK KANG[1], MIN HO HAN[2], GI-YOUNG KIM[3], CHEOL MIN KIM[4], HAE YOUNG CHUNG[5], HYE JIN HWANG[1,6], BYUNG WOO KIM[1,7] and YUNG HYUN CHOI[1,2]

[1]Blue-Bio Industry RIC and Anti-Aging Research Center, Dongeui University, Busan 614-714; [2]Department of Biochemistry, Dongeui University College of Korean Medicine, Busan 614-052; [3]Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756; [4]Department of Biochemistry, Busan National University College of Medicine, Yangsan 626-870; [5]Molecular Inflammation Research Center for Aging Intervention (MRCA), Department of Pharmacy, Pusan National University, Busan 609-735; Departments of [6]Food and Nutrition, and [7]Life Science and Biotechnology, College of Natural Sciences and Human Ecology, Dongeui University, Busan 614-714, Republic of Korea

Received July 27, 2014; Accepted November 26, 2014

DOI: 10.3892/ijmm.2014.2028

Abstract. The aim of the present study was to examine the cytoprotective effects of Schisandrae semen essential oil (SSeo), purified from Schisandrae fructus, against oxidative stress-induced cell damage in C2C12 myoblasts. SSeo attenuated hydrogen peroxide (H_2O_2)-induced growth inhibition and exhibited scavenging activity against the intracellular reactive oxygen species (ROS) that were induced by H_2O_2. SSeo also inhibited comet tail formation, chromatin condensation and phospho-histone γH2A.X expression, suggesting that it prevents H_2O_2-induced cellular DNA damage and apoptotic cell death. Furthermore, SSeo significantly enhanced the expression of heme oxygenase-1 (HO-1) associated with the induction of nuclear factor erythroid-2-related factor 2 (Nrf2) in a time- and concentration-dependent manner. In addition, the protective effect of SSeo on H_2O_2-induced C2C12 cell damage was significantly inhibited by zinc protoporphyrin IX, an HO-1 competitive inhibitor, in C2C12 cells. These findings suggest that SSeo augments the cellular antioxidant defense capacity through intrinsic free radical scavenging activity and activation of the Nrf2/HO-1 pathway, thereby protecting the C2C12 cells from H_2O_2-induced oxidative cytotoxicity. As a result, SSeo may have therapeutic potential in the development of functional foods and as the raw material for medicines to protect against oxidative stress.

Correspondence to: Professor Yung Hyun Choi, Department of Biochemistry, Dongeui University College of Korean Medicine, San 45, Yangjung-dong, Busanjin-gu, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

Key words: Schisandrae semen essential oil, oxidative stress, reactive oxygen species, apoptosis, nuclear factor erythroid-2-related factor 2/heme oxygenase-1

Introduction

Oxidative stress, a normal phenomenon in the body, is caused by an imbalance between the production of reactive oxygen species (ROS) and the protective action of the antioxidant system that is responsible for their neutralization and removal. Under normal conditions, the physiologically important intracellular levels of ROS are maintained at low levels by various enzyme systems participating in redox homeostasis (1,2). However, disturbances in the normal redox state of cells and/or a concomitant decline in the antioxidant scavenging capacity can cause toxic effects through the production of peroxides and free radicals that damage all the components of the cell, including proteins, lipids and nucleic acids. Therefore, oxidative stress can cause disruptions in the normal mechanisms involved in cellular signaling pathways. Additionally, the elevated production of ROS increases oxidative stress and causes a pathological response leading to cellular dysfunction, and eventually, to apoptotic cell death (3-5).

As ROS formation occurs naturally, mammalian cells have developed several adaptive mechanisms to limit ROS formation or to detoxify ROS. These mechanisms employ antioxidant enzymes or antioxidant compounds. The nuclear factor erythroid-2-related factor 2 (Nrf2), a basic leucine zipper-transcription factor, is a master cellular sensor for oxidative stress and represents the primary response to changes in the cellular redox state (6-8). Nrf2 acts as the regulator of antioxidant-related genes such as heme oxygenase 1 (HO-1) and nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase 1 (NQO1) through binding to the antioxidant response element (ARE), a *cis*-acting enhancer present in the promoter region of a large and distinct set of target genes, which aims to restore redox homeostasis. However, under

pathological conditions and periods of chronic inflammation, Nrf2 activity and expression of its cytoprotective target genes can be significantly decreased, often contributing to the progression of disease (6,9). Therefore, pharmacological activation of Nrf2 has been shown to be critical for the protection of cells under conditions of oxidative stress.

Numerous traditional medicines have been considered as potential therapeutic candidates for the management of intracellular oxidative balance due to their decreased cytotoxicities and potent pharmacological features. Among them, *Schisandra chinensis* Baill. fruits (Schisandrae fructus) as one of the most well-known herbal medicines has been extensively used in Asia, including Korea, China, Japan and Russia (10,11). Schisandrae fructus is often used to increase physical working capacity and it produces a stress-protective effect. Previous studies suggest that the major bioactive constituents of Schisandrae fructus are the essential oil (12-14) and lignans belonging to the dibenzocyclooctadiene type (15,16), and this has been intensively studied from the pharmacological and phytochemical perspectives. Previously, an essential oil purified from Schisandrae fructus, whose main chemical components are monoterpenes, sesquiterpenes and aromatic compounds (17), has been shown to have various pharmacological potentials, such as antioxidant, antimicrobial, and antiseptic effects (13,18-20). However, the seed sections of *S. chinensis* (Schisandrae semen) have been disregarded and unused. Additionally, to the best of our knowledge, no studies have documented the protective action of Schisandrae semen essential oil (SSeo), an essential oil purified from Schisandrae fructus, against oxidative stress. Therefore, the aim of the present study was to examine the ability of SSeo to protect cells from hydrogen peroxide (H_2O_2)-induced cell damage and to elucidate the mechanism underlying these protective effects using a C2C12 murine skeletal-muscle cell line.

Materials and methods

Preparation of SSeo. S. chinensis Baillon seeds were collected around Mungyeong City (Gyeongbuk, Korea) and were completely dried at 180˚C in a furnace (Daihan Scientific Co., Seoul, Korea). The dried seeds were pulverized and lyophilized in a programmable freeze dryer (Freezone 1, Labconco Co., Kansas City, MO, USA). Lyophilized materials were extracted with 100% ethanol at room temperature for 24 h, filtered, and were subsequently concentrated using a rotary vacuum evaporator (Buchi Rotavapor R-144, BÜCHI Labortechnik, Flawil, Switzerland). Finally, the SSeo was isolated by hydrodistillation using a Clevenger-type apparatus for 3 h according to the method in a previous study (21). The oil was stored in a refrigerator at 4˚C to protect it from light and degeneration. The yield of the oil based on the dried weight of the Schizandrae semen was 0.66%.

Cell culture and treatment. The C2C12 myoblast cell line was obtained from the American Type Culture Collection (Manassa, VA, USA) and was maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL), 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin fungizone at 37˚C in a humid atmosphere of 5% CO_2 in air. SSeo was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as a stock solution at a 100 mg/ml concentration, and the stock solution was diluted with medium to the desired concentration prior to use.

Cell viability assay and morphological imaging. C2C12 cells were seeded in 6-well plates at a density of $1x10^5$ cells per well. After a 24-h incubation, the cells were treated with various concentrations of the SSeo in the absence or presence of H_2O_2 and/or zinc protoporphyrin IX (ZnPP; Sigma-Aldrich) for the indicated times. The medium was removed, and the cells were incubated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution for 2 h. The supernatant was discarded and the formazan blue, which was formed in the cells, was dissolved in DMSO. The optical density was measured at 540 nm with a microplate reader (Dynatech Laboratories, Chantilly, VA, USA) and growth inhibition was assessed as the percentage viability in which the vehicle-treated cells were considered as 100% viable. Morphological changes were monitored by obtaining photomicrographs under an inverted phase contrast microscope (Carl Zeiss, Oberkochen, Germany).

Flow cytometric detection of apoptosis. The cells were trypsinized and washed with phosphate-buffered saline (PBS) and resuspended in 100 μl of binding buffer containing annexin-V-fluorescein isothiocyanate and propidium iodide (PI) (BD Sciences, Heidelberg, Germany) for 15 min at room temperature in the dark, as according to the manufacturer's instructions. The cells were immediately analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA). The percentages of apoptotic cells (annexin-V^+ cells) are presented as the mean ± standard deviation (SD), as described previously (22).

Morphological observation of nuclear change. The cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS and were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) solution for 10 min at room temperature. The cells were analyzed using a fluorescence microscope (Carl Zeiss).

Measurement of intracellular ROS. The measurement of ROS was performed using the ROS sensitive 2',7',-dichlorofluorescein-diacetate (H2DCFDA, Molecular Probes, Eugene, OR, USA). Briefly, the cells were incubated with 10 μM H2DCFDA for 30 min at room temperature in the dark. Spectrofluorimetry analysis using a microplate reader was performed to quantitate the intracellular ROS using excitation and emission wavelengths at 488 and 525 nm, respectively.

Comet assay (single-cell gel electrophoresis). The cell suspension was mixed with 0.5% low melting agarose (LMA) at 37˚C, and the mixture was spread on a fully-frosted microscopic slide precoated with 1% normal melting agarose. After the solidification of the agarose, the slide was covered with 0.5% LMA and was subsequently immersed in a lysis solution [2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO (pH 10)] for 1 h at 4˚C. The slides were placed in a gel electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow for DNA unwinding and the expression

of alkali-labile damage, and subsequently an electrical field was applied (300 mA, 25 V) for 20 min at 4˚C to draw the negatively-charged DNA toward the anode. After electrophoresis, the slides were washed three times for 5 min at 4˚C in a neutralizing buffer [0.4 M Tris, (pH 7.5)], followed by staining with 20 μg/ml PI (Sigma-Aldrich). The slides were examined under a fluorescence microscope (Carl Zeiss).

Protein extraction and western blot analysis. The cells were collected by trypsin-EDTA and lysed with lysis buffer [20 mM sucrose, 1 mM EDTA, 20 μM Tris-Cl (pH 7.2), 1 mM dithiothreitol, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin and 2 μg/ml aprotinin] containing protease inhibitors for 30 min at 4˚C. The mixtures were centrifuged (10,000 x g) for 10 min at 4˚C and the supernatants were collected as whole-cell extracts. The protein content was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as a standard, as according to the manufacturer's instructions, to determine the protein concentrations. Following normalization, an equal amount of protein was subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and was subsequently transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) by electroblotting. The blots were blocked with 5% skimmed milk for 1 h at room temperature. The blots were incubated overnight with primary antibodies, followed by horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse immunoglobulin for 1 h. The immunoreactive bands were revealed by enhanced chemiluminescence (ECL) with a commercially available ECL kit (Amersham, Arlington Heights, IL, USA). Anti-Nrf2 (SC-13032), anti-NQO1 (SC-16464), anti-TrxR1 (SC-28321) and anti-actin (SC-1616) antibidoes were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). Antibodies against HO-1 (CS #5061S) and p-γH2A.X (CS #9718S) were obtained from Cell Signaling Technology (Danvers, MA, USA).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using the TRIzol reagent according to the manufacturer's instructions, and 2 μg of RNA was used for cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA). RT-generated cDNA encoding the *HO-1* gene was amplified by PCR using a specific primer (forward, 5'-CGA CAG CAT GTC CCA GGA TT-3' and reverse, 5'-CTG GGT TCT GCT TGT TTC GC-3'). The amplified cDNA products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide (Sigma-Aldrich). In a parallel experiment, glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and reverse, 5'-AGC CTT CTC CAT GGT GAA GAC-3') was used as an internal control.

Statistical analysis. Data are presented as the means ± SD. Statistical significance was determined using an analysis of variance followed by a Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of SSeo on the cell viability in C2C12 cells. The effect of increasing concentrations of SSeo was first evaluated on the viability of 2C12 cells. As shown in Fig. 1, treatment of the cell cultures with 10-80 μg/ml of SSeo for 24 h had no effect on cell viability. However, 100 μg/ml induced a significant reduction in the number of cells after 24 h, suggesting that SSeo may be toxic to the cells at this concentration. Therefore, 80 μg/ml SSeo was chosen as the optimal dose for studying the cytoprotective effect of SSeo against H_2O_2-induced cell damage.

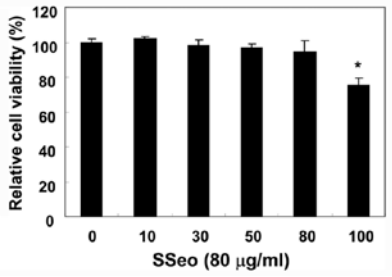

Figure 1. Effect of SSeo on the cell viability of C2C12 cells. Cells were treated with various concentrations of SSeo for 24 h. Cell viability was assessed using an MTT reduction assay. Data are expressed as mean ± standard deviation of three independent experiments. Significance was determined by the Student's *t*-test (*P<0.05 vs. untreated control). SSeo, Schisandrae semen essential oil.

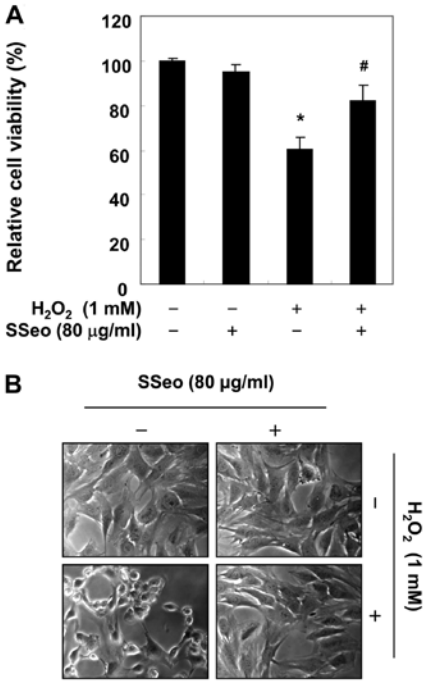

Figure 2. Effect of SSeo on H_2O_2-induced growth inhibition and morphological changes in C2C12 cells. Cells were pretreated with 80 μg/ml SSeo for 1 h and subsequently were incubated with and without 1 mM H_2O_2 for 6 h. The (A) cell viability and change in (B) cell morphology were measured. The results are the mean ± standard deviation values obtained in three independent experiments (*P<0.05 compared to control group; #P<0.05 compared to H_2O_2-treated group). SSeo, Schisandrae semen essential oil.

Effects of SSeo on H_2O_2-induced C2C12 cell growth inhibition. Subsequently, the potential protective effect of SSeo was evaluated against oxidative stress-induced cytotoxicity (Fig. 2A). The cell viability was significantly reduced to 60.2% in 1 mM H_2O_2-treated cells in the absence of SSeo; however, this was increased to 82.2% in H_2O_2-treated cells that were pretreated

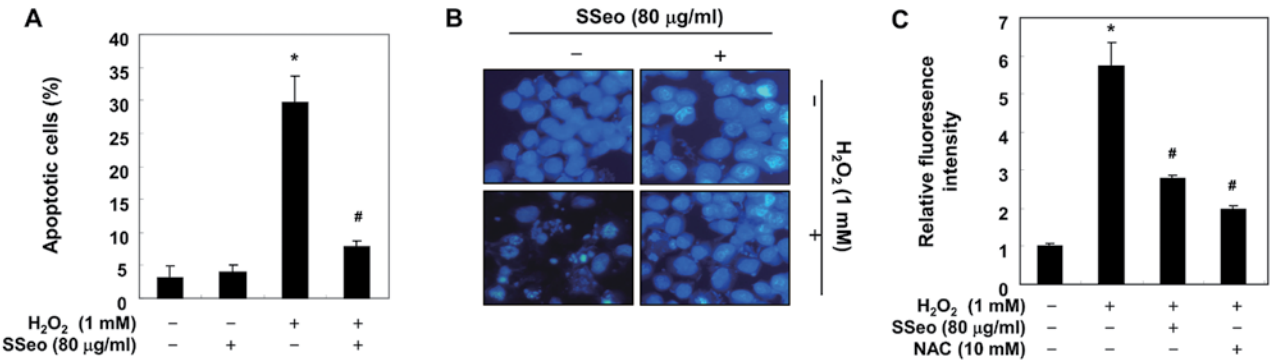

Figure 3. Protection of H_2O_2-induced apoptosis and ROS generation by SSeo in C2C12 cells. (A) Cells were pretreated with 80 μg/ml SSeo for 1 h and were subsequently incubated with and without 1 mM H_2O_2 for 6 h. To quantify the degree of apoptosis, cells were stained with annexin-V-FITC and PI for flow cytometry analysis. (B) The cells grown under the same conditions as (A) were sampled, fixed and stained with DAPI solution. The stained nuclei were observed under a fluorescent microscope (original magnification, x400). (C) Cells were pretreated with 80 μg/ml SSeo or 10 mM NAC for 1 h and were subsequently stimulated with and without 1 mM H_2O_2 for 6 h. The fluorescence intensity of H2DCFDA taken up by the treated cells was measured by spectrofluorimetry. Data are presented as mean ± standard deviation of three independent experiments (*P<0.05 compared to control group; $^\#$P<0.05 compared to H_2O_2-treated group). ROS, reactive oxygen species; SSeo, Schisandrae semen essential oil; FITC, fluorescein isothiocyanate; PI, propidium iodide; DAPI, 4,6-diamidino-2-phenylindole; NAC, N-acetyl-L-cysteine; H2DCFDA, 2',7',-dichlorofluorescein-diacetate.

with 80 μg/ml SSeo. In addition, H_2O_2 stimulation significantly induced morphological changes, including extensive cytosolic vacuolization and the presence of irregular cell-membrane buds, which were effectively attenuated by SSeo pretreatment (Fig. 2B).

Inhibition of H_2O_2-induced apoptosis and ROS generation by SSeo in C2C12 cells. To evaluate the potential effect of SSeo on H_2O_2-apoptosis, the proportion of apoptotic cells was further determined by staining the cells with annexin V and PI. Flow cytometry analysis showed that the proportion of apoptotic cells increased significantly from 3.1% in the untreated control to 29.7% in the cells treated with 1 mM H_2O_2; however, the enhanced ratio of apoptosis was significantly alleviated by pre-incubation with SSeo (Fig. 3A). Furthermore, DAPI staining revealed nuclei with chromatin condensation and the formation of apoptotic bodies, characteristic morphological changes of apoptosis, in cells cultured with 1 mM H_2O_2. By contrast, extremely few apoptotic cells were observed in the control culture, and pretreatment of the cells with SSeo significantly abrogated these apoptotic characteristics (Fig. 3B). The scavenging effect of SSeo against H_2O_2-induced ROS was examined using the H2DCFDA assay. These results indicated that the ROS levels were increased in H_2O_2-treated cells compared to non-treated cells, whereas SSeo decreased the ROS generation in H_2O_2-treated cells (Fig. 3C). As a positive control, an ROS scavenger of 10 mM N-acetyl-L-cysteine also markedly attenuated H_2O_2-induced ROS generation, indicating that SSeo scavenged H_2O_2-induced ROS.

Reduction of H_2O_2-mediated DNA damage by SSeo in C2C12 cells. As oxidative stress-induced damage to DNA produces lesions that are responsible for the loss of cell viability, H_2O_2-mediated damage to C2C12 cell DNA was detected using the alkaline comet assay and western blot analysis. As assessed using the comet assay, a longer comet tail moment (DNA migration) occurred with an increase in H_2O_2-treated cells (Fig. 4A), and the untreated control cells only showed typical representative nuclei. In addition, the levels of phosphorylation of nuclear histone H2A.X (Ser139) (p-γH2A.X), a sensitive marker for DNA double-strand break (DSB) formation (23), increased in the H_2O_2-treated cells, as shown by western blotting (Fig. 4B). However, pretreatment with SSeo resulted in a significant decrease in comet tails and in the expression of p-γH2A.X. The results suggest that SSeo has a protective property against DNA damage induced by H_2O_2 treatment.

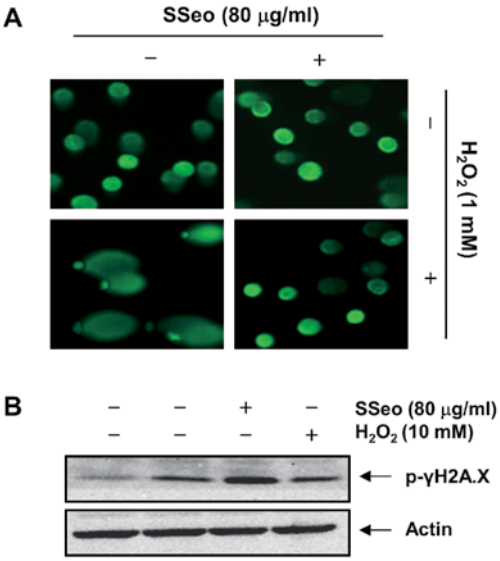

Figure 4. Attenuation of H_2O_2-induced DNA damage by SSeo in C2C12 cells. Cells were pretreated with 80 μg/ml SSeo for 1 h and were subsequently incubated with and without 1 mM H_2O_2 for 6 h. (A) To detect cellular DNA damage, the comet assay was performed and representative images of the comets were obtained using a fluorescence microscope (original magnification, x200). (B) The cells were lysed and subsequently equal amounts of cell lysates (30 μg) were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against phospho-histone γH2A.X and actin, as an internal control, and the proteins were visualized using an ECL detection system. SSeo, Schisandrae semen essential oil; ECL, enhanced chemiluminescence.

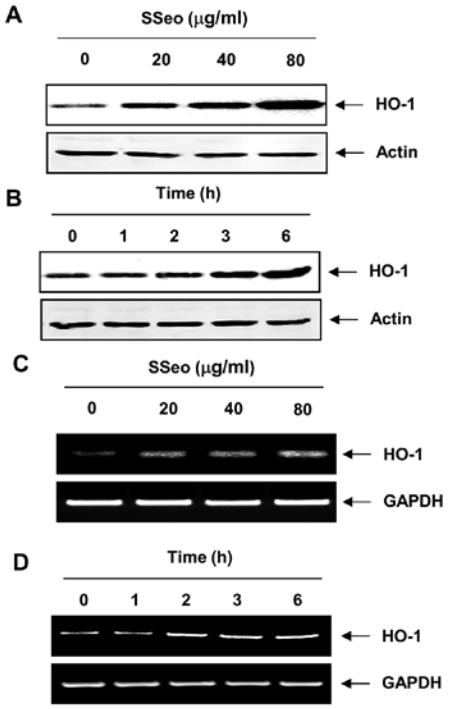

Figure 5. Induction of HO-1 expression by SSeo in C2C12 cells. Cells were incubated with (A and C) various concentrations of SSeo for 6 h or for (B and D) indicated periods with 80 μg/ml SSeo. (A and B) Cellular proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and subsequently transferred onto nitrocellulose membranes. The membranes were probed with the specific antibody against HO-1. Proteins were visualized using an ECL detection system. (C and D) Total RNA was isolated and reverse-transcribed using an *HO-1* primer. The resulting cDNAs were subsequently subjected to PCR. The reaction products were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Actin and GAPDH were used as internal controls for the western blot analysis and RT-PCR assay, respectively. HO-1, heme oxygenase-1; SSeo, Schisandrae semen essential oil; ECL, enhanced chemiluminescence; RT-PCR, reverse transcription-polymerase chain reaction.

Induction of HO-1 expression by SSeo in C2C12 cells. As HO-1 is an important component of the cellular defense against oxidative stress (7,24), whether non-cytotoxic concentrations of SSeo induced HO-1 protein expression was accessed in association with its antioxidant activity. Treatment of C2C12 cells with SSeo induced a concentration- and time-dependent enhancement in HO-1 protein expression (Fig. 5A and B). Additionally, the increased HO-1 expression correlated with *HO-1* mRNA levels in a concentration- and time-dependent manner in SSeo-treated cells (Fig. 5C and D), suggesting that the increased HO-1 expression at the transcriptional level contributed to the enhanced expression of HO-1 protein.

Effect of SSeo on the levels of Nrf2 in C2C12 cells. As several studies have reported that Nrf2 is an important transcription factor for the regulation of a number of antioxidants and phase II detoxifying enzymes, including HO-1 (6,8,9), whether SSeo was able to induce the expression of Nrf2 in C2C12 cells was further examined. Following exposure to SSeo, the C2C12 cells showed a gradual increase in Nrf2 levels in a concentration- and time-dependent manner, which was strongly correlated with the increase in HO-1 expression (Fig. 6). However, the levels of other Nrf2 target genes, such as antioxidant enzyme thioredoxin reductase-1 (*TrxR1*) and *NQO1*, remained unchanged in SSeo-treated C2C12 cells.

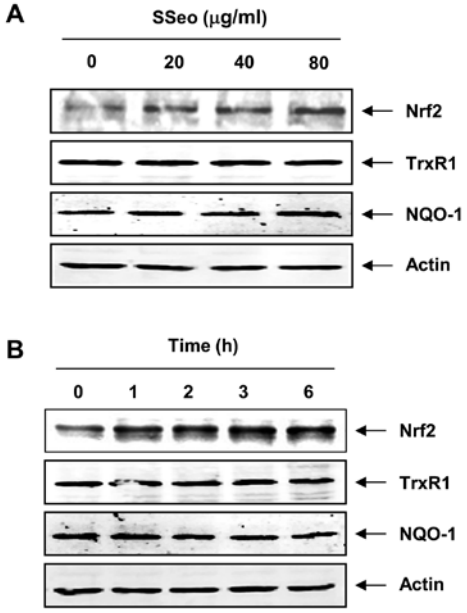

Figure 6. Induction of Nrf2 expression by SSeo in C2C12 cells. Cells were incubated with (A) various concentrations of SSeo for 6 h or for (B) indicated periods with 80 μg/ml SSeo. Cellular proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and were subsequently transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control. Nrf2, nuclear factor erythroid-2-related factor 2; SSeo, Schisandrae semen essential oil.

Involvement of HO-1 in C2C12 cell damage induced by H_2O_2 treatment. To identify whether the HO-1 protein is involved in the protective effect of SSeo on oxidative stress induced in H_2O_2-treated C2C12 cells, a selective inhibitor of HO-1, ZnPP, was applied in the present study. As shown in Fig. 7A, ZnPP significantly reversed the inhibition of ROS generation by SSeo in H_2O_2-stimulated C2C12 cells. In addition, the results of the MTT assay showed that the protective effect of SSeo on H_2O_2-induced cytotoxicity was blocked by the addition of ZnPP (Fig. 7B), suggesting that the cytoprotective effect of SSeo is partly mediated through HO-1 induction.

Discussion

Under normal conditions, the cells of aerobic organisms contain safe levels of ROS, which are counterbalanced by biochemical antioxidants. However, excess ROS generation and/or antioxidant depletion under pathological conditions lead to oxidative stress, along with direct or indirect ROS-mediated damage to nucleic acids, proteins, and lipids. Although the cell and tissue defense systems against ROS consist of various antioxidant enzymes and antioxidants, these defense systems are overpowered in the face of high levels of ROS. Accumulating evidence indicates that ROS-provoked oxidative stress can kill cells via necrosis and/or apoptosis through a variety of overlapping signaling pathways and cascades (1,6,9,25). Apoptosis, or a programmed cell death, is a complex process characterized

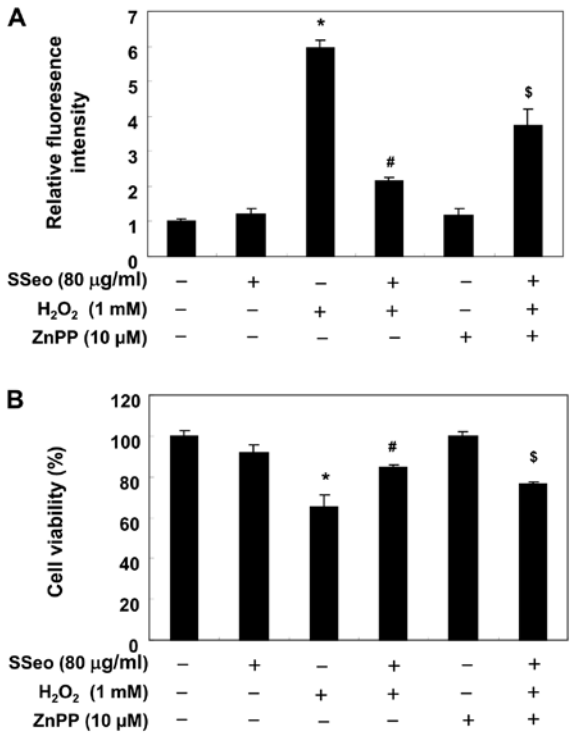

Figure 7. Effects of an inhibitor of HO-1 on SSeo-mediated attenuation of ROS formation and growth inhibition by H_2O_2 in C2C12 cells. Cells were pretreated for 1 h with 80 μg/ml SSeo and were subsequently treated for 6 h with or without 1 mM H_2O_2 in the absence or presence of 10 μM ZnPP. Subsequently (A) ROS generation and (B) cell viability were estimated, respectively. The results were the mean ± standard deviation values obtained in three independent experiments ($^*P<0.05$ compared with control group; $^\#P<0.05$ compared to H_2O_2-treated group; $^\$P < 0.05$ compared to H_2O_2 and SSeo-treated group). HO-1, heme oxygenase-1; SSeo, Schisandrae semen essential oil; ROS, reactive oxygen species; ZnPP, zinc protoporphyrin IX.

by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and the formation of apoptotic bodies. Furthermore, the addition of exogenous H_2O_2 is sufficient to trigger the apoptotic process, with different concentrations required depending on the cell type under investigation (26). Thus, the inhibition of oxidative stress is theoretically an expeditious method for the management of disorders associated with ROS-induced cell injury and apoptosis (6,8,9). For this reason, investigators are searching for natural antioxidants that have safe and effective pharmacological activity with low cytotoxicity for the prevention of oxidative stress-mediated cellular damage. In the present study, as a part of the screening program for therapeutic anti-oxidative agents from traditional medicine resources, whether SSeo, an essential oil purified from Schisandrae fructus, has protective effects against oxidative stress-induced cytotoxicity was investigated. Consequently, H_2O_2 was selected as an oxidative inducer of ROS in a C2C12 myoblast model due to its high diffusion capacity and rapid membrane permeability. Furthermore, the mechanisms by which H_2O_2 induces alterations in cellular components and regulates cell fate are well understood (25,27), which makes this ROS particularly of interest for study. The present study showed that C2C12 cells exposed to H_2O_2 exhibited significantly decreased cell viability and increased apoptosis; however, SSeo markedly increased cell viability by inhibiting H_2O_2-induced apoptosis and reduced ROS generation generated by H_2O_2 treatment in C2C12 cells (Figs. 2 and 3). In addition, H_2O_2 treatment increased the expression of p-γH2A.X, a sensitive marker for DNA DSBs (23), and increased the tail length of DNA in the comet assay, whereas each event was mitigated in C2C12 cells by treatment with SSeo prior to H_2O_2 exposure (Fig. 4). The results suggest that SSeo prevented ROS-mediated DNA damage and apoptosis via the attenuation of oxidative stress.

Among the various antioxidant enzymes, the protective functions of HO-1, an inducible isoform of the first and rate-limiting enzyme of heme degradation, against oxidative stress have recently been demonstrated (7,24). Transcriptional regulation of the *HO-1* gene is linked to the transcription factor Nrf2, which plays a crucial role in cellular defense (6,8,9). Nrf2 is ubiquitously expressed at low levels in the cytoplasm under normal physiological conditions and is constantly bound to the repressor protein Kelch-like ECH-associated protein-1 (Keap1), a special molecular 'sensor' for changes in intracellular homeostasis. In response to oxidative stress, Nrf2 is released from Keap1 and transmits the stress signal to the nucleus for the activation of a distinct set of genes encoding phase II detoxifying enzymes, as well as several stress-responsive proteins, including HO-1 (6,7,24,28). Therefore, the present study further determined the potential role of the Nrf2/HO-1 pathway in H_2O_2-induced C2C12 cell damage and SSeo-mediated cytoprotection. Evidence for the induction of HO-1 by SSeo has been demonstrated and it was shown that SSeo-induced HO-1 protein expression occurred in a concentration- and time-dependent manner, with a concomitant increase in Nrf2 expression, but not TrxR1 or NQO-1 expression (Fig. 6). Therefore, exogenous induction of *HO-1* by SSeo was further confirmed and was useful in H_2O_2-induced oxidative damage of C2C12 cells. The data indicated that the inhibition of *HO-1* function through using an HO-1 inhibitor, ZnPP, effectively reduced the protective effect of SSeo against H_2O_2-induced ROS generation, as well as cytoprotection (Fig. 7). The present results clearly demonstrate that *HO-1* induction by SSeo is responsible for protecting C2C12 cells against H_2O_2-induced oxidative stress, and also suggest that SSeo-induced cytoprotection of C2C12 cells against oxidative stress is critically dependent on the activation of the Nrf2/HO-1 pathway.

In conclusion, the present study identified SSeo as an antioxidant with the ability to scavenge intracellular ROS in C2C12 myoblasts. In addition, SSeo prevented cell damage resulting from H_2O_2 exposure and increased C2C12 cell survival by boosting HO-1 induction for ROS detoxification. Although further investigation to elucidate the detailed mechanism by which SSeo mitigates apoptosis associated with the Nrf2/HO-1 signaling pathway is required, these data suggest that SSeo may find utility as a therapeutic agent for the management of ROS-linked clinical conditions and disorders.

Acknowledgements

The present study was supported by the R&D program of MOTIE/KIAT (grant no. 10040391; Development of Functional Food Materials and Device for Prevention of Aging-associated Muscle Function Decrease). We thank the Aging Tissue Bank for providing research materials.

References

1. Zhang Y, Du Y, Le W, Wang K, Kieffer N and Zhang J: Redox control of the survival of healthy and diseased cells. Antioxid Redox Signal 15: 2867-2908, 2011.
2. Piantadosi CA: Carbon monoxide, reactive oxygen signaling, and oxidative stress. Free Radic Biol Med 45: 562-569, 2008.
3. Ray PD, Huang BW and Tsuji Y: Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal 24: 981-990, 2012.
4. Ott M, Gogvadze V, Orrenius S and Zhivotovsky B: Mitochondria, oxidative stress and cell death. Apoptosis 12: 913-922, 2007.
5. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J: Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39: 44-84, 2007.
6. Chapple SJ, Siow RC and Mann GE: Crosstalk between Nrf2 and the proteasome: therapeutic potential of Nrf2 inducers in vascular disease and aging. Int J Biochem Cell Biol 44: 1315-1320, 2012.
7. Alam J and Cook JL: Transcriptional regulation of the heme oxygenase-1 gene *via* the stress response element pathway. Curr Pharm Des 9: 2499-2511, 2003.
8. Jeong WS, Jun M and Kong AN: Nrf2: a potential molecular target for cancer che-moprevention by natural compounds. Antioxid Redox Signal 8: 99-106, 2006.
9. Chen XL and Kunsch C: Induction of cytoprotective genes through Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. Curr Pharm Des 10: 879-891, 2004.
10. Panossian A and Wikman G: Pharmacology of *Schisandra chinensis* Bail.: an overview of Russian research and uses in medicine. J Ethnopharmacol 118: 183-212, 2008.
11. Lu Y and Chen DF: Analysis of *Schisandra chinensis* and *Schisandra sphenanthera*. J Chromatogr A 1216: 1980-1990, 2009.
12. Ma CH, Liu TT, Yang L, Zu YG, Chen X, Zhang L, Zhang Y and Zhao C: Ionic liquid-based microwave-assisted extraction of essential oil and biphenyl cyclooctene lignans from *Schisandra chinensis* Baill fruits. J Chromatogr A 1218: 8573-8580, 2011.
13. Ma CH, Yang L, Zu YG and Liu TT: Optimization of conditions of solvent-free microwave extraction and study on antioxidant capacity of essential oil from *Schisandra chinensis* (Turcz.) Baill. Food Chem 134: 2532-2539, 2012.
14. Ikeya Y, Taguchi H, Yosioka I and Kobayashi H: The constituents of *Schizandra chinensis* Baill. I. Isolation and structure determination of five new lignans, gomisin A, B, C, F and G, and the absolute structure of schizandrin. Chem Pharm Bull (Tokyo) 27: 1383-1394, 1979.
15. Lee YW, Voyksner RD, Pack TW, Cook CE, Fang QC and Ito Y: Application of countercurrent chromatography/thermospray mass spectrometry for the identification of bioactive lignans from plant natural products. Anal Chem 62: 244-248, 1990.
16. Opletal L, Sovová H and Bártlová M: Dibenzo[a,c]cyclooctadiene lignans of the genus *Schisandra*: importance, isolation and determination. J Chromatogr B Analyt Technol Biomed Life Sci 812: 357-371, 2004.
17. Wang L, Chen Y, Song Y, Chen Y and Liu X: GC-MS of volatile components of *Schisandra chinensis* obtained by supercritical fluid and conventional extraction. J Sep Sci 31: 3238-3245, 2008.
18. Liu CJ, Zhang SQ, Zhang JS, Liang Q and Li DS: Chemical composition and antioxidant activity of essential oil from berries of *Schisandra chinensis* (Turcz.) Baill. Nat Prod Res 26: 2199-2203, 2012.
19. Chen X, Zhang Y, Zu Y and Yang L: Chemical composition and antioxidant activity of the essential oil of *Schisandra chinensis* fruits. Nat Prod Res 26: 842-849, 2012.
20. Song L, Ding JY, Tang C and Yin CH: Compositions and biological activities of essential oils of *Kadsura longepedunculata* and *Schisandra sphenanthera*. Am J Chin Med 35: 353-364, 2007.
21. Minaiyan M, Ghannadi AR, Afsharipour M and Mahzouni P: Effects of extract and essential oil of *Rosmarinus officinalis* L. on TNBS-induced colitis in rats. Res Pharm Sci 6: 13-21, 2011.
22. Lee SJ, Hwang SO, Noh EJ, Kim DU, Nam M, Kim JH, Nam JH and Hoe KL: Transactivation of bad by vorinostat-induced acetylated p53 enhances doxorubicin-induced cytotoxicity in cervical cancer cells. Exp Mol Med 46: e76, 2014.
23. Xie H, Wise SS, Holmes AL, Xu B, Wakeman TP, Pelsue SC, Singh NP and Wise JP Sr: Carcinogenic lead chromate induces DNA double-strand breaks in human lung cells. Mutat Res 586: 160-172, 2005.
24. Lee DS, Li B, Kim KS, Jeong GS, Kim EC and Kim YC: Butein protects human dental pulp cells from hydrogen peroxide-induced oxidative toxicity *via* Nrf2 pathway-dependent heme oxygenase-1 expressions. Toxicol In Vitro 27: 874-881, 2013.
25. Hampton MB and Orrenius S: Redox regulation of apoptotic cell death. Biofactors 8: 1-5, 1998.
26. Chandra J, Samali A and Orrenius S: Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 29: 323-333, 2000.
27. Nguyen TT, Park HJ, Kim JY, Kim HE, Lee H, Yoon J and Lee C: Microbial inactivation by cupric ion in combination with H_2O_2: role of reactive oxidants. Environ Sci Technol 47: 13661-13667, 2013.
28. Lee EK, Kim JA, Park SJ, Kim JK, Heo K, Yang KM and Son TG: Low-dose radiation activates Nrf1/2 through reactive species and the Ca^{2+}/ERK1/2 signaling pathway in human skin fibroblast cells. BMB Rep 46: 258-263, 2013.