Protective Effects of Aqueous *O. gratissimum* Extract on Peroxide-induced Cell Death of H9c2 Myocardiac Cells

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Running head: Protection of cardiomyocyte by OGE

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Abstract

Increased cell death of cardiomyocyte by oxidative stress is known to cause dysfunction of heart. Previous studies have demonstrated that extracts of *Ocimum* species are able to protect different cells from oxidative stress-induced damage and the following cell death. Among the *Ocimum* species, *O. gratissimum* is a well-known medicinal plant and widely used in treatment of inflammatory diseases. Therefore, we hypothesized that aqueous extract of *O. gratissimum* leaf (OGE) may have protective effects on cardiomyocyte. Our findings revealed that hydrogen peroxide (H_2O_2) treatment significantly decreased cell viability of H9c2 myocardiac cell, and the viability was dose-dependently restored by OGE. Condensed staining of nucleus by DAPI implied that H_2O_2 treatment led to apoptosis and the apoptosis was attenuated by OGE. Further investigation showed that OGE inhibited H_2O_2–induced activation of caspas-3 and caspase-9, but little affected the activation of caspase-8. The H_2O_2–induced Apaf-1 and cytochrome c, upstream of caspase-9 in mitochondrial pathway, were also decreased by OGE. Additionally, Bcl-2 was significantly induced by OGE. Analysis of mitogen-activated protein kinase (MAPK) signaling revealed that OGE mainly induced the activation of AKT and little affected the activation of ERK1/2, p38 MAPK and JNK. Taken together, our findings revealed that OGE

effectively inhibited the mitochondrial pathway and increased the Bcl-2 expression level, which may play important roles in protecting H9c2 cell from H_2O_2-induced cell death.

Key words: *Ocimum gratissimum*, Apoptosis, Bcl-2, Mitochondrial pathway, Akt, ERK

Introduction

Cardiac cell apoptosis plays an important role in heart development and pathogenesis of heart dysfunctions related with ischemia-reperfusion, pressure overload, and chronic heart failure (14). Loss of contractile tissue, compensatory hypertrophy, and reparative fibrosis caused by cardiac apoptosis is also being reported to contribute to the development of cardiovascular diseases (24). Therefore, signaling pathways leading to modification of cardiomyocyte apoptosis have become a major area of both clinical interest and basic research.

Oxidative stress has been known as an imbalance of oxidant/antioxidant which could result in cell damage. It is believed that oxidative stress plays a crucial role in cardiac cell apoptosis and in pathology associated with cardiovascular diseases (20).

Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2), has been demonstrated as an important oxidative stress (15). Increase of intracellular ROS leads to irreversible damage of various cellular components, such as lipids, proteins and DNA, and accumulation of the cellular damages is able to further result in cell apoptosis, a programmed cell death being characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and formation of apoptotic bodies (18,22).

The genus *Ocimum*, belonging to the family *Labiatae*, is widely found in tropical and subtropical regions. The widespread plant is commonly used as not only a fresh and dried food spice, but also a traditional herb in European and Asian countries for the treatment of various ailments since ancient times. The ethanolic extract of *Ocimum* leaf has shown significant modulatory influence on carcinogen metabolizing enzymes including cytochrome P450, cytochrome b5 and aryl hydrocarbon hydroxylase, glutathione-s-transferase. Additionally, the aqueous extract of *Ocimum sanctum* is reported to have a more profound effect than both the fresh paste and the ethanolic extract on reducing the chemical-induced papillomagenesis (17,25). However, the functions and mechanisms for therapeutic or protective effects of aqueous extract of *Ocimum gratissimum* (OG) remain unclear.

In this study, we aimed to examine the protective effects of aqueous extract of OG leaf (OGE) on H9c2 myocardiac cells against H_2O_2-induced cell death, and to investigate the mechanisms induced by OGE. Cell viability was determined by MTT assay. Nucleus was monitored by DAPI staining. Activation of caspase and mitogen-activated protein kinase (MAPK) signaling was determined by immunoblots probed with specific antibodies.

Materials and Methods

Chemicals

H_2O_2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), penicillin and streptomycin were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and trypsin-EDTA were purchase from Gibco BRL (Gaithersburg, MD, USA). Antibodies against caspase-3, caspase-9, apaf-1, cytochrome c, Bcl-2, ERK1/2, JNK, p38 and AKT were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against β-actin, mouse IgG and rabbit IgG were purchased from Abcam Inc. (Cambridge, UK). The myocardiac cell H9c2 was obtained from

American Type Culture Collection (ATCC; Rockville, MD).

Preparation of OGE

Leaves of *Ocimum gratissimum* Linn were harvested, washed with distilled water and then homogenized with distilled water by using polytron. The homogenate was incubated at 95^{o}C for 1 hour (h) and then filtered through two layers of gauze. The filtrate was centrifuged to remove insoluble pellets (20,000 g for 15 min at 4ºC) and the supernatant (OGE) was collected, lyophilized and stored at -70^{o}C until use.

Cell culture and experimental treatments

H9c2 cells were maintained in DMEM supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin at 37^{o}C in a humidified atmosphere containing 10% CO_2. In all conditions, H9c2 cells were seeded in 6-well culture plates at an initial density of $1x10^5$ cells/ml and grown to approximately 80% confluence. Oxidative stress was induced by treating with freshly prepared H_2O_2. Cells were pretreated with OGE at indicated concentration for 3 hrs, and then the medium containing H_2O_2 was added (final concentration at 200 μM) and incubated for 24 h. After the incubation, the cells were washed with phosphate-buffered saline (PBS; 25 mM sodium phosphate, 150 mM NaCl, pH 7.2) and then collected for

the subsequent analysis.

DAPI staining

H9c2 cells (5 x10^4 cells/ml) were pretreated with 0, 50 and 100 μg/ml OGE for 3 hrs and then incubated with 200 μM H_2O_2 for 24 h. After the treatment, the cells were stained with DAPI and photographed using a fluorescence microscope as previously described (7,13).

MTT assay for cell viability

Cell viability was determined by MTT assay (11) in the absence or presence of 50 or 100 μg/ml OGE. After the 24 h treatments, medium was removed and the H9c2 cells were incubated with MTT (0.5 mg/ml) at 37^{o}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 (SpectraMAX 360 pc, Molecular Devices, Sunnyvale, CA).

Immunoblotting

The treated H9c2 cells were washed with PBS and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 10 μg/ml aprotinin and leupeptin). The lysates

were incubated on ice for 30 min and centrifuged at 20,000g for 15 min. The supernatants were collected and followed by protein quantitation using Bradford method. Crude proteins (30 μg per lane) were electrophoresed on 12.5% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane (Millipore, Bedford ,MA) as previously described.(19) The blotted membrane was blocked with 5% w/v skimmed milk in PBS, and then incubated for 2 h with 1/1000 dilution of the specific antibodies against human caspase-3, caspase-9, apaf-1, cytochrome c, Bcl-2, ERK1/2, JNK, p38, PI3K/Akt and β-actin. Bound antibodies were detected using 1/2000 dilution of peroxidase-conjugated secondary antibodies and ECL chemiluminescence reagent (Millipore) as the substrate system (8).

Statistical analysis

Statistical analysis was performed using the SigmaStat version 3.5 for Windows (Systat Software Inc. San Jose, CA). The results are presented as mean ± SD. The statistical significance between groups was determined using Student's t test. A *p* value less than 0.05 was considered statistically significant.

Results

OGE alleviates H9c2 cells H_2O_2-induced cell death

Prior to investigate the protective effects OGE on peroxide-induced cell death, the cytotoxicity of OGE alone was examined. H9c2 cells were treated with a series concentration of OGE (10-300 μg/ml) for 24 h, and the following MTT assay for cell viability was performed. As shown in Fig. 1A, although the cell viability was slightly increased by 10 and diminished by 50, 100, 200, and 300 μg/ml OGE, the changes were not statistically significant as comparing to control. Therefore, the protective effects of OGE on H_2O_2-induced cell death were then examined. The cell viability of H9c2 cells treated with 200 μM H_2O_2 containing 0, 50, 100 and 150 μg/ml OGE was determined. As shown in Fig. 1B, the findings showed that H_2O_2 effectively diminished the cell viability to 19.5 ± 0.7 % of control, and H_2O_2 combining OGE (50, 100 and 150) co-treatment reduced the cell viability to 41.3 ± 2.6, 39.7 ± 8.1, 68.6 ± 0.8 and 88.4 ± 3.1 % of control respectively. Together, OGE alone treatment showed no significant cytotoxicity to H9c2 myocardiac cells and the OGE co-treatment dose-dependently recovered the cell viability diminished by H_2O_2.

OGE attenuates the DNA fragmentation of H9c2 cells induced by H_2O_2

To investigate whether apoptosis contributed to H_2O_2-induced cell death, DNA

fragmentation was monitored by DAPI staining. As shown in Fig. 2, H9c2 cells treated with H_2O_2 revealed the condensed DAPI staining which probably resulting from H_2O_2-induced DNA fragmentation. Pretreated with 50 and 100 μg/ml OGE significantly attenuated the ratio of condensed DAPI-stained cells as comparing to the treated with H_2O_2 alone, and the attenuation of condensed DAPI-stained cells by OGE pretreatment was dose-dependent. Therefore, these findings indicated that apoptosis may involve in the H_2O_2-induced cell death and OGE pretreatment may attenuate the apoptosis of H9c2 cells induced by H_2O_2.

OGE inhibits the mitochondrial pathway induced by H_2O_2

To further investigate the apoptotic pathways induced by H_2O_2 and the effects of OGE on these pathways, activation of intrinsic (mitochondrial) pathway and extrinsic pathway was examined. As shown in Fig. 3, H_2O_2 treatment reduced the level of caspase-3 (precursor form, 32 kDa) and increased the level of cleaved caspase-3 (active form, 17 kDa). OGE pretreatment restored the level of caspase-3 and decreased the level of cleaved caspase-3 induced by H_2O_2. The activation of the upstream effectors of caspase-3, caspase-9 and caspase-8, was also determined. Interestingly, the levels of cleaved form/active form of caspase-9 and caspase-8 were both increased by H_2O_2 treatment; however, only the level of cleaved form/active

form of caspase-9 was decreased by OGE pretreatment (Fig. 3). Therefore, the levels of the upstream effectors of caspase-9, including Bcl-2, Apaf-1 and cytpchrome c, were further determined. As shown in Fig. 4, H_2O_2 treatment alone decreased the level of anti-apoptotic Bcl-2 and increased the levels of Apaf-1 and cytochrome c, the activators for caspase-3. OGE pretreatment significantly increased the level of anti-apoptotic Bcl-2 and diminished the levels of Apaf-1 and cytochrome c. Taken together, these findings suggested that OGE attenuated the H_2O_2-induced apoptosis of H9c2 cells through inhibiting mitochondrial pathway.

OGE induces the activation of Akt but not affects the other MAPKs

To investigate the anti-apoptotic mechanisms induced by OGE, kinase-mediated survival signaling and apoptotic signaling was investigated. As shown in Fig. 5, phosphorylation of Akt (pAkt) and ERK 1/2 (p-ERK 1/2) was increased by H_2O_2 treatment alone as comparing to control. OGE pretreatment significantly increased the phosphorylation of Akt as comparing to both H_2O_2 treatment alone and control. Interestingly, OGE pretreatment slightly decreased the phosphorylation of ERK 1/2 as comparing to H_2O_2 treatment alone. Additionally, the phosphorylation of p38 MAPK (p-p38) and JNK (p-JNK) was not affected by neither H_2O_2 treatment alone nor OGE pretreatment. Taken together, these findings suggested that OGE may increase the

expression of anti-apoptotic Bcl-2 through activating Akt-mediated signaling and may suppress the mitochondrial pathway through inhibiting ERK-mediated apoptosis.

Discussion

Direct treatment of cells with oxidants such as H_2O_2 was thought to cause necrosis, but recent studies have shown that ROS can induce cellular senescence and apoptosis under certain circumstances (22,28). In this study, it is found that the H_2O_2 treatment significantly diminishes the viability of H9c2 cells to 18.7 ± 0.6% and leads to DNA fragmentation, the characteristics of apoptosis, but the 150 μg/ml OGE pretreatment only recovered the cell viability to 67.8 ± 2.6%. It is suggested that the H_2O_2 treatment causes both apoptosis and necrosis of H9c2 cells, but OGE pretreatment may attenuate the apoptosis and may have little effect on the necrosis.

Mitochondria are important targets of ROS and the interaction leads to dysfunction of mitochondria and the subsequent cell apoptosis. In situ generated ROS can open the permeability transition (PT) pore with subsequent mitochondrial membrane potential and can cause cytochrome c release into the cytosol, which is required for the formation of the apoptosome and the resultant activation of procaspase-9. Activated caspase-9 in turn cleaves and activates caspase-3 (4,21),

which leads to apoptosis. On the contrary, Bcl-2 inhibits apoptosis, PT pore opening and cytochrome c release (Schlottmann and Schölmerich, 1999), which leads to anti-apoptosis. Our findings reveal that OGE pretreatment effectively inhibits the mitochondrial pathway and increases Bcl-2 level, suggesting that OGE pretreatment should be beneficial to ROS-induced apoptosis,

Polyphenols have been demonstrated to be the important and the major components in plant extracts for their therapeutic effects. Although the cellular mechanisms underlying the actions of flavonoids and their metabolites remain unclear, it is believed that antioxidant activity, free radical scavenging, and MAPK signaling pathways should be involved (2,9,26). MAPK family, comprising ERKs, JNK and p38, is activated in response to various stress stimuli. Recently, ERKs, associating with a variety of biological responses such as proliferation, migration and differentiation, have also been reported to mediate apoptosis in cultured cells (3,12,29).

It has been demonstrated that inhibition of ERK1/2 blocks caspase-3 activation showing both cytochrome c release dependent and independent (23,29). Therefore, ERK1/2 may act on mitochondria to cause cytochrome c release and/or may affect activation of caspase-3 downstream of cytochrome c release. Additionally, ERK may

also induce apoptosis through regulating the level of caspase-8, an initial caspase in extrinsic apoptotic pathway (5). Our findings reveal that OGE pretreatment diminishes the phosphorylation of ERK1/2 induced by H_2O_2 and the level of caspase-8 is not affected by OGE pretreatment, suggesting that OGE may predominantly inhibit ERK1/2 activation and the subsequent mitochondrial pathway.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays an important role in the regulation of cell survival, and most growth and survival factors activate the pathway (1). Moreover, activated PI3K/Akt promotes survival via the direct regulation of anti-apoptotic Bcl-2 and apoptotic proteins including BAD, BCL-X_L and caspase-9 (6,10,16). It is also reported that withdrawal of soluble growth factors from primary cultured cells leads to activation of ERK1/2, which is accompanied by a great decrease in Akt activity (27). In this study, both the H_2O_2 treatment and the OGE pretreatment are performed without serum deprivation, and the findings indicate that OGE pretreatment significantly induces the activation of Akt and increases the level of Bcl-2, suggesting that OGE may also protect H9c2 cells from H_2O_2 damage through enhancing survival signal pathway.

In conclusion, the present study provides evidences that OGE attenuated the H_2O_2-induced apoptosis of H9c2 myocardiac cell, which may result from the

inhibition of apoptotic ERK1/2 activity and mitochondrial signaling as well as from the enhancement of PI3K/Akt survival signaling and the increase of anti-apoptotic Bcl-2. These findings indicate that OGE should be beneficial to protect cardiomyocyte from oxidative stress induced by H_2O_2.

Acknowledgements

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Figure legends

Fig. 1. Effects of OGE on cell viability of H9c2 treated with H_2O_2. (A) The cell viability of H9c2 cells treated with a series concentration of OGE (10, 50, 100, 200 and 300 μg/ml) for 24 h. (B) The cell viability of H9c2 cells pretreated with a series concentration of OGE (50, 100, and 150 μg/ml) for 3 h and then treated with 200 μM H_2O_2. Three independent experiments were performed for statistic analysis. NS, not significant; ##, p <0.01 as comparing to control (C); *, p <0.05 and **, p <0.01 as comparing to 0 μg/ml of OGE.

Fig. 2. Effects of OGE on H_2O_2-induced DNA fragmentation. H9c2 cells were pretreated with 0, 50 and 100 μg/ml OGE for 3 h and then treated with H_2O_2 for 24 h. After the treatments, the H9c2 cells were stained with DAPI and photographed by fluorescence microscopy (200X). The cells presented DNA fragmentation were indicated by arrow.

Fig. 3. Effects of OGE on mitochondrial and extrinsic pathway. The protein levels of caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, caspase-8, and cleaved caspase-8 were determined by immunoblotting. β-actin was used as control. The apparent molecular weights for detected proteins were indicated.

Fig. 4. Effects of OGE on protein expression of Bcl-2, Apaf-1 and cytochrome c. The protein expression of Bcl-2, Apaf-1 and cytochrome c was determined by immunoblotting. β-actin was used as control. The apparent molecular weights for detected proteins were indicated.

Fig. 5. Effects of OGE on kinase-mediated pathways. The levels of phosphorylated-Akt (p-Akt), Akt, phosphorylated-ERK (p-ERK), ERK, phosphorylated-p38 (p-p38) and phosphorylated-JNK (p-JNK) were determined by immunoblotting. β-actin was used as control.

Fig. 1.

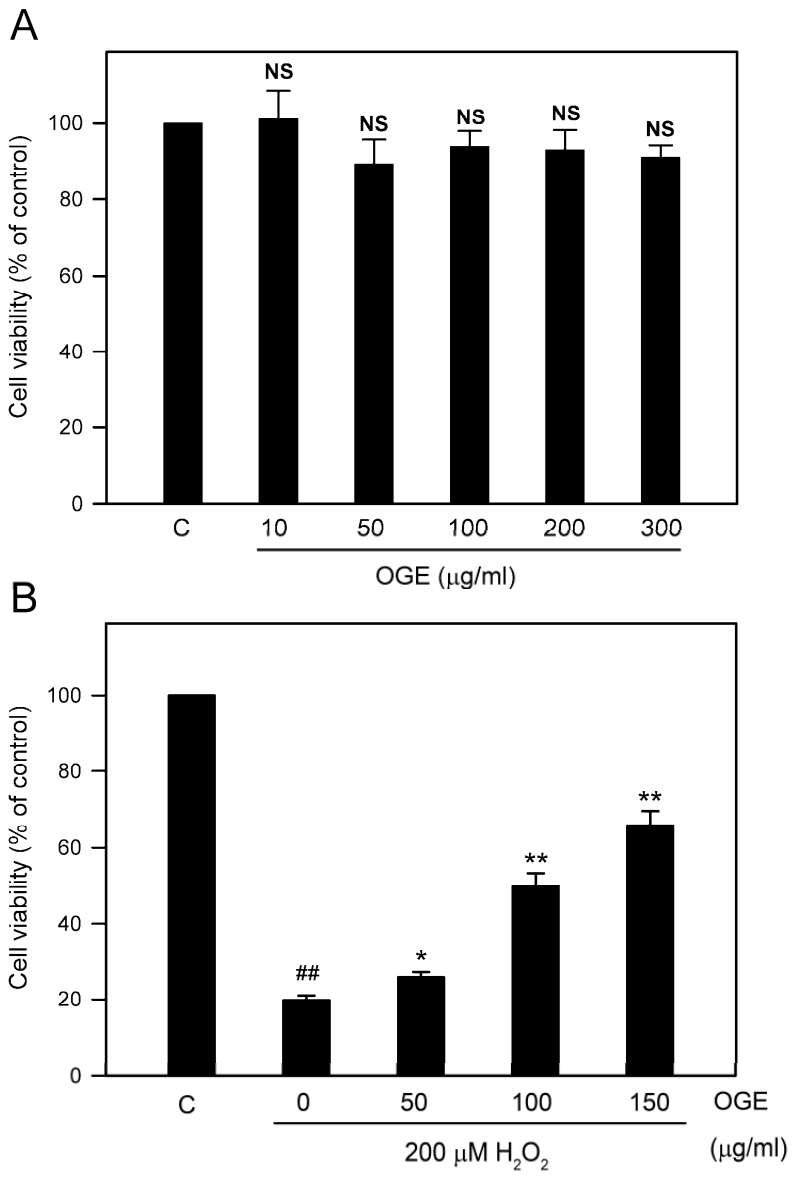

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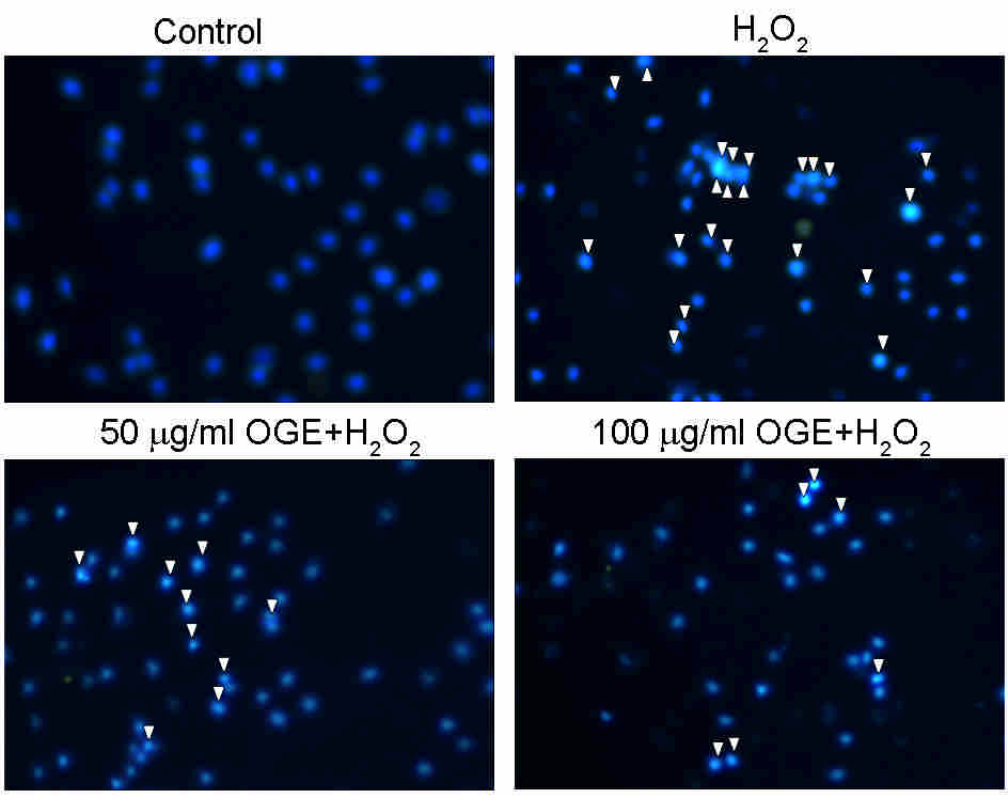
Fig. 2.



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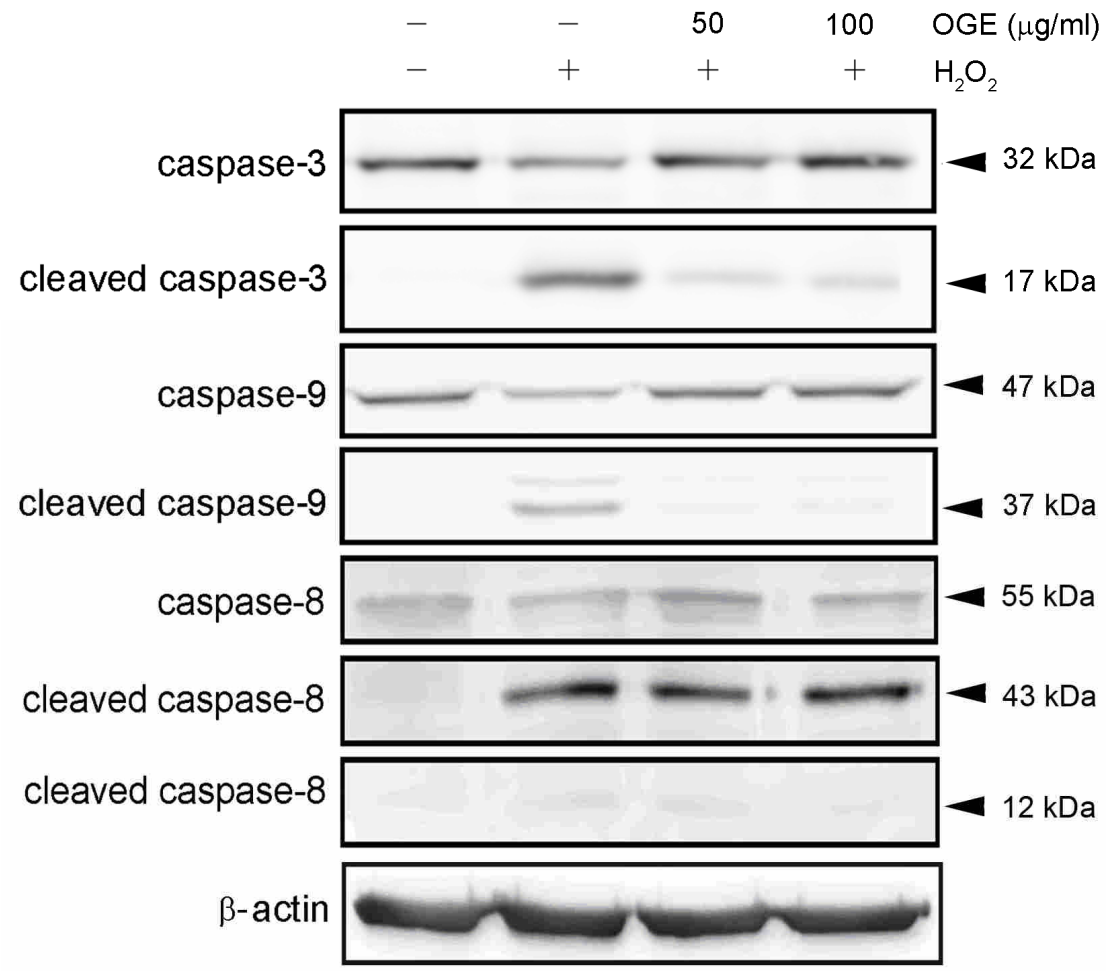

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Fig. 4.

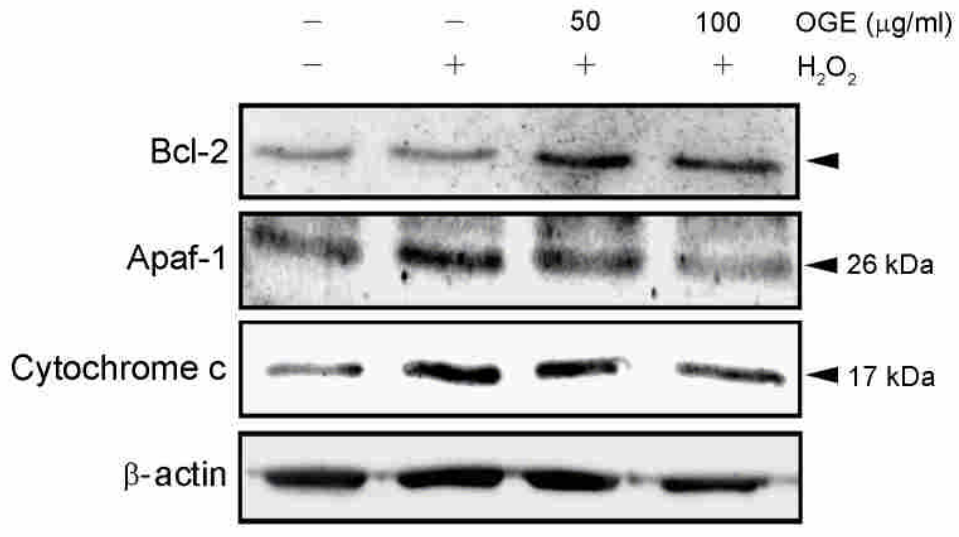

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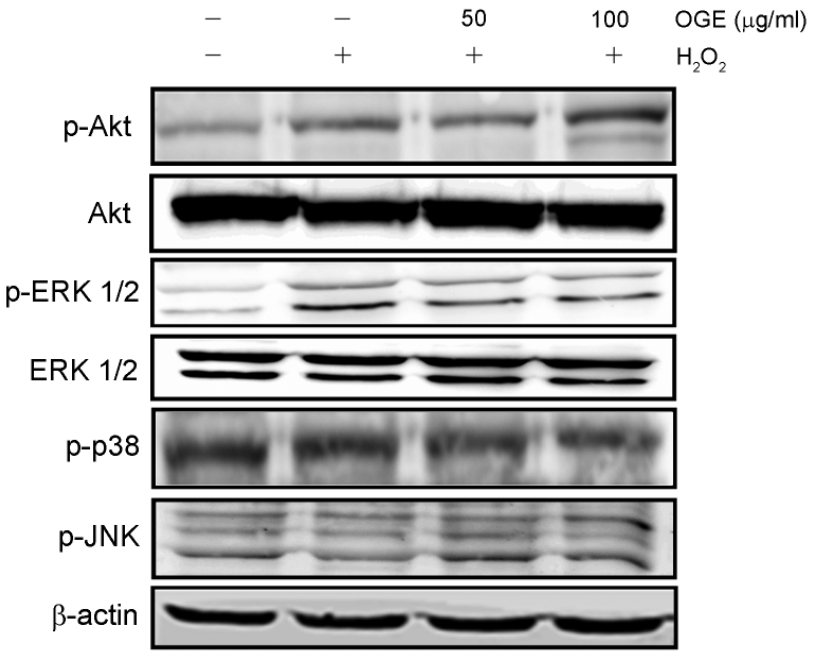
Fig. 5.



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