Resveratrol induces human colorectal cancer cell apoptosis by activating the mitochondrial pathway via increasing reactive oxygen species

YUN FU[1,2], YUEDIAN YE[1,3], GUIFANG ZHU[1,4], YANGWEI XU[1,2], JINGBO SUN[1,2], HONGMEI WU[1,2], FEIYAN FENG[1,2], ZHIHUI WEN[1,2], SHANSHAN JIANG[1,2], YANYAN LI[1,2] and QINGLING ZHANG[1,2]

[1]Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong 510515; [2]Department of Pathology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510080; [3]Nanfang Hospital/First Clinical Medical School, Southern Medical University, Guangzhou, Guangdong 510515; [4]Department of Pathology, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510282, P.R. China

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Abstract. Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide according to Global Cancer Statistics 2018. Resveratrol (RSV) is a phenolic compound that possesses anticancer functions against various types of cancer, including breast and gastric cancer. However, the functions and mechanism underlying RSV in CRC are not completely understood. The present study aimed to investigate the anticancer effects and mechanism underlying RSV in CRC cells by conducting Cell Counting Kit-8, apoptosis, reactive oxygen species (ROS) and western blotting assays. The results suggested that RSV dose-dependently inhibited CRC cell viability, and increased cell apoptosis and ROS levels compared with the control group. The protein expression levels of Bax, cytochrome *c*, cleaved caspase-9 and cleaved caspase-3 were upregulated, whereas Bcl-2 expression levels were downregulated in RSV-treated CRC cells compared with control cells. The results indicated that RSV might activate the mitochondrial apoptotic pathway by increasing ROS release. The present study suggested that RSV possessed antitumour activity against CRC by modulating an ROS-mediated mitochondrial apoptotic pathway.

Correspondence to: Professor Qingling Zhang, Department of Pathology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, 106 Zhongshan Second Road, Guangzhou, Guangdong 510080, P.R. China
E-mail: zqllc8@126.com

Abbreviations: CRC, colorectal cancer; RSV, resveratrol; CCK, Cell Counting Kit; ROS, reactive oxygen species; 5-FU, 5-fuorouracil

Key words: resveratrol, colorectal cancer, apoptosis, reactive oxygen species, mitochondrial pathway

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide, resulting in an estimated 1.8 million new cases and 881,000 deaths in 2018 (1). Despite advances in curative surgical resection and chemotherapy, the 5-year survival rate is ~65% (2). Moreover, the recurrence rates of stage I-III and stage IV CRCs are 30 and 65%, respectively (3), and most patients with metastatic CRC cannot be treated (4). Therefore, a major challenge in the treatment of CRC is identifying effective and low toxicity chemotherapeutic agents.

Resveratrol (RSV) is a stilbenoid (3,4',5-trihydroxy-trans-stilbene) found in the skin of red grapes and other fruits; thus, red wine is the primary source of RSV (5). RSV has attracted considerable attention in the last two decades due to its numerous proposed health benefits, including anticancer (6-8), chemopreventive and chemotherapeutic (9), cardio-neuro-protective (10) and antidiabetic (11) effects. RSV is a prospective multitarget anticancer agent, which displays therapeutic potential in all three stages of carcinogenesis (initiation, promotion and progression) (12). RSV in combination with 5-fuorouracil (5-FU) increased the inhibitory effects of 5-FU on CRC cells (13). Moreover, RSV in combination with oxaliplatin synergistically suppressed CRC cell proliferation (14). RSV also displays low renal and hepatic toxicity both *in vivo* and *in vitro* (15,16).

Oxidative stress serves an important role in colorectal carcinogenesis (17). Reactive oxygen species (ROS), a group of highly reactive ions and molecules that are generated by mitochondria and participate in redox signalling pathways, influence the regulation of cell function, including proliferation (18). Excessive ROS generation or the failure of oxidant-scavenging systems in cancer cells can destroy the balance between Bcl-2 and Bax, and induce mitochondrial oxidative damage, leading to the release of cytochrome *c*, which is required for the activation of caspases (19). Therefore, stimulation of mitochondrial ROS production may serve as a potential anticancer strategy. However,

the modulatory mechanisms underlying RSV in human cancer cells are not completely understood, and relevant results reported in certain scientific literatures are controversial. For example, RSV suppresses reactive superoxide species, including ROS, in the mitochondria (20), whereas various RSV-treated cancer cells display elevated ROS generation, which induces extensive apoptosis (21-23).

In view of the aforementioned data, the aim of the present study was to investigate whether pharmacological concentrations of RSV could induce the apoptosis of HCT116 and SW620 human CRC cell lines and to explore the possible underlying mechanisms.

Materials and methods

Cell culture. Human CRC cells HCT116 and SW620 were purchased from American Type Culture Collection and maintained in the Department of Pathology of Southern Medical University (Guangzhou, China). Cells were cultured in RPMI-1640 (Biological Industries) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37˚C. The culture medium was changed every other day.

Cell Counting Kit-8 (CCK-8) assay. Cell viability was assessed by performing the CCK-8 assay (Dojindo Molecular Technologies, Inc.). CRC cells were seeded ($1x10^4$ cells) into 96-well plates with 100 μl medium per well and cultured for 24 h. Cells were cultured with different concentrations of RSV (0, 2, 4, 8, 16, 32, 64, 125, 250 or 500 μg/ml; Sino Biological, Inc.) at 37˚C for 48 h. Different doses of RSV were added. After 48 h, 10 μl CCK-8 buffer was added to each well and incubated at 37˚C for 2 h in the dark. Absorbance was measured at a wavelength of 450 nm using a microplate autoreader (Bio-Rad Laboratories, Inc.).

Cell apoptosis assay. To assess cell apoptosis, CRC cells were incubated with RSV (0, 6 or 12 μg/ml) at 37˚C for 48 h. The control group was treated with 0.2% DMSO. Cells ($1x10^5$) were harvested and washed with cold PBS. Cells were resuspended in 500 μl binding buffer supplemented with 5 µl FITC-Annexin V and 5 μl PI (Annexin V-FITC/PI Apoptosis Detection Kit; cat. no. KGA106; Nanjing KeyGen Biotech Co., Ltd.) and incubated in the dark at room temperature for 15 min. Flow cytometry was performed to measure apoptotic cells using a FACSAria™ flow cytometer (BD Biosciences) and FlowJo software (version 7.6; FlowJo LLC). Cell apoptosis was calculated as a sum of early and late apoptotic cells.

Investigation of intracellular ROS. Intracellular ROS levels were measured by performing 2',7'-dichlorofluorescein-diacetate (DCFH-DA; Beyotime Institute of Biotechnology) staining according to the manufacturer's instructions. CRC cells at a density of $1x10^5$/ml were exposed to RSV (0, 6 or 12 μg/ml) at 37˚C for 48 h, washed with PBS and incubated with 10 μM DCFH-DA at 37˚C for 30 min. Subsequently, flow cytometry was performed to measure fluorescence intensity in the FITC channel.

Western blotting. Total protein was isolated from cells using radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) on ice. Cell lysates were sonicated and centrifuged at 10,000 x g for 15 min at 4˚C. Total protein was quantified by performing a bicinchoninic acid assay. Proteins (40 μg/lane) were separated via 12% SDS-PAGE and transferred to 0.45 μm PVDF membranes. After washing with PBS, membranes were blocked with 5% milk at room temperature for 1 h. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies targeted against: Bax (1:500; cat. no. ab182734; Abcam), Bcl-2 (1:1,000; cat. no. 26593-1-AP; ProteinTech Group, Inc.), cytochrome *c* (1:1,000; cat. no. BSM-52050R; BIOSS), cleaved caspase-3 (1:1,000; cat. no. AF1150; Beyotime Institute of Biotechnology), caspase-3 (1:1,000; cat. no. AF0081; Beyotime Institute of Biotechnology), cleaved caspase-9 (1:1,000; cat. no. PB0285; Boster Biological Technology), caspase-9 (1:1,000; cat. no. bs-0049R; BIOSS) and β-actin (1:2,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.). Following primary incubation, the membranes were incubated with a HRP conjugated secondary goat anti rabbit antibody (1:100,000; cat. no. FD0128) or HRP conjugated goat anti mouse antibody (1:100,000; cat. no. FD0142; both purchased from Hangzhou Fude Biological Technology Co., Ltd.) for 1 h at room temperature. Following washing with PBS with 0.1% Tween, protein bands were visualized using enhanced chemiluminescence (cat. no. 36208ES60; Shanghai Yeasen Biotechnology Co., Ltd.) and photographed using a Tanon-5200 image analyser (Tanon Science and Technology Co., Ltd.). Protein expression levels were semi-quantified with β-actin as the loading control. Semi-quantitative analysis of protein signals were measured using ImageJ software (version 1.52; National Institutes of Health).

Statistical analysis. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS software (version 26.0; IBM Corp.). The unpaired Student's t-test was used to analyse comparisons between two groups. Comparisons among multiple groups were analysed using one-way ANOVA followed by Dunnett's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

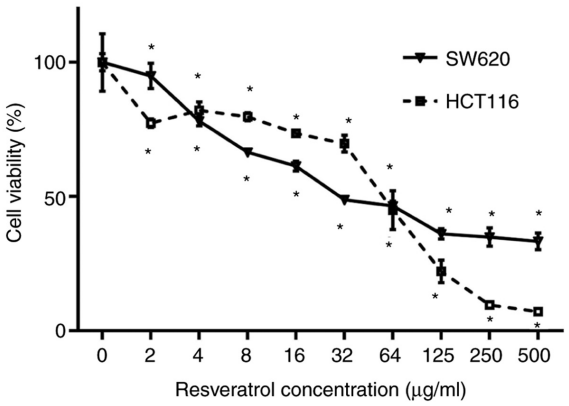

Figure 1. RSV inhibits SW620 and HCT116 cell viability. SW620 and HCT116 cells were treated with RSV (0, 2, 4, 8, 16, 32, 64, 125, 250 or 500 μg/ml) in 96-well plates for 48 h. The IC_{50} of RSV for SW620 and HCT116 cells at 48 h were 51.75 and 43.54 μg/ml, respectively. *P<0.05 vs. 0 μg/ml RSV. RSV, resveratrol.

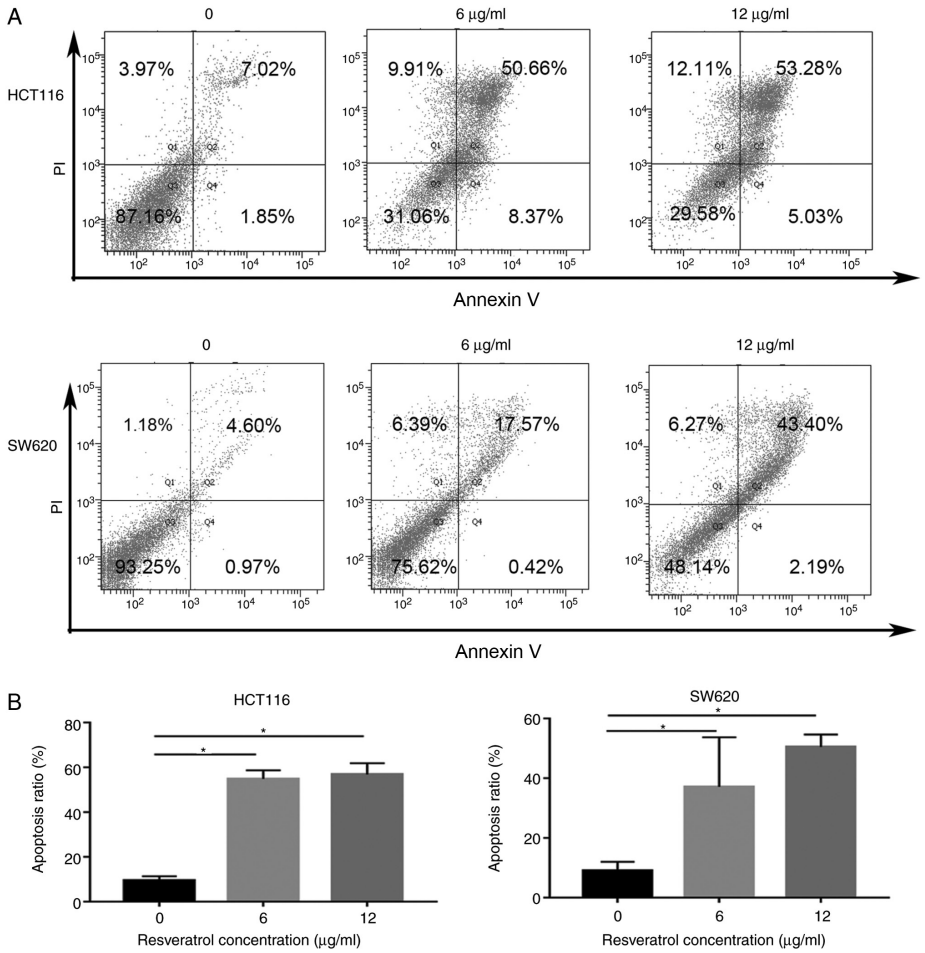

Figure 2. Effect of RSV on HCT116 and SW620 cell apoptosis. Cell apoptosis was (A) determined via flow cytometry and (B) quantified. Cells in the lower right quadrant were defined as Annexin-positive, early apoptotic cells and cells in the upper right quadrant were defined as Annexin-positive/PI-positive, late apoptotic cells. Cell apoptosis was calculated as a sum of early and late apoptotic cells. *P<0.05 vs. 0 μg/ml RSV. RSV, resveratrol.

Results

RSV inhibits CRC cell viability. The CCK-8 assay was performed to assess the potential therapeutic effect of RSV on CRC. HCT116 and SW620 cells were treated with different concentrations of RSV (0, 2, 4, 8, 16, 32, 64, 125, 250 or 500 μg/ml) for 48 h (Fig. 1). Exposure to RSV significantly reduced HCT116 and SW620 cell viability in a dose-dependent manner compared with the 0 μg/ml RSV group. The dose-dependent decrease in SW620 cell viability was more obvious in the lower concentration range (≤32 μg/ml) compared with the higher concentration range (>32 μg/ml). By contrast, the dose-dependent decrease in HCT116 cell viability was more obvious in the higher concentration range (>32 μg/ml) compared with the lower concentration range (≤32 μg/ml). At 48 h, the 50% maximal inhibition concentration (IC_{50}) values of RSV for HCT116 and SW620 cells were 43.54 and 51.75 μg/ml, respectively. For subsequent experiments, the 1/8 and 1/4 IC_{50} value doses (6 and 12 μg/ml, respectively) were selected.

RSV induces extensive CRC cell apoptosis. Annexin V/PI double staining and flow cytometry were performed to evaluate apoptosis in RSV-treated CRC cells (Fig. 2). In HCT116 cells, apoptosis was significantly increased in the 6 (54.82±3.866%) and 12 μg/ml (56.84±5.087%) RSV groups compared with the control group (9.42±1.935%). Similarly, the proportion of apoptotic SW620 cells in the control group was 9.023±2.991%, which was significantly increased to 37.13±16.59% and 50.46±4.225% in the 6 and 12 μg/ml RSV groups.

RSV enhances ROS generation in CRC cells. Whether RSV enhanced ROS accumulation in CRC cells was assessed via flow cytometry (Fig. 3). In HCT116 cells, ROS generation was significantly increased in the 6 (1,124±99.05) and 12 μg/ml (841±132.1) RSV groups compared with the control group (514.7±168.3). Similarly, in SW620 cells, ROS generation was significantly increased in the 6 (3,767±828.5) and 12 μg/ml (5,412±792.4) RSV groups compared with the control group (2,173±208.2). The results suggested that RSV remarkably enhanced ROS generation in CRC cells.

RSV triggers the mitochondrial pathway in CRC cells. HCT116 and SW620 cells treated with 0, 6 and 12 μg/ml RSV for 48 h were subjected to western blotting to assess the expression levels of proteins involved in the mitochondrial apoptotic pathway (Fig. 4). The protein expression levels of Bax and Bcl-2 in CRC cells were analysed because Bcl-2 family proteins serve an essential regulatory role in the mitochondrial pathway (24). The expression level of Bax was upregulated, whereas the expression level of Bcl-2 was downregulated in

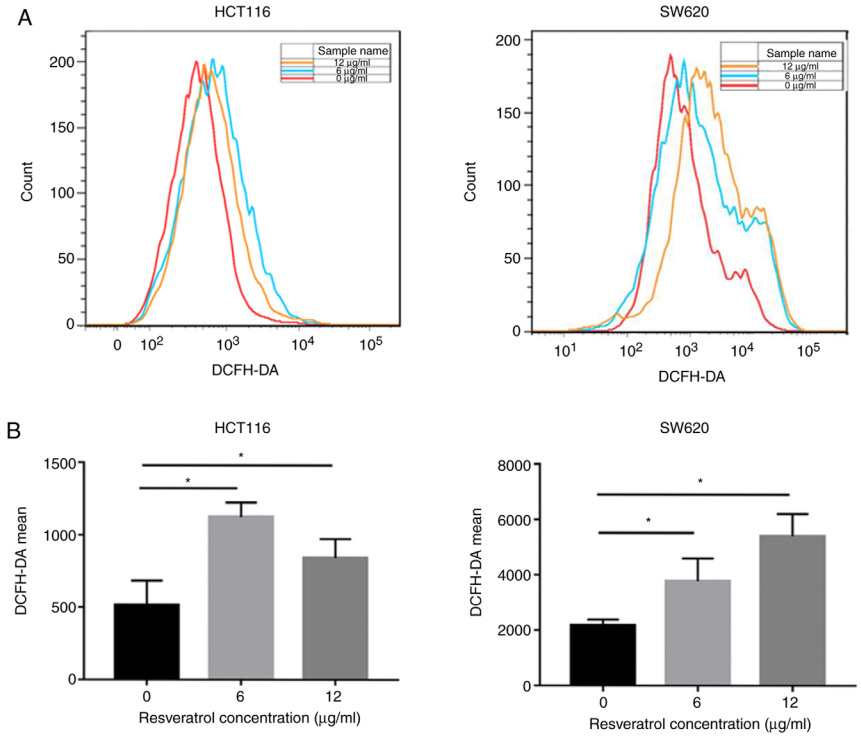

Figure 3. RSV enhances ROS generation in HCT116 and SW620 cells. Intracellular ROS levels were (A) determined via flow cytometry and (B) quantified. *P<0.05 vs. 0 μg/ml RSV. RSV, resveratrol; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein-diacetate.

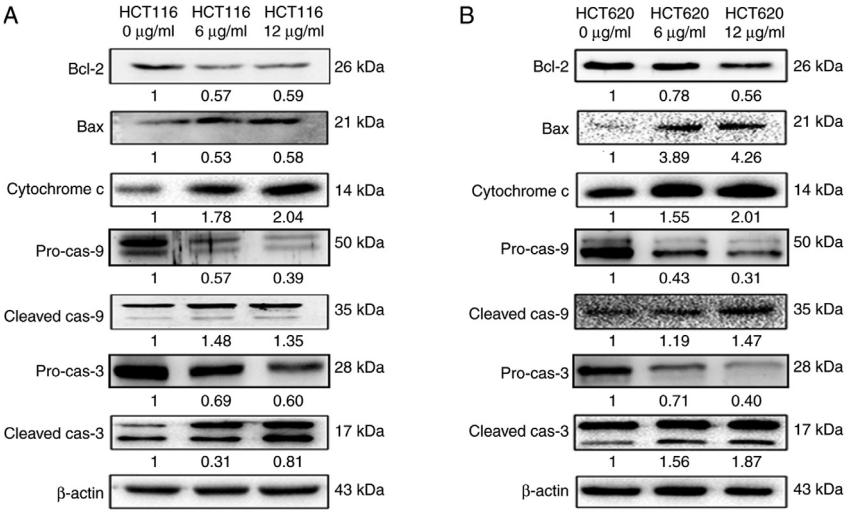

Figure 4. Effects of resveratrol on the expression levels of apoptosis-related proteins in colorectal cancer cells. Protein expression levels of apoptosis-related genes in (A) HCT116 and (B) SW620 cells. Cas, caspase.

the 6 and 12 μg/ml RSV groups compared with the control group. Moreover, the expression levels of cytochrome *c*, cleaved caspase-9 and cleaved caspase-3 were increased in the 6 and 12 μg/ml RSV groups compared with the control group. The results indicated that the activation of the mitochondrial pathway by oxidative stress is the underlying mechanism by which RSV kills CRC cells, as illustrated in Fig. 5.

Discussion

Phenolic compounds possess potential inhibitory effects on cancer invasion and metastasis (25,26). The anticancer effect of RSV has been associated with its proliferation-inhibiting and apoptosis-inducing activities, antioxidant properties and inhibition of stress induction in multiple types of cancer, such as breast and prostate cancer (27-29). However, the functions and mechanism underlying RSV in CRC are not completely understood. In the present study, the results indicated that the anticancer effects of RSV on CRC cell lines were related to activation of the mitochondrial apoptotic pathway via enhanced ROS generation.

Apoptosis is an important means of eliminating most tumorigenic cells for tissue homeostasis (30). Further understanding apoptosis will provide molecular basis for

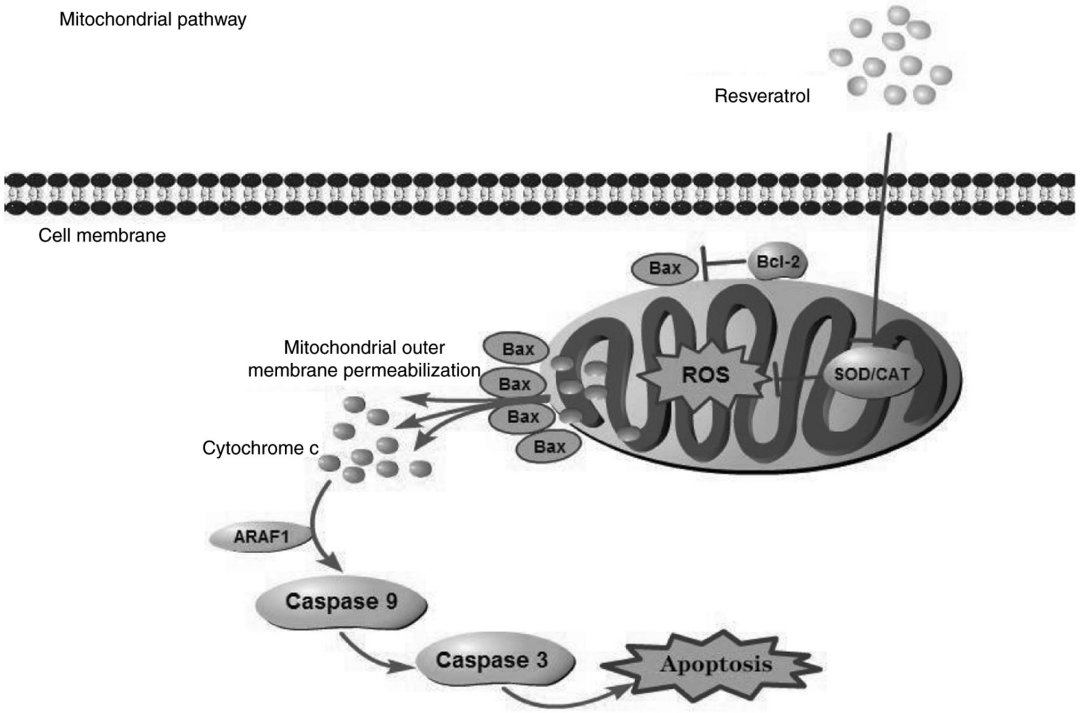

Figure 5. Summary of RSV-induced cytotoxicity. RSV induced intracellular ROS accumulation, which resulted in mitochondrial outer membrane permeabilization. Subsequently, cytochrome *c* was released to the cytoplasm and combined with ARAF1, which activated caspase cascades, leading to cell apoptosis. RSV, resveratrol; ROS, reactive oxygen species; ARAF1, A-Raf proto-oncogene, serine/threonine kinase; SOD, superoxidedismutase; CAT, catalase.

novel targeted therapies that can induce cancer cell death or sensitise cancer cells to other established chemotherapeutic agents (31). In the present study, the Annexin-V/PI double staining results indicated that RSV significantly increased apoptosis in CRC cell lines compared with the control group. The dynamic balance between proliferation and apoptosis is tightly regulated by specific signalling pathways (32). The apoptotic pathways include extrinsic (cytoplasmic) and intrinsic (mitochondrial) pathways (31). The former is triggered via the Fas death receptor whereas the latter is triggered via cellular stress, including DNA damage, oxidative stress and growth factor deprivation, resulting in mitochondrial depolarisation and the release of cytochrome *c* from the mitochondria to the cytosol (24). Both pathways converge to a common pathway involved in the activation of the caspase cascade. Proapoptotic caspases, including caspase-3/8/9, serve an important role in mediating the apoptosis signalling pathway (33). Caspase-3 can be activated by caspase-8 or caspase-9, which are important markers of the extrinsic and intrinsic pathways, respectively (34). One of the key regulators of the intrinsic pathway is the Bcl-2 family of proteins, which includes proapoptotic members, such as Bax, and antiapoptotic members, such as Bcl-2 (35). The Bcl-2 family members regulate apoptosis by controlling the release of cytochrome *c*, and a direct interaction between Bcl-2 and Bax has been observed in individual mitochondria (36). Cytochrome *c* released from the mitochondria binds to apoptosis protease-activating factor 1 to activate caspase-9 and caspase-3, which induce the terminal events in cell apoptosis (37). The western blotting results of the present study indicated that RSV increased the expression levels of cytochrome *c*, Bax, cleaved caspase-9 and cleaved caspase-3, but decreased the expression of Bcl-2 compared with the control group. The results indicated that RSV induced CRC cell apoptosis by activating the intrinsic apoptotic pathway.

Elevated ROS generation is related to RSV-induced apoptosis in numerous cancer cells (38,39). ROS overproduction is the early step involved in the mitochondrial apoptotic pathway (40). In the present study, RSV increased ROS generation in HCT116 and SW620 cells compared with the control group; thus, indicating that apoptosis in RSV-treated cells may be increased by oxidation. ROS accumulation in cancer cells can induce genomic damage, such as double-strand breaks and chromosome damage, accompanied by apoptosis (41). Moreover, excessive ROS leads to mitochondrial swelling, the permeabilization of outer mitochondrial membrane and the release of cytochrome *c*. Therefore, a pro-oxidant action serves an important role in the anticancer effect of RSV.

The present study had several limitations. First, only the anticancer effect of RSV on CRC cells was investigated in the present study, but the toxicity of RSV on normal cell lines remains unclear. Secondly, the results indicated that RSV dose-dependently inhibited CRC cell viability by performing the CCK-8 assay. However, RSV did not display dose-dependent effects on cell apoptosis and intracellular ROS among the three groups (0, 6 and 12 μg/ml).

In conclusion, the present study suggested that RSV-induced apoptosis in CRC cells involved activation of the ROS-mediated mitochondrial pathway. Therefore, RSV may serve as a potential therapeutic agent for patients with CRC.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ conceived and designed the study, and wrote the manuscript. YF, YY, GZ, YX, JS, HW, FF, ZW, SJ and YL performed the experiments. YF analysed the data and revised the manuscript. YY helped with the writing of the manuscript. All authors discussed the results, and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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