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Original Article: Laboratory Investigation

Resveratrol induces apoptosis associated with mitochondrial dysfunction in bladder carcinoma cells

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Abbreviations & Acronyms
ATP = adenosine 5′-triphosphate
BBN = N-butyl-N-(4-hydroxybutyl nitrosamine)
cyt c = cytochrome c
DCFH-DA = 2′,7′-dichlorfluorescein-diacetate
DMSO = dimethylsulfoxide
ΔΨm = mitochondrial membrane potential
PBS = phosphate buffer saline
ROS = reactive oxygen species
TBST = Tris-buffered saline with Tween
TUNEL = transferase dUTP nick end labeling

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Objective: Resveratrol shows chemopreventive activity in a variety of human cancers by targeting mitochondria and triggering apoptosis. The purpose of this study was to investigate the antitumor action of resveratrol in bladder cancer and its underlying mechanism.

Methods: Using two different bladder cell lines, BTT739 and T24, the cytotoxicity of resveratrol were determined by MTT assay. The apoptosis induced by resveratrol was assayed by transferase dUTP nick end labeling staining. To show whether the mitochondrial dysfunction involved in the effects of resveratrol, mitochondrial function was detected by mitochondrial membrane potential, reactive oxygen species production and adenosine 5′-triphosphate content. In addition, the markers of apoptosis in the intrinsic mitochondrial-dependent pathway were analyzed by the release of cytochrome c and the activities of caspase-9 and caspase-3.

Results: Resveratrol effectively decreased cell viability and induced apoptosis in a concentration- and time-dependent manner. In addition, resveratrol significantly disrupted the mitochondrial membrane potential in both intact cells and isolated mitochondria. Resveratrol also increased reactive oxygen species production and reduced adenosine 5′-triphosphate concentrations. Western blot analysis showed that resveratrol provoked the release of cytochrome c from mitochondria to the cytosol. Furthermore, resveratrol significantly promoted the activation of caspase-9 and caspase-3.

Conclusions: These findings suggest that resveratrol efficiently triggers apoptosis in bladder cancer cells through the intrinsic mitochondrial-dependent pathway, which is associated with mitochondrial dysfunction. Resveratrol might have great pharmacological promise in the treatment of bladder cancer.

Key words: apoptosis, bladder carcinoma, mitochondrial dysfunction, resveratrol.

Introduction

Bladder cancer is the second most common tumor of the genitourinary tract. Approximately 80% of patients with primary bladder cancer superficial to mucosa are traditionally treated by transurethral resection. However, 50–80% of patients will have tumor recurrence after transurethral resection, one-third of which will progress to more invasive and metastatic cancers.[1,2] Currently, intravesical chemotherapy and immunotherapy with bacillus Calmette–Guérin are widely applied to reduce the recurrence and progression of superficial bladder cancer. Although efficacious, these approaches are usually associated with serious adverse effects and fail to significantly improve overall survival.[1,2] Therefore, there is an urgent need to explore drugs that are both effective and non-toxic for bladder carcinoma therapy.

Resveratrol, a natural grape-derived polyphenolic phytoalexin, possesses pleiotropic effects including anticancer, anti-aging, anti-inflammatory and anti-oxidant activities, as well as cardioprotection and neuroprotection.[3,4] On one hand, resveratrol effectively scavenges superoxide and peroxynitrite radicals generated from enzymatic and non-enzymatic systems, and affords protection against DNA damage caused by these ROS in normal cells.[5]

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On the other hand, mounting evidence indicates that resveratrol has cancer chemopreventive activity in a variety of cancers, both in animal models and in cell cultures.[6–13] The possible mechanisms underlying the anticancer activity of resveratrol include interference with many of the intracellular signaling pathways that regulate cell survival or apoptosis.[3] Resveratrol-mediated apoptosis has been associated with p53 activation in various human cancer cells, such as breast cancer cells.[14] Resveratrol might also show prooxidant properties, catalyzing cellular DNA degradation in the presence of transition metal ions, such as copper.[15] Elevated copper levels occur in hepatocellular carcinoma cells as compared with normal cells, and copper metabolism is upregulated in many other tumors.[16] Therefore, these cancer cells might be more susceptible to ROS generation by resveratrol. In addition, resveratrol has been shown to modulate the major cell cycle mediators at micromolar concentrations arresting cancer cells at the G1/S phase of the cell cycle.[17] Furthermore, resveratrol was reported to enhance the efficacy of anticancer drugs in inducing apoptosis and to sensitize tumor cells to death receptor-mediated apoptosis.[18,19] Despite these findings, little work has been carried out to explore the effects of resveratrol on bladder carcinoma.

Mitochondria are regarded as important targets for cancer therapy, including bladder cancer therapy.[20–23] Mitochondria mediate resveratrol-induced apoptosis in many tumor cells in both *in vivo* and *in vitro* studies.[3,6–13] Resveratrol is able to modulate the mitochondrial permeability transition pore,[10] increase mitochondrial superoxide production,[24] disrupt the mitochondrial membrane potential,[6,8,13] disturb the mitochondrial respiratory chain[25,26] and interact with Bcl-2 family proteins in cancer cells.[9] Thus, resveratrol likely triggers apoptosis by promoting both the release of cyt c and Smac/Diablo from mitochondria, and the activation of caspase-9 and caspase-3.[21] Here, we sought to detect the antitumor effects of resveratrol in bladder carcinoma cells and to determine the underlying mechanism of its activity, which involves apoptosis associated with mitochondrial dysfunction.

Methods

Cell culture and treatment

The mouse bladder cancer cell line, BTT739, and the human bladder cancer cell line, T24, were used in these experiments. The BTT739 mouse bladder transitional carcinoma cell line was provided by Professor Wensen Wu (Pathology Department, Shanghai Hospital, China). The BTT739 cells were induced by BBN and cultured as previously described.[27] The human T24 bladder cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The T24 cells were cultured in McCoy's medium and modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum (HyClone, Logan, UT, USA) and 50 mg/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA). Both cell lines were maintained at 37°C and 5% CO_2. At 80% confluency, the cells were treated with increasing concentrations of resveratrol (Sigma) (12.5 μmol/L, 25 μmol/L, 50 μmol/L or 100 μmol/L) for 24 h or 50 μmol/L resveratrol for various periods (0 h, 6 h, 12 h, 24 h or 48 h).

Cell viability assay

The effect of resveratrol on the cell viability of bladder cancer cells was evaluated *in vitro* using the MTT assay (Sigma). Briefly, 1×10^5 cells were seeded into 96-well plates. After treatment with resveratrol, 5 mg/mL MTT (15 μL/well) was added to the cells. A volume of 20 μL of 10 mmol/L PBS was added to the control cells. Cells were cultured for 4 h at 37°C. Then the culture medium was removed, and 150 μL of DMSO was added to each well. The plates were shaken on a swing bed for 10 min, and the optical density at 492 nm was detected using an enzyme-linked immunosorbent assay plate reader at 490 nm and compared with that of the untreated controls.

Apoptosis detection

Apoptotic bladder cancer cells were identified using TUNEL staining (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, cells were fixed on glass slides at a density of 3×10^5 cells/mL. After treatment with resveratrol, cells were washed with NaCl/Pi and fixed with 4% formaldehyde. Apoptotic cells (TUNEL-positive) were identified by 3,3′-diaminobenzidine staining and quantified using a fluorescence microscope (TCS SP2, Leica, Solms, Germany) equipped with a CCD camera. The experiments were repeated three times with two cover slips for each condition, and 600–800 nuclei were counted for each cover slip. The percentage of apoptotic cells was obtained by dividing the number of TUNEL-positive cells by the total number of cells in a defined area.

Measurement of $\Delta\Psi m$

The effect of resveratrol on mitochondrial membrane potential was determined in both intact cells and isolated mitochondria using the JC-1 probe (Invitrogen Corp, Carlsbad, CA, USA). For intact cells, 1×10^6 bladder cancer cells were trypsinized, resuspended and stained with JC-1 at 37°C for 15 min. The fluorescence intensity of the JC-1 aggregate (red) and the JC-1 monomer (green) was analyzed with a flow cytometer using emission wavelengths at 590 nm and 530 nm. Each experiment was repeated four times. To isolating mitochondria, $5–6 \times 10^7$ treated cells were lysed for

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isolating mitochondria.[28] After treatment with resveratrol, the isolated mitochondria were stained with JC-1 at 37°C for 15 min. Because no cytosolic compartment was presented, red fluorescence was measured on a spectrofluorometer at 590 nm to reflect the changes in ΔΨm induced by resveratrol.[13]

Determination of ROS levels

The levels of ROS were monitored by DCFH-DA (Sigma). Bladder cancer cells (1×10^5) were seeded into 96-well plates. After treatment with resveratrol, the cells were cultured with DCFH-DA at 37°C for 30 min. Fluorescence was generated as a result of the reaction of DCFH with ROS to form the fluorescent product, DCF, which was trapped inside the cells. Values are given as the mean fluorescence intensity and were determined at 485 nm using a fluorescence microplate reader.

Determination of ATP concentration

ATP concentrations were measured using the ATP Determination Kit (Invitrogen Corp) following the instructions of the manufacturer.

Western blot analysis

A total of 30 mL of protein from the cytosolic and mitochondrial fractions were applied to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After protein transfer, nitrocellulose membranes were blocked in 5% (w/v) non-fat dry milk in TBST. Next, the membranes were incubated overnight at 4°C with the primary antibodies rabbit anti-cytochrome c (Abcam, Cambridge, UK) and mouse anti-β-actin (Santa Cruz Technologies, Santa Cruz, CA, USA). After washing at least three times in TBST, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase at 37°C for 1 h. Blots were developed using Biomax-Light films and an enhanced chemiluminescence system.

Caspase activity assay

The activities of caspase-9 and caspase-3 were determined according to the manufacturer's instructions (Beyotime Company, Shanghai, China).

Statistical analysis

The results are presented as the means ± SE. Each experiment was carried out at least three times. Statistically significant differences between two groups were determined using a two-way analysis of variance (ANOVA). All *P*-values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Resveratrol induced cytotoxicity and apoptosis in bladder cancer cells

Figure 1a and b show that resveratrol efficiently decreased the cell viability of BTT739 and T24 cells in a concentration- and time-dependent manner. At the highest concentration of resveratrol (100 μmol/L), 24-h treatment decreased cell viability by approximately 70% in the two bladder cancer cell lines (Fig. 1a). Treatment with 50 μmol/L resveratrol for 48 h resulted in a 73% and 66% reduction in the cell viability of BTT739 and T24 cells, respectively (Fig. 1b). TUNEL staining showed that the percentage of apoptotic BTT739 and T24 cells treated with 100 μmol/L resveratrol for 24 h markedly increased up to 30% and 25%, respectively (Fig. 1c). The time course analysis showed that treatment with 50 μmo/L resveratrol for 48 h led to significant cell death as a result of apoptosis, 35% in BTT739 cells and 31% in T24 cells (Fig. 1d).

Resveratrol disrupted the mitochondrial membrane potential in bladder cancer cells

As shown in Figure 2a, treatment with increasing concentrations of resveratrol, ranging from 25 μmol/L to 100 μmol/L, for 24 h led to a gradual increase in a cell population characterized by a depolarized ΔΨm, which was shown by an increase in green fluorescence and a decrease in red fluorescence. In fresh isolated mitochondria, we found that the ΔΨm significantly decreased in a dose-dependent manner. Treatment with 100 μmol/L resveratrol led to a 69% and 66% reduction in red fluorescence intensity in BTT739 cells and T24 cells, respectively (Fig. 2b).

Resveratrol increased ROS production in bladder cancer cells

As Figure 3 shows, treatment with increasing concentrations of resveratrol (12.5 μmol/L, 25 μmol/L, 50 μmol/L or 100 μmol/L) for 24 h obviously increased ROS production up to 1.5-, 1.8-, 2.3- and 2.8-fold of that in BTT739 control cells, respectively. Similar results were obtained in T24 cells.

Resveratrol reduced the ATP concentration in bladder cancer cells

Treatment with increasing concentrations of resveratrol (25 μmol/L, 50 μmol/L or 100 μmol/L) for 24 h signifi-

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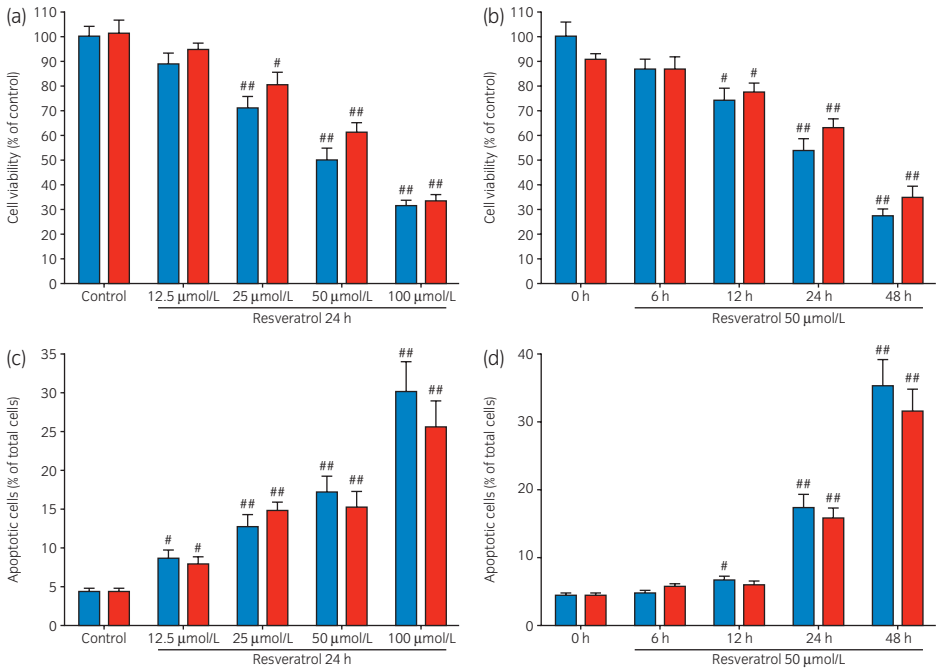

Fig. 1 Resveratrol induced cytotoxicity and apoptosis in bladder cancer cells. After treatment with resveratrol (12.5 μM, 25 μM, 50 μM, or 100 μM) for 24 h or 50 μM resveratrol for various periods (0 h, 6 h, 12 h, 24 h, or 48 h), the bladder cancer cell lines BTT739 and T24 showed an increase in cytotoxicity and apoptosis. The cytotoxicity of resveratrol presented in a concentration-dependent manner (a) and a time-dependent manner (b) based on the MTT assay. The results are expressed as a percentage of the control, which is set at 100%. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group. Values are means ± SE, $n = 4$. Resveratrol-induced apoptosis evaluated by TUNEL staining was also concentration-dependent (c) and time-dependent (d). Apoptotic cells were identified by 3,3′-diaminobenzidine staining and quantified using a fluorescence microscope equipped with a CCD camera. Data are presented as the percentage of apoptotic cells relative to the total number of cells in a defined area (10 mm^2). $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group. Values are means ± SE. (■) BTT739; (■) T24.

cantly reduced the ATP concentration by 26%, 38% and 62% compared with that of BTT739 control cells, respectively. Consistent with this result, the ATP concentration in similarly treated T24 cells also presented a significant reduction (Fig. 4).

Resveratrol promoted the mitochondrial release of cyt c in bladder cancer cells

Western blot analysis showed that cyt c gradually accumulated in the cytosol, whereas the protein levels of cyt c in the mitochondria progressively decreased after treatment with various concentrations of resveratrol (12.5 μmol/L, 25 μmol/L, 50 μmol/L or 100 μmol/L). Similar results were observed for T24 cells, as shown in Figure 5b. These results show that resveratrol induced apoptosis in bladder cells through mitochondria-dependent signaling pathways.

Resveratrol activated caspase-9 and caspase-3 in bladder cancer cells

As shown in Figure 6a, treatment with increasing concentrations of resveratrol (25 μmol/L, 50 μmol/L or 100 μmol/L) for 24 h obviously promoted the activation of caspase-9 in bladder cancer cells. At the highest concentration, resveratrol (100 μmol/L) increased the activity of caspase-9 up to 2.2-fold and 1.9-fold of that in BTT739 control cells and T24 control cells, respectively (Fig. 6a). Treatment with resveratrol also increased the activity of caspase-3 in bladder cancer cells (Fig. 6b). As anticipated, these results support the hypothesis that resveratrol triggers apoptosis through the intrinsic mitochondrial-dependent pathway, after mitochondrial dysfunction and cyt c release.

Discussion

Mitochondria play a central role in the intrinsic apoptotic pathway. The unique structural and functional characteristics of mitochondria enable the selective targeting of drugs designed to modulate the function of this organelle for cancer therapy.[21,22] In the present study, we provide evidence that resveratrol administration efficiently induced apoptosis in two bladder cancer cell lines. The chemotherapeutic effects of resveratrol are accompanied by effects on mitochondria, including a significant disruption of the mitochondrial membrane potential both in intact cells and isolated

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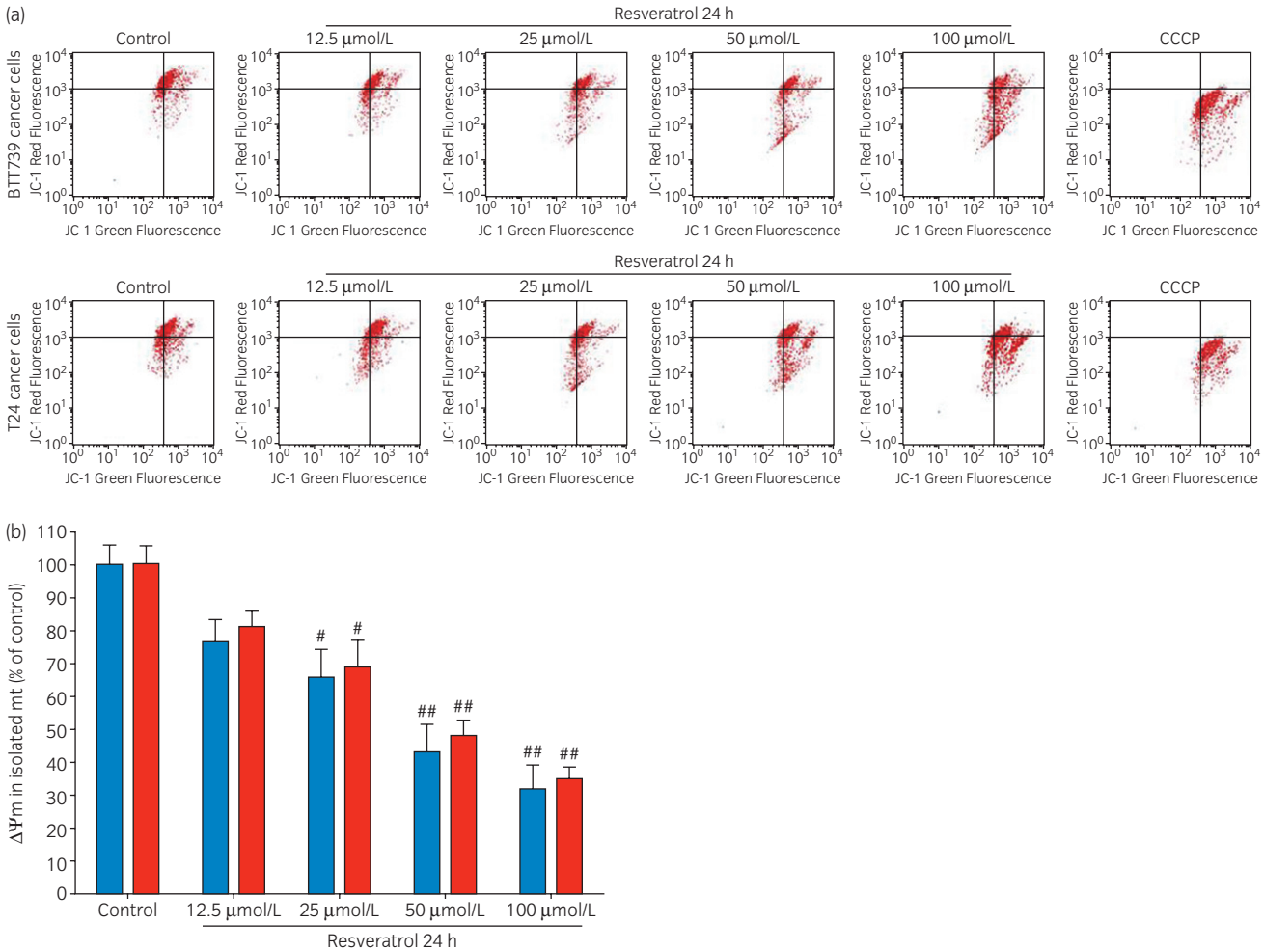

Fig. 2 Resveratrol disrupted the mitochondrial membrane potential in bladder cancer cells. JC-1 was used to detect the effect of resveratrol on the ΔΨm in bladder cancer cells. (a) After treatment with resveratrol (12.5 μM, 25 μM, 50 μM, or 100 μM) for 24 h, intact BTT739 and T24 bladder cancer cells were trypsinized, centrifuged, and resuspended in preheated culture medium. The treated cells were incubated in JC-1 in growth medium at 37°C for 15 min. The changes in ΔΨm were then analyzed by flow cytometry. Cells with depolarized mitochondria were established by treatment with 50 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 37°C for 5 min and were used as a positive control. Each experiment was repeated three times. The data are representative of three experiments. (b) Fresh mitochondria isolated from BTT739 and T24 bladder cancer cells were treated with resveratrol (12.5 μM, 25 μM, 50 μM, or 100 μM) for 1 h. Then the treated mitochondria were incubated with JC-1 at 37°C for 15 min. Red fluorescence was measured with a spectrofluorometer at 590 nm to reflect the changes in ΔΨm induced by resveratrol. The results are expressed as a percentage of the control, which is set at 100%. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group. Values are means ± SE, $n = 4$. (■) BTT739; (■) T24.

mitochondria, an elevation in ROS production and a reduction in ATP concentration. These results suggest that resveratrol has great pharmacological potential for the treatment of bladder cancer by inducing apoptosis mediated by mitochondrial dysfunction. To our knowledge, the present study is the first to explore the chemotherapeutic effects of resveratrol in bladder cancer cells and its underlying mechanism.

Previous reports show that loss of mitochondrial membrane polarization is an early event in resveratrol-induced apoptosis.[6,9,13] Consistent with this observation, the present study showed that resveratrol treatment obviously dissipated the ΔΨm in intact bladder cancer cells. Compared with intact cells, treatment with lower concentrations of resveratrol (12.5 μmol/L) for 1 h resulted in a rapid loss of membrane potential in isolated mitochondria, suggesting that resveratrol induced apoptosis by directly targeting the mitochondria. The disruption of mitochondrial membrane depolarization appears to be at least partially the result of the ability of resveratrol to interact with mitochondrial components,[3,21,22] such as F1 complex of the F0/F1 ATPase proton pump[21] and anti-apoptotic Bcl-2 proteins in a variety of

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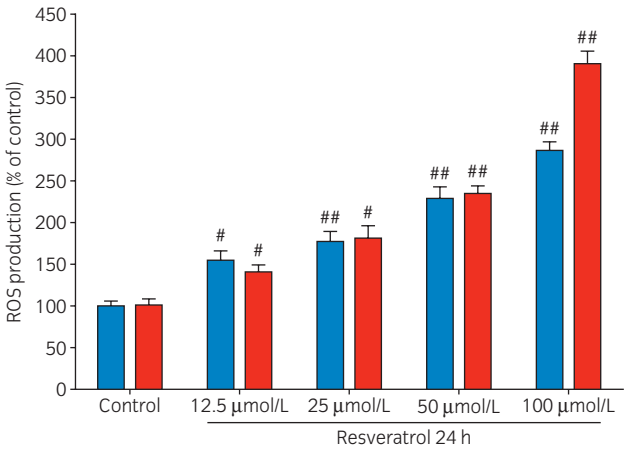

Fig. 3 Resveratrol increased ROS production in bladder cancer cells. After treatment with resveratrol (12.5 μM, 25 μM, 50 μM, or 100 μM) for 24 h, the bladder cancer cells BTT739 and T24 were incubated with DCFH-DA at 37°C for 20 min. Fluorescence was generated due to the reaction of DCFH with ROS to form the fluorescent product DCF, which was trapped inside the cells. Values are given as the mean fluorescence intensity, obtained at 485 nm using a fluorescence microplate reader. Cellular fluorescence intensity is expressed in multiples of that of the control groups. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group. Values are means ± SE, $n = 4$. (▬) BTT739; (▬) T24.

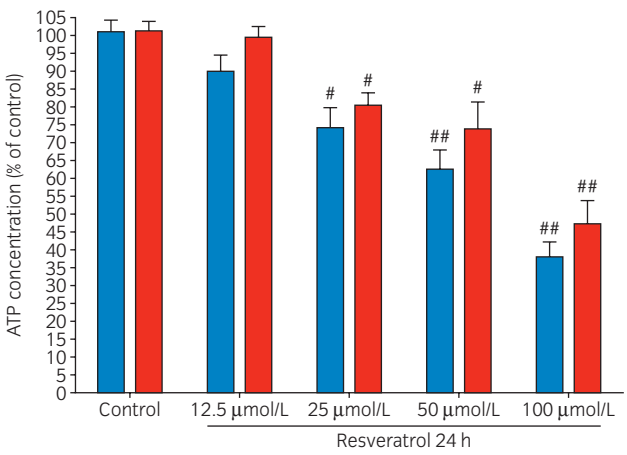

Fig. 4 Resveratrol reduced the ATP concentration in bladder cancer cells. After treatment with resveratrol (12.5 μM, 25 μM, 50 μM or 100 μM) for 24 h, the bladder cancer cells BTT739 and T24 were lysed in RIPA buffer and diluted 1:10 in ATP Determination Kit reaction buffer. The ATP concentration was determined using the ATP Determination Kit. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group. The results are expressed as a percentage of the control, which is set at 100%. Values are means ± SE, $n = 4$. (▬) BTT739; (▬) T24.

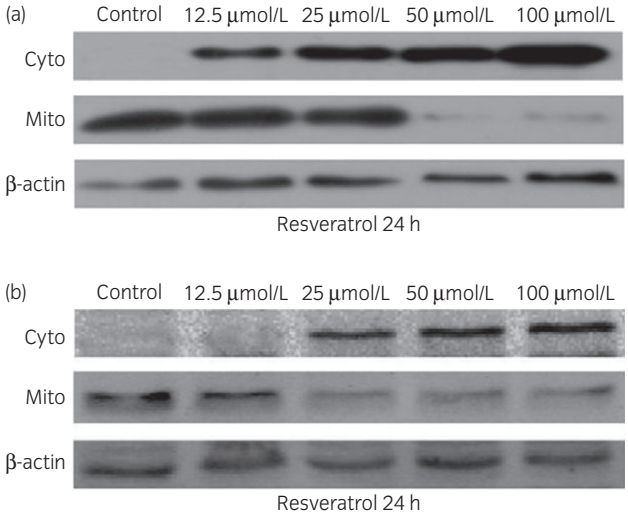

Fig. 5 Resveratrol promoted the mitochondrial release of cytochrome c in bladder cancer cells. After treatment with resveratrol (12.5 μM, 25 μM, 50 μM, or 100 μM) for 24 h, the bladder cancer cells BTT739 and T24 were separated into cytosolic and mitochondrial fractions. Thirty milligrams of protein from the cytosolic and mitochondrial fractions were applied to western blot analysis as described in Materials and Methods. Blots were developed using Biomax-Light films and an enhanced chemiluminescence system. The data represent three experiments.

cancers.[9,11,24] Disruption of the ΔΨm usually accompanies or initiates apoptosis. In line with this fact, we found that the disruption of the ΔΨm by resveratrol seems to precede the release of cyt c from mitochondria, followed by the activation of caspase-9 and caspase-3. This result suggests that resveratrol promoted cell death through the mitochondrial intrinsic apoptosis pathway in early-stage bladder carcinoma cells, supporting previous findings on other tumor cell lines treated with resveratrol.[6–8,13]

ROS are thought to play multiple roles in tumor initiation, progression and maintenance. However, excess ROS also activate and modulate apoptosis in tumor cells, especially under conditions of oxidative stress in mitochondria.[29] In addition to the disruption of the ΔΨm, we found that resveratrol markedly increased ROS production in bladder carcinoma cells. The excessive ROS within mitochondria might further induce oxidative modification to mitochondrial membrane lipids and change the permeability of the mitochondrial outer membrane, aggravating the disruption of the ΔΨm.[30] A model has been proposed in which an early transient ΔΨm breakdown causes mitochondrial generation of ROS in tumor cells, which then further induces an ongoing loss of ΔΨm in a positive feedback loop.[9] However, it seems paradoxical that as an anti-oxidant, resveratrol could enhance ROS overproduction and induce oxidative stress. In fact, resveratrol is a polyphenol, a compound that can work

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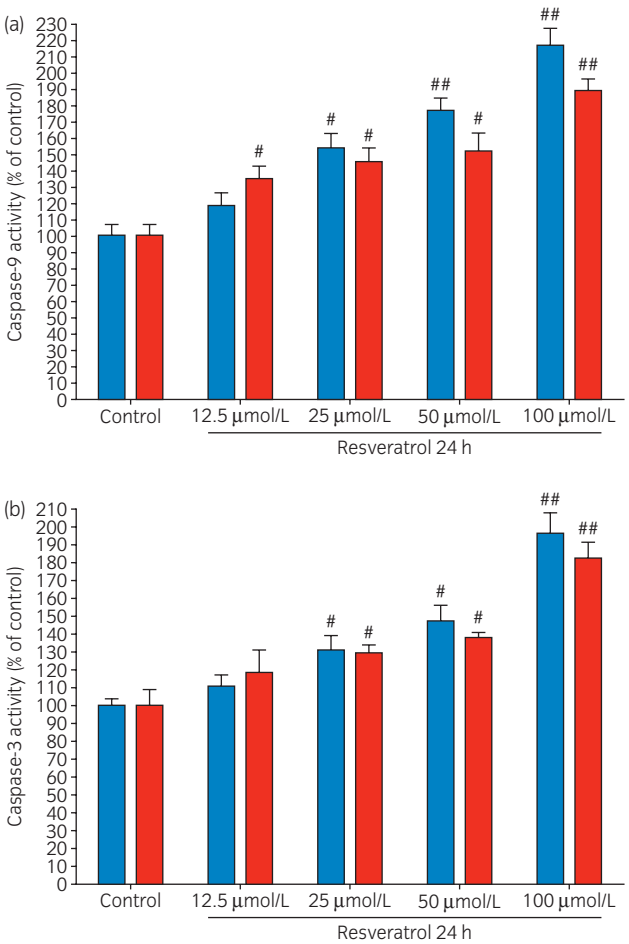

Fig. 6 Resveratrol activated caspase-9 and caspase-3 in bladder cancer cells. After treatment with resveratrol (12.5 μM, 25 μM, 50 μM, or 100 μM) for 24 h, the bladder cancer cells BTT739 and T24 were lysed and incubated at 37°C for 1 h with caspase-9 substrate (Ac-LEHD-pNA) and caspase-3 substrate (Ac-DEVD-pNA), respectively. The activities of caspase-9 and caspase-3 were monitored by measuring the relative fluorescence intensity at 405 nm. The results are expressed as a percentage of the control, which is set at 100%. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group. Values are means ± SE, $n = 4$. (■) BTT739; (■) T24.

either as an inhibitor or enhancer of oxidative and radical chain processes, depending on the concentration of the polyphenol, the redox potential of the polyphenol, the abundance of metal ions, the pH and the subcellular compartment.[31] Recent studies confirmed that a high dose of resveratrol induces excessive ROS generation, and the chemotherapeutic effects of resveratrol were found to be a result of its activity as a pro-oxidant rather than as an anti-oxidant.[9,11,12,24]

Furthermore, the present study found that resveratrol obviously reduced the ATP concentration in bladder carcinoma cells. Considering the high metabolic rate of cancer cells, ATP depletion might impair downstream ATP-dependent processes, leading to cell death.[32] Previous studies showed that resveratrol inhibits mitochondrial proton F0F1-ATPase/ATP synthase in a concentration-dependent manner both in the rat brain and liver.[25] In addition, resveratrol decreases mitochondrial complex III activity by competing with coenzyme Q.[26] Thus, mitochondrial ATP synthesis might be blocked by resveratrol through a disruption of the mitochondrial respiratory chain. However, compared with non-malignant cells, tumor cells, including bladder cancer cells, depend more on glycolysis for ATP production, suggesting that resveratrol might also disturb bladder cancer cell metabolism at the level of glycolysis. This possibility is supported by reports that resveratrol inhibits glycolysis or increases the rate limiting steps in glycolysis through the PI3K/Akt/mTOR signaling pathway in human cancer cells.[33,34]

In summary, the present study showed that resveratrol displays effective chemotherapeutic effects by triggering apoptosis in bladder cancer cells by promoting mitochondrial dysfunction. Combined with its wide range of biological activities, resveratrol has great pharmacological promise not only to prevent and treat the initiation, promotion and progression of bladder cancer,[3–5] but also to enhance the efficacy of anticancer drugs and sensitize tumor cells to chemotherapeutic agents and radiation therapy.[18,19] Future studies must be carried out to explore the effects of resveratrol on bladder cancer *in vivo*.

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Conflict of interest

None declared.

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