

Berberine Induced Apoptosis *via* Promoting the Expression of Caspase-8, -9 and -3, Apoptosis-inducing Factor and Endonuclease G in SCC-4 Human Tongue Squamous Carcinoma Cancer Cells

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Abstract. Many phytochemicals have been recognized to have potential chemopreventive or chemotherapeutic efficacy in cancer treatment. In this study, we hypothesized that berberine would have anticancer activities in SCC-4 human tongue cancer cells. Results indicated that berberine reduced the viability of SCC-4 cells, which was initiated by the generation of reactive oxygen species, via an increase in cytosolic Ca^{2+} . Berberine-induced apoptosis was associated with a reduction of the mitochondrial membrane potential associated with changes in the Bax/Bcl-2 ratio, release of cytochrome c from mitochondria and activation of down stream caspase-3. Real-time PCR showed that berberine stimulated gene expression of caspase-8, -9 and -3, apoptosis-inducing factor and endonuclease G. The present study demonstrated that berberine-mediated apoptosis of SCC-4 cells is regulated by ROS, mitochondria, caspase-3-dependent and mitochondria-dependent pathways, suggesting that berberine may be considered for future studies as a promising therapeutic candidate for human tongue cancer.

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Oral and oropharyngeal carcinomas total over 300,000 cases annually throughout the world and are more common among males than females (1). Tobacco and alcohol consumption are reported to be the major factors for the development of oral cancer (2, 3), with other contributing factors such as diets low in carotenoids and vitamin A, poor oral hygiene and indoor air pollution (4-6). Diets deficient in vegetables, fruits, and micronutrients are important in the pathogenesis of esophageal cancer (7, 8). Betel quid chewing is one of the important causes of oral cancer in Taiwan, and 9.6 persons per 100 thousand die annually from oral cancer based on reports from the Peoples Health Bureau of Taiwan. Typical treatments of patients with oral cancer include surgery, radiotherapy and chemotherapy; however, the cure rates are not satisfactory. Therefore, many investigators are focusing on identifying new agents and novel targets for treating oral cancer.

Berberine, a natural alkaloid, possesses antibacterial (9), antioxidative (10) and anti-inflammatory (11) activities anti-carcinogenic activity in skin (12), inhibits cyclooxygenase-2 transcriptional activity in human colon cancer cells (13-15) and has antimetastatic properties in non-small cell lung cancer cells (16). Berberine was also found to inhibit activator protein-1, which is a key transcription factor in inflammation and carcinogenesis in human cell lines (13) and DNA topoisomerase II (17). Other reports showed that berberine had cytotoxic activity in human leukemia U937 and murine melanoma B16 cells (18), and inhibited growth in human epidermoid carcinoma A431 cells (19) and prostate cancer cells (20) which was associated with activation of caspases.

The induction of apoptosis is recognized as an important mechanism of action for an anticancer agent. We have shown previously that berberine induced cell cycle arrest and apoptosis in HSC-3 human oropharyngeal cancer cells (21) but there is no information on apoptotic induction in human tongue cancer cells. Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity (22, 23). The purpose of this study was to investigate the effects of berberine on the induction of apoptosis in the human tongue cancer SCC-4 cell line, which is an oral squamous cell carcinoma.

Materials and Methods

Materials and chemicals. Berberine, dimethyl sulfoxide (DMSO), trypan blue, propidium iodide (PI) and triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), Indo 1/AM and DiOC₆ were from Calbiochem (La Jolla, CA, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. SCC-4 human tongue squamous carcinoma cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were plated in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 1% penicillin-streptomycin (100 units/ml penicillin and 100 µg/ml streptomycin) and 1% L-glutamine under an atmosphere of humidified 5% CO₂ and 95% air grown at 37°C and one atmosphere in an incubator.

Viable cell determination. In all treatments, berberine (Sigma Chemical Co.) was dissolved initially in a small amount of DMSO. Approximately 2×10⁵ SCC-4 cells/well on a 12-well plate were treated with 0, 15, 25, 50, 75 or 100 µM berberine, or only with vehicle (DMSO, 1% in culture media) and all cells were incubated for 12, 24 or 48 h. For determining cell viability, the trypan blue exclusion and flow cytometric protocol were used, as previously described (21, 24, 25). An aliquot of the total cell suspension from each sample was mixed with an equal volume of trypan blue in PBS and incubated for 5 min at room temperature. Under a microscope, viable cells are a white color and dead cells are a light blue color. A Neubauer chamber was used for counting total viable cells.

Apoptosis determination by flow cytometric assay. Berberine-induced apoptosis in human tongue cancer cells was quantitatively determined by flow cytometry as described elsewhere (26). Briefly, SCC-4 cells were treated with berberine (0, 15, 25, 50, 75 and 100 µM) for 48 h. Cells were then harvested by brief trypsinization, washed with PBS, and incubated with PI for cellular staining in binding buffer at room temperature for 30 min in the dark. The stained cells were analyzed using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software. The sub-G₁ group was representative of mean apoptosis.

DAPI staining. Detection of apoptotic cells was accomplished by fluorescence staining. Approximately 2×10⁵ SCC-4 cells/well on 6-well plates were treated with 75 µM berberine or vehicle only

Table I. Primers used in PCR analysis of SCC-4 cells.

| Primer name | Primer sequence |
|-----------------|---------------------------|
| homo caspase3-F | CAGTGGAGGCCGACTTCTTG |
| homo caspase3-R | TGGCACAAAGCGACTGGAT |
| homo caspase8-F | GGATGGCCACTGTGAATAACTG |
| homo caspase8-R | TCGAGGACATCGCTCTCTCA |
| homo caspase9-F | TGTCCTACTCTACTTTCCAGGTTTT |
| homo caspase9-R | GTGAGCCCACTGCTCAAAGAT |
| homo AIF-F | GGGAGGACTACGGCAAAGGT |
| homo AIF-R | CTTCCTTGCTATTGGCATTCTG |
| homo EndoG-F | GTACCAGGTCATCGGCAAGAA |
| homo EndoG-R | CGTAGGTGCGGAGCTCAATT |
| homo GAPDH-F | ACACCCACTCCTCCACCTTT |
| homo GAPDH-R | TAGCCAAATTCGTTGTCATACC |

Each assay was conducted at least twice to ensure reproducibility; F, Forward; R, Reverse.

(DMSO) and incubated for 24, 48 or 72 h under 5% CO₂ and 95% air at 37°C. Cells were individually stained by 4'-6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) and then examined and photographed using fluorescence microscopy as described elsewhere (24).

Comet assay. Approximately 2×10⁵ SCC-4 cells/well of on 12-well plates were incubated with berberine at final concentrations of 0, 50, 75 or 100 µM, or vehicle (DMSO) and grown at 37°C in 5% CO₂ and 95% air. Cells were harvested for the examination of DNA damage using the comet assay as described elsewhere (27, 28).

Assay for reactive oxygen species (ROS), cytosolic Ca²⁺ concentration and mitochondrial membrane potential ($\Delta\Psi_m$). Approximately 2×10⁵ SCC-4 cells/well on 12-well plates were incubated with 75 µM berberine for 0, 0.1, 0.5, 1, 3, 6, 12 or 24 h to determine the production of ROS and cytosolic Ca²⁺ and the level of $\Delta\Psi_m$. Cells were harvested, washed twice by PBS, then were re-suspended in 500 µl of DCFH-DA (10 µM) for ROS, in Indo 1/AM (3 µg/ml) for cytosolic Ca²⁺ production, and in 500 µl of DiOC₆ (4 µmol/l) for the level of $\Delta\Psi_m$ in a dark room for 30 min at 37°C, then were analyzed immediately by flow cytometry (Becton Dickinson FACS Calibur) as described previously (21, 29).

Western blotting analysis. Levels of proteins (Bcl-2, Bcl-xL, Bax, Bad, Bak, cytochrome c, Apaf-1, caspase-9, caspase-3, Fas, FADD and caspase-8) associated with apoptosis were determined in cells incubated with 75 µM berberine for 0, 12, 24, 48 and 72 h. Lysates of treated cells were prepared using lysis buffer as described previously (30, 31). Each sample was incubated with primary antibody (Santa Cruz Biotechnology) then for secondary antibody then was detected by ECL kit and autoradiography using X-ray film. To ensure equal protein loading, each membrane was stripped and reprobed with anti-β actin antibody.

Assay for mRNA levels of AIF, caspase-3, -8 and -9 and Endo G. Cells were treated with 75 µM berberine for 0, 24 or 48 h and total RNA was extracted using the Qiagen RNeasy Mini Kit as described previously (32, 33). RNA samples were reverse-transcribed with High Capacity cDNA Reverse Transcription Kit

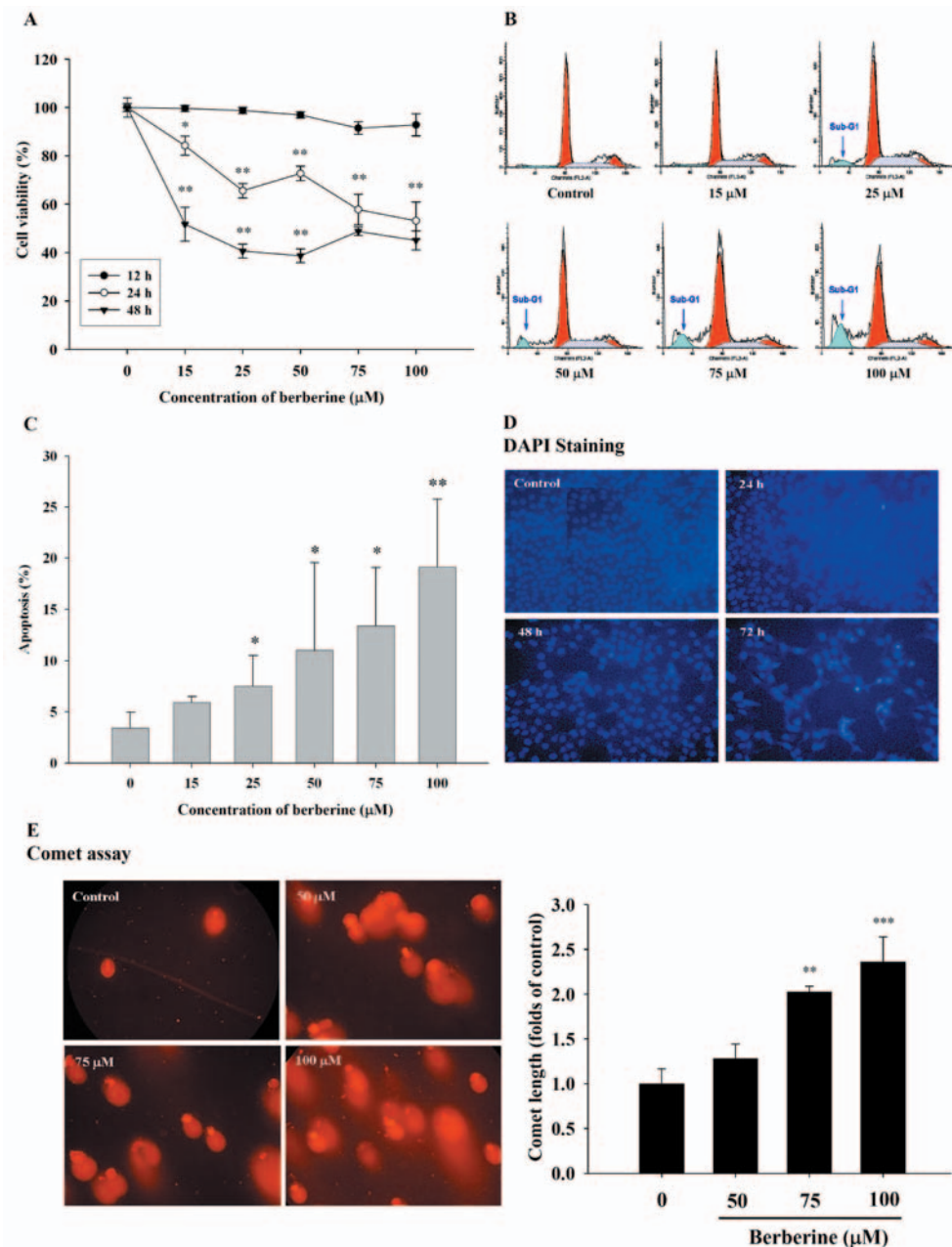


Figure 1. Berberine-induced apoptosis and DNA damage in SCC-4 cells. Cells were treated with 0, 15, 25, 50, 75 and 100 μM berberine for 12, 24 and 48 h. Viable cells (A), sub- G_1 phase (B) and apoptosis (C) of SCC-4 cells were determined by flow cytometric assay. Cells were treated with 75 μM berberine for 0, 24, 48 and 72 h. DNA condensation, apoptosis and DNA damage were examined by DAPI staining (D) and comet assay (E), respectively, as described in Materials and Methods. Each point is the mean \pm S.D. of three experiments. * $p < 0.05$; ** $p < 0.01$ Significantly different from the control.

at 42°C for 30 min according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, 1 min at 60°C using 1 μl of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers (Table I). Each assay was run on an Applied Biosystems 7300 real-time PCR system in

triplicates and expression fold-changes were derived using the comparative C_T method.

Statistical analysis. The statistical differences between the berberine-treated and control samples were calculated by Student's *t*-test. A *p*-value of < 0.05 was considered significant. The results from the *in vitro* studies are representative of at least two or three independent experiments. The quantitative data are shown as mean \pm SD.

Results

Berberine-induced apoptosis and DNA damage in SCC-4 cells. The potential cytotoxic effects of berberine on SCC-4 human tongue cancer cells were investigated by trypan blue exclusion assay and also confirmed by flow cytometric assay. Berberine reduced the percentage of viable cells in a dose and time-dependent manner, with an IC_{50} (50% inhibitory concentration) value of 75 μ M after 48 h of treatment (Figure 1A). The cells were treated with berberine (0-100 μ M) for 48 h. The number of cells in each compartment of the cell cycle and sub- G_1 phase were expressed as a percentage of the total number of cells. The data showed that berberine induced apoptosis (sub- G_1 phase) in a dose-dependent manner (Figure 1B and C). When SCC-4 cells were treated with 75 μ M berberine for 0, 24, 48 and 72 h, DAPI assay demonstrated that berberine induced apoptosis in a time-dependent manner (Figure 1D). Comet assays also demonstrated that berberine (50, 75 and 100 μ M) for 24 h induced DNA damage in a dose-dependent manner (Figure 1E).

The effects of berberine on reactive oxygen species, cytosolic Ca^{2+} concentration and $\Delta\Psi_m$ level in SCC-4 cells. The results from flow cytometric analysis for ROS production, cytosolic Ca^{2+} concentration and $\Delta\Psi_m$ level are shown in Figure 2A, B and C. A significant increase in intracellular ROS was observed in the berberine-treated cells but after 12 h of berberine treatment, ROS levels were somewhat lower than those of the control (Figure 2A). Berberine initially significantly increased cytosolic Ca^{2+} levels, but after 6 h of incubation, cytosolic Ca^{2+} levels were not significantly different from that in controls (Figure 2B). It was also observed that berberine significantly reduced the level of $\Delta\Psi_m$ in SCC-4 cells in a time-dependent manner (Figure 2C).

Effects of berberine on mRNA expression and levels of apoptosis-associated proteins in SCC-4 cells. Levels of anti apoptosis proteins Bcl-2 and Bcl- x_L (Figure 3A) decreased and the levels of pro-apoptosis proteins Bax, Bad and Bak (Figure 3A) were up-regulated in SCC-4 cells treated with berberine. Cytochrome *c*, Apaf-1, caspase-9 and -3 (Figure 3B) and Fas, FADD and caspase-8 (Figure 3C) were also up-regulated, which could contribute to apoptosis. Expression levels of *AIF*, *caspase-3*, -8 and -9 and *EndoG* mRNA assembly by real-time PCR were increased in SCC-4 cells treated with 75 μ M of berberine for 24 and 48 h. These effects were time dependent (Figure 3D).

Discussion

Cancer is the major cause of death in humans, resulting from a stepwise and progressive disruption of cellular signaling cascades controlling cell proliferation, survival and differentiation (34).

Inducing apoptosis in cancer cells is one of the major strategies of cancer therapeutics. It is well known that apoptosis can be divided into the extrinsic, caspase-dependent pathway and the mitochondria-dependent pathway (35-37). Caspase-dependent pathways include activation of caspase-8, -9 and -3 while mitochondrial pathways are involved in the efflux of cytochrome *c* from mitochondria to the cytosol, forming apoptosomes with Apaf-1 and caspase-9, and leading to the activation of caspase-3 and apoptosis (38). In fact, both pathways are intricately related. There is evidence that phytochemicals have potential chemopreventive or chemotherapeutic efficacy against various types of (39). Berberine has been shown to induce toxicity in cells including some types of cancer cells (10, 19, 40, 41) and the purpose of the present study was to determine if berberine would induce apoptosis in SCC-4 oral squamous cell carcinoma human tongue cancer cells. Oral squamous cell carcinoma is the most common malignancy of the oral cavity (42, 43).

Figure 1A indicated that the berberine-induced decrease in the percentage of viable cells may be due to apoptotic cell death. The results shown in Figures 1B and 1C demonstrated that berberine does indeed induce the apoptosis of SCC-4 cells. These observations were verified by the increase of sub- G_1 DNA content (Figure 1B), DAPI staining (Figure 1D) and it also showed that berberine induced DNA damage (Figure 1E) in SCC-4 cells.

For further investigations of the molecular mechanism involved in apoptosis caused by berberine, the expression of the apoptosis-related proteins Bcl-2, Bcl- x_L , Bax, Bad, Bak, caspase-3, -8 and -9, cytochrome *c*, Apaf-1, Fas, FADD and changes in $\Delta\Psi_m$ were assessed in SCC-4 cells. A decrease in the ratio of Bax/Bcl-2 may cause the collapse of $\Delta\Psi_m$, resulting in the release of cytochrome *c* thus causing apoptosis (44). Our results from flow cytometric analysis demonstrated that berberine reduced the levels of $\Delta\Psi_m$ (Figure 2C). The results from Western blotting also showed that berberine promoted the levels of cytochrome *c* (Figure 3B), promoted pro-apoptotic proteins such as Bax, Bad and Bak and inhibited the levels of anti-apoptotic proteins such as Bcl-2 and Bcl- x_L . There are data indicating that Bax can disrupt mitochondrial membrane integrity and also that formation of the mitochondrial permeability transition (MPT) pore (45) can occur, resulting in the release of cytochrome *c*. Previous work has shown that the MPT is accompanied by mitochondrial depolarization, respiratory inhibition or stimulation, matrix swelling, matrix pyridine nucleotide depletion, and release of intermembrane proteins, including cytochrome *c* (46).

The cell death receptor pathway is mediated distinctively by active/cleaved caspase-8 that is characterized by binding cell death ligand and cell death receptors followed by activation of caspase-8 and caspase-3 (47) for apoptosis to occur. The results from this study indicated that cleaved caspase-8, -9 and -3 (Figure 3B and C) increased in SCC-4 cells treated with berberine. Moreover, the increase of cleaved caspase-9 led to

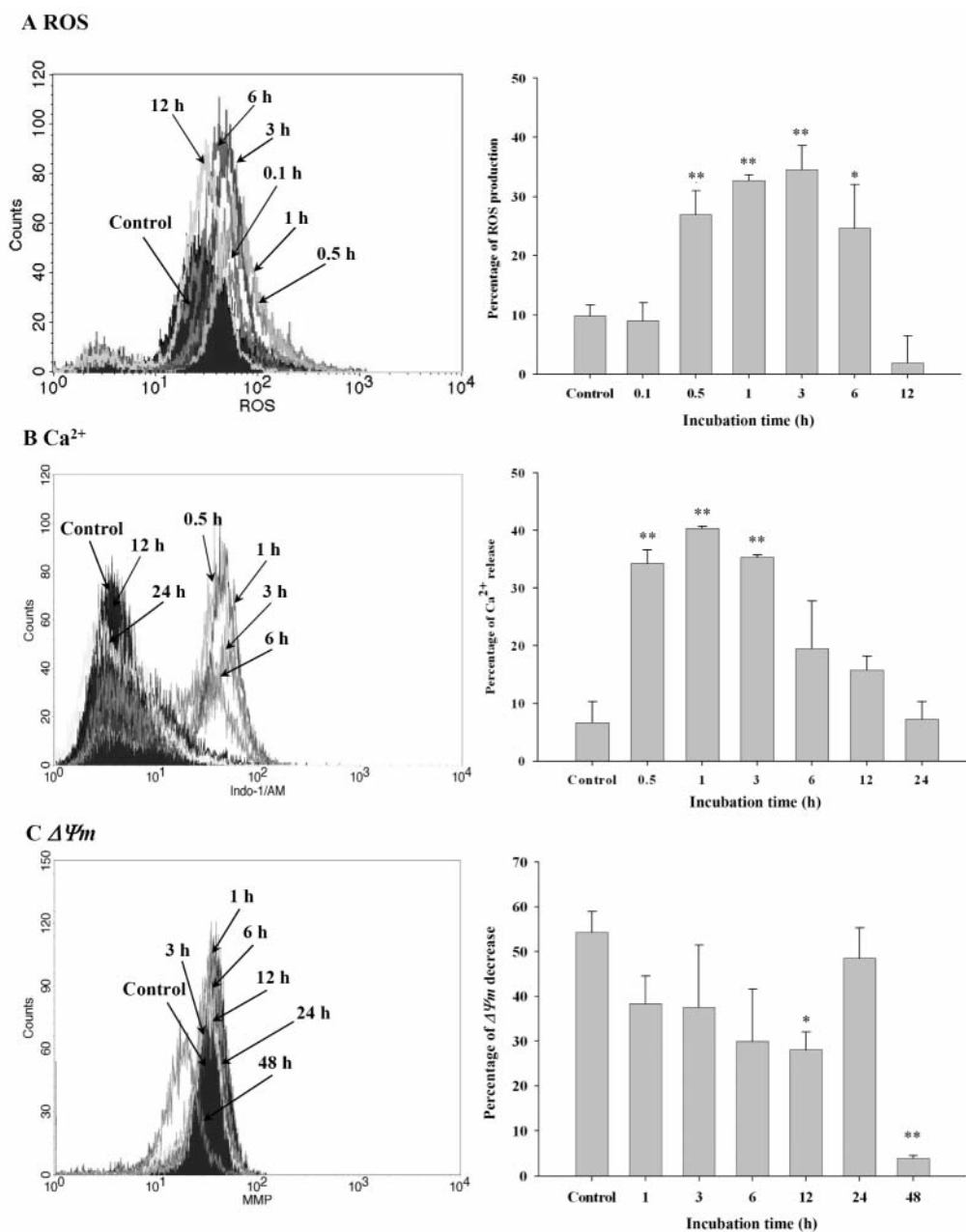


Figure 2. Effects of berberine on ROS, cytosolic Ca²⁺ concentrations and $\Delta\Psi_m$ levels in SCC-4 cells. Cells were incubated with 75 μ M berberine for different time periods. The percentage of cells were stained individually by DCFH-DA for ROS (A), Indo1/AM dye for cytosolic Ca²⁺ (B) and DiOC₆ for $\Delta\Psi_m$ (C), and were quantified by flow cytometry as described in the Materials and Methods. * $p < 0.05$; ** $p < 0.01$ Significantly different from the control group.

subsequent activation of the downstream caspase-3 (an apoptotic executioner) (Figure 3B). Our result also showed that berberine promoted the expression of Apaf-1 which led to promotion of caspase-3 activation thus causing apoptosis.

In summary, berberine appears to induce apoptosis in SCC-4 human tongue cancer cells *via* a mechanism shown in

Figure 4. Fas (adaptor proteins) appears to act as a receptor for berberine.

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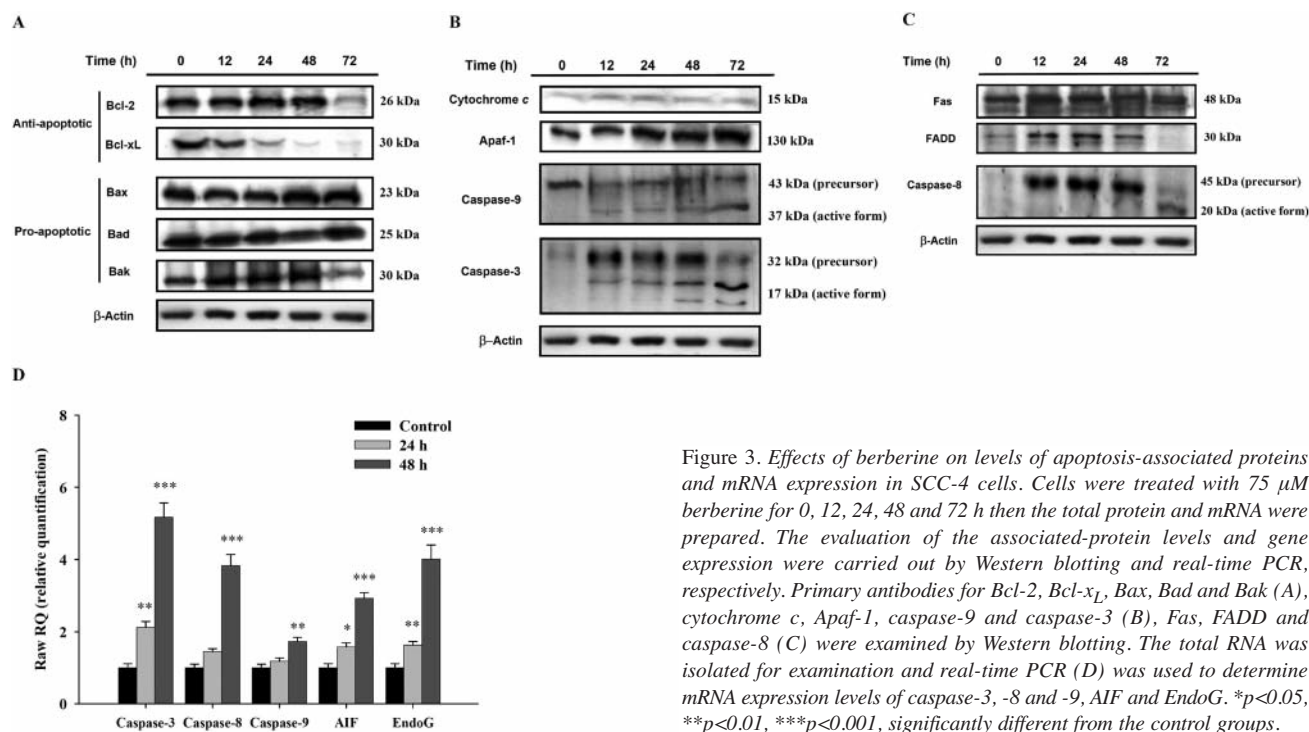


Figure 3. Effects of berberine on levels of apoptosis-associated proteins and mRNA expression in SCC-4 cells. Cells were treated with 75 μ M berberine for 0, 12, 24, 48 and 72 h then the total protein and mRNA were prepared. The evaluation of the associated-protein levels and gene expression were carried out by Western blotting and real-time PCR, respectively. Primary antibodies for Bcl-2, Bcl-xL, Bax, Bad and Bak (A), cytochrome c, Apaf-1, caspase-9 and caspase-3 (B), Fas, FADD and caspase-8 (C) were examined by Western blotting. The total RNA was isolated for examination and real-time PCR (D) was used to determine mRNA expression levels of caspase-3, -8 and -9, AIF and EndoG. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from the control groups.

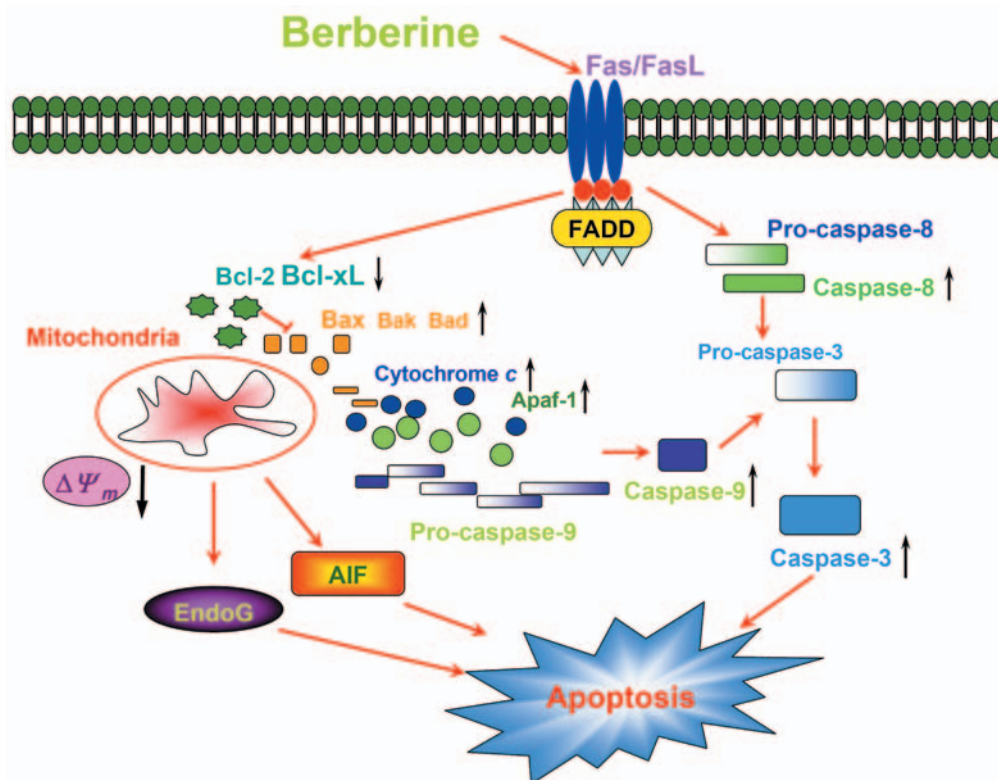


Figure 4. Proposed model for berberine-mediated apoptosis in SCC-4 human tongue cancer cells. Berberine increases production of ROS and cytosolic Ca^{2+} levels, resulting in stimulation of Bax protein expression and reduction of Bcl-2. Mitochondrial membrane potential ($\Delta\Psi_m$) is then reduced and cytochrome c is released, which activates caspases -9 and -3 producing apoptosis in the SCC-4 human tongue squamous cell carcinoma cancer cell line.

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