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Juglone-induced apoptosis in human gastric cancer SGC-7901 cells via the mitochondrial pathway

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ABSTRACT

This study was designed to investigate the effect of juglone on the apoptosis of human gastric cancer SGC-7901 cells. The cytotoxic activity of juglone on SGC-7901 cells was tested by the sulforhodamine B (SRB) assay. The morphological changes in the cells were observed by transmission electron microscopy (TEM). The apoptotic rate, the level of reactive oxygen species (ROS), mitochondrial transmembrane potential and the expression of cytochrome c protein were detected by flow cytometry (FCM). The expression of Bcl-2 and Bax proteins were examined by Western blot. Caspase 3 activity was determined with a microplate reader. Our results were as follows: the GI_{50} values for SGC-7901 cells were 36.51 ± 1.05 μmol/L (24 h) and 25.37 ± 1.19 μmol/L (48 h). After 24 h of exposure to juglone (5, 10, 15 and 20 μmol/L), the cells presented the typical morphological changes of apoptosis, and the rate of apoptosis was found to increase in a dose-dependent manner. After cells were treated with juglone at the same dose for 24 h, the level of ROS was significantly higher, the expression of Bcl-2 was significantly down-regulated and the expression of Bax was significantly up-regulated compared to the control. The mitochondrial transmembrane potential was significantly lower, and the expression of the cytochrome c protein was significantly higher relative to the control. Caspase 3 was activated in a concentration-dependent manner. In conclusion, juglone can induce apoptosis in SGC-7901 cells through a mitochondrial pathway that seems to be mediated by the generation of ROS and a reduction in the Bcl-2/Bax ratio.

Introduction

Gastric cancer is one of the most common malignancies in the world. The highest incidence rates occur in Eastern Asia (China, Japan, Republic of Korea, Democratic Republic of Korea and Mongolia), where the rates are 46 per 100,000 males and 21 per 100,000 females (Ferlay et al., 2004). Although the surgical treatment of gastric cancer is currently the main therapy, chemotherapy still plays an important role in comprehensive therapy. The therapeutic effect of chemotherapy drugs is limited, however, due to their adverse reactions and the resistance of tumor cells to chemotherapeutic agents. In the last few decades, natural products have become an increasingly important source of potential anticancer agents (Gatti and Perego, 2009; Johnson et al., 2008).

Juglone (5-hydroxy-1,4-naphtha-quinone) is a naturally-occurring naphthoquinone found in the roots, leaves, nut-hulls, bark and wood of Manchurian walnut (*Juglands mandshurica*), black walnut (*Junglans nigra*), walnut (*Junglans regia*) and butternut (*Junglans cinerea*) (Funt and Martin, 1993) trees. The bark, branches and exocarp of the immature green fruit of these medicinal plants have been used to treat gastric cancer, liver cancer, lung cancer and other types of cancer for a long time in China (Liu et al., 2004). Juglone has been reported to inhibit intestinal carcinogenesis induced by azoxymethane in rats and might be a promising chemopreventive agent in human intestinal neoplasia (Sugie et al., 1998). Juglone was also proven to be a potent cytotoxic agent *in vitro* in human tumor cell lines, including human colon carcinoma (HCT-15) cells, human leukemia (HL-60) cells and doxorubicin-resistant human leukemia (HL-60R) cells (Kamei et al., 1998; Segura-Aguilar et al., 1992). Our previous results suggested that juglone can inhibit the growth and induce apoptosis of sarcoma 180 cells *in vivo*. We observed the typical morphological changes that occur during apoptosis of sarcoma 180 cells by transmission electron microscopy (TEM) and detected the apoptosis rate by flow cytometry (FCM) to be $10.27 \pm 1.05\%$ in a group treated with 8 μmol/kg of juglone

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(Ji et al., 2008b). The results from our *in vivo* work indicate that the mechanism of juglone's antitumor effect in human tumor cell lines is worth further study. In this work, we studied the growth inhibition and induction of apoptosis by juglone in human gastric cancer SGC-7901 cells and demonstrate that the antitumor effect is associated with the generation of reactive oxygen species (ROS), expression of Bcl-2 and Bax protein, mitochondrial depolarization, release of cytochrome c into the cytosol and activation of the caspase-3 cascade.

Materials and methods

Cell lines and cell culture

Human gastric cancer SGC-7901 cells were purchased from the Institute for Cancer Research, Heilongjiang Cancer Hospital. SGC-7901 cells of a suitable concentration were inoculated in culture flasks containing RPMI-1640 solution with 10% of fetal bovine serum. The cultures were then incubated at 37 °C, with 5% CO_2 and saturated humidity; culture transfer was performed once every 2–3 days.

Test drugs and chemical reagents

Juglone with a purity of 97% was purchased from Sigma-Aldrich Corporation; Hydroxycamptothecine Injection (HCPT, 20070430) was purchased from Harbin Shengtai Pharmaceutical Co., Ltd. RPMI-1640 culture medium (Invitrogen Corporation); fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.); pancreatin (Gibco); Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology); propidium iodide (PI, Sigma); Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology); Mouse anti-human Bcl-2 Antibody (Santa Cruz Biotechnology); Mouse anti-human Bax Antibody (Santa Cruz Biotechnology); AP-labeled Horse Anti-Mouse IgG(H+L) (Zhongshan Golden Bridge BioTechnology Co., Ltd.); BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology); Mouse anti-β-Actin Antibody (Santa Cruz Biotechnology); Coomassie brilliant blue G-250(Tianjin Resent Chemicals CO., Ltd.); Cell lysis buffer for Western and IP (Beyotime Institute of Biotechnology); Rhodamine 123 (Sigma); Triton X-100 (Watson Biotechnologies, Inc., Shanghai); bovine serum albumin (BSA, Sigma); Mouse Anti-Human cytochrome C (Santa Cruz Biotechnology); FITC-Goat Anti-Mouse IgG (Wuhan Boster Biological Technology Co., Ltd.); Caspase 3 Activity Assay Kit (Beyotime Institute of Biotechnology).

Detection of the cytotoxic effect of juglone in SGC-7901 cells with the sulforhodamine B (SRB) assay

The cytotoxic effect of juglone in SGC-7901 cells was detected with the SRB assay. SGC-7901 cells in logarithmic growth phase were digested with 0.25% pancreatin diluted with RPMI-1640 culture medium containing 10% fetal bovine serum; the cell concentration was adjusted to 1×10^5 cells/mL. Cells were plated at 100 μL/well in 96-well plates and incubated. Juglone and HCPT (positive control drug) were dissolved in ethanol and then diluted with the medium; the final ethanol content was no more than 2%. After the cells had been incubated for 24 h, juglone was added to the wells in a volume of 100 μL a final concentration of 6.25, 12.5, 25, 50 or 100 μmol/L. HCPT was also added to the wells in a volume of 100 μL to a final concentration of 0.2, 2, 20 or 200 μmol/L. The same volume of medium was added to the wells in a control group. After the cells were further incubated for 24 or 48 h, the medium was discarded and replaced with ice-cold 10% Trichloroacetic Acid (TCA), and the cells were kept for 1 h at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionized water. After 10 min of air drying, 0.4% SRB was added, and the cells were allowed to stain for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. The plates were then air-dried. Bound stain was dissolved by adding 200 μL of 10 mM Tris base to each well. The 96-well plates were shaken on a mini vibrator for 5 min, and the optical density of each plate was read at 490 nm with a microplate reader. Each experiment was repeated three times. Cytotoxicity was evaluated from the cell-growth inhibition in the treated cultures and untreated controls. GI_{50} (concentration that causes 50% growth inhibition) and TGI (concentration that causes total growth inhibition) were determined by linear regression analysis.

Observation of the ultrastructure of SGC-7901 cells using TEM

The changes in ultrastructure of the SGC-7901 cells were observed under TEM (Krysko et al., 2008). Briefly, 1×10^6 cells in the logarithmic growth phase were plated in culture flasks and allowed to attach overnight. After treating the cells with juglone (5, 10, 15 and 20 μmol/L), HCPT (60 μmol/L) or medium for 24 h, cells were fixed in suspension with 3% glutaraldehyde in 0.1 M PBS for 10 min at 4 °C and as a pellet for 2 h. Subsequently, the pellets were postfixed using 1% osmic acid in 0.1 M sodium cacodylate for 30 min at ambient temperature; they were then washed again in distilled water, stained *en bloc* with aqueous uranyl acetate, washed in distilled water, dehydrated in a graded series of ethanol and embedded in Epon812 resin. Ultra-thin sections were cut using an ultramicrotome, equipped with a diamond knife, and counterstained with lead citrate. Cells were examined under TEM (JEM-1220, Japan) operating at 60 kV.

Detection of apoptotic rate of SGC-7901 cells induced by juglone with FCM

The apoptotic rate of SGC-7901 cells was detected using FCM with the Annexin V-FITC/PI double labeling method (Brumatti et al., 2008). Briefly, SGC-7901 cells in logarithmic growth phase (3×10^5 cells/mL) were plated at 1 mL/well in 6-well plates and allowed to attach overnight. After 24 h, juglone was added to the wells in a volume of 1 mL per well to a final concentration of 5, 10, 15 or 20 μmol/L. HCPT was also added to the wells in a volume of 1 mL per well to a final concentration of 60 μmol/L in the positive control group. An equal volume of medium was added to the wells in control group. The dishes were incubated in 5% CO_2 at 37 °C for 24 h. Then, cells were harvested, washed with pre-chilled PBS (4 °C) and adjusted to 1×10^5 cells/mL. The cell suspension (100 μL) was centrifuged at 1000 × g for 5 min. After, the supernatant was discarded, the pellet was re-suspended gently in 195 μL Annexin V-FITC binding buffer and incubated with 5 μL Annexin V-FITC in the dark at ambient temperature for 10 min. Cells were then centrifuged at 1000 × g for 5 min, and the pellet was re-suspended in 190 μL binding buffer. Cells were then incubated with 10 μL PI solution on an ice bath in the dark. After filtration (300 apertures), the suspension of each group was analyzed by FCM (EPICS XL-MCL, Beckman Coulter, US). Each experiment was repeated three times.

Determination of ROS generation in SGC-7901 cells using FCM

Levels of ROS in cells of the control and treatment groups were determined by staining cells with DCFDA. DCFDA is cell

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permeable; it is cleaved by nonspecific esterases and oxidized by peroxides in the cells to form fluorescent DCF. The intensity of DCF fluorescence is proportional to the amount of peroxide produced in the cells (Das et al., 2008). Briefly, SGC-7901 cells were plated in 6-well plates (3×10^5 cells/well) and allowed to attach overnight. After treating the cells with juglone (5, 10, 15 or 20 μmol/L) or medium for 24 h, cells were further incubated with 10 μmol/L DCFDA at 37 °C for 20 min. In the positive control group, 3×10^5 cells labeled with DCFH-DA were treated with 1 μL Rosup for 20 min. Subsequently, cells were removed, washed, re-suspended in PBS, filtered with 300 apertures and analyzed for DCF fluorescence by FCM. Approximately 10,000 cells were evaluated in each sample. Each experiment was repeated three times.

Determination of Bcl-2 and Bax protein expression in SGC-7901 cells using Western blots

SGC-7901 cells were plated in culture flasks (1×10^6 cells/flask) and allowed to attach overnight. After treating the cells with juglone (5, 10, 15 or 20 μmol/L), HCPT (60 μmol/L) or medium for 24 h, cells were harvested, removed to a 1.5 mL centrifuge tube and centrifuged at 1500 rpm for 5 min, after which the sample was rinsed twice with PBS. Ice-cold cell lysis buffer was added (100 μL) to the sample for 30 min in an iced water bath. Lysates were centrifuged (25,600 × g, 10 min at 4 °C). The supernatant fluids were collected and stored at −20 °C until assayed. Electrophoresis was performed using a Bio-Rad mini-Protean 3 apparatus. An equal amount of protein for each sample was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred onto nitrocellulose membrane (0.22 μm, Sigma) for 2 h at 250 mA. Membranes were incubated in a blocking buffer (5% nonfat dry milk in TBST) for 2 h at room temperature, and then the blots were incubated with a mouse anti-human Bcl-2 antibody (1:200) or mouse anti-human Bax antibody (1:200) overnight at 4 °C. The membranes were rinsed twice, 10 min each time, with TBST and then rinsed once for 10 min with TBS at room temperature. Then, the anti-murine IgG antibody labeled with alkaline phosphatase (1:500) was added. The membrane was taken out after 2 h, rinsed twice with TBST, 10 min each time, and then rinsed once with TBS for 10 min. Coloration was conducted after 3.3 μL of BCIP and 6.6 μL of NBP were mixed with 1 mL of alkaline phosphatase buffer; photographs were taken using the gel imaging system (GIS-2019, Tannon, Shanghai, China), and the protein content was quantified. The protein content of the control was taken to be 100%; the protein content in the remaining samples was calculated relative to the control. These experiments were performed three times independently.

Detection of changes in mitochondrial transmembrane potential in SGC-7901 cells using FCM

Rhodamine 123 was used to evaluate perturbations in mitochondrial transmembrane potential ($\Delta\Psi_m$). SGC-7901 cells were plated in 6-well plates (3×10^5 cells/well) and allowed to attach overnight. After treating the cells with juglone (5, 10, 15 or 20 μmol/L), HCPT (60 μmol/L) or medium for 24 h, cells were collected in a 10 mL centrifuge tube and re-suspended in 500 μL PBS; then, 500 μL of 20 μg/mL of Rhodamine 123 was gently added to the tube, so that the final concentration was 10 μg/mL. Cells were then incubated for 30 min in the dark. Cells were centrifuged at 1500 rpm for 5 min; the supernatant was removed; pellets were gently rinsed once with PBS and then re-suspended in 800 μL PBS. After filtration (300 apertures), the suspension was analyzed by FCM. Each experiment was repeated three times.

Determination of expression of the cytochrome c protein in SGC-7901 cells using FCM

The expression of the cytochrome c protein in cells was determined using FCM (Bouchier-Hayes et al., 2008). Briefly, SGC-7901 cells were plated in culture flasks (1×10^6 cells/flask) and allowed to attach overnight. After treating the cell with juglone (5, 10, 15 or 20 μmol/L), HCPT (60 μmol/L) or medium for 24 h, cells were collected and fixed in 4% (w/v) paraformaldehyde for 40 min. Then, cells were centrifuged and rinsed twice with PBS. After permeabilization with 2 mL of 0.1% Triton X-100 for 15 min, the samples were centrifuged and rinsed twice with PBS. Then, 1 mL of 1% BSA was added to the centrifuge tube for 1 h; samples were centrifuged, and the supernatant was discarded. Mouse anti-cytochrome c antibody (1:200) was added to the samples, which were incubated at 37 °C for 1 h. After the samples were centrifuged and rinsed once with PBS, FITC-goat anti-mouse IgG antibody diluted 1:50 was added and the samples were incubated in the dark at ambient temperature for 30 min. Then, the samples were centrifuged and re-suspended in 800 μL PBS. After filtration (300 apertures), the suspension was analyzed by FCM. Each experiment was repeated three times.

Determination of caspase 3 activity in SGC-7901 cells using a microplate reader

The activity of caspase-3 was determined using the caspase-3 activity kit. Briefly, SGC-7901 cells were plated in culture flasks (1×10^6 cells/flask) and allowed to attach overnight. After treating the cells with juglone (5, 10, 15 or 20 μmol/L), HCPT (60 μmol/L) or medium for 24 h, cell lysates were prepared by incubating 2×10^6 cells in 100 μL lysis buffer for 15 min on ice. Cell lysates were centrifuged at 20,000 × g for 15 min at 4 °C. Supernatants were collected and added to an ice-cold centrifuge tube. A blank solution containing 90 μL reaction buffer and 10 μL Ac-DEVD-pNA and the sample solution for each group including 80 μL reaction buffer, 10 μL sample and 10 μL Ac-DEVD-pNA were incubated in a 96-well microplate for 2 h at 37 °C. Caspase-3 activity was measured at 405 nm using a microplate reader (680, Bio-Rad, US) (Belmokhtar et al., 2001). The active unit of caspase 3 was calculated. One unit is the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-DEVD-pNA per hour at 37 °C under saturated substrate concentrations. These experiments were performed three times independently.

Statistical analysis

Data are expressed as mean ± SD and were analyzed with SPSS for Windows Version 15.0. Comparisons of data from different groups were performed using a one-way ANOVA test. A P value less than 0.05 was considered statistically significant.

Results

The cytotoxic effect of juglone on SGC-7901 cells

Using the SRB assay, we examine the effect of juglone on the growth of SGC-7901 cells. Juglone was found to significantly inhibit cell growth in a dose-dependent manner. The GI_{50} value indicates the high sensitivity of this human gastric cancer line to juglone (Table 1). Juglone was found to potently inhibit the growth of SGC-7901 cells *in vitro*.

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Effect of juglone on the ultrastructure of SGC-7901 cells

The results are shown in Fig. 1. In the control group, we observed the morphological characteristics of tumor cells, such as clear cellularity, integrated structure of the organelles, uniform distri bution of chromatin and microvilli on the cell membrane. After being exposed to different concentrations of juglone for 24 h, SGC-7901 cells displayed the typical apoptotic morphology, including a decrease in microvilli, chromatin condensation, crescent margination of chromatin against the nuclear envelope, enlargement of the perinuclear space and the formation of an apoptotic body. In the 5 μmol/L juglone dosage group, the microvilli of the tumor cells were smaller. The modalities of early apoptosis, such as the disappearance of microvilli and margination of chromatin with crescent formation, were observed. With increasing concentrations of juglone, the number of apoptotic cells was found to increase; a few cells formed apoptosis bodies. The numbers of early and late apoptotic cells were higher in the 20 μmol/L dosage group of juglone.

Rate of apoptosis in SGC-7901 cells after exposure to juglone

Translocation of phosphatidylserine (PS) to the outer leaflet of the cellular membrane seems to be a key step in the early stages of apoptosis. Annexin V has a strong affinity for PS. Because Annexin V is conjugated to FITC, it is possible to identify and quantitate apoptotic cells on a single-cell basis by FCM.

Early apoptotic and late apoptotic cells will bind Annexin V-FITC; necrotic cells have permeable membranes and will also bind Annexin V-FITC. PI is used to distinguish between viable, early apoptotic and necrotic, late apoptotic cells. PI is excluded by viable cells (FITC-negative) and early apoptotic cells (FITC-positive). Late apoptotic and necrotic cells stain with both Annexin V-FITC and PI. We measured the rates of apoptosis and generation of necrotic cells of SGC-7901 by FCM at 24 h after double staining with Annexin V-FITC and PI. We found that juglone could induce apoptosis in SGC-7901 cells. Also, a greater number of early apoptotic cells were found after exposure to increasing concentrations of juglone ($P < 0.01$) (Table 2. and Fig. 2).

Table 1
The GI_{50} and TGI of juglone on SGC-7901 cells (mean ± SD, $n=3$).

Compounds	24 h		48 h	
	GI_{50} (μM)	TGI (μM)	GI_{50} (μM)	TGI (μM)
Juglone	38.57 ± 0.93	174.65 ± 6.58	9.69 ± 0.21	1931.49 ± 56.96
HCPT	26.51 ± 1.09	107.16 ± 8.04	7.17 ± 0.22	1736.58 ± 31.04

Effect of juglone on the generation of ROS in SGC-7901 cells

We found that the level of ROS after treatment with 5, 10, 15 and 20 μmol/L of juglone was 34.83 ± 1.45%, 42.43 ± 1.36%, 53.73 ± 1.38% and 68.67 ± 1.33%, respectively; these values are significantly higher than those of the control group (27.70 ± 1.06%, $P < 0.01$). The level of ROS in the positive control group was 72.53 ± 1.27% (Fig. 3).

Effect of juglone on the expression of Bcl-2 and Bax proteins in SGC-7901 cells

The results are shown in Figs. 4 and 5. The expression of the Bcl-2 protein in SGC-7901 cells is significantly lower upon exposure to increasing concentrations of juglone than in the control ($P < 0.01$), while the expression of the Bax protein was significantly higher after juglone exposure than in the control group ($P < 0.01$). Protein quantities relative to the control were calculated and plotted as histograms.

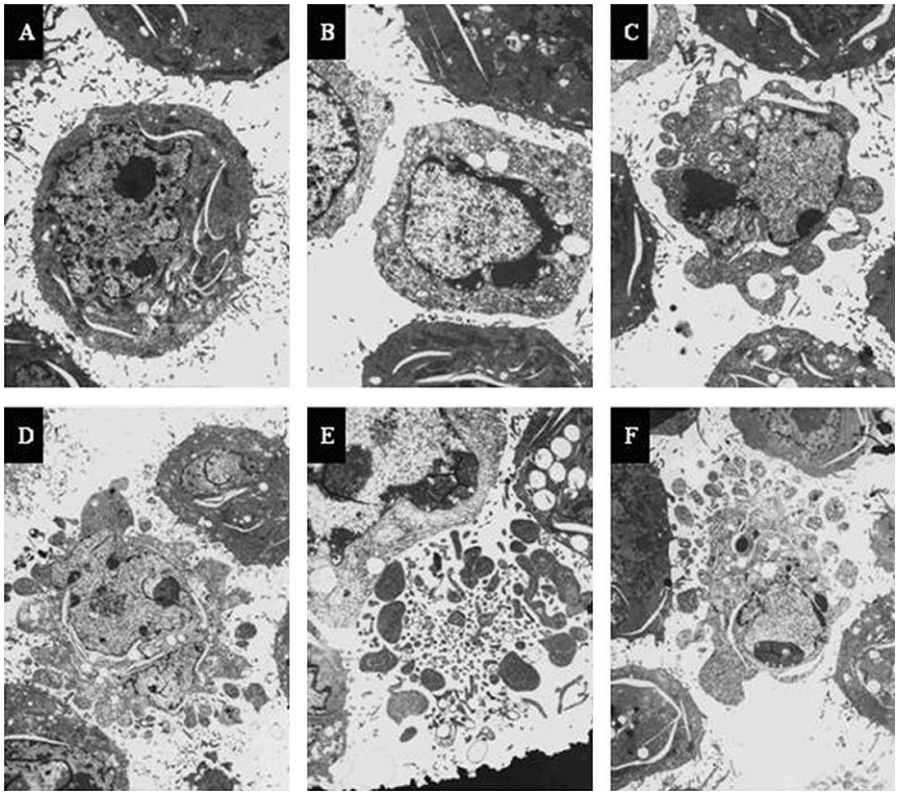

Fig. 1. Electron micrographs of SGC-7901 cells stained with uranyl acetate and lead citrate. A: control (× 6000); B: 5 μmol/L juglone (× 6000); C: 10 μmol/L juglone (× 6000); D: 15 μmol/L juglone(× 6000); E: 20 μmol/L juglone (× 6000). F: 60 μmol/L HCPT (× 4000).

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Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) in SGC-7901 cells upon exposure to juglone

In Fig. 6, we show that juglone can decrease the mitochondrial transmembrane potential of SGC-7901 cells. The mitochondrial transmembrane potentials in SGC-7901 cells exposed to 5, 10, 15 and 20 μmol/L of juglone were significantly lower than that of the control group ($85.53 \pm 1.82\%$, $53.57 \pm 2.48\%$, $46.33 \pm 1.46\%$, $36.43 \pm 2.64\%$ vs. 95.57 ± 1.14, $P < 0.01$). We observed a negative correlation between the mitochondrial transmembrane potential and the concentration of juglone. The mitochondrial transmembrane potential in the positive control group was $38.30 \pm 1.61\%$.

Table 2
Effect of juglone on the cell apoptosis of SGC-7901 cells (24 h) (mean ± SD, $n=3$).

Group	Dose (μM)	Apoptosis rate (%)	Normal rate (%)	Necrosis rate (%)
Control	–	0.17 ± 0.06	95.13 ± 1.23	0.13 ± 0.06
Juglone	5	7.27 ± 0.15**	82.90 ± 0.90**	2.3 ± 0.36**
	10	10.47 ± 0.15**	83.73 ± 0.35**	5.13 ± 0.32**
	15	16.13 ± 0.25**	75.33 ± 0.25**	5.97 ± 0.25**
	20	27.60 ± 0.70**	63.07 ± 0.77**	7.27 ± 0.15**
HCPT	60	32.83 ± 0.35**	55.43 ± 0.67**	5.80 ± 0.30**

*Compared with control $P < 0.05$.

** Compared with control $P < 0.01$.

Effect of juglone on the expression of cytochrome c protein in SGC-7901 cells

We found that every concentration of juglone could expedite the release of cytochrome c. The expression of cytochrome c was significantly higher after exposure to any concentration of juglone (5, 10, 15, 20 μmol/L) compared to the control ($16.70 \pm 0.82\%$, $21.43 \pm 0.97\%$, $26.27 \pm 0.68\%$, $29.27 \pm 0.55\%$ vs. $6.9 \pm 0.20\%$, $P < 0.01$). We observed a positive correlation between cytochrome c level and the concentration of juglone (Fig. 7). The expression of cytochrome c in the positive control group was $21.67 \pm 0.35\%$.

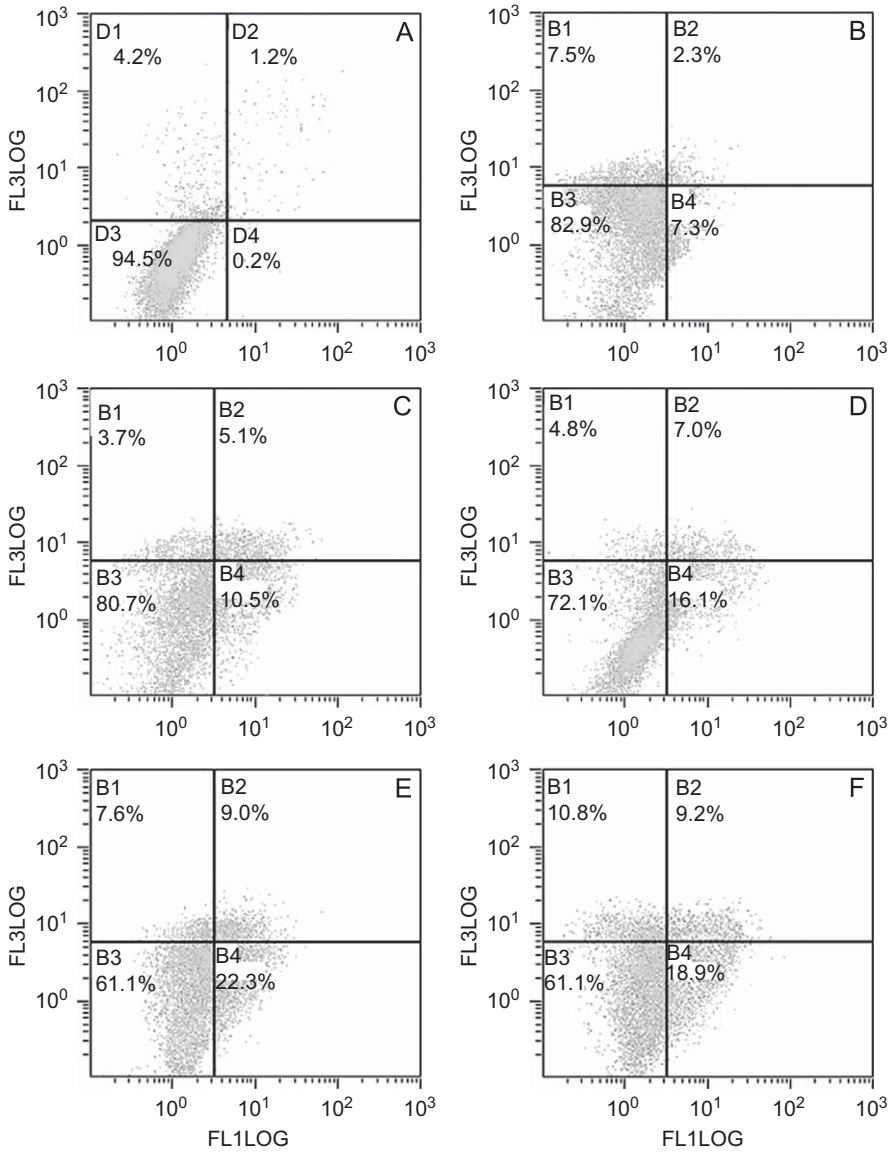

Fig. 2. The apoptosis rate of SGC-7901 cells determined using FCM with annexin V-FITC and PI double labeling. A: control; B: 5 μmol/L juglone; C: 10 μmol/L juglone; D: 15 μmol/L juglone; E: 20 μmol/L juglone. F: HCPT.

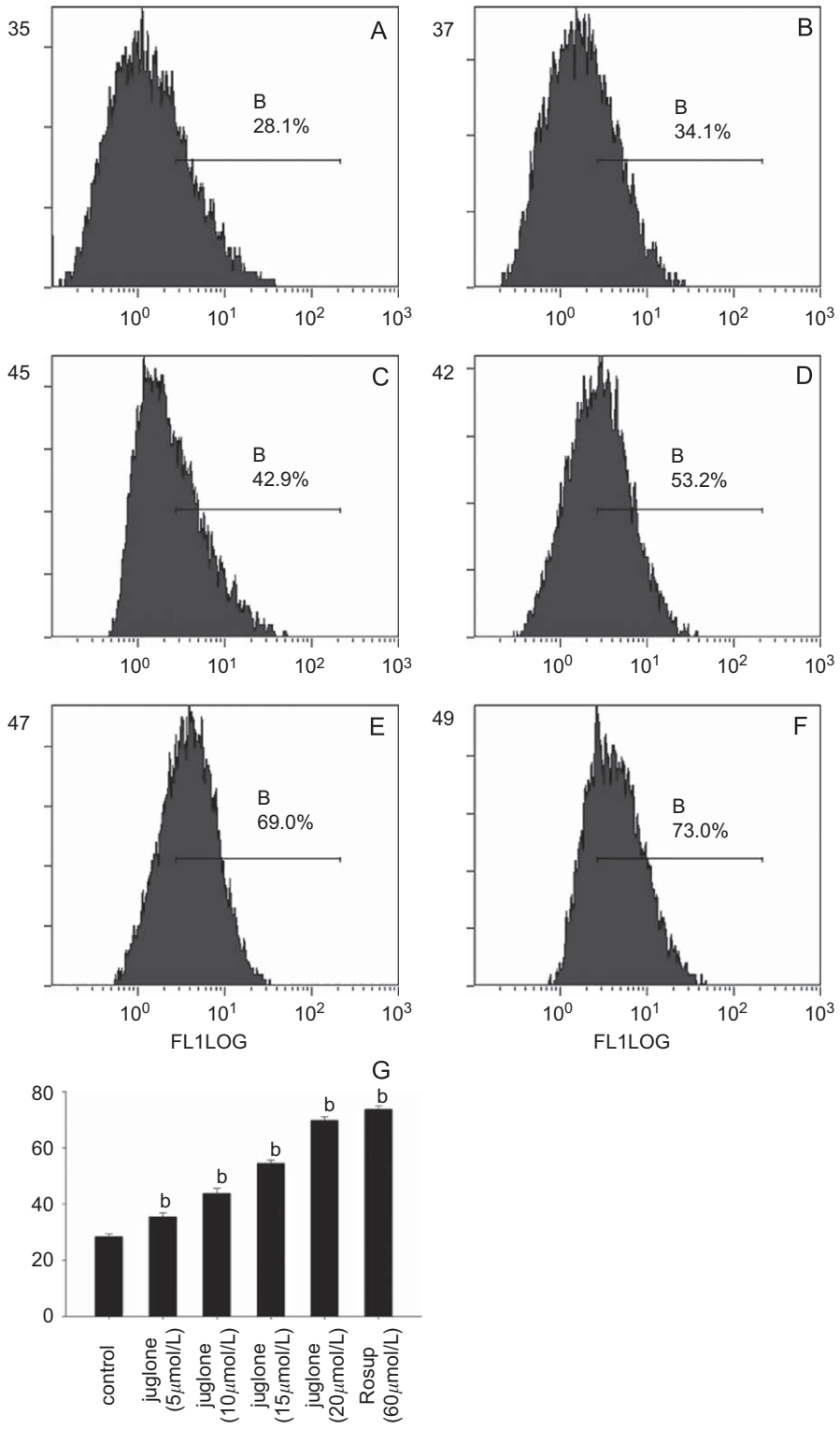

Fig. 3. The levels of ROS in SGC-7901 cells after incubation with juglone. A control; B: 5 μmol/L juglone; C: 10 μmol/L juglone; D: 15 μmol/L juglone; E: 20 μmol/L juglone. F: Rosup. [a]$P < 0.05$ vs. control; [b]$P < 0.01$ vs. control.

Effect of juglone on the caspase 3 activity in SGC-7901 cells

We found that juglone can activate caspase 3 in a concentration-dependent manner. The activity of caspase 3 of the experimental groups and the control group was calculated. The activity of caspase-3 was significantly higher after exposure to any concentration of juglone (5, 10, 15, 20 μmol/L) compared to the control (5.44 ± 0.48, 5.65 ± 0.79, 7.00 ± 0.83, 10.44 ± 0.63 vs.2.73 ± 0.48, $P < 0.01$). The activity of caspase 3 in the positive control group was 10.45 ± 0.83 (Fig. 8).

Discussion

Quinones are widely distributed in nature. Quinones have many biological functions and are applied as antibacterial agents,

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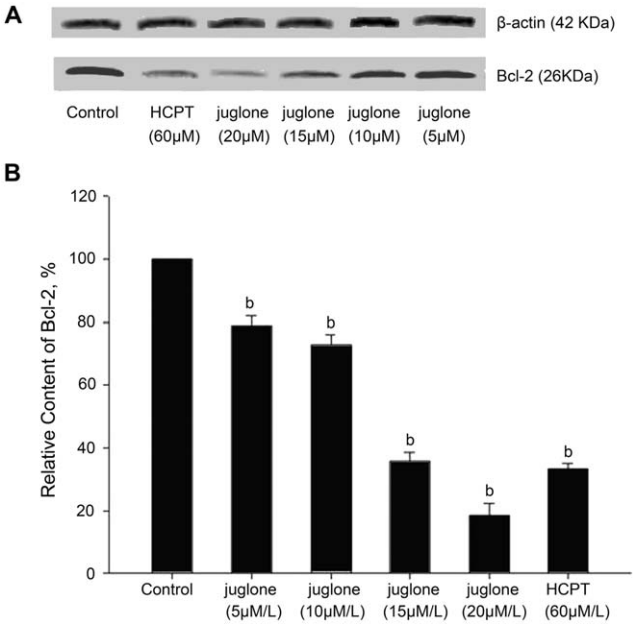

Fig. 4. Down-regulation of Bcl-2 expression in SGC-7901 cells treated with juglone visualized using the GIS-2019 system (A) and plotted as a relative level (B). $^{a}P < 0.05$ vs. control; $^{b}P < 0.01$ vs. control.

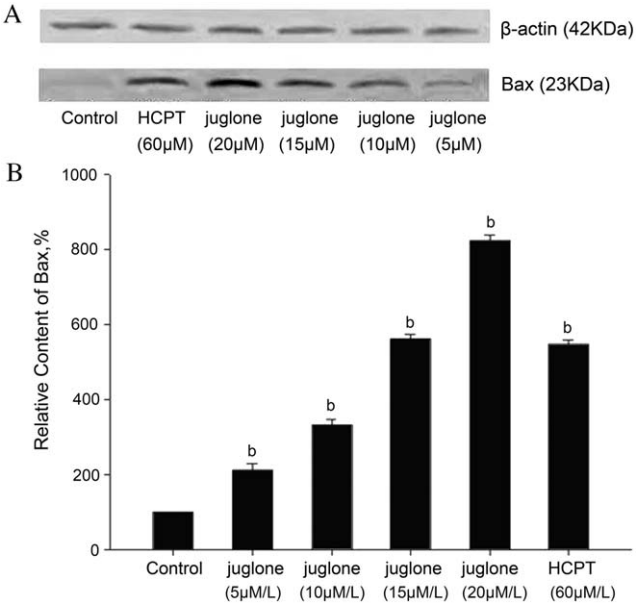

Fig. 5. Up-regulation of Bax expression in SGC-7901 cells treated with juglone visualized using the GIS-2019 system (A) and plotted as relative level (B). $^{a}P < 0.05$ vs. control; $^{b}P < 0.01$ vs. control.

fungicides, antimalarials and anticancer drugs. The biological effects of quinones are largely mediated by the formation of reactive oxygen species through redox activation and the covalent modification of free thiols to form thioethers (Bolton et al., 2000). Juglone is 5-hydroxy-1,4-naphthoquinone and is a strong cytotoxic agent. Its cytotoxicity is based on its high reactivity with oxygen, and it is frequently applied as a free radical enhancer (Bhuyan et al., 1991). It was found that juglone undergoes rapid single-electron reduction to semiquinone radicals. The relevant enzymes for this process are microsomal NADPH-cytochrome P450 reductase, microsomal NADH-cytochrome b_5 reductase and mitochondria1 NADH-ubiquinone oxidoreductase (Monks et al., 1992). The semiquinone radicals of juglone rapidly reduce molecular oxygen to form the superoxide anion radical ($O_2^{\bullet -}$) and thereby regenerate the quinone. Subsequent enzymatic or spontaneous dismutation of $O_2^{\bullet -}$ yields hydrogen peroxide (H_2O_2), which participates in additional reactions to form the hydroxyl radical ($OH^{\bullet}$) (Babich and Stern, 1993). ROS are the known mediators of intracellular signaling cascades. The excessive production of ROS, such as superoxide anion radical, hydrogen peroxide and the hydroxyl radical, leads to oxidative stress, loss of cell function and ultimately apoptosis or necrosis (Ito et al., 2004; Zhang et al., 2008). ROS can also induce lipid peroxidation or the cross-linking of thiol groups in proteins; both of these processes cause the opening of the mitochondrial permeability transition pore (PTP) (Kroemer et al., 1998). We speculated that ROS production by redox-cycling quinones could be involved in juglone-induced apoptosis. Therefore, we determined the levels of ROS in cells treated with juglone by staining cells with DCFDA. As shown in Fig. 3, the level of ROS significantly increases in a concentration-dependent manner after cells are exposed to juglone for 24 h. Thus, juglone can promote the generation of ROS in SGC-7901 cells.

Bcl-2 family proteins are involved in the response to apoptosis. They include a wide variety of anti-apoptotic proteins but also pro-apoptotic proteins, such as Bax (Ji et al., 2008a; O'Neill and Hockenbery, 2003). Bax is soluble in the cytosol under normal conditions. In the presence of apoptotic stimuli, such as the excess production of ROS, it translocates to the outer mitochondrial membrane (OMM) and inserts into the OMM. Then, Bax forms oligomers that are thought to be important in the formation of the PTP (Antonsson et al., 2001; Harris and Thompson, 2000; Yethon et al., 2003). Bcl-2 inhibits apoptosis by negatively regulating the apoptotic activity of Bax and forming Bcl-2/Bax heterodimers. The Bcl-2/Bax ratio is a measure of the cell death switch, which determines whether a cell will live or die upon being exposed to an apoptotic stimulus (Dias and Bailly, 2005; Sambaziotis et al., 2003). Thus, the expression of the Bcl-2 and Bax proteins in the cells of each group were measured using Western blots. Our results indicate that upon exposure to increasing levels of juglone, the expression of the Bcl-2 protein in SGC-7901 cells gradually decreases, while the expression of the Bax protein gradually increases (Figs. 4 and 5). We next detected the mitochondrial transmembrane potential. A lower membrane potential was measured in cells of the treatment groups, as shown in Fig. 6, suggesting that the PTP was open. Therefore, we conclude that juglone can promote the opening of the mitochondrial PTP by decreasing the expression of Bcl-2 and increasing the expression of Bax.

Mitochondria play a pivotal role in signal transduction during apoptosis; its insult or failure could rapidly lead to the inhibition of cell survival and proliferation. Inducing mitochondrial impairment may be a successful anticancer strategy (Heimlich et al., 2004). The opening of the mitochondrial PTP can lead to a release of cytochrome c and other pro-apoptotic molecules from the intermembrane space. Once cytochrome c is released into the cytosol, it forms an apoptosome with Apaf-1 and procaspase-9. This release causes the activation of caspase-9, which further activates caspase-3 (Launay et al., 2005; Li et al., 1997; Suzuki et al., 1998). Caspase 3 has been identified as a key mediator of apoptosis of mammalian cells (Kothakota et al., 1997). Its activity is considered to be an appropriate measure of cytotoxic responsiveness (Sonnemann et al., 2006). We investigated the effect of juglone on the release of cytochrome c and the activation of caspases-3 in SGC-7901 cells. As shown in Figs. 7 and 8, juglone

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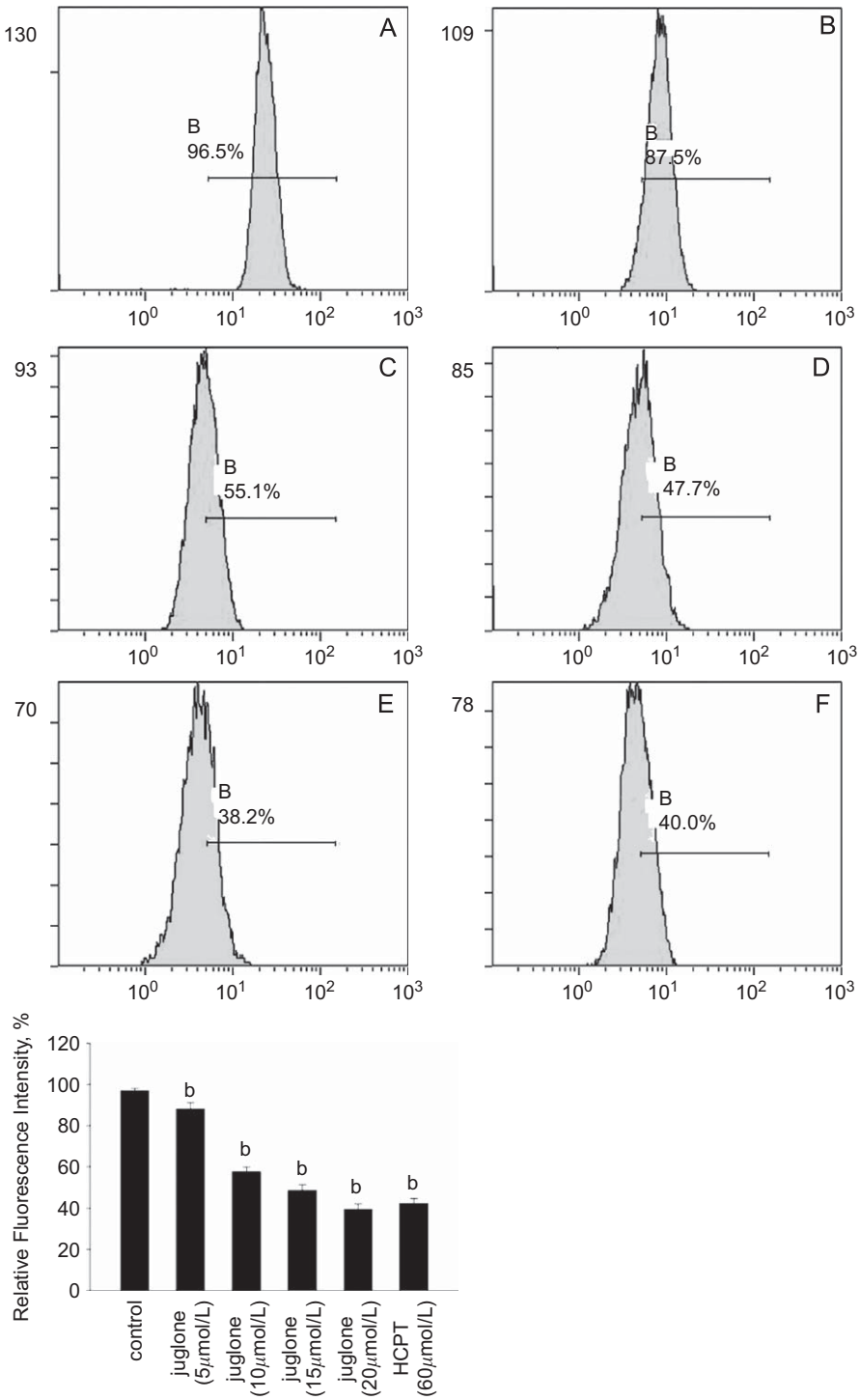

Fig. 6. The mitochondrial transmembrane potential in SGC-7901 cells after incubation with juglone. A: control; B: 5 μmol/L juglone; C: 10 μmol/L juglone; D: 15 μmol/L juglone; E: 20 μmol/L juglone. F: HCPT. $^{a}P < 0.05$ vs. control; $^{b}P < 0.01$ vs. control.

can expedite the release of cytochrome c and activate caspase 3 in a concentration-dependent manner. Our results indeed suggest that juglone can induce apoptosis in SGC-7901 cells via the mitochondrial apoptosis pathway.

Bcl-2 protein is a scavenger of ROS, and can effectively remove superoxide anion radicals, inhibit the generation of hydrogen peroxide and reduce the impairment to the cells caused by ROS. Meanwhile, Bcl-2 protein played an important role in regulating and maintaining activity of intracellular antioxidants. Bcl-2 protein was mainly distributed in the locations where ROS was produced, such as, mitochondrial membrane, ER, and nuclear envelope, suggesting that Bcl-2 protein can regulate the production of ROS generated by cells or mitochondria (Hochman et al., 1998; Zimmermann et al., 2007). Previous findings indicated that juglone straightly produced active oxygen, and affected the tumor cells. However, this study found that the ROS elevation of SGC-7901 cells induced by juglone is accompanied with the down-regulation of Bcl-2 protein. This phenomenon suggested

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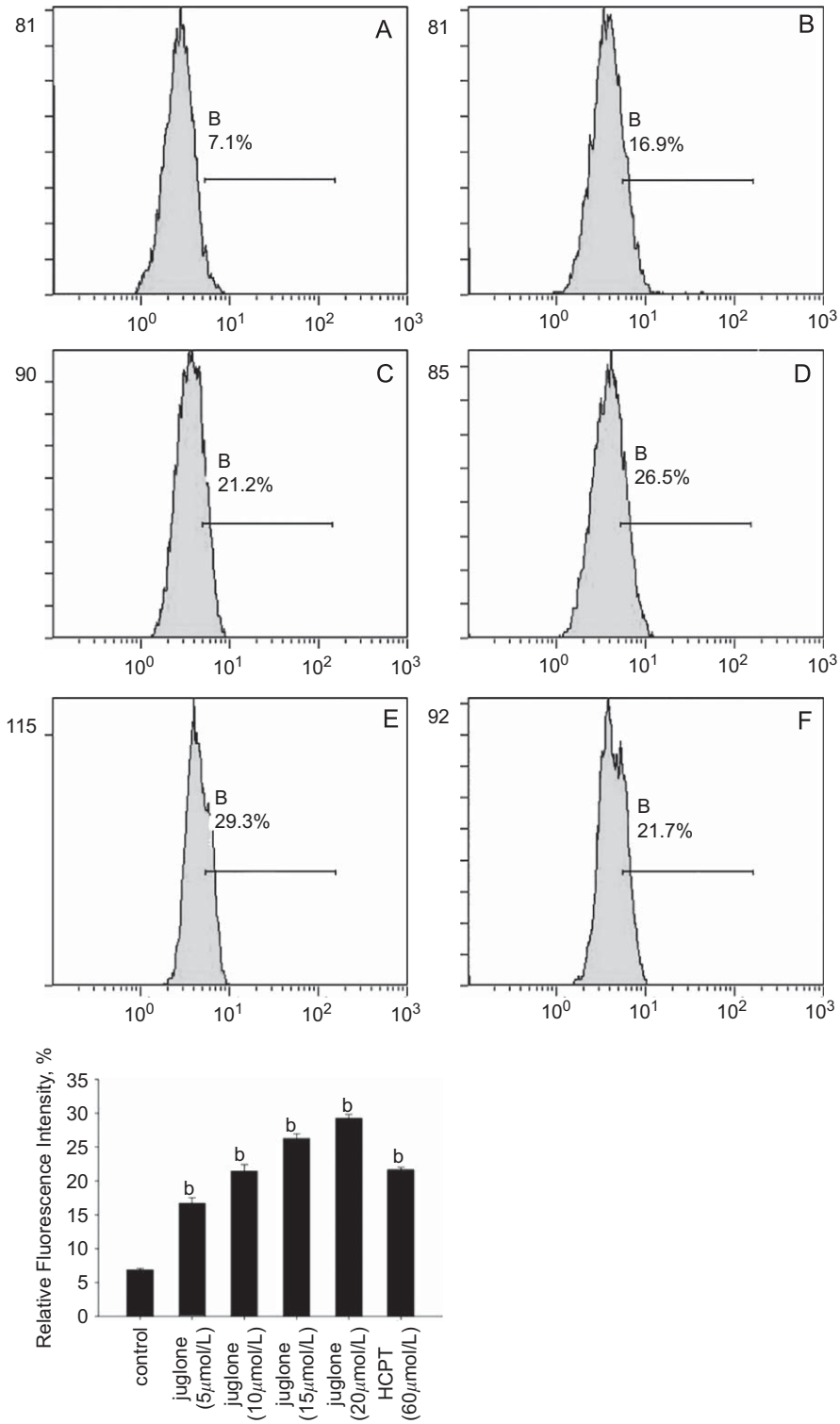

Fig. 7. Up-regulation of cytochrome c expression in SGC-7901 cells treated with juglone, determined using FCM. A: control; B: 5 μmol/L juglone; C: 10 μmol/L juglone; D: 15 μmol/L juglone; E: 20 μmol/L juglone. F: HCPT. $^{a}P < 0.05$ vs. control; $^{b}P < 0.01$ vs. control.

that the regulation of Bcl-2 protein is one of the mechanisms by which juglone increased ROS level in SGC-7901 cells.

Conclusion

Juglone can potently inhibit the growth and induce apoptosis of SGC-7901 cells. The mechanism is mediated by the activation of the mitochondrial death pathway, which requires the generation of ROS, down-regulation of Bcl-2 protein expression and up-regulation of Bax protein expression.

Competing interests

The authors declare that they have no competing interests.

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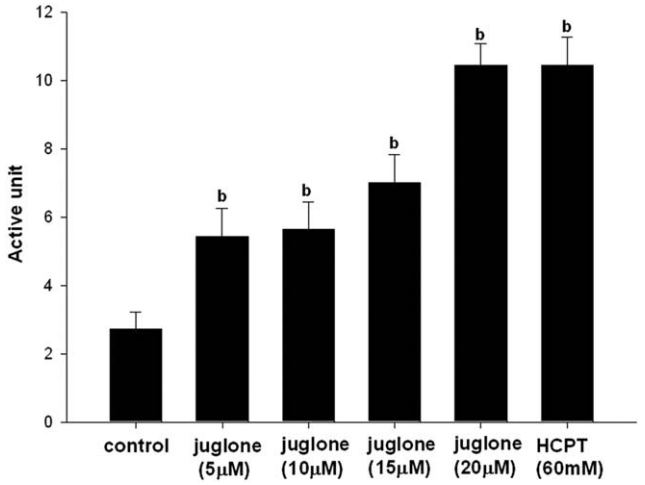

Fig. 8. Caspase 3 activity of SGC-7901 cells after incubation with juglone. $^{a}P < 0.05$ vs. positive control group (HCPT); $^{b}P < 0.01$ vs. HCPT.

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