Bee Venom Induced Cell Cycle Arrest and Apoptosis in Human Cervical Epidermoid Carcinoma Ca Ski Cells

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Abstract. *Although it has been previously reported that bee venom (BV) can induce apoptosis in many cancer cell lines, there is no information on the effect of BV on human cervical cancer cells and its molecular mechanisms of action are not fully elucidated. In this study, the possible mechanisms of apoptosis by which BV acts on human cervical cancer Ca Ski cells were investigated. BV induced morphological changes and decreased the percentage of viable Ca Ski cells in a dose- and time-dependent manner. Flow cytometric analysis demonstrated that BV induced the production of reactive oxygen species, increased the level of cytoplasmic Ca^{2+}, reduced mitochondrial membrane potential which led to cytochrome c release, and promoted the activation of caspase-3 which then led to apoptosis. BV also induced an increase in the levels of Fas, p53, p21 and Bax, but a decrease in the level of Bcl-2. The activities of both caspase-8 and caspase-9 were enhanced by BV, promoting caspase-3 activation, leading to DNA fragmentation. Based on the DNA fragmentation and DAPI staining, BV-induced apoptosis was mitochondrial-dependent and caspase-dependent. BV also promoted the expression of AIF and Endo G in the Ca Ski cells. Both AIF and Endo G proteins were released from the mitochondria, and then induced apoptosis which was not through activation of caspase. In conclusion, our data demonstrated that BV-induced apoptosis occurs via a Fas receptor pathway involving mitochondrial-dependent pathways and is closely related to the level of cytoplasmic Ca^{2+} in Ca Ski cells.*

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Key Words: Bee venom (BV), apoptosis, caspase, reactive oxygen species, cytoplasmic calcium (Ca^{2+}), mitochondria.

Bee venom (BV) has been used in oriental medicine for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, and in relief of pain (1-3). BV has been demonstrated to possess biological activities, such as inducing analgesic and anti-inflammatory effects (1, 4-6), a recognized property of non-steroidal anti-inflammatory drugs (NSAIDs). It has been reported that BV induced apoptosis in synovial fibroblasts *via* caspase-3 activation (7) and inhibition of cyclooxygenase (Cox)-2 expressions in human lung cancer cells (8). Furthermore, it has been determined that BV inhibited mammary carcinoma cell proliferation and tumor growth *in vivo*. However, it also caused tumor rejection by stimulation of the local cellular immune responses in lymph nodes (9, 10). BV has been reported to induce apoptosis in proliferating vascular smooth muscle cells *via* suppression of NF-κB and Akt activation, and down-regulation of Bcl-2 (11). Recently, it has been reported that the key regulators in BV-induced apoptosis in human leukemia U937 cells are Bcl-2 and caspase-3 through down-regulation of the ERK and Akt signal pathway (12, 13).

Apoptosis plays an important role in the development and homeostasis of multicellular organisms and it is a well regulated and organized death process that occurs under a variety of physiological and pathological conditions (13). It is well-known that apoptotic features include cellular morphological change, membrane blebbing, chromatin condensation, oligonucleosomal DNA cleavage, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet, dysfunction of mitochondria and activation of caspases (14-16). Caspase activation and dysfunction of mitochondria are generally recognized to be the key hallmarks of apoptosis, but there is no available information on the effect of BV on human cervical cancer cells. Therefore, in this study the molecular mechanisms of action and the role of mitochondria in the induction of apoptosis in Ca Ski cells caused by BV were investigated.

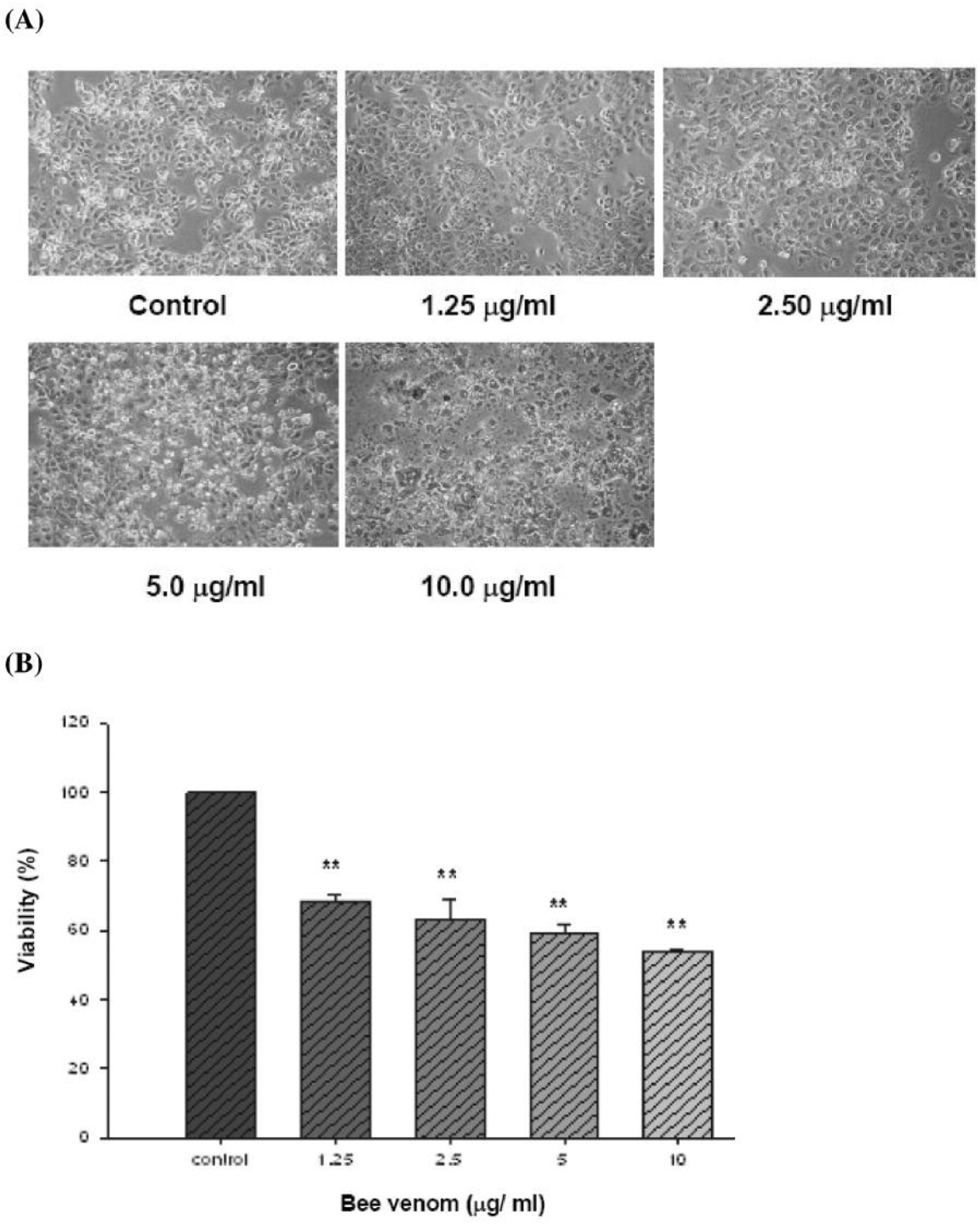

Figure 1. *continued*

Materials and Methods

Chemicals and reagents. The bee venom, dichlorodihydro-fluorescein diacetate (DCFH-DA), propidium iodide (PI), RNase, trypan blue, Tris-HCl and Triton X-100, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $DiOC_6$, Indo 1/AM, potassium phosphates and dimethyl sulfoxide (DMSO) were obtained from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was bought from OncoImmunin, Inc. (Gaithersburg, MD, USA).

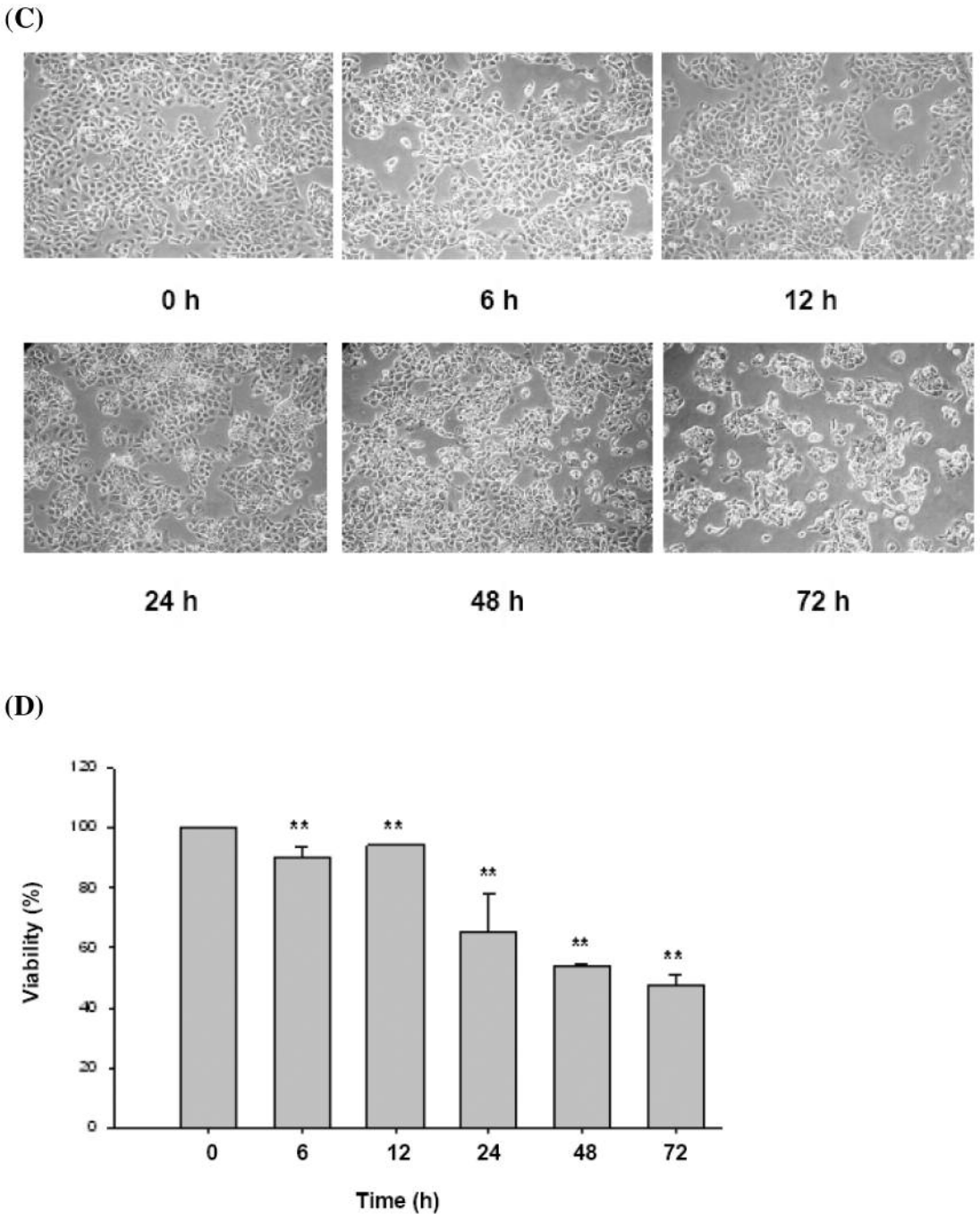

Figure 1. *BV induced morphological changes and decreased the percentage of viable Ca Ski cells. Ca Ski cells were incubated with different concentrations of BV for 48 h or 10 μg/ml BV for various times contrast. The cells were photographed under phase microscopy (Panel A and C). Viable cells were determined using trypan blue exclusion, propidium iodine and flow cytometry (panel B and D). *P<0.05, **p<0.01 compared to control.*

Human cervical epidermoid carcinoma Ca Ski cell line. The Ca Ski cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air at 1 Atm in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% L-glutamine. The cells were cultured for several generations and the viability of each generation was checked (17).

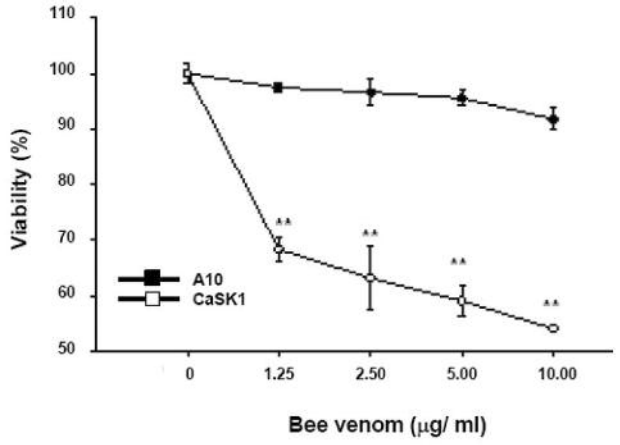

Figure 2. *BV effect on the percentage of viable Ca Ski and normal fibroblast cells. Viable cells were determined after incubation with different concentrations of BV for 48 h using trypan blue exclusion, propidium iodine and flow cytometry. *P<0.05, **p<0.01 compared to control.*

Morphological changes and cell viability. The Ca Ski cells were plated in 12-well plates at a density of $2x10^5$ cells/well and grown for 24 h. The cells were incubated for various time periods with BV dissolved in DMSO at a final concentration of 0 (control, solvent alone), 1.25, 2.5, 5.0 or 10 μg/ml. To determine the morphological changes and cell viability, phase-contrast microscopy and flow cytometric assay were used as described previously (17-21).

Cell cycle and apoptosis. The Ca Ski cells were plated in 12-well plates at a density of $2x10^5$ cells/well and grown for 24 h. The cells were incubated with dissolved in DMSO BV at a final concentration of 0, 1.25, 2.5, 5.0 or 10 μg/ml, for different periods of time. For cell cycle analysis the cells were harvested and fixed gently with 70% ethanol (in PBS), maintained at 4˚C overnight and then re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in a dark room. After 30 min at 37˚C, the cells were analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wavelength. Then the cell cycle and sub-G1 group were determined and analyzed as described previously (17-21). Alternatively the cells were stained by DAPI, and photographed using a fluorescence microscope as described previously (17-21), or isolated for examination of DNA damage by using the Comet assay as described previously (17-21).

Flow cytometry examining the levels of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi m$), Ca^{2+} and caspase-3 activity. Approximately $5x10^5$ Ca Ski cells/ml were treated with 10 μg/ml BV for various time periods. For ROS examination the cells were harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma) and incubated at 37˚C for 30 min before flow cytometry analysis, as described previously (17-21). To detect changes in the mitochondrial membrane potential (MMP), the cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L), incubated at 37˚C for 30 min and analyzed by flow cytometry as described previously (17-21). To detect any changes in Ca^{2+} concentration the cells were harvested by centrifugation, washed twice and re-suspended in Indo 1/AM (3 μg/ml) (fluorescent dye for staining Ca^{2+}) (Calbiochem, La Jolla, CA, USA), they were then incubated at 37˚C for 30 min and analyzed by flow cytometry as described previously (17-21). For the analysis of caspase-3 activity. After adding 50 μl of 10 μM substrate solution (PhiPhilux, a unique class of substrates for caspase-3) to the cell pellet ($1x10^5$ cells per sample), caspase-3 activity was analyzed by flow cytometry (Becton Dickinson FACS Calibur) as described previously (17-21).

Western blotting of cell cycle and apoptosis associated proteins. The total proteins were collected from approximately $5x10^5$ Ca Ski cells treated with 10 μg/ml BV for 0, 6, 12, 24 or 48 h. The Cdk2, 4 and 6, cyclin D3, cyclin E, pRb, E2F-1, p53, p21, p27, p18, p19, Bax, Bad, Bcl-2, cytochrome c, caspase-9, AIF, Endo G, caspase-3, caspase-7, PARP, Fas, Fas-L, caspase-8, Bid, Ras, Raf-1, JNK1/2, ERK1/2, p38, p38-p, C-jun, C-jun-p, COX-2, NF-κB p65 and p50 were measured by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously (17-21).

Results

Morphological changes and decreased percentage of viable cells. BV induced morphological changes (Figure 1A and C) and decreased the percentage of viable cells (Figure 1B and D) in a dose- and time-dependent manner.

Percentage of viable Ca Ski and normal A10 fibroblast cells. Ca Ski cells and normal fibroblast cells incubation with various concentrations of BV for 24 h demonstrated the decrease in the percentage of viable Ca Ski cells, but it did not significantly decrease the percentage of viable normal fibroblast cells (Figure 2).

Cell cycle arrest and apoptosis. The BV induced G0/G1 arrest (Figure 3A and C) and sub-G1 group (apoptosis) (Figure 3B and D). Both effects were dose- and time-dependent. The comet assay demonstrated the BV-induced DNA damage (Figure 4), and those effects were dose-dependent. DAPI staining demonstrated that BV induced apoptosis (Figure 5) and those effects were dose-dependent.

DNA gel electrophoresis. BV induced DNA fragmentation (Figure 6) and the effect was dose-dependent.

DCFH-DA staining of reactive oxygen species (ROS). The flow cytometric assay indicated that BV induced the production of ROS in the Ca Ski cells (Figure 7A) in a time-dependent manner.

Indo 1/AM staining of Ca^{2+}. Flow cytometric assay indicated that BV induced the production of Ca^{2+} in Ca Ski cells (Figure 7C) in a time-dependent manner.

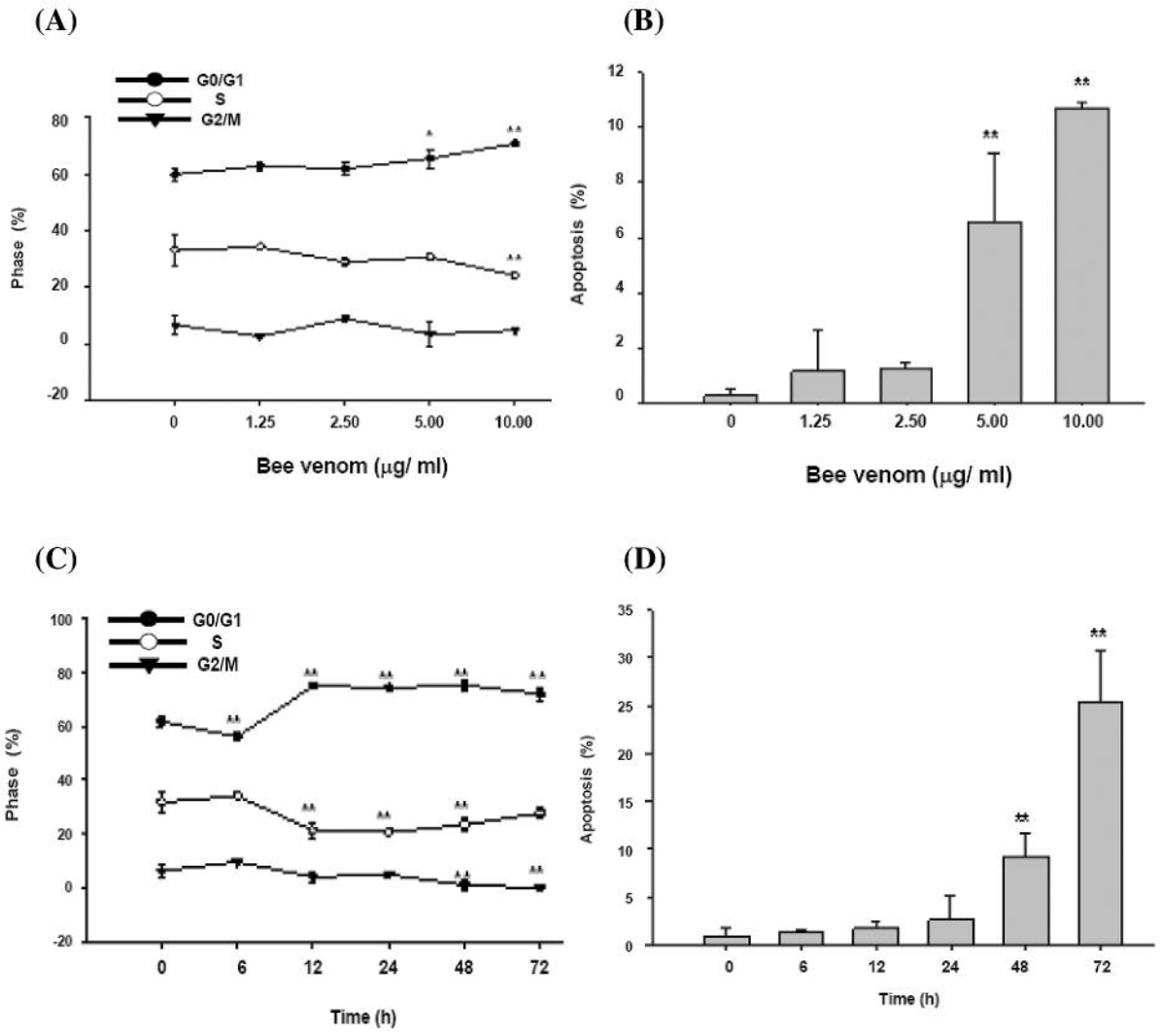

Figure 3. *BV effects on cell cycle and apoptosis in Ca Ski cells. The Ca Ski cells were incubated for various times with various concentrations of BV, and cell cycle and apoptosis were determined by flow cytometric analysis. Panel A and C: percentage of cells in different phases; panel B and D: percentage of apoptotic cells. **P<0.01 compared to control.*

$DiOC_6$ staining for determination of MMP. The flow cytometric assay indicated that BV induced the production of MMP in the examined cells (Figure 7B). These effects had a time-dependent manner.

Flow cytometric assay of caspase-3 activity. The flow cytometric assay indicated that BV promoted the caspase-3 activation in Ca Ski cells (Figure 7D) in a time-dependent manner.

Western blotting of proteins associated with G0/G1 arrest and apoptosis. BV promoted the expression of Cdk4, -6, Cyclin D3, pRb, p53, p21, p27, p18 and p19 (Figure 8A and B) but inhibited the expression of Cdk2, Cyclin E, E2F-1 (Figure 8A and B) which may have led to G0/G1 arrest in the Ca Ski cells. BV also promoted the expression of Bax, Bad, cytochrome c, caspase-9, AIF, Endo G, caspase-3, -7 and PARP (Figure 8C and D), but it inhibited Bcl-2 levels (Figure 8C). BV also promoted the expression of Fas, Fas-L, caspase-8, Bid, Ras, ERK1/2, p38-p and C-jun-p (Figure 8E and F), but it did not affect the levels of Raf-1, JNK1/2, p38 and C-jun (Figure 8E and F).

Discussion

The present study is the first to show the induction of apoptosis in human cervical cancer Ca Ski cells by BV. BV induced apoptosis through a decrease in Bcl-2 expression and an increase in Bax, dysfunction of mitochondria

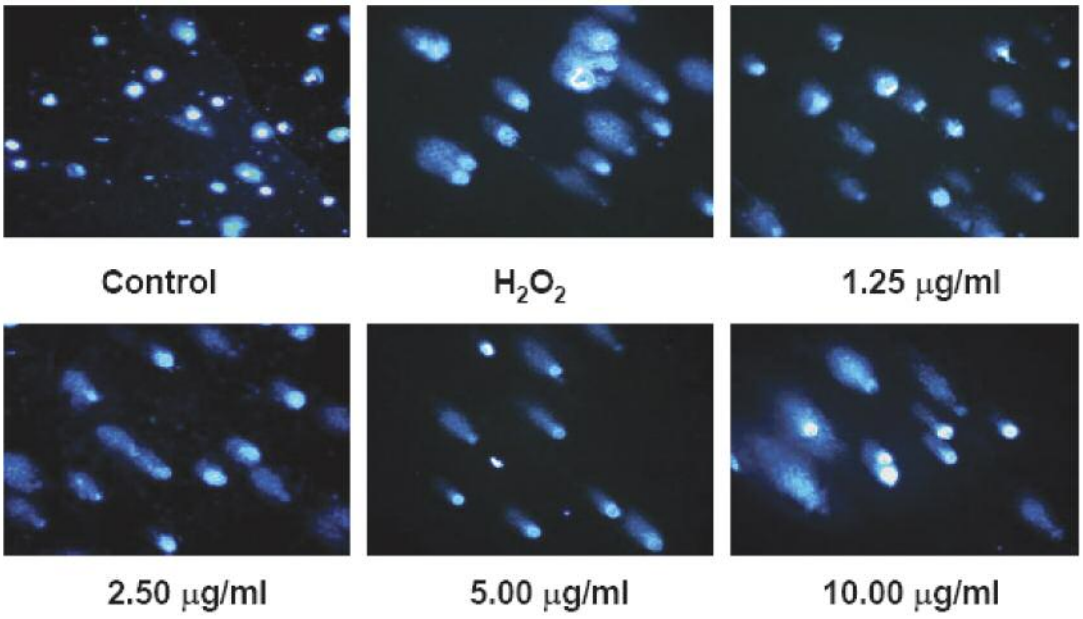

Figure 4. *Comet assay of the effects of BV on Ca Ski cell DNA damage. The Ca Ski cells were incubated with various concentrations of BV for 48 h and DNA damage was determined using the Comet assay.*

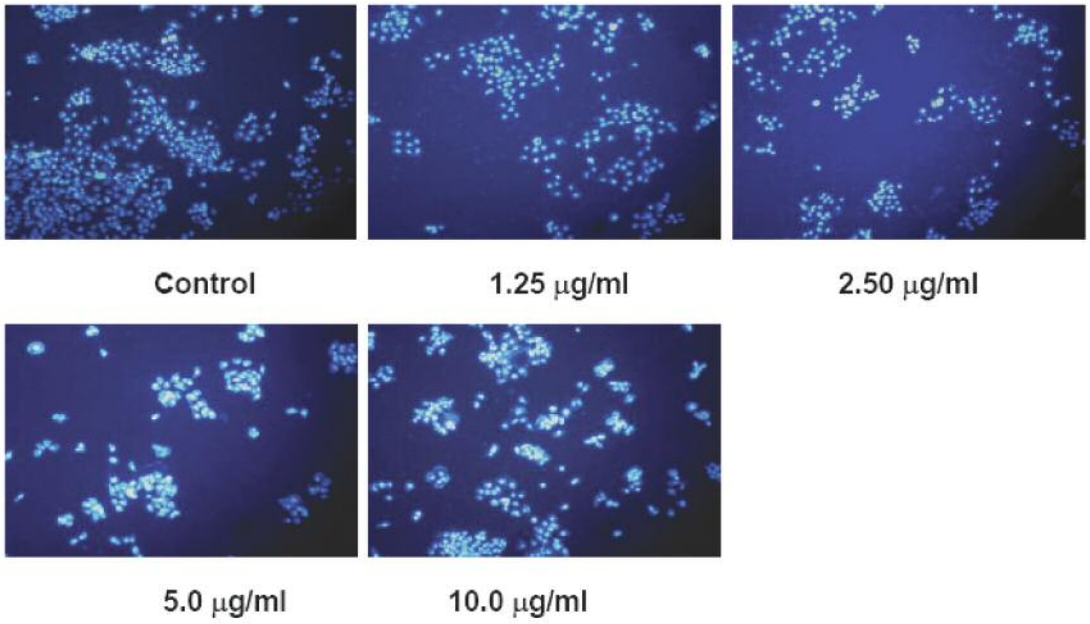

Figure 5. *DAPI staining of the effects of BV on Ca Ski cell apoptosis. The Ca Ski cells were incubated with various concentrations of BV for 48 h and apoptosis was determined using DAPI staining. Data represent mean±S.D. of three experiments. *P<0.05.*

(decreased MMP), cytochrome c release and caspase-3 expression in the Ca Ski cells. BV has been previously shown to induce apoptosis in the osteosarcoma MG-63 cell line (22), the breast cancer MCF-7 cell line (23), the lung cancer NCI-H1299 cell line (24, 25) and the human leukemia U937 cell line (12). Our results demonstrated that BV induced morphological changes (cell shrinkage, cytoplasmic condensation and irregularity of cell shape) and decreased the percentage of viable Ca Ski cells in a dose- and time-dependent manner. Cells undergoing apoptosis

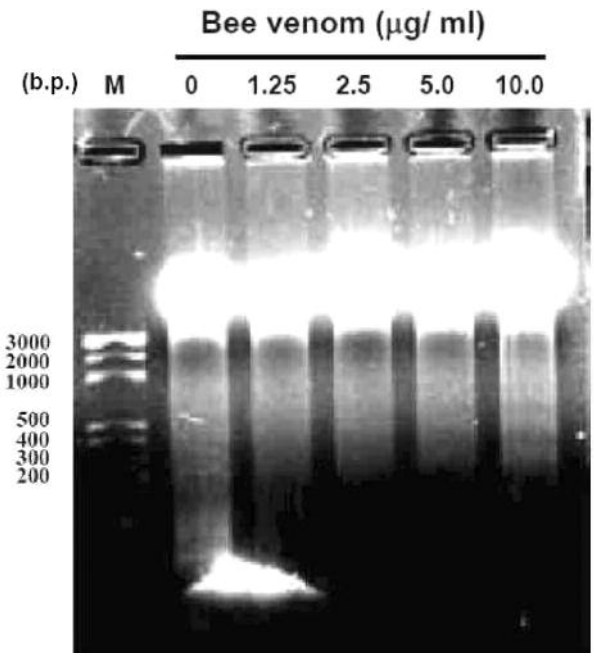

Figure 6. *DNA fragmentation in Ca Ski cells treated with BV. The Ca Ski cells were incubated with various concentrations of BV for 48 h, and DNA fragmentation was determined using DNA gel electrophoresis.*

exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape, and the retraction of processes (26) and DNA strand breaks can be detected *via* TUNEL or DAPI assay (27). Our results showed strand breaks by DAPI staining (Figure 5) and DNA fragmentation was also clearly detected by DNA agarose gel electrophoresis (Figure 6). DNA fragmentation into well defined fragments is linked to the activation of endonucleases and can be seen upon electrophoretic examination as a characteristic ladder pattern (28).

In the present study, BV treatment resulted in a decrease in Bcl-2 (an anti-apoptotic protein) expression and an increase in Bax (a pro-apoptotic protein) expression in the Ca Ski cells. Members of the Bcl-2 family of proteins are characterized by their ability to form a complex combination of heterodimers with Bax and homodimers with itself (29). Therefore, the ratio of Bax to Bcl-2 is thought to determine the susceptibility of cells to apoptosis (30). Caspases, a family of cysteine proteases, are also involved in different apoptotic pathways. In particular when caspase-3, is activated it has many cellular targets that produce the morphological features of apoptosis (26). Our results demonstrated that BV promoted caspase-8 and caspase-3 in the Ca Ski cells.

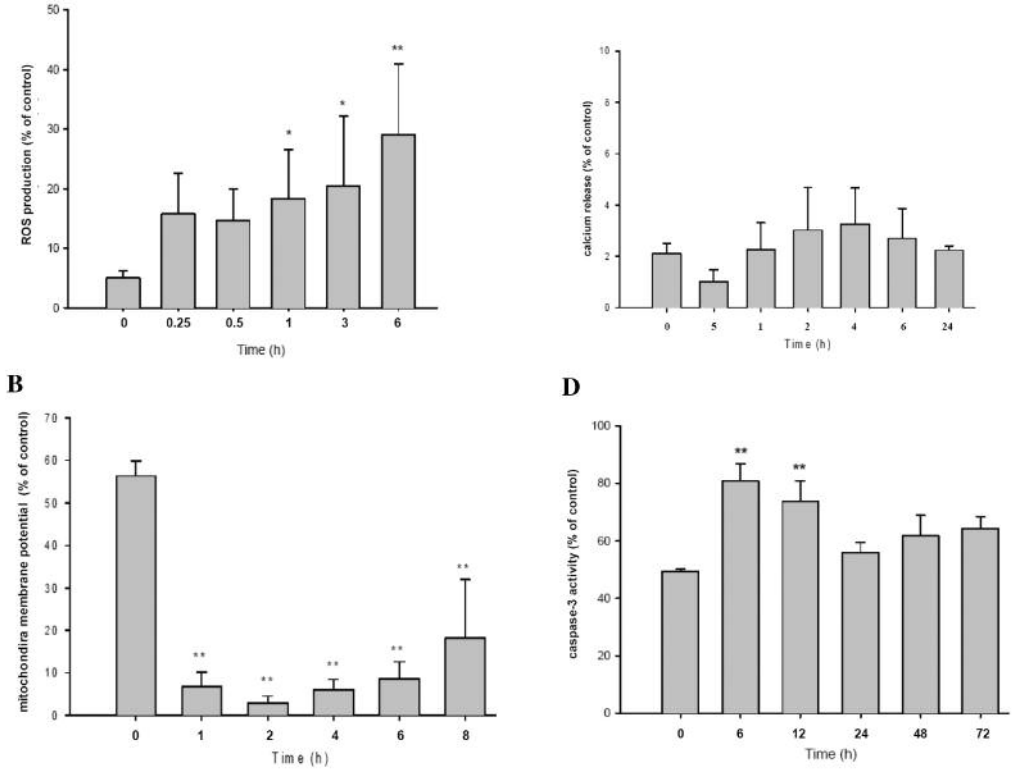

Figure 7. *Flow cytometric analysis of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), cytoplasmic Ca^{2+} levels and caspase-3 activity in human Ca Ski cells after exposure to BV. Ca Ski cells ($5x10^5$ cells/ml) were treated with 10 μg/ml of BV for various time periods. No BV was added to the controls. A) ROS were stained by DCFH-DA dye, B) MMP was detected using $DiOC_6$ dye, C) cytoplasmic Ca^{2+} was detected with Indo 1/AM and D) PhiPhilux, a unique class of substrates for caspase-3 was used for caspase-3 determination. *P<0.05, **p<0.01, significant difference between the BV-treated cells and the control.*

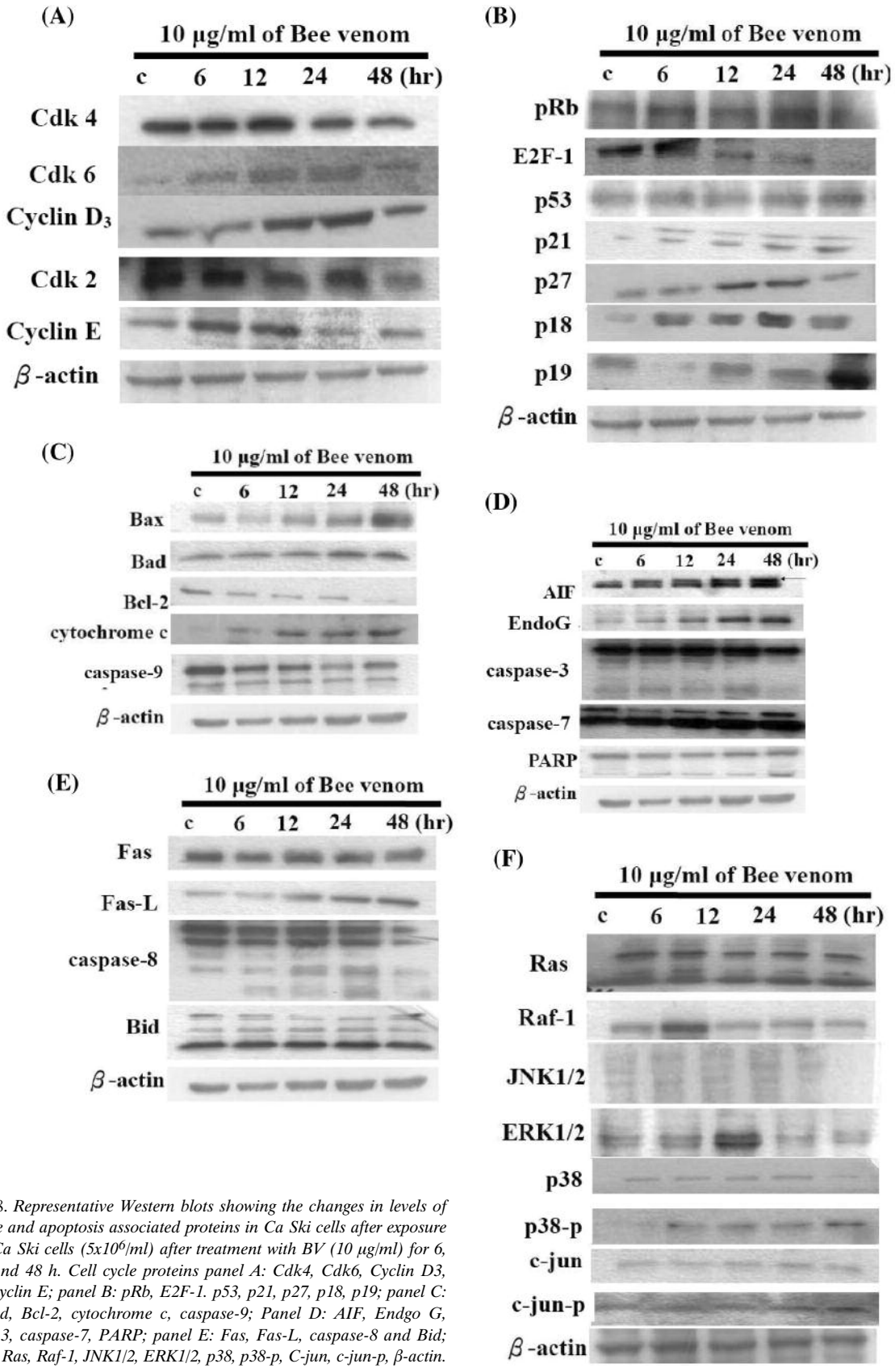

Figure 8. *Representative Western blots showing the changes in levels of cell cycle and apoptosis associated proteins in Ca Ski cells after exposure to BV. Ca Ski cells (5x10^6/ml) after treatment with BV (10 μg/ml) for 6, 12, 24 and 48 h. Cell cycle proteins panel A: Cdk4, Cdk6, Cyclin D3, Cdk2, Cyclin E; panel B: pRb, E2F-1. p53, p21, p27, p18, p19; panel C: Bax, Bad, Bcl-2, cytochrome c, caspase-9; Panel D: AIF, Endgo G, caspase-3, caspase-7, PARP; panel E: Fas, Fas-L, caspase-8 and Bid; panel F: Ras, Raf-1, JNK1/2, ERK1/2, p38, p38-p, C-jun, c-jun-p, β-actin.*

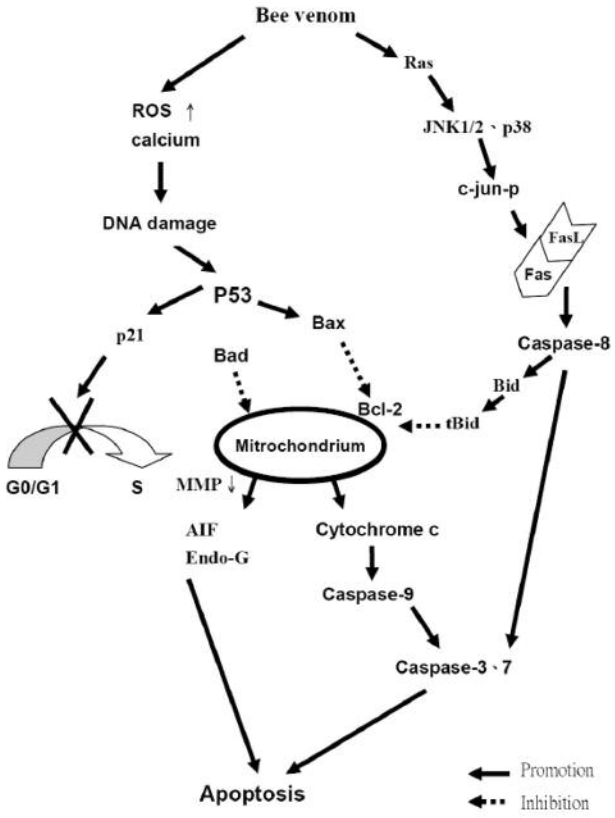

Figure 9. *Proposed model of BV mechanism of action for apoptosis in Ca Ski cells. BV increases the production of ROS and Ca^{2+}, affects the ratio of Bcl-2/Bax and decreases MMP levels leading to increased caspase-3 activity causing apoptosis in Ca Ski cells.*

In conclusion, our results showed that BV induced apoptosis through caspase-8 up-regulation, changes in the ratio of Bcl-2/Bax, dysfunction of the mitochondria, cytochrome c release and caspase-9 and -3 activation leading to the release of Endo G and AIF (Figure 9). Apparently, ROS and DNA damage are also involved in BV-induced apoptosis in Ca Ski cells.

Acknowledgements

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