Quercetin induced apoptosis of human oral cancer SAS cells through mitochondria and endoplasmic reticulum mediated signaling pathways

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Abstract. Oral cancer is a cause of cancer-associated mortality worldwide and the treatment of oral cancer includes radiation, surgery and chemotherapy. Quercetin is a component from natural plant products and it has been demonstrated that quercetin is able to induce cytotoxic effects through induction of cell apoptosis in a number of human cancer cell lines. However, there is no available information to demonstrate that quercetin is able to induce apoptosis in human oral cancer cells. In the present study, the effect of quercetin on the cell death via the induction of apoptosis in human oral cancer SAS cells was investigated using flow cytometry, Annexin V/propidium iodide (PI) double staining, western blotting and confocal laser microscopy examination, to test for cytotoxic effects at 6-48 h after treatment with quercetin. The rate of cell death increased with the duration of quercetin treatment based on the results of a cell viability assay, increased Annexin V/PI staining, increased reactive oxygen species and Ca^{2+} production, decreased the levels of mitochondrial membrane potential ($\Delta\Psi_m$), increased proportion of apoptotic cells and altered levels of apoptosis-associated protein expression in SAS cells. The results from western blotting revealed that quercetin increased Fas, Fas-Ligand, fas-associated protein with death domain and caspase-8, all of which associated with cell surface death receptor. Furthermore, quercetin increased the levels of activating transcription factor (ATF)-6α, ATF-6β and gastrin-releasing peptide-78 which indicated an increase in endoplasm reticulum stress, increased levels of the pro-apoptotic protein BH3 interacting-domain death antagonist, and decreased levels of anti-apoptotic proteins B-cell lymphoma (Bcl) 2 and Bcl-extra large which may have led to the decreases of $\Delta\Psi_m$. Additionally, confocal microscopy suggested that quercetin was able to increase the expression levels of cytochrome *c*, apoptosis-inducing factor and endonuclease G, which are associated with apoptotic pathways. Therefore, it is hypothesized that quercetin may potentially be used as a novel anti-cancer agent for the treatment of oral cancer in future.

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Introduction

Oral cancer is a global public health problem standing among the 10 primary causes of cancer-associated mortality globally (1). The incidence of oral cancer remains high in Asian and Western countries (2). Oral cancer affects a notable number of patients <40 years of age (3). In Taiwan, oral cancer is the fourth most common cancer type in males based on a report in 2011 from the Department of Health, which indicated that 7.9/100,000 individuals succumb annually to oral cancer (4). It has been reported that oral cancer result in high rates of mortality and markedly affects patient's quality of life (5). The World Health Organization has recognized the importance of health education, prevention, tracking, early diagnosis and treatment in dealing with oral cancer (6). Scientific investigators have thus far focused on the compounds from natural products for treatment of patients with oral cancer.

The major treatment option for patients with oral cancer with stage I and II disease (7) is surgery and radiotherapy, which results in a 5-year survival rate of >70% (8,9). The application of concurrent chemoradiation presents an attractive alternative

to traditional surgical management in advanced squamous cell carcinoma of head and neck, as it has been identified to improve loco-regional control, disease free survival and overall survival (10,11). Quercetin [2-(3,4-dihydroxy-phenyl)-3,5, 7-trihydroxy-4H-1-benzopyran-4-one] is a polyphenolic flavonoid present in plants including fruits, vegetables and several other human dietary sources including seeds and nuts (12,13), and has been identified to exert various beneficial health effects, including the suppression of inflammation, promotion of immune function and anti-aging effects (14,15). Quercetin has been demonstrated to induce cytotoxic effects on human breast (16), lung (17) and prostate (18) cancer cell lines through the induction of apoptosis. Furthermore, it was identified that quercetin suppresses the proliferation of gastric (19), colon (20), liver (21) and cervical (22) cancer cells.

Although numerous studies have identified that quercetin has an impact on a number of human cancer cell lines, including oral cancer cells lines SCC-9 (23), SCC-1483, SCC-25, SCC-QLL1 (24) and HSC-3 (25), potential actions of quercetin on the oral cancer cells SAS have been poorly explored. The underlying molecular mechanism is not fully understood. Therefore, in the present study, the effect of quercetin on the human oral cancer SAS cell line was investigated. It was identified that quercetin induced apoptosis of SAS cells *in vitro* via endoplasmic reticulum (ER) stress- and mitochondria-signaling pathways.

Materials and methods

Chemicals and reagents. Quercetin (cat. no. Q4951; ≥95%), propidium iodide (PI), Trypsin-EDTA, L-glutamine and penicillin-streptomycin were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fluo-3/AM, dihexyloxacarbocyanine iodide ($DiOC_6$) and dichloro-dihydro-fluorescein diacetate (H_2DCF-DA) were obtained by Invitrogen; Thermo Fisher Scientific, Inc.

Cell culture. Human oral cancer cells SAS cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). These cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine, and were cultured at 37˚C in a humidified incubator in an atmosphere containing 5% CO_2 (26,27).

Cell morphology and viability assays. SAS cells ($1x10^5$ cells/well) were placed in 12-well plates with DMEM for 24 h then quercetin (40 μM) or 1% dimethyl sulfoxide as a vehicle control was added to each well for 0, 12, 24 and 48 h. In order to examine morphological changes, cells in each well were examined and images were captured using contrast phase microscopy at a magnification, x400. To measure the percentage of viable cells, cells were collected from each treatment well, counted and stained with PI (5 μg/ml) at room temperature in the dark then immediately analyzed using a Flow Cytometry system (BD Biosciences, San Jose, CA, USA) assay as previously described (26,28).

Annexin V/PI staining. Cell apoptosis was measured using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences) as described previously (29,30). Briefly, SAS cells ($5x10^4$ cells/ml) in 12-well culture plates were treated with quercetin (40 μM) for 24 and 48 h or 1% DMSO as a vehicle control. Cells were harvested and then re-suspended in Annexin V binding buffer, followed by incubation with Annexin V-FITC/PI in the dark for 15 min according to the manufacturer's protocol for labeling of apoptotic cells (29,30). In each experiment, $1x10^4$ cells were analyzed using Cell Quest™ program (Version 5.2.1; BD Biosciences). Experiments were performed in triplicate.

Measurement of reactive oxygen species (ROS), intracellular Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$). Flow cytometry was used to measure the levels of ROS, Ca^{2+} and MMP in SAS cells following exposure to quercetin. SAS cells ($1x10^5$ cells/well) were placed in 12-well plates and were treated with 40 μM quercetin or 1% DMSO as a vehicle control for various time periods (1, 3, 6, 9, 12, 24 and 48 h). Cells were isolated and re-suspended in 500 μl H_2DCF-DA (10 μM), then kept in darkness for 30 min at 37˚C to measure the levels of ROS (H_2O_2); re-suspended in 500 μl $DiOC_6$ (4 μmol/l) and maintained in darkness for 30 min at 37˚C to measure the levels of MMP; or re-suspended in 500 μl of Fluo-3/AM (2.5 μg/ml) and kept in darkness for 30 min at 37˚C for intracellular Ca^{2+} concentrations measurement. After 30 min of incubation, all samples were analyzed using flow cytometry as described previously (30,31).

Caspase-3, caspase-8 and caspase-9 activities assay. Flow cytometry was used to measure the activities of caspase-3, caspase-8 and caspase-9. SAS cells ($1x10^5$ cells/well) were incubated with 40 μM quercetin or 1% DMSO as a vehicle control for 6, 24 and 48 h, harvested, washed with 1X PBS and re-suspended in 25 μl 10 μM substrate solution sourced from caspase activity assay kits (PhiPhiLux-G1D1 for caspase-3, CaspaLux8-L1D2 for caspase-8 and CaspaLux9-M1D2 for caspase-9, OncoImmunin, Inc., Gaithersburg, MD, USA) prior to incubation at 37˚C for 60 min. Following incubation, cells were washed with PBS and were immediately analyzed using flow cytometry as described previously (29,30).

Western blotting analysis. SAS cells ($1.5x10^6$ cells/dish) were incubated in a 10-cm dish for 24 h at 37˚C, then incubated with 40 μM quercetin for 6, 12, 24 and 48 h or 1% DMSO as a vehicle control. Cells were collected then lysed and denatured using ice-cold lysis buffer for 1 h (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.3 mM PMSF, 0.2 mM sodium orthovanadate, 0.1% SDS, 1 mM EDTA, 1% NP-40, 10 mg/ml leupeptin and 10 mg/ml aprotinin). Total protein quantity was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as described previously (29). Equal amounts of total protein (40 μg) were separated using SDS-PAGE (12% v/v) and then transferred onto polyvinylidene difluoride membranes. Following blocking with 5% non-fat dried milk in PBS with Tween-20 for 1 h at room temperature, membranes were immunoblotted with specific primary antibodies [Apoptosis-inducing factor

(AIF; cat. no. sc-13116), activating transcription factor-6α (ATF-6α; cat. no. sc-22799), ATF-6β (cat. no. sc-30597), cytochrome *c* (Cyto c; cat. no. sc-13560), endonuclease G (Endo G; cat. no. sc-365359), gastrin-releasing peptide-78 (GRP-78; cat. no. sc-13968), iron-responsive element-1α (IRE-1α; cat. no. sc-20790), X-box binding protein 1 (XBP-1; cat. no. sc-7160), TNF-related apoptosis-inducing ligand (TRAIL; cat. no. sc-80393) antibodies were supplied by Santa-Cruz Biotechnology, Inc. (Dallas, TX, USA; all dilution, 1:1,000); apoptotic protease activating factor 1 (Apaf-1; cat. no. 8723), Bcl-2-associated death promoter (Bad; cat. no. 9239), Bcl-2 homologous antagonist killer (Bak; cat. no. 12105), B-cell lymphoma 2 (Bcl-2; cat. no. 2870), Caspase-3 (cat. no. 9665s), Caspase-8 (cat. no. 9746), Caspase-9 (cat. no. 9508) antibodies were supplied by Cell Signaling Technology (Danvers, MA, USA; all dilution, 1:1,000); Caspase-6 (cat. no. AB10512; dilution, 1:2,000), BH3 interacting-domain death antagonist (Bid; cat. no. AB1730; dilution, 1:500), Fas-associated protein with death domain (FADD; cat. no. 05-486; dilution, 1:1,000), Fas ligand (Fas-L; cat. no. 05-571; dilution, 1:2,000) antibodies were supplies by EMD Millipore (Billerica, MA, USA); Caspase-4 (cat. no. 556459; dilution, 1:500) and Fas (cat. no. 610198, dilution, 1:5,000) antibodies were obtained from BD Biosciences (Bedford, MA, USA); poly(ADP-ribose) polymerase (PARP; cat. no. ab6079-1; dilution, 1:400) and Caspase-7 (cat. no. ab181579; dilution, 1:5,000) antibodies were obtained from Abcam (Cambridge, MA, USA); Bcl-extra large (Bcl-x; cat. no. B9304; dilution, 1:2,000), Caspase-2 (cat. no. C7349; dilution, 1:200) and β-actin (cat. no. A5316; dilution, 1:10,000) antibodies were supplied by Sigma-Aldrich; Merck KGaA] for 1 h at room temperature (25˚C). Subsequently, the membranes were incubated with secondary antibodies [horseradish peroxidase (HRP)-conjugated mouse immunoglobulin G (IgG; cat. no. GTX213112) and rabbit HRP-conjugated IgG secondary antibodies (cat. no. GTX213110) at dilution, 1:5,000 (GeneTex, Inc., Irvine, CA, USA)] for 1 h at room temperature. Chemiluminescence signals were enhanced using enhanced chemiluminescence reagent (EMD Millipore) (27,29,30), and images were captured using the MultiGel-21 Image system (TOP BIO CO., Taipei, Taiwan).

Confocal laser scanning microscopy assay. SAS cells ($1x10^5$ cells/well) were maintained on 6-well chamber slides with or without 40 μM quercetin or 1% DMSO as a vehicle control for 48 h and then fixed in 4% formaldehyde in PBS for 15 min at room temperature. Triton X-100 in PBS (0.1%) was added to the cells for 15 min at room temperature, followed by 2% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature to block non-specific binding sites. Cells were then incubated overnight with primary antibodies, including anti-Cyto c (cat. no. sc-13560; 1:250 dilution), AIF (cat. no. sc-13116; 1:250 dilution) and anti-Endo G (cat. no. sc-365359; 1:250 dilution) (all in green fluorescence) at 4˚C, and then followed by incubation with a secondary antibody (FITC-conjugated goat anti-mouse IgG; cat. no. 115-545-003; 1:100 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature, and PI (red fluorescence) staining for examination as described previously (30). Slides were mounted, examined with oil immersion and images (magnification, x630) were captured using a Leica TCS SP2 Confocal Spectral Microscope (Leica Microsystems GmbH, Mannheim, Germany).

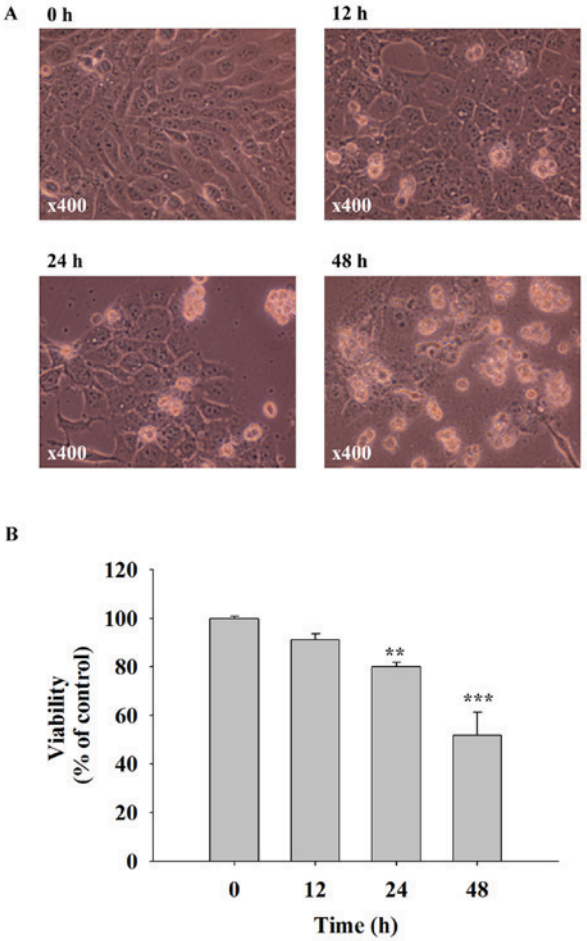

Figure 1. Quercetin induced cell morphological changes and decreased the percentage of viable cells in human oral cancer SAS cells. Cells ($1x10^5$ cells/well) were placed in a 12-well plate for 24 h and 40 μM quercetin was added to well for 0, 12, 24 and 48 h. (A) Cells were examined and images were captured using a contrast phase microscope. (B) Assays were performed to assess the percentages of viable cells. Each point is mean ± SD of three experiments. **P<0.01 and ***P<0.001 vs. vehicle control (1% DMSO).

Statistical analysis. All data are expressed as the mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance followed by the Dunnett's method. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using SigmaPlot for Windows (version 12.0; Systat Software, Inc., San Jose, CA).

Results

Quercetin induces cell morphological changes and decreases the cell viability of SAS cells. SAS cells were treated with 40 μM of quercetin for 0, 12, 24 and 48 h. Cells were examined for morphological changes and the percentage of viable cells. The results indicated that quercetin induced cell morphological changes including cell shrinkage and cell floating, and significantly decreased the quantity of viable cells (Fig. 1).

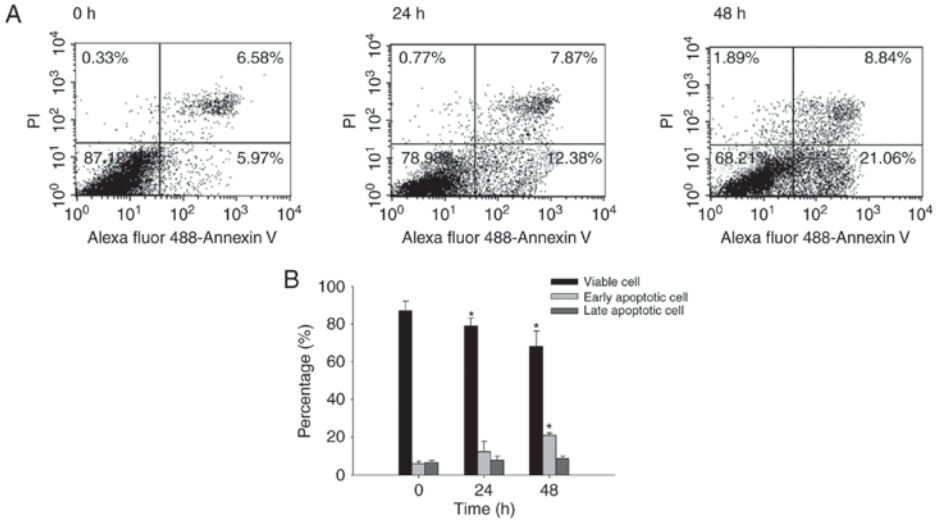

Figure 2. Quercetin induced apoptosis in human oral cancer SAS cells. SAS cells ($1x10^5$ cells/ml) in 12-well culture plates were treated with 40 μM quercetin for 0, 24 and 48 h. Results of (A) flow cytometry and (B) assay for percentage of apoptotic cells. *P<0.05 vs. vehicle control (0 h).

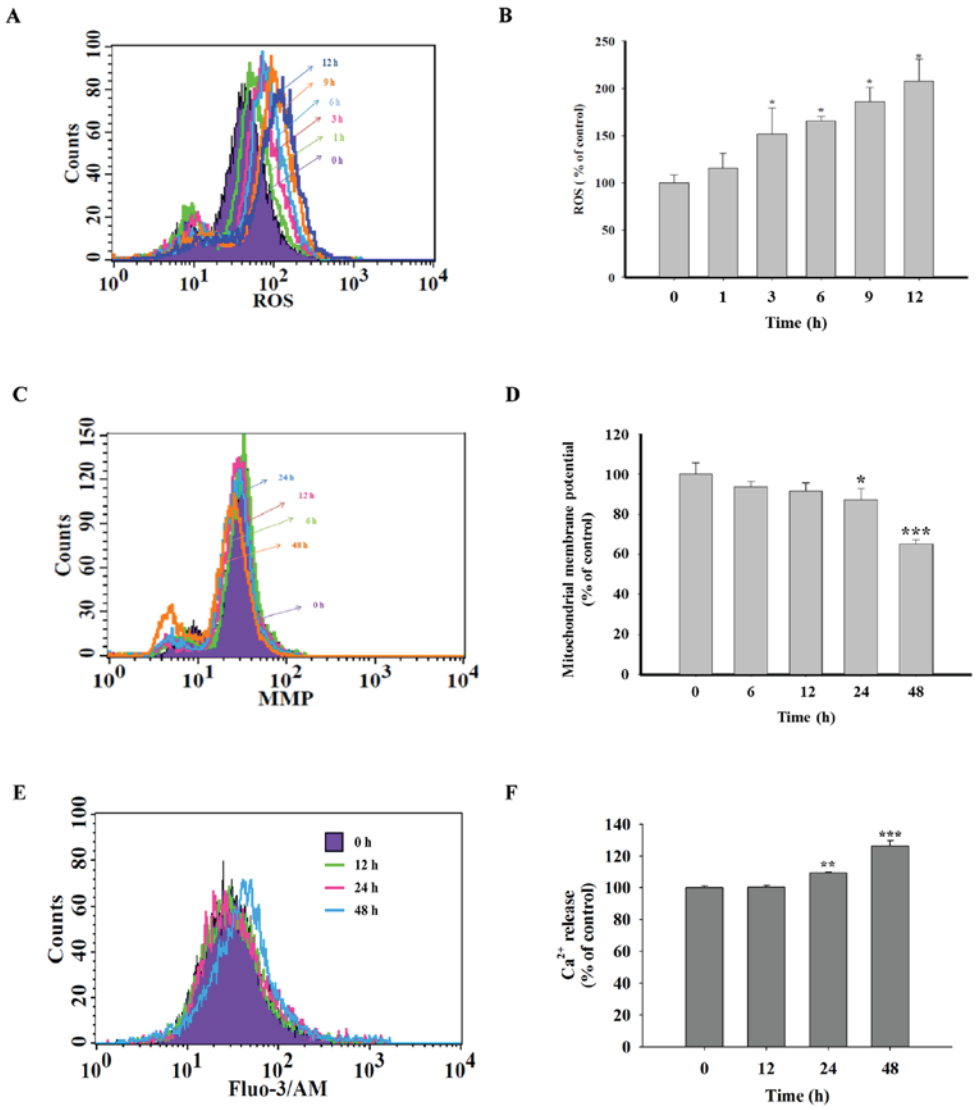

Figure 3. Quercetin affected the production of ROS and Ca^{2+} and the levels of MMP in human oral SAS cells. Cells ($1x10^5$ cells/well) were treated with 40 μM of quercetin for various time periods prior to being (A) stained by 2,7-dichlorodihydrofluorescein diacetate and (B) ROS levels quantified, (C) stained by $DiOC_6$ and (D) the MMP levels quantified, and (E) stained by Fluo-3/AM and (F) the Ca^{2+} levels quantified. Data represents mean ± SD of three experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle control (0 h). ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential; Fluo-3/AM, Fluo-3 acetoxymethyl ester.

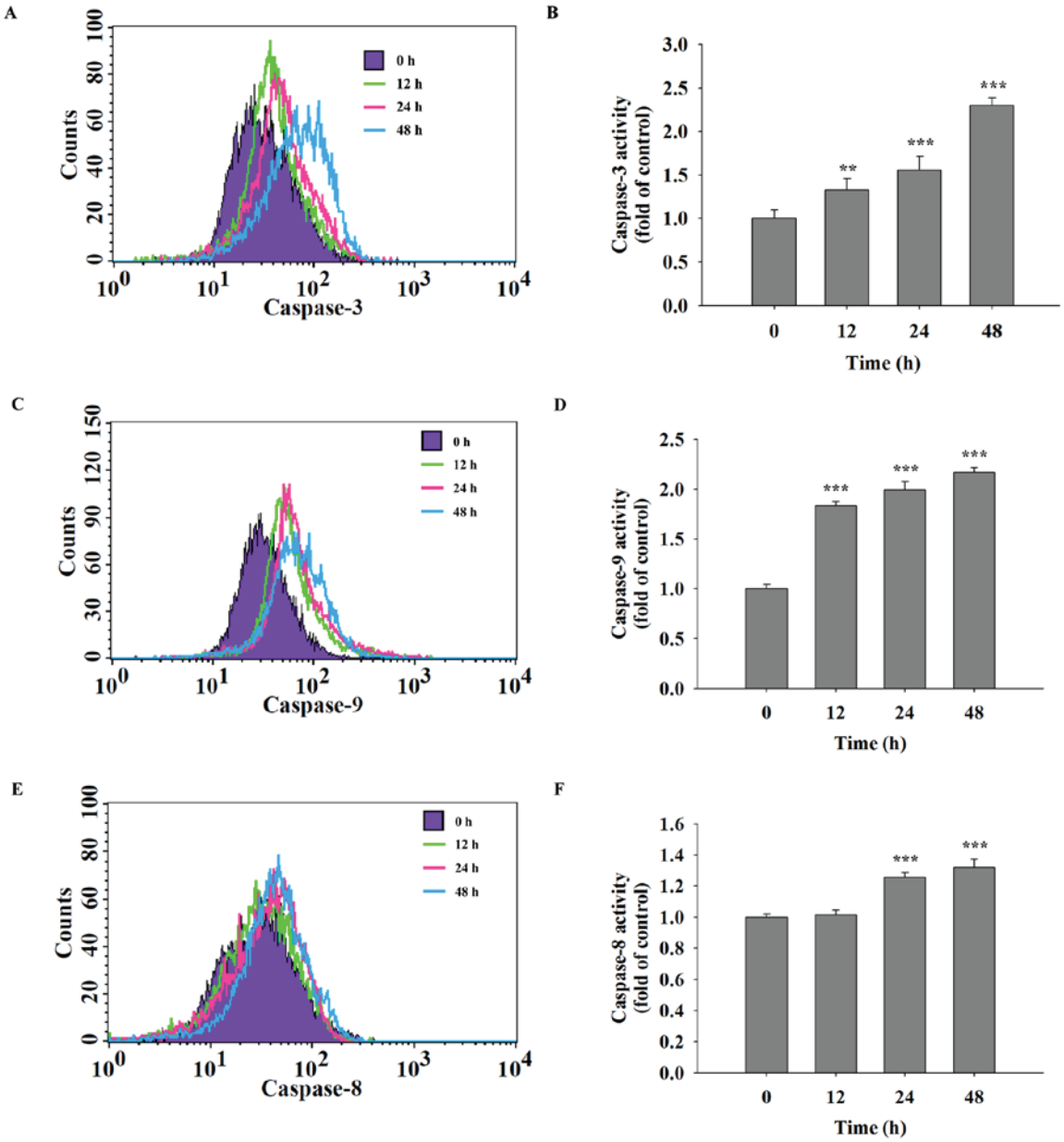

Figure 4. Quercetin stimulated the activities of caspase-3, caspase-9 and caspase-8 in SAS cells. Cells were treated with quercetin, and the activities of caspase-3, presented as (A) histogram of FACS and (B) Bar chart; caspase-9, presented as (C) histogram of FACS and (D) Bar chart; and caspase-8, presented as (E) histogram of FACS and (F) Bar chart, were determined by flow cytometric assay. **P<0.01 and ***P<0.001 vs. vehicle control (0 h).

These effects were time-dependent. A 40 μM dose of quercetin decreased cell viability to ~50% when compared with the control group (0 h) that was selected for all experiments.

Quercetin induces apoptosis in SAS cells. In order to investigate the effects of quercetin on apoptosis in SAS cells, Annexin V/PI-double staining was performed. The results of dot plots (Fig. 2A) indicated that quercetin induced apoptosis of cells, with a increase in the percentage of early apoptotic cells detected at 24 h (5.97%) and 48 h (21.06%). The percentage of apoptotic cells at different times are presented in Fig. 2B; the results indicate that quercetin significantly induced early-stage apoptosis in a time-dependent manner.

Quercetin induces ROS and Ca^{2+} production and decreases the levels of $\Delta\Psi_m$ in SAS cells. In order to investigate whether the cell apoptosis induced by quercetin in SAS cells involve the production of ROS and Ca^{2+} or dysfunction of mitochondrial, cells were treated with quercetin for various time periods and then analyzed using a flow cytometric assay. Fig. 3A and B identified that quercetin significantly increased ROS production following 3 h treatment. Fig. 3C and D showed that quercetin treatment significantly decreased the levels of $\Delta\Psi_m$ following 24 h treatment. In addition, it was demonstrated that quercetin significantly promoted Ca^{2+} production following 24 h treatment (Fig. 3E and F).

Quercetin increases the activities of caspase-3, caspase-8 and caspase-9 in SAS cells. It was hypothesized that quercetin was able to induce cell apoptosis in SAS cells through the activation of caspases. Following treatment of SAS cells with quercetin (40 μM) for various time periods, caspase-3, -9 and -8 activities

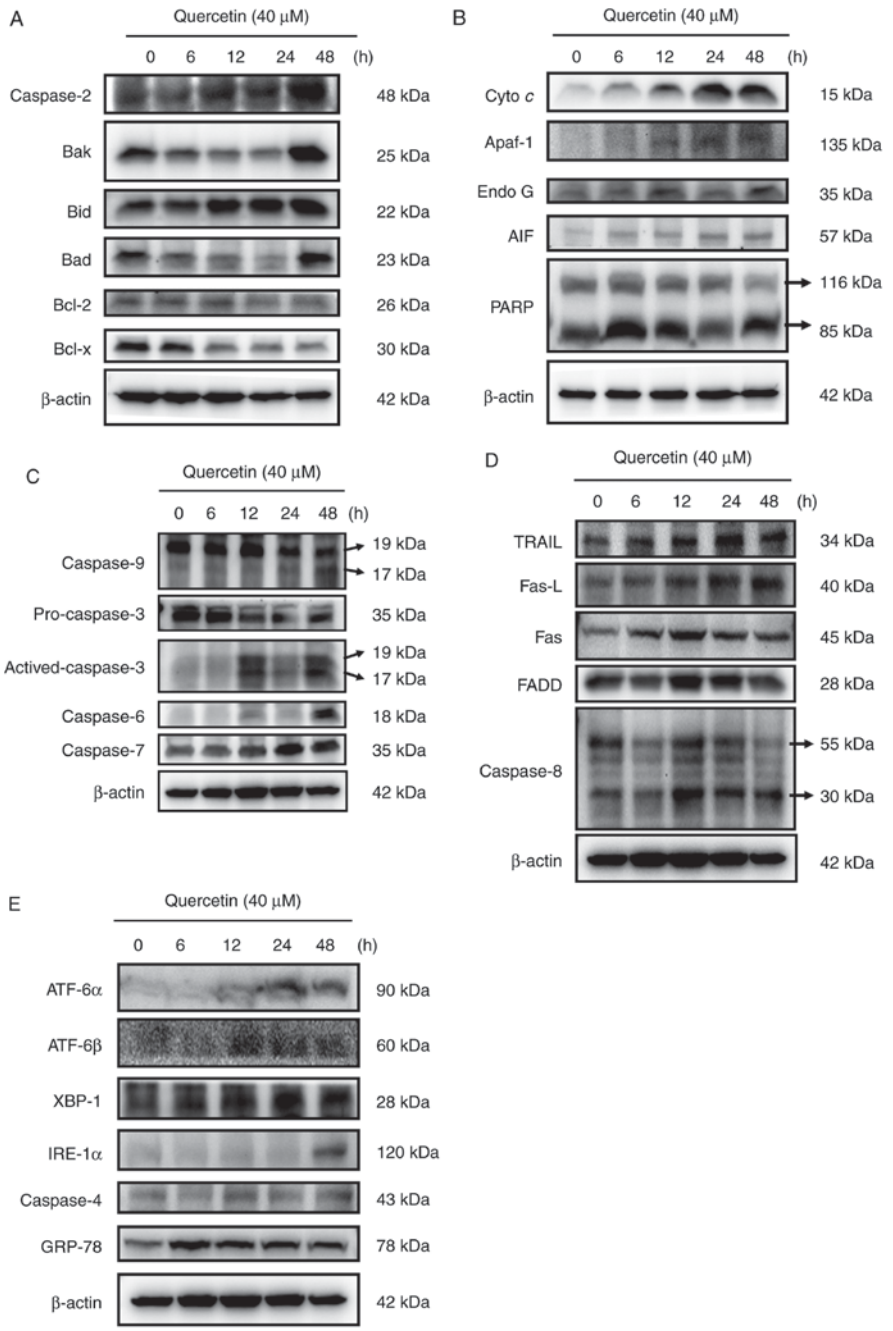

Figure 5. Quercetin affected the levels of apoptosis-associated proteins of SAS cells. Cells were treated with quercetin and the total protein levels were determined and used for SDS page gel electrophoresis. The levels of (A) caspase-2, Bak, Bid, Bad, Bcl-2 and Bcl-x; (B) cytochrome c, Apaf-1, Endo G, AIF and PARP; (C) caspase-9, pro-caspase-3, active-caspase-3, caspase-6 and caspase-7; (D) TRAIL, Fas-L, Fas, FADD and caspase-8; (E) ATF-6α, ATF-6β, XBP-1, IRE-1α and GRP-78 were examined. Bak, Bcl-2 homologous antagonist killer; Bid, BH3 interacting-domain death antagonist; Bad, Bcl-2-associated death promoter, Bcl-2, B cell lymphoma 2; Bcl-xl, Bcl-extra large; Apaf-1, apoptotic protease activating factor; Endo G, endonuclease G; AIF, apoptosis-inducing factor; PARP, poly(ADP-ribose) polymerase; TRAIL, TNF-related apoptosis-inducing ligand; Fas-L, Fas ligand; FADD, fas-associated protein with death domain; ATF-6β, activating transcription factor-6β; XBP-1, X-box binding protein 1; IRE-1α, iron responsive element-1α; GRP-78, gastrin releasing peptide-78.

were assayed using flow cytometric analysis. The results indicated that quercetin significantly increased the activities of caspase-3 (Fig. 4A and B), caspase-9 (Fig. 4C and D) and caspase-8 (Fig. 4E and F) in a time-dependent manner.

Quercetin alters apoptosis-associated protein expression in SAS cells. In order to understand whether quercetin induced the apoptosis of SAS cells through the effects of apoptosis-associated proteins, SAS cells were treated with quercetin (40 μM) for various time periods and then the levels of apoptosis-associated proteins were measured using western blotting. The results demonstrated that quercetin markedly increased the expression of caspase-2, Bak, Bid and Bad (Fig. 5A), Cyto c, Apaf-1, Endo G, AIF and PARP (Fig. 5B), active form of caspase-9, caspase-3, caspase-6 and -7 (Fig. 5C), TRAIL, Fas-L, Fas, FADD, and active form of caspase-8 (Fig. 5D), ATF-6α, ATF-6β, XBP-1, IRE-1α, caspase-4 and GRP-78 (Fig. 5E), but inhibited the expression of Bcl-2

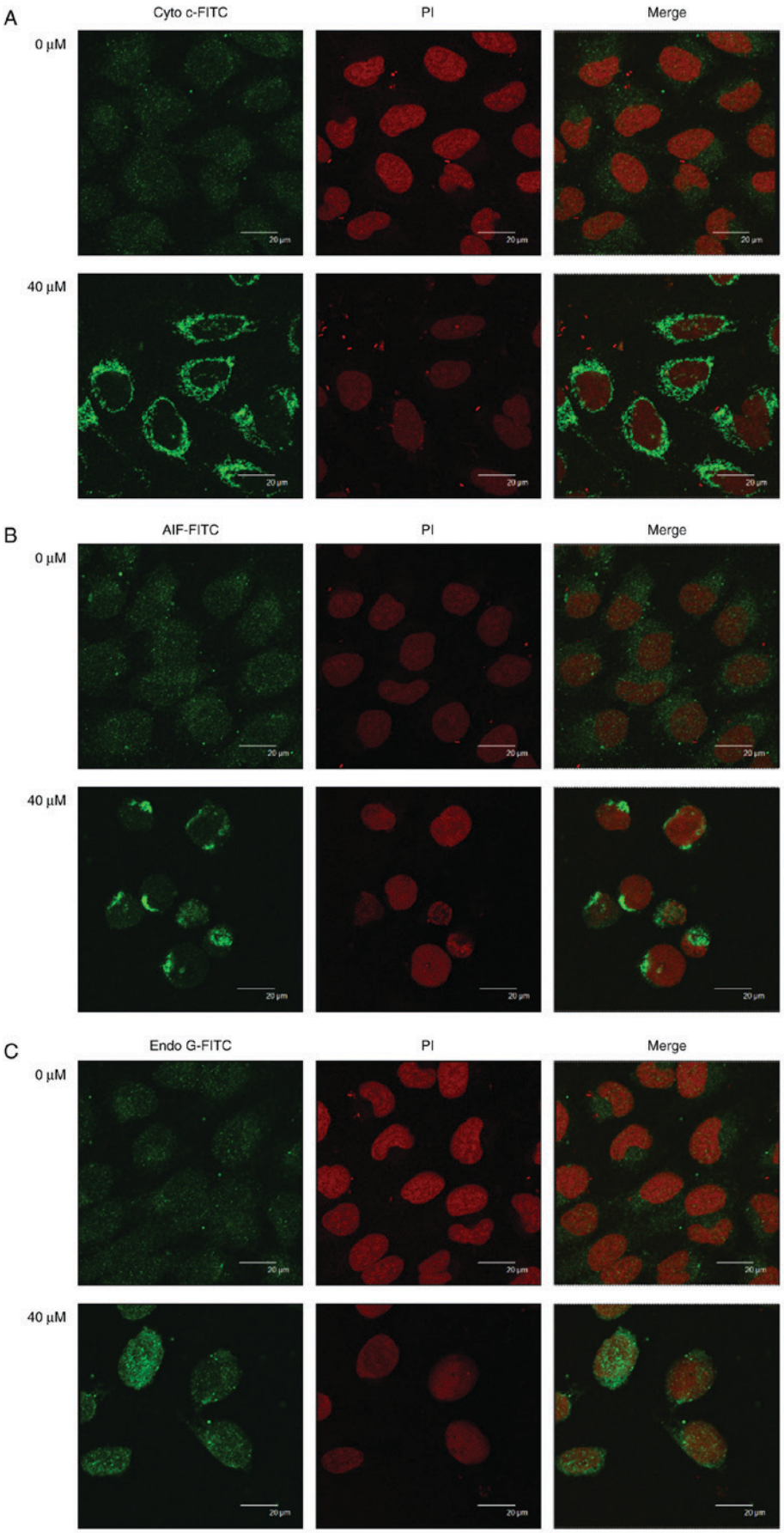

Figure 6. Quercetin promoted the levels of Cyto c, AIF and Endo G in SAS cells. Cells were incubated quercetin, fixed and stained with (A) anti-Cyto c, (B) anti-AIF and (C) anti-Endo G which were then stained by FITC-labeled secondary antibodies (green fluorescence) and the nuclei were stained by PI (red fluorescence). All stained proteins were examined and images were captured using a confocal laser microscopic system. Scale bar, 20 μm. Cyto C, cytochrome *c*; AIF, apoptosis-inducing factor; Endo G, endonuclease G; FITC, fluorescein isothiocyanate; PI, propidium iodide.

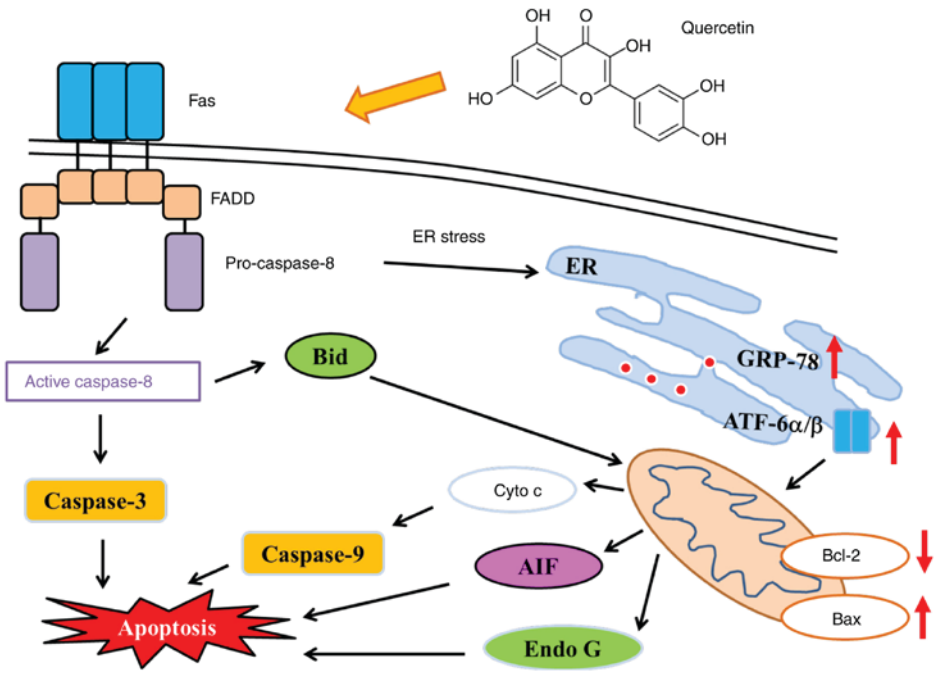

Figure 7. Proposed signaling pathways for quercetin induced apoptosis in SAS cells. FADD, fas-associated protein with death domain; ER, endoplasmic reticulum; Cyto c, cytochrome *c*; AIF, apoptosis-inducing factor; Endo G, endonuclease G; Bcl-2, B cell lymphoma 2; Bax, Bcl-associated X protein; Bid, BH3 interacting-domain death antagonist; GRP-78, gastrin-releasing peptide-78; ATF-6α/β, activating transcription factor-6α/β.

and Bcl-x (Fig. 5A), pro-caspase-3 (Fig. 5C). These results suggested that quercetin induced apoptosis of SAS cells via cell surface receptor (Fas-L and Fas) and mitochondria-dependent pathways.

Quercetin alters the translocation of apoptotic-associated proteins in SAS cells. In order to investigate the effect of quercetin on the release and translocation of Cyto c, AIF, and Endo G during apoptosis of SAS cells, cells were treated with or without 40 μM of quercetin for 48 h, then stained by anti-Cyto c, -AIF and -Endo G. Images were captured using a confocal laser microscopic system. The results revealed that quercetin markedly increased cytochrome *c* (Fig. 6A), AIF (Fig. 6B) and Endo G (Fig. 6C) release from mitochondria to the cytoplasm in SAS cells compared with the corresponding control group.

Discussion

Numerous studies have demonstrated that quercetin is able to induce cell death through cell cycle arrest and the induction of apoptosis in a number of human cancer cell lines (19-25). However, there remains a lack of available information to demonstrate quercetin-induced cell apoptosis of human oral cancer cells; thus, in the present study, the cytotoxic effects of quercetin on human oral cancer SAS cells were investigated *in vitro*. Notably, induction of cancer cell apoptosis has been recognized to be an optimal strategy of anti-cancer drugs (32,33). In the present study it was identified that quercetin: i) induced cell morphological changes and decreased total viable cell numbers; ii) induced apoptotic cell death; iii) increased ROS and Ca^{2+} levels, but decreased the $\Delta\Psi_m$; iv) increased the activities of caspase-3, caspase-9 and caspase-8; v) increased the levels of pro-apoptotic proteins, including Bak, Bid and Bad and cell surface receptors, including Fas-L and Fas, but decreased anti-apoptotic proteins including Bcl-2 and Bcl-x; and vi) induced Cyto c, AIF and Endo G release from mitochondria to cytoplasm which was confirmed by confocal laser microscopy.

The cell viability assay results indicated that quercetin induced cytotoxic effects based on the morphological changes observed and decreased the total number of viable cells. Furthermore, Annexin V/PI staining demonstrated that quercetin induced cell apoptosis. These results demonstrated that quercetin may have decreased cell numbers partially through the induction of cell apoptosis. Numerous studies have identified that DNA fragmentation is one of the characteristics of apoptotic cell death (33-35). Annexin V/PI staining was used to confirm that quercetin induced apoptosis of SAS cells. Annexin V/PI staining is recognized as a protocol for measuring and quantifying the percentage of apoptotic cells (36,37).

It was hypothesized that quercetin-induced apoptotic cell death involved ROS and Ca^{2+} production, and decreased the levels of $\Delta\Psi_m$ in SAS cells. It was identified that quercetin increased the production of ROS and Ca^{2+}. It has been reported that ROS production is involved in agent-induced cancer cell apoptosis (38-40). It has also been reported that agent-induced ER stress leads to Ca^{2+} release from the endoplasm leading to cell apoptosis (41). Based on the data of the present study, it is hypothesized that quercetin-induced apoptotic cell death involves ROS and Ca^{2+} production in SAS cells. Numerous studies have identified that agent-induced cell apoptosis may be due to a dysfunction in mitochondria or decreased levels of $\Delta\Psi_m$ (13,42,43). The results in the current study also identified that quercetin decreased the levels of $\Delta\Psi_m$ in SAS cells. The results from western blotting revealed that quercetin increased

the protein expression of TRAIL, Fas-L, Fas, FADD and caspase-8, which indicated that quercetin induced apoptosis of SAS cells through cell surface death receptors and mitochondria-dependent pathways. It has also been reported that quercetin treatment resulted in apoptosis in human oral cancer HSC-3 and TW206 cells through Fas, and caspase-3 activation (25). It was also identified that quercetin increased the active form of caspase-3 and caspase-9 in SAS cells in the current study.

It is well documented that the balance between pro- and anti-apoptotic proteins regulate cell viability (44), and drug-induced apoptotic pathways modulate pro- and anti-apoptotic protein expression (45). Western blotting was used for additional examination of the effects of apoptosis-associated protein expression, with results indicating that quercetin induced upregulation of major pro-apoptotic proteins including Bak, Bid and Bad downregulation of major anti-apoptotic proteins including Bcl-2 and Bcl-x in SAS cells. The results of the present study indicated that the anti-apoptotic/pro-apoptotic protein ratio was markedly decreased at 48 h post-treatment in SAS cells. It has been reported that decreased levels of $\Delta\Psi_m$ during apoptosis may be associated with drug-induced Bcl-2/Bax imbalance (46). The results of the present study demonstrate that quercetin decreased the levels of $\Delta\Psi_m$ in SAS cells at 24-48 h treatment. The western blotting results indicated that quercetin increased the expression of Cyto c, AIF and Endo G, which was confirmed using confocal laser microscopy. These data suggest that Cyto c, AIF and Endo G expression levels are markedly increased in SAS cancer cells. Based on the aforementioned results, it is hypothesized that quercetin-induced cell apoptosis occurs partially via mitochondria-mediated signaling pathways in SAS cells.

In conclusion, quercetin affects the ratio of anti-/pro-apoptotic proteins, which may lead to dysfunction of mitochondria (decreased levels of $\Delta\Psi_m$) followed by the release of Cyto c, AIF and Endo G from mitochondria, inducing cell-destruction by triggering apoptosis (Fig. 7). An understanding of the underlying molecular mechanism of the action of quercetin in human oral SAS cells *in vitro* may provide valuable information for its potential application in oral cancer prevention and therapy in the future.

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Availability of data and materials

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

Authors' contributions

YSM, CNY, HCL, FSC and JGC conceived and designed the experiments. YSM, CNY and HCL performed the experiments. YSM, HCL, FSY, JJL, KWL and CLL analyzed the data and contributed in reagents/materials/analysis tools. FSC and JGC wrote the paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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