

Research Paper

Attenuation of Oxidative Stress-Induced Cell Apoptosis in Schwann RSC96 Cells by *Ocimum Gratissimum* Aqueous Extract

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Abstract

Objectives: Cell transplantation therapy of Schwann cells (SCs) is a promising therapeutic strategy after spinal cord injury. However, challenges such as oxidative stress hinder satisfactory cell viability and intervention for enhancing SCs survival is critical throughout the transplantation procedures. *Ocimum gratissimum*, widely used as a folk medicine in many countries, has therapeutic and anti-oxidative properties and may protect SCs survival.

Methods: We examined the protective effects of aqueous *O. gratissimum* extract (OGE) against cell damage caused by H₂O₂-induced oxidative stress in RSC96 Schwann cells.

Results: Our results showed that the RSC96 cells, damaged by H₂O₂ oxidative stress, decreased their viability up to 32% after treatment with different concentrations of up to 300 μM H₂O₂, but OGE pretreatment (150 or 200 μg/mL) increased cell viability by approximately 62% or 66%, respectively. Cell cycle analysis indicated a high (43%) sub-G1 cell population in the H₂O₂-treated RSC96 cells compared with untreated cells (1%); whereas OGE pretreatment (150 and 200 μg/mL) of RSC96 cells significantly reduced the sub-G1 cells (7% and 8%, respectively). Furthermore, Western blot analysis revealed that OGE pretreatment inhibited H₂O₂-induced apoptotic protein caspase-3 activation and PARP cleavage, as well as it reversed Bax up-regulation and Bcl-2 down-regulation. The amelioration of OGE of cell stress and stress-induced apoptosis was proved by the HSP70 and HSP72 decrease.

Conclusion: Our data suggest that OGE may minimize the cytotoxic effects of H₂O₂-induced SCs apoptosis by modulating the apoptotic pathway and could potentially supplement cell transplantation therapy.

Key words: *Ocimum gratissimum*; Schwann cells; oxidative stress, apoptosis.

Introduction

Schwann cells (SCs), the myelinating glia of the peripheral nervous system, ensheath individual axons, promote axonal growth and maintain normal electric conductivity. Damage to SCs will likely cause faulty neuronal maintenance or demyelination and

reduces axonal conductivity [1]. During peripheral nervous system regeneration, SCs can express various types of neurotrophic factors and adhesion molecules that support axonal regrowth and myelin sheath reconstruction. Apoptosis of SCs may promote axonal

degeneration by reducing the neurotrophic support for axons from SCs [2, 3]. Unlike neurons, which are not endogenously replaced after injury, SCs proliferate immediately after injury and infiltrate the lesion site to myelinate [4]. The SCs ability to promote nerve regeneration has increased interest in cell transplantation therapy for nervous system repair. Several previous studies have demonstrated that SC transplantation post-spinal cord-injury induces remyelination, promotes axonal regeneration, and enhances functional recovery [5, 6].

The efficacy of such cell-based therapies depends greatly on transplanted cell survival [7, 8]. In rat models of contused spinal cord, the dramatic losses of implanted SCs via necrotic and apoptotic cell deaths occur largely 3 weeks post-implantation [9-11]. Therefore, proper intervention by strategies that limit necrosis and/or apoptosis is being considered to enhance overall transplanted cell survival. Disturbances in the normal redox state of cells can cause toxic effects by producing peroxides and free radicals that damage cell components, including proteins, lipids, and DNA.

The genus *Ocimum* (*O.*), collectively called basil, is a popular culinary and medicinal plant. *O. gratissimum*, of the Lamiaceae family, is a small shrub known as "scent leaf" in Africa and "Chit-Chan-Than" in Taiwan. Phytochemically, *O. gratissimum* contains a high quantity of essential oils (3.2%–4.1%), ocimol, gratissimin, β -sitosterol, flavonoids, linolenic acid, and polyphenolic compounds [12-14]. A significant quantity of simple phenols and flavonoids, including catachin, caffeic acid, epicatechin, and rutin, have been discovered in aqueous *Ocimum gratissimum* extract (OGE) [14-17]. Our recent study has shown that OGE can effectively inhibit the apoptotic mitochondrial pathway and protect nearby cells from oxidative stress-induced cell damage *in vitro* [15] and *in vivo* [17, 18]. Furthermore, OGE significantly decreases stress-related proteins, including HSP70 and iNOS, in livers of CCl₄-administrated rats [14], indicating the anti-oxidative capabilities of OGE. Moreover, OGE is an anti-depressant, sedative, and anxiolytic [18, 19].

This study aimed to examine the effect of direct H₂O₂ oxidative injury on Schwann RSC96 cell survival and whether and how OGE could modulate the RSC96 cell survival. We found that H₂O₂-induced oxidative stress promotes RSC96 cell apoptosis and OGE minimizes the cytotoxic effects of H₂O₂ by modulating the apoptotic mitochondrial pathway. Moreover, we investigated how OGE prevents H₂O₂-induced apoptotic cell death in RSC96 cells and elucidated the underlying molecular mechanisms.

Methods and Materials

Preparation of *O. gratissimum* extract

The *O. gratissimum* leaves were harvested, washed in running water, and air-dried for 1 week to create a coarse powder. The powdered vegetal material (400 g) was homogenized with distilled water (1000 mL) using a polytron. The homogenate was incubated at 95 °C for 1 h and filtered through a two-layer gauze. The filtrate was centrifuged at 20,000 g, 4 °C for 15 min to remove insoluble pellets, and the supernatant was thereafter collected, lyophilized, and stored at –20 °C until use. Before the assays, the extract powder (OGE) was dissolved at required concentrations. The total polyphenol content was analyzed according to Folin–Ciocalteu method [20, 21], and total flavonoid content was determined using the Lamaison method [22]. The final OGE was composed of 11.1% phenolic acid and 4.5% flavonoids, as quantitatively measured and indicated in our previous paper [14-17].

Cell culture

RSC96 cells were obtained from Bioresources Collection and Research Center and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% v/v fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 100 µg/mL penicillin/streptomycin (Sigma-Aldrich Chemie, Munich, Germany) at 37 °C in a humidified atmosphere containing 5% CO₂. RSC96 cells were seeded in 24-well culture plates at an initial density of 2 × 10⁵ cells/mL and grown to approximately 80% confluence. Oxidative stress was induced using fresh H₂O₂. Cells were pretreated with OGE at indicated concentrations for 24 h, and the medium containing H₂O₂ (final concentration at 5.6%) was added and incubated for indicated amounts of time. After incubation, the cells were washed with phosphate-buffered saline (PBS; 25 mM sodium phosphate, 150 mM NaCl, pH 7.2) and collected for subsequent analysis. For morphological analysis, the cells were observed for size and number changes under an inverted Microscope (Olympus Corp., Japan) at 100× magnification.

MTT assay

Cell viability was determined using MTT assay. RSC96 cells were exposed to H₂O₂ or with or without test sample pretreatments (OGE). To determine the hydrogen peroxide cytotoxicity, RSC96 cells were treated with five H₂O₂ concentrations (0, 100, 200, 300, and 400 µM), according to our pilot study. The 300 µM H₂O₂ caused over 30% cell death after 6 h compared with untreated control cells. Thus, 400 µM H₂O₂

concentration was deemed appropriate for subsequent experiments in this study. RSC96 cells were starved for 6 h and pretreated with various indicated concentrations of OGE for 24 h and treated with H₂O₂ for 24 h. After treatments, the medium was removed, and RSC96 cells were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT 0.5 µg/mL) at 37 °C for 4 h. The viable cell number was directly proportional to the production of formazan, which was dissolved in isopropanol and determined by measuring the absorbance at 570 nm using a microplate reader.

Flow cytometry

Cells were suspended in phosphate buffered saline (PBS, pH 7.4) and fixed with 75% (v/v) ethanol at -20 °C for 12–16 h. After ethanol removal, the cells were washed with PBS and stained for 30 min with 0.005% propidium iodide (PI). Analysis was performed immediately in FACSCalibur flow cytometer and Cellquest software (Becton Dickinson, San Jose, CA).

Reverse transcription (RT)-PCR

Total RNA was isolated from cell specimens by the guanidinium thiocyanate-phenol method. The extract integrity was assessed by 1.5% agarose gel electrophoresis and RNA was visualized by ethidium bromide staining. The total amount of RNA was determined spectrophotometrically. For each experiment, the number of PCR cycles was titrated to avoid reaching the amplification plateau. 25 cycles of PCR was used for an internal control GAPDH, and 30 cycles for other selected genes. The cDNA was synthesized using 500 ng of total RNA (Omniscript RT Kit, Qiagen). PCR was performed in an Eppendorf PCR machine with the following conditions: denaturation/polymerase activation at 95 °C for 5 min, followed by the indicated cycles of 95 °C for 15 s, 50 °C for 20 s, and 72 °C for 30 s. The 20 µL final reaction volume consisted of a pre-made reaction mix containing 5 pM of each primer and 1 µL cDNA in water. The following mouse targets were amplified using the indicated primer pairs: GAPDH, 5'-TCACTCAAGATTGTCAGCAA-3' and 5'-AGATC CACGACGGACACATT-3' (307 bp fragment 476–783, NCBI Reference Sequence: XM_001476707.5); HSP70, 5'-ATGAAGGAGATCGCCGAGG-3' and 5'-GTCAA AGATGAGCACGTTG-3' (218 bp fragment 595–813, NCBI Reference Sequence: NM_005346.4); HSP72, 5'-CTGGGCACCACCTACTCCTG-3' and 5'-CTCCTTCATCTTCGTCAGCA -3' (356 bp fragment 262–618, NCBI Reference Sequence: NM_010478.2).

Western blot

Cultured RSC96 cells were scraped and washed once with PBS. The cell suspension was spun down, and cell pellets were lysed for 30 min in the lysis buffer (50 mM Tris (pH 7.5), 0.5 M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol, 1 mM BME, 1% IGEPAL-630, and proteinase inhibitor cocktail (Roche, Mannheim, Germany)) and centrifuged at 12,000 g for 10 min. The supernatants were removed and placed in new Eppendorf tubes for Western blot analysis. Proteins from the RSC96 cells were separated in 12% gradient SDS-PAGE and transferred onto nitrocellulose membranes. Nonspecific protein binding was blocked in the blocking buffer at room temperature for 1 h (5% milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). The membranes were incubated in 4 °C blocking buffer overnight with specific antibodies (1:1000) caspase-3, PI3K, and Bcl2 (BD Biosciences, San Jose, CA); and Bax and tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For repeated blotting, nitrocellulose membranes were stripped with restore Western blot stripping buffer (Pierce Biotechnology, Inc, Rockford, IL, USA) at room temperature for 30 min. Densitometric analysis of immunoblots was performed using the AlphaImager 2200 digital imaging system (Digital Imaging System, CA, USA). Experiments were performed in triplicate.

Statistical analysis

Statistical differences were assessed using one-way ANOVA. $p < 0.05$ was considered statistically significant. Student's t-test was used in two-group comparisons. Data are expressed as mean ± SD.

Results

OGE attenuates H₂O₂-induced cell death in RSC96 cells

To examine the effects of OGE on H₂O₂-induced cell death, RSC96 cells were pretreated with 0, 50, 100, 150, and 200 µg/mL of OGE and challenged with 0, 100, 200, 300, and 400 µM H₂O₂. The results showed that administration of H₂O₂ (200, 300, and 400 µM) significantly diminishes RSC96 cell viability (53%, 32%, and 23%, respectively) (Fig. 1). However, OGE pretreatment (150 and 200 µg/mL) significantly elevates cell viability by approximately 62% or 66%, respectively. Treatment with various OGE concentrations without the H₂O₂ challenge did not affect cell viability. The data therefore indicated that OGE pretreatment enhances the viability of cells affected by H₂O₂ challenge in a dose-dependent manner; moreover, OGE can protect RSC96 cells from H₂O₂-induced cell death.

OGE inhibits cell cycle transition

Effect of OGE on cell proliferation was determined via cell cycle analysis using flow cytometry. RSC96 cells challenged with 300 μM H_2O_2 showed a higher (43%) sub-G1 population compared with the untreated control group (1%), indicating an accumulation in the sub-G1 population. However, OGE pretreatment (150 and 200 $\mu\text{g}/\text{mL}$) significantly reduced the sub-G1 cells in a dose-dependent manner (7% and 8%, respectively) and facilitated the cell cycle progression in RSC96 cells, as reflected by the reduced sub-G1 and increased G0/G1 populations (Fig. 2).

OGE reverses the apoptotic protein expressions

To observe whether OGE treatment could suppress caspase-3 activation and arrest H_2O_2 -induced apoptotic cell death in RSC96 cells, the changes in caspase-3 levels and downstream PARP cleavage levels were examined. As shown in Fig. 3, an increase in caspase-3 activity was observed in H_2O_2 -treated cells. However, the phenomenon was attenuated upon OGE pretreatment. Moreover, the Bcl-2 protein expression level was significantly decreased and Bax protein expression level was

significantly increased in the H_2O_2 -treated group compared with the untreated control group. Treatment with OGE significantly attenuated the effects of H_2O_2 on the expression levels of the two proteins. Adding OGE normalized the Bcl-2 and Bax levels in all conditions of H_2O_2 toxicity (Fig. 3).

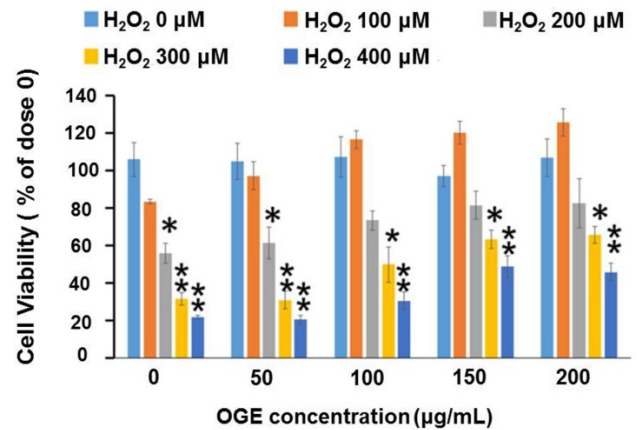


Figure 1. Effects of OGE on H_2O_2 -induced cytotoxicity in RSC96 cells. The RSC96 cells were pretreated with OGE (0, 50, 100, 150, and 200 $\mu\text{g}/\text{mL}$) and then exposed to H_2O_2 (0, 100, 200, 300, and 400 μM). The cell viability was estimated using MTT assay. The results are expressed in percentage of untreated cells. Data are means \pm S.E. (n=3). * p <0.05 indicates significant difference compared with the untreated group. ** p <0.01 indicates significant difference compared with the untreated group.

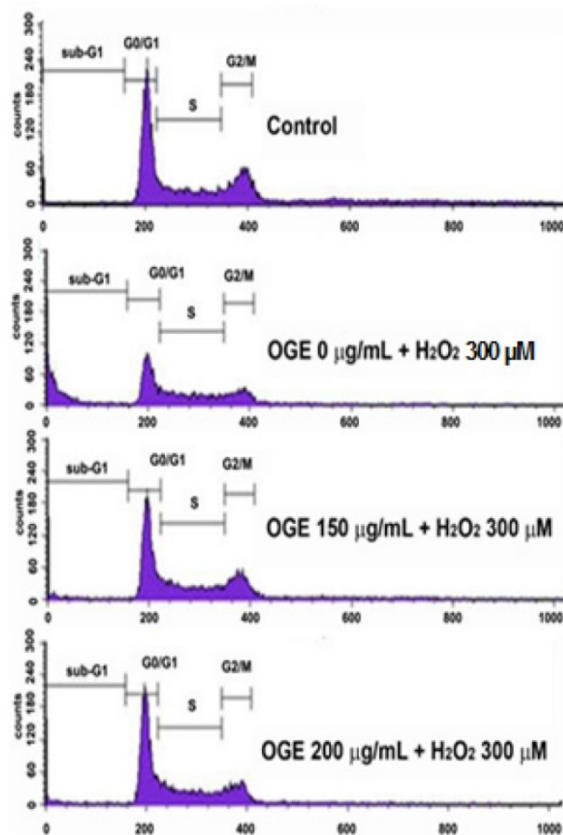
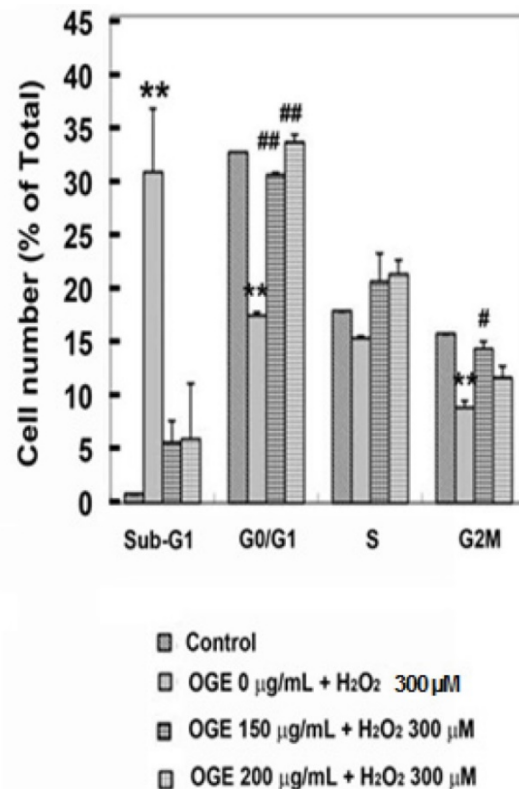


Figure 2. Effects of OGE on the cell cycle distribution in H_2O_2 -treated cells. RSC96 cells were pretreated with 150 and 200 $\mu\text{g}/\text{mL}$ OGE for 24 h and challenged with 300 μM H_2O_2 for 12 h. Data are means \pm S.E. (n=3). ** p <0.01 indicates significant difference compared with the control group; # p <0.05 and ## p <0.01 indicate significant compared with the H_2O_2 group



Protective effects of OGE is associated with PI3K- and ERK-independent signaling pathways

RSC96 cells were exposed to specific PI3K and ERK inhibitors to identify the potential role of PI3K/ERK survival signaling cascades in the OGE-established protection against H₂O₂ toxicity in SCs. Upon MTT assay, the administration of PI3K and ERK inhibitors did not interfere in the cytoprotection of OGE against H₂O₂ toxicity in RSC96 cells (Fig. 4). Therefore, the enhanced cell survival by OGE is not dependent on PI3K- and ERK signaling pathways.

HSP70 expression plays a compensative protection against H₂O₂-induced oxidative stress

Induced expression or overexpression of Hsp70 can inhibit caspase activation by directly interacting

with caspases [23, 24]; moreover, the protective effect of Hsp72 occurs upstream of the mitochondria [25]. To determine whether OGE administration has a stress-ameliorative effect, RSC96 cells were incubated in OGE and then exposed to H₂O₂ (300 μM) for 8 h. The HSP70 and HSP72 expression levels were determined post-H₂O₂ treatment: the H₂O₂ challenge increased the HSP70 and HSP72 mRNA levels (Fig. 5), indicating the development of a compensative mechanism against H₂O₂-induced stress. However, OGE treatment resulted in a dose-dependent reduction of HSP70 and HSP72 mRNA levels due to oxidative stress suppression and reduction of the associated compensative HSP70 and HSP72 mechanism.

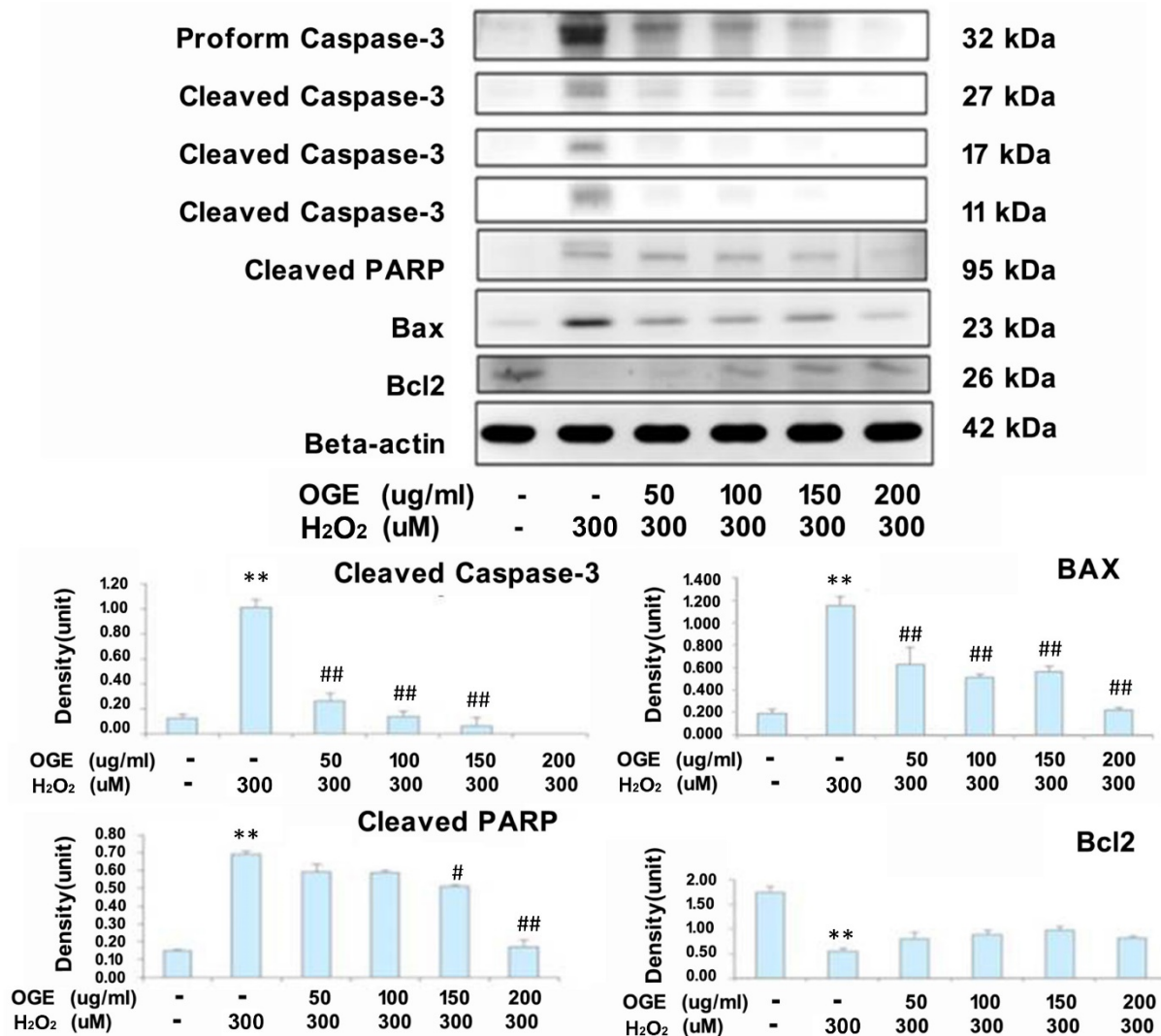


Figure 3. Effects of OGE on the expression levels of apoptosis-related proteins. The cells were pretreated with different OGE concentrations and then treated with H₂O₂ for 24 h. The protein levels of cleavage caspase-3, PARP, Bax, and Bcl-2 were measured using Western blot, and the statistical analysis (n=3) are presented in the panels below. Data are presented in means ± S.E. (n=3). **p<0.01 indicates significant difference compared with the untreated control group. #p<0.05 and ##p<0.01 indicate significant difference compared with the H₂O₂-treated group.

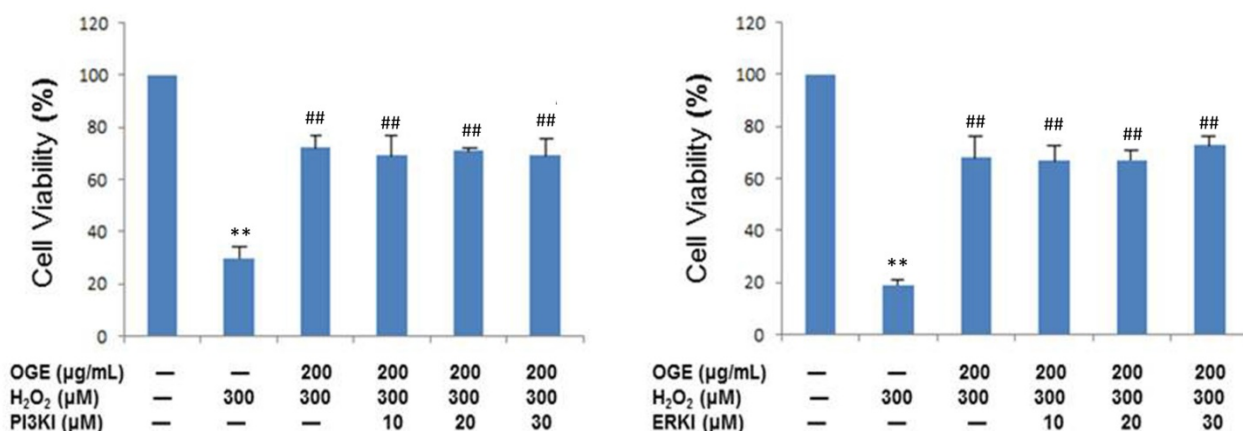


Figure 4. OGE enhances cell viability in the PI3K- and ERK-independent signaling pathways. The RSCs cells were treated with H₂O₂, OGE, and one inhibitor (PI3K and ERK). The cell viability was measured using MTT assay. The untreated cells were designated as control. The results were expressed as percentage of the untreated cells. Data are means ± S.E. (n=3). **p<0.01 indicates significant difference compared with the untreated control group. ##p<0.01 indicates significant difference compared with the H₂O₂-treated group.

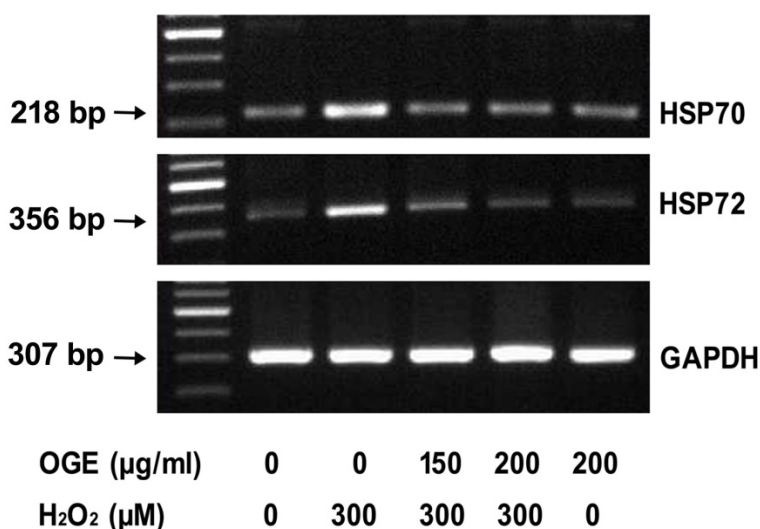


Figure 5. The HSP70 and HSP72 expressions in H₂O₂-treated RSC cells. RSC96 cells were pretreated with different OGE concentrations for 24 h and challenged with 300 μM H₂O₂ for 24 h. The expressions HSP70, HSP72 and GAPDH were detected using RT-PCR analysis.

Discussion

Oxidative stress, which is characterized by overwhelming ROS, is among the mechanisms that induce cell apoptosis and is believed to cause neurodegenerative diseases. In neuronal cells, ROS results in membrane lipid peroxidation, protein nitration, and DNA degradation, all of which are associated with apoptosis [26]. Among the ROS stress mediators, H₂O₂ plays a pivotal role because it is generated from nearly all sources of oxidative stress and diffuses freely in and out of cells and tissues [27]. Schwann cells are exposed to various oxidative stress-mediated processes during nerve repair [28, 29]. Our results showed that H₂O₂ could induce cellular apoptosis in RSC96 cells.

Flow cytometry analysis showed that OGE pretreatment attenuates H₂O₂-induced sub-G1 phase accumulation, which may indicate cell apoptosis [28].

Moreover, OGE pretreatment reduces the level of cleaved caspase-3 and only slightly affected the level of cleaved caspase-8, suggesting that the OGE mainly protects the mitochondrial pathway.

The caspases are cysteine proteases involved in the execution of cellular apoptosis in all most all cell types. They are expressed as pro-enzymes and are then activated following proteolytic cleavage. Cleaved caspases further cleave their target proteins as a part of the apoptosis-signaling mechanism. Caspase-3 is a major downstream caspase, activated by cleavage to produce at least a 12 kD and a 17 kD subunit. The un-cleaved pro-enzyme is expressed in various tissue types with different levels of expression. Caspase-3 is highly expressed in tissues with a greater tendency to apoptosis and the effects of caspase-3 expression on apoptosis are depended on the tissue type and the stimulus that induces apoptosis [29, 30].

In our results the increase in the level of pro-caspase expression shows that H₂O₂ treatment on RSC96 cells triggers the cells to be more inclined to apoptotic assault. Further increase in the levels in cleaved caspases also reveals the elevated apoptosis event in the RSC96 cells.

The mechanism of apoptosis is regulated by the Bcl-2 family of proteins, which includes the death agonists (e.g., Bax, Bak, and Bad) and antagonists (e.g., Bcl-2 and Bcl-xL) [31, 32]. Among these, Bax and Bcl-2 play key roles in regulating cell apoptosis. The balance between proapoptotic proteins, such as Bax, and antiapoptotic proteins, such as Bcl-2, is deemed critical in regulating apoptosis [33]. Our results indicated that OGE significantly enhances Bcl-2 levels in RSC96 cells and down-regulates the level of Bax, thereby suggesting that the antiapoptotic effects of OGE on RSC96 cells are correlated with Bcl-2 changes.

Upon studying, ERK pathway has been proven important in cell survival [15, 27]. Moreover, PI3K/Akt activation promotes cell survival via direct regulation of antiapoptotic Bcl-2 and apoptotic proteins, including BAD, BCL-XL, and caspase-9. In previous papers [15], evidence showed that OGE activates PI3K/Akt and can protect H9c2 cells from H₂O₂-induced cell death. The PI3K and ERK inhibitors showed not much effect on the OGE mediated reduction of RSC96 cell death. Therefore the results suggests that either OGE competitively enhances the activation of PI3K and ERK or acts on other survival factors downstream to compensate the loss of PI3K and ERK activation.

Hsp70 protects SCs against apoptotic death from both H₂O₂ and serum withdrawal [34]. When ROS stress exceeds a threshold level, the preexisting antioxidant pathways may be complemented by cytoprotective mechanisms represented by heat shock proteins (HSPs) [35]. To test this hypothesis, we analyzed the HSP70/HSP72 expressions and found that they can be induced in H₂O₂-challenged RSC96 cells, suggesting that HSP70/HSP72 cause a complementary protective mechanism in RSC96 cells. OGE did not provide additional HSP70/HSP72 expressions, and we speculate that the reduced oxidative stress due to OGE treatment triggered the decrease in HSP70/HSP72 expressions however further studies should be performed to confirm such understanding.

In conclusion, OGE pretreatment prevents RSC96-cell apoptosis against oxidative stress in a dose-dependent manner. Cell transplantation of SCs is a promising modality for promoting neuronal survival and neural regeneration in spinal cord injury; therefore, these findings indicate that OGE could

potentially confer neuroprotection to peripheral nerves by preserving SCs and diminishing oxidative stress-mediated injuries.

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Competing Interests

The authors have declared that no competing interest exists.

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