



# Mitochondrial dysfunction in resveratrol-induced apoptosis in QGY-7701 cells

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**ABSTRACT.** This study aims to evaluate the cytotoxicity of resveratrol on QGY-7701 cells via a cell viability assay, and determine the cytological alterations and damages that result. Resveratrol was found to inhibit QGY-7701 cell growth and decrease their viability in a remarkably dose-dependent manner. Resveratrol exposure also induced an increase in Caspase-3 activity and a decrease in Bcl-2, which caused an increase in membrane permeability, and the opening of mitochondrial permeability transition pores and mitochondrial depolarization. Cellular ATP is thus exhausted, and apoptosis is induced via the change in mitochondrial membrane permeability and mitochondrial dysfunction.

**Key words:** Resveratrol; QGY-7701 cells; Mitochondrial dysfunction; Membrane permeability; Apoptosis

## INTRODUCTION

Resveratrol (3,4',5-trihydroxystilbene) is a type of polyphenolic compound present in a variety of plants such as grapes, peanuts, berries, and especially in the dried roots of a traditional Chinese medicine *Polygonum cuspidatum* Sieb. et Zucc (Hu et al., 2013; Han et al., 2015). Previous studies have demonstrated that resveratrol possesses multiple bioactivities, including anti-oxidative, anti-inflammation, estrogen-like, growth-inhibitory, immune-regulatory, chemoprophylactic, and antitumor activities, as well as a cardio-protective effect (Lamont et al., 2011; Yang et al., 2011). It has attracted much because not only as a natural compound with few side effects, but also because it has an anti-tumor effect against various cancers.

Cancer is becoming an increasingly severe health threat. After cardiovascular disorders, it is the second leading cause of death worldwide (Shukla and Singh, 2011). Cancer is characterized by uncontrolled cell growth and acquisition of metastatic properties (Sarkar et al., 2013). To our best knowledge, no studies on the cytotoxicity and apoptotic effect of resveratrol on QGY-7701 cells have been performed. Therefore, the present study evaluated the cytotoxicity of resveratrol on QGY-7701 cells by determining cell viability, plasma membrane permeability, mitochondrial depolarization, the activity of mitochondrial permeability transition pores (MPTPs), and ATP levels, to illuminate the mechanism of action. This study may aid in the evaluation of resveratrol for cancer chemoprevention and chemotherapy.

## MATERIAL AND METHODS

### Chemicals and reagents

Resveratrol (purity 99%; Sigma), RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Invitrogen (Shanghai, China).

MTT Cell Proliferation and Cytotoxicity Assay Kit (catalog No. C0009), Caspase-3 antibody (catalog No. AC033), Bcl-2 antibody (catalog No. AB112), secondary antibody [HRP-labeled goat anti-rabbit IgG (H+L)] (catalog No. A0208), JC-1 probe (catalog No. C2006), ATP assay kit (catalog No. S0026), and BCA protein assay kit (catalog No. P0012) were purchased from Beyotime Institute of Biotechnology (Haimen, China) and MPTP assay kit (catalog No. GMS10095.1) from Genmed Scientifics Inc. (USA) Fluorescein diacetate (FDA) was obtained from Keeasy Economic & Trade Co., Ltd. All other reagents were of analytical grade and were obtained from commercial sources.

### Cell culture

The QGY-7701 cell line was purchased from the Institute of Cell Biology, Chinese Academy of Science (CAS), and cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS. Cells were maintained at 37°C in an incubator with 5% (v/v) CO<sub>2</sub> and the water-saturated atmosphere.

### Cell viability assay

Cytotoxicity tests were conducted on a 96-well culture plate seeded with 1 x 10<sup>4</sup> QGY-7701 cells. Following a 6-h incubation, the cells were washed with fresh medium and treated with resveratrol at concentrations of 0.1, 0.5, 1, 4, 8, 16, 32, 64, 128, 200 μM. Cells cultured in complete

medium without resveratrol served as the control. Each test was conducted in triplicate. After 24 h of treatment, cell viability was determined using a colorimetric assay with MTT reagent according to the previous study (Li et al., 2012a). The percent viability of cells was calculated by comparing treated cells with untreated control cells. The  $EC_{50}$  value was calculated using the Matlab software.

### **Determination of Caspase-3 activity and Bcl-2 level**

The Caspase-3 activity assay was based on the cleavage of the chromogenic Caspase substrate Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide); the assay conducted according to the manufacturer's instructions (Li et al., 2012a). The QGY-7701 cells were cultured for 6 h on 48-well plates ( $1 \times 10^5$  cells per well). The cells were exposed to resveratrol at 0, 6, 12, and 24  $\mu$ M for 24 h. The cells were then fixed with 4% paraformaldehyde and treated with 10% horse serum for 1 h at room temperature, before being incubated overnight with Caspase-3 antibodies (1:400) at 4°C. The cells were washed three times with 0.01 M PBS containing 0.3% Triton X-100, and incubated with the secondary antibodies [HRP-labeled goat anti-rabbit IgG (H+L)] (1:250) for 1 h. After washing the cells three times with 0.01 M PBS, 200  $\mu$ L TMB chromogenic reagent was added into each well and incubated for 30 min at room temperature. An equal volume of  $H_2SO_4$  (1 M) was added into each well to stop the reaction and the absorbance at 450 nm was monitored. Cells incubated in PBS instead of the primary antibody were used as the negative control.

The Bcl-2 ELISA procedure was also the same as that for Caspase-3 except that the primary antibody used was Bcl-2 antibodies.

### **Alteration of plasma membrane permeability determination**

FDA-staining is often used to determine the alteration of plasma membrane permeability (Zhou et al., 2010; Li et al., 2012b). Briefly, the QGY-7701 cells were cultured on 6-well plates ( $1 \times 10^6$  cells per well) with 0, 6, 12, and 24  $\mu$ M resveratrol for 24 h, and then cells were collected and washed twice with PBS. FDA was added to the cell suspensions at a final concentration of 20  $\mu$ g/mL and the cells were incubated at 37°C for 10 min. After incubation, the cells were centrifuged at 1000 g, the supernatant was removed, and the cells were finally resuspended in 0.5 mL PBS. The cell suspensions were transferred to a 96-well black plate, and then the fluorescence intensity (Ex: 485, Em: 538) was detected by fluorescent chemical analyzer (Thermo Scientific Fluoroskan Ascent FL). Each test was conducted in quadruplicate. The results were presented as NRFU (normalized relative fluorescence units) (U/cell).

### **Mitochondrial depolarization assay**

QGY-7701 cells were cultured for 6 h on 12-well plates ( $5 \times 10^5$  cells per well). The cells were exposed for 24 h to resveratrol at 0, 6, 12, and 24  $\mu$ M. The cells were stained with a JC-1 probe to measure mitochondrial membrane depolarization, according to the manufacturer's instructions. The treated cells were incubated with an equal volume of JC-1-staining solution at 37°C for 20 min and rinsed twice with PBS. The mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from both mitochondrial JC-1 monomers and aggregates using a fluorescence microscope (Nikon, Japan). Mitochondrial depolarization is expressed as an increase in the intensity ratio of green/red fluorescence.

## Detection of opened MPTPs

Opened MPTPs in the QGY-7701 cells were detected using an MPTP assay kit according to the manufacturer's instructions. The cell culture and resveratrol exposure were performed as when determining plasma membrane permeability. Cells were collected, rinsed twice with PBS, then washed with Reagent A from the kit, incubated in Reagents B and C of the kit (1:50) at 37°C for 20 min, and washed twice again with Reagent A. Fluorescence intensity of the cell solutions was measured by fluorescent chemical analyzer (Ex: 485, Em: 538). The results were presented as NRFU (U/cell).

## Cellular ATP level determination

ATP level was measured using an ATP assay kit according to the manufacturer's instructions. QGY-7701 cell culture and resveratrol treatment were performed as when determining plasma membrane permeability. After rinsing with PBS, the cells were schizolysed by solution and then centrifuged at 12,000 *g* for 5 min. The supernatant was collected. In a 1.5 mL tube, 100  $\mu$ L of each supernatant was mixed with 100  $\mu$ L ATP detection solution. Luminance (RLU) was immediately measured using a fluorescent chemical analyzer. Standard curves were also generated and the protein concentration of each treatment group was determined using the BCA protein assay kit. Total ATP levels are reported as nmol/mg protein (Chen et al., 2009).

## Statistical analysis

Each value is reported as means  $\pm$  standard deviation. Statistical analysis was performed using SPSS 13.0 for Windows. The data were analyzed using one-way ANOVA followed by a least significant difference determination. Differences were considered significant when the calculated *P* value was <0.05.

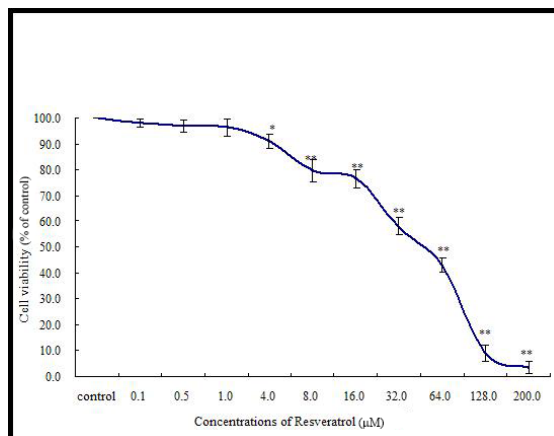
## RESULTS

### QGY-7701 cell viabilities

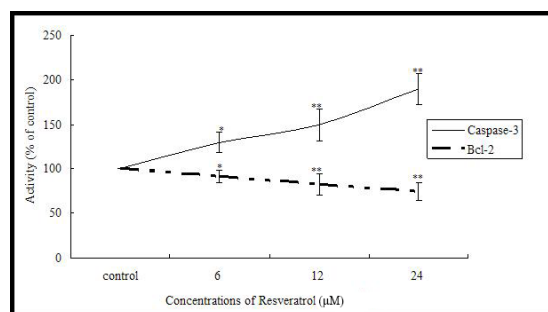
Viabilities of the QGY-7701 cells are shown in Figure 1. After 24-h treatment, differences between groups with lower concentrations of resveratrol (0.1, 0.5, 1  $\mu$ M) and the control groups were not significant (*P* > 0.05). However, when resveratrol concentrations were higher than 4  $\mu$ M, remarkable differences were observed (Figure 1). Furthermore, the resveratrol cytotoxicity on QGY-7701 cells presented in a dose-dependent pattern. The 24-h EC<sub>50</sub> was calculated to be around 48  $\mu$ M. Thus, resveratrol concentrations of 6, 12, and 24  $\mu$ M were adopted for further experiments.

### Resveratrol increased Caspase-3 activity and decreased Bcl-2 level

The activity of Caspase-3 and the level of Bcl-2 were examined to gain insight into the molecular signaling pathway of apoptosis. The results are displayed in Figure 2. Resveratrol treatment caused an increase in Caspase-3 activity and a decrease in Bcl-2 levels, indicating that resveratrol induces apoptosis in QGY-7701 cells.



**Figure 1.** Viabilities of QGY-7701 cells exposed to various concentrations of resveratrol. Data are reported as means  $\pm$  SD from three independent experiments. Asterisks denote responses that are significantly different from the control (\*P < 0.05, \*\*P < 0.01).



**Figure 2.** Caspase-3 activity and Bcl-2 level in QGY-7701 cells. Data are reported as means  $\pm$  SD from three independent experiments. Asterisks denote responses that are significantly different from the control (\*P < 0.05, \*\*P < 0.01).

### Plasma membrane permeability

Compared to the control group, the results of the FDA staining in all resveratrol-treated groups showed that an obvious fluorescein leakage was present (Figure 3), indicating that resveratrol exposure may increase membrane permeability in QGY-7701 cells.

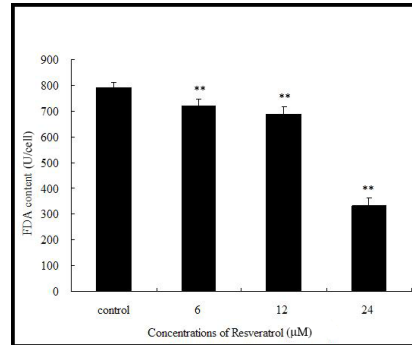
### Mitochondrial depolarization

As for the JC-1 staining, a significant increase in the green (low  $\Delta\Psi_m$ ) to red (high  $\Delta\Psi_m$ ) ratio was found in resveratrol-treated cells when compared to the control groups (Figure 4).

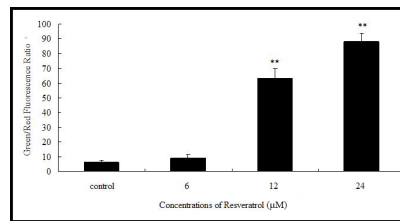
### Opening of MPTPs

The results of the MPTP assay showed a marked decrease in the fluorescence intensity

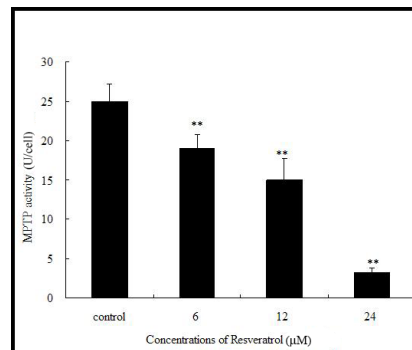
of resveratrol-untreated cells (Figure 5), which indicates that resveratrol may promote the opening of MPTPs in QGY-7701 cells.



**Figure 3.** Fluorescein diacetate (FDA) contents of QGY-7701 cells exposed to various concentrations of resveratrol. Data are reported as means  $\pm$  SD from three independent experiments. Asterisks denote responses that are significantly different from the control (\*\* $P < 0.01$ ).



**Figure 4.** Mitochondrial depolarization of QGY-7701 cells. Data are reported as means  $\pm$  SD from three independent experiments. Asterisks denote responses that are significantly different from the control (\*\* $P < 0.01$ ).

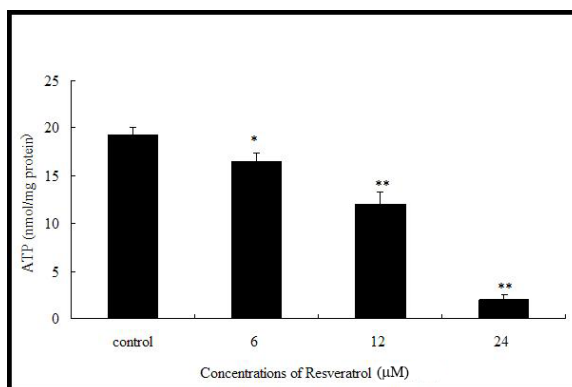


**Figure 5.** Opening of mitochondrial permeability transition pores (MPTPs) in QGY-7701 cells. Data are reported as means  $\pm$  SD from three independent experiments. Asterisks denote responses that are significantly different from the control (\*\* $P < 0.01$ ).

### Cellular ATP levels

After 24 h of resveratrol exposure, a significant decrease in cellular ATP levels was detected in all treatment groups (Figure 6). Cellular ATP was almost exhausted when the concentration

of resveratrol reached 24  $\mu\text{M}$ . These findings provide direct evidence that resveratrol induces mitochondrial dysfunction.



**Figure 6.** ATP levels of QGY-7701 cells. Data are reported as means  $\pm$  SD from three independent experiments. Asterisks denote responses that are significantly different from the control (\* $P < 0.05$ , \*\* $P < 0.01$ ).

## DISCUSSION

In recent years, resveratrol has attracted much attention in the field of tumor prevention. In the present study, an MTT assay revealed that after treatment with resveratrol at a concentration of 4  $\mu\text{M}$  or more, QGY-7701 viability decreases significantly. The cytotoxicity of resveratrol has been previously evaluated in different cell lines, such as U20S (human osteosarcoma cell line) (Liu et al., 2012), 786-0 (human renal cancer cell line) (Yang et al., 2011), and A431 (human epidermoid carcinoma cells) (Kim et al., 2006). The results of these studies indicate that resveratrol indeed has cytotoxicity.

Apoptosis, one of the major patterns of cell death, is characterized by the expression of apoptosis-associated genes (Fraser and Evan, 1996; Liu et al., 2013). Caspase-3, a member of the Caspase family, is an important upstream effector of apoptosis, whereas Bcl-2, the anti-apoptotic protein, is a downstream effector. Apoptosis can be easily detected via some cytological and biochemical alterations in treated cells. In the present study, Caspase-3 activity and Bcl-2 level assays revealed that resveratrol exposure induces typical apoptosis in QGY-7701 cells.

Mitochondrion has an inner and outer membrane separated by the intermembrane space. The inner membrane is the site of oxidative phosphorylation, which produces ATP. Mitochondrial permeability transition (MPT) can disrupt the permeability barrier of the inner membrane, thus dissipating the membrane potential and pH gradient that together drive ATP synthesis, causing the impairment of mitochondrial function (Li et al., 2012b). Thus, MPT induction is a catastrophic event that can lead to apoptosis (Eaton and Klaassen, 2001). According to previous studies, many types of chemicals such as methylmercury, silver nanoparticles, and ionic liquids can induce MPT and lead to mitochondrial dysfunction (Polunas et al., 2011; Teodoro et al., 2011; Li et al., 2012b). Our results reveal that resveratrol may promote the opening of MPTs and induce MPT in the treated QGY-7701 cells, causing mitochondrial depolarization and ATP depletion. Mitochondria are one of the most important organelles, and dysfunction leads to the mitochondrial apoptotic pathway.

Resveratrol is a non-flavone polyphenolic compound, so it can be easily adsorbed onto the surface of cells, act on biomembranes (Roberts and Costello, 2003), and increase cellular membrane permeability, as verified by the FDA assay in our study. Therefore, it can be hypothesized

that resveratrol might enter cytoplasm, act on the mitochondrial membrane, and initiate MPT, which induces mitochondrial depolarization and the sustained decrease of ATP. This ultimately further promotes MPT and results in the apoptosis of QGY-7701 cells.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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