Apoptosis of Human Leukemia HL-60 Cells and Murine Leukemia WEHI-3 Cells Induced by Berberine through the Activation of Caspase-3

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Abstract. *The aim of this study was to clarify the mechanisms of apoptosis, cytotoxicity, DNA damage and fragmentation, as well as the production of reactive oxygen species (ROS) and Ca^{+2}, induced by berberine in human promyelocytic leukemia HL-60 and murine myelomonocytic leukemia WEHI-3 cells. The levels of Bcl-2 and Bax, the changes of mitochondria membrane potential (MMP), cytochrome c release and activation of caspase-3 were also investigated in both cell lines. The flow cytometry and DAPI staining assays indicated that berberine induced cytotoxicity in both cell lines examined. Flow cytometry assay also showed that berberine induced ROS and Ca^{+2} production, decreased the levels of MMP and increased the activity of caspase-3 in both cell lines examined. Berberine-induced apoptosis was accompanied by increased levels of Ca^{+2} and a decrease in the mitochondrial membrane potential, leading to the release of cytochrome c and the cleavage of pro-caspase-3. Western blotting also showed that berberine increased the levels of Bax and cytochrome c and decreased the levels of Bcl-2 in both cell lines. Inhibition of caspase-3 activation (z-VAD-fmk: cell-permeable broad-spectrum caspase inhibitor) completely blocked berberine-induced apoptosis in both HL-60 and WEHI-3 cells. Therefore, berberine induced apoptosis in both examined cell lines through the activation of caspase-3.*

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Key Words: Berberine, apoptosis, leukemia cells, caspase-3, mitochondria membrane potential.

Berberine is the major constituent of Coptis Chinese, a yellow benzylisoquinoline alkaloid, which plays an important role as a biological barrier protecting plant tissue from pathogens and is commonly used in Chinese herbal medicine to treat patients with gastrointestinal disorders. It has anti-inflammatory, antidiarrhetic, antimalarial and antimicrobial activity (1-5), as well as antineoplastic properties (6-10). To date, the effects of berberine on human and mouse leukemia cells have not been well investigated. In our screening test, we found berberine induced cytotoxicity, cell cycle arrest and apoptosis by using flow cytometry analysis in human and mouse leukemia cells.

The modulated expression of cell cycle-regulatory molecules on cell cycle arrest or apoptosis have been investigated in many cell types (11-13). Cyclins and cyclin-dependent kinases (cdks) are evolutionarily-conserved proteins that are essential for cell cycle control. It is well known that distinct pairs of cyclins and cdks regulate progression through different stages of the cell cycle. Therefore, we investigated the molecular mechanism of berberine-induced apoptosis in human and mouse leukemia cell lines.

Apoptosis is programmed cell death through a series of stereotypic molecular events, including the expression and translocation of the Bcl-2 family of proteins, and the release of cytochrome c from mitochondria and activation of caspases. Two major mechanisms, by caspase cascade, lead to activation of caspase-3 for the induction of apoptosis, one involving caspase-8 and the other involving caspase-9 (14, 15). The activation of caspase-3 is required for apoptosis, either by the caspase-3 or caspase-9 pathways. The Bcl-2 family proteins, such as Bax, Bak, Bcl-2 and Bcl-XL, are well-characterized regulators of apoptosis and are associated with mitochondia (15, 16). The effects of berberine on the expression of the Bcl-2 family in both leukemia cell lines were also examined.

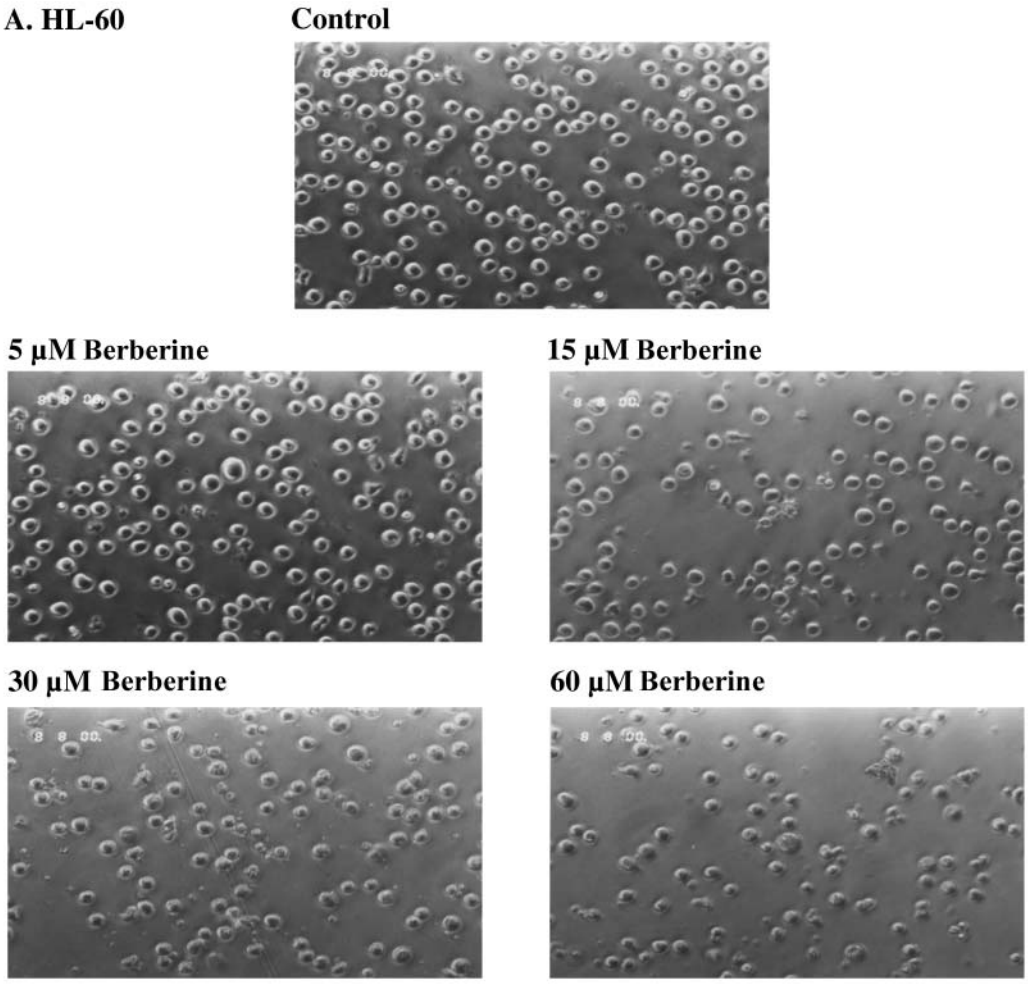

Figure 1. *Morphological changes of HL-60 and WEHI-3 cells in response to berberine. HL-60 (panel A) and WEHI-3 (panel B) cells were treated with various concentrations of berberine for 24 h. The cells were examined under a contrast-phase microscope and photographed.*

Materials and Methods

Chemicals and reagents. Berberine, Tris-HCl, Triton X-100, propidium iodide (PI), ribonuclease-A and Trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was from Boehringer Mannheim (Mannhein, Germany).

Leukemia cell lines. The human HL-60 and murine WEHI-3 leukemia cell lines were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C, in a humidified 5% CO_2 and 95% air atmosphere, in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine. All the data presented in this report are from at least 3 independent experiments.

Cell viability as determined by flow cytometry. HL-60 or WEHI-3 cells were plated in 12-well plates at a density of $5x10^3$ cells/well and grown for 24 h. The various concentrations of berberine (0, 5, 15, 30 and 60 μM) were then added and the cells were grown for different periods of time. DMSO (solvent) was used as control. For determining cell viability, the flow cytometric protocol was used, as previously described (17).

Flow cytometric analysis of DNA content for apoptosis studies in HL-60 and WEHI-3 cells treated with different concentrations of berberine. Approximately $5x10^5$ HL-60 or WEHI-3 cells/well in 12-well plates were incubated with various concentrations of berberine at 0, 5, 15, 30 and 60 μM for different time-periods before the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/mL

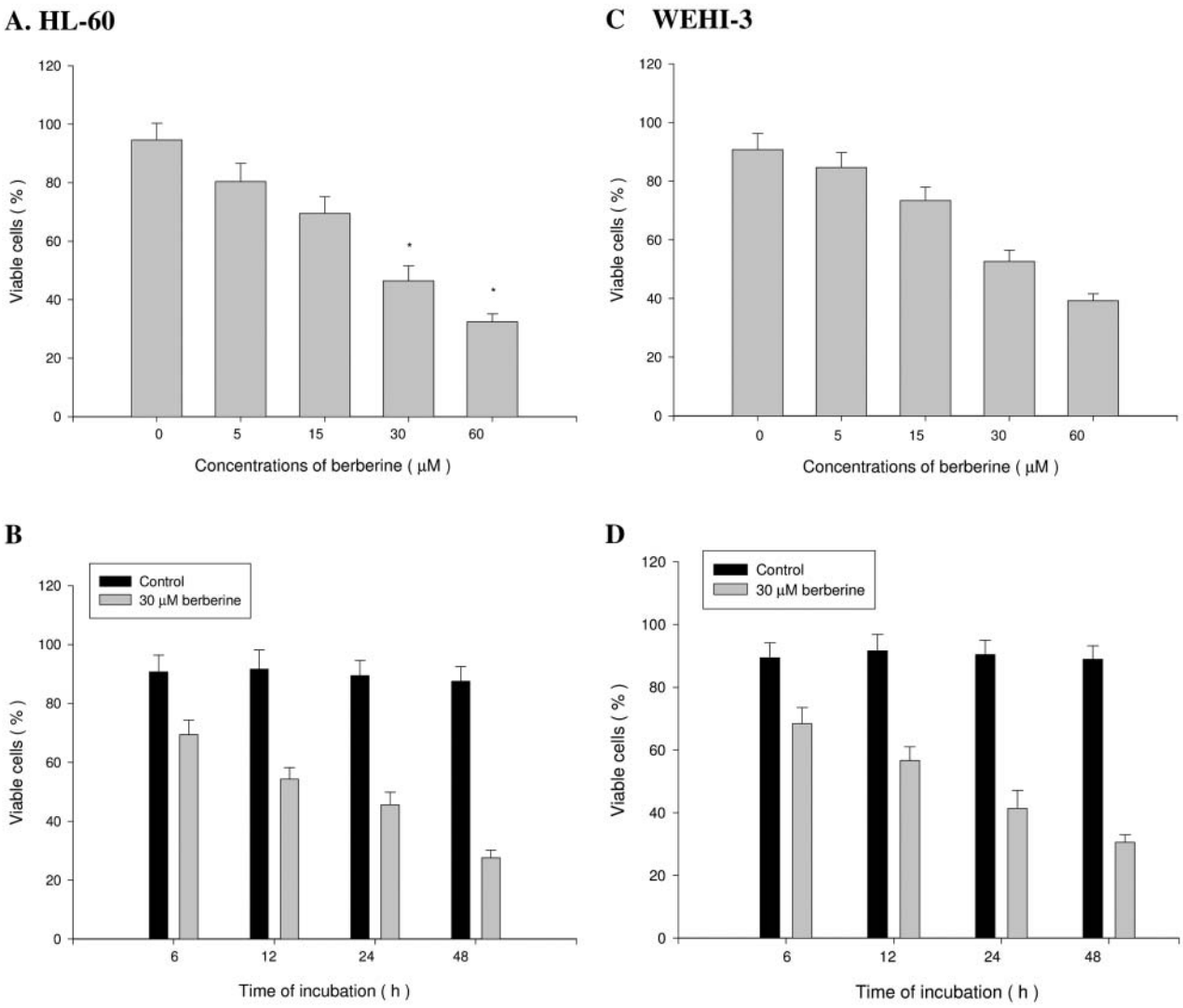

Figure 2. *Percentage of viable HL-60 and WEHI-3 cells after berberine treatment. HL-60 (panel A and B) and WEHI-3 (panel C and D) cells (5x10^3 cells/well; 12-well plates) were cultured in RPMI 1640 medium + 10% FBS with berberine for 24 h. The cells were then collected and determination of viable cells was conducted by flow cytometry, as described in the Materials and Methods. The data represent mean±S.D. of three experiments *p<0.05.*

PI, 0.1 mg/mL RNase (Sigma) and 0/1% Triton X-100. After 30 min at 37˚C in the dark, the cells were analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA, USA), equipped with an argon laser, at 488 nm (17). The percentage of cells that had undergone apoptosis was assessed as the ratio of the fluorescence area (sub-G1) less than the G0/G1 peak to the total area of fluorescence.

Examination of apoptosis by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. 5x10^4 cells/ml were treated with or without berberine (0, 5, 15, 30 and 60 μM) for 48 h and were then isolated for DAPI staining. After treatment, the cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml DAPI for 5 min at 37˚C. The cells were then washed with PBS and examined by fluorescence microscopy (Nikon, 200X).

Detection of reactive oxygen species (ROS) in HL-60 or WEHI-3 cells by flow cytometry. The levels of ROS of the HL-60 or WEHI-3 cells were examined by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). 5x105 cells/ml were treated with or without berberine (0, 5, 15, 30 and 60 μM) for 24 h. The changes in ROS were detected in isolated cells. The cells were harvested and washed twice, re-suspended in 500 μL of DCFH-DA (10 μM) and incubated at 37˚C for 30 min, before analysis by flow cytometry (18).

Detection of Ca^{+2} concentrations in HL-60 or WEHI-3 cell lines by flow cytometry after treatment with berberine. The Ca^{+2} levels in the HL-60 or WEHI-3 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using Indo 1/AM (Calbiochem, La Jolla, CA, USA). 5x10^5 cells/ml were treated with or without berberine (0, 5, 15, 30 and 60 μM) for 24 h and

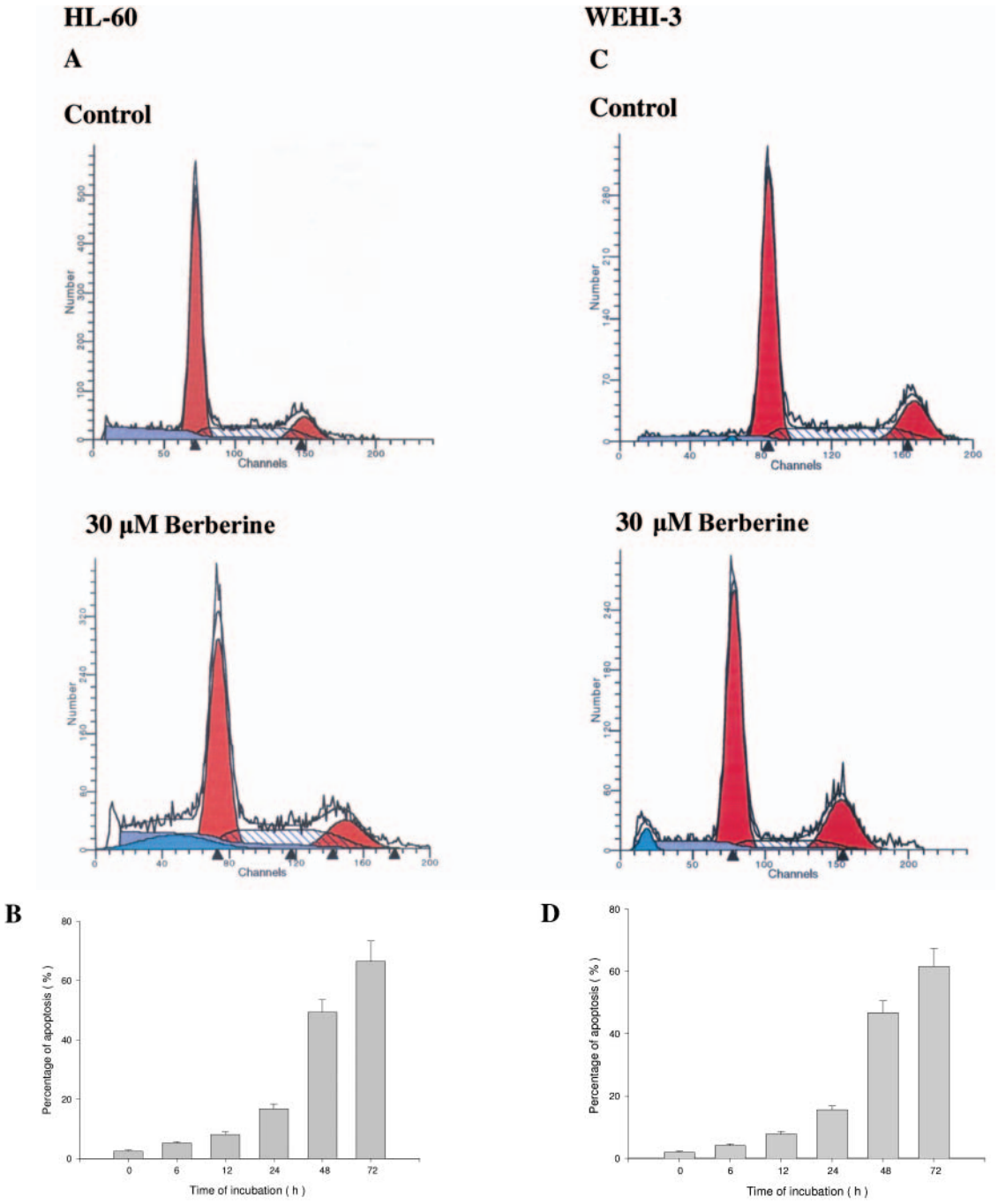

Figure 3. *Effects of berberine on apoptosis in HL-60 and WEHI-3 cell lines. HL-60 (panel A and B) and WEHI-3 (panel C and D) cells were cultured with various concentrations of berberine (0, 5, 15, 30 and 60 μM) for 48 h, and the cells were harvested and analyzed for the sub-G1 group. The percentage of apoptosis was evaluated by flow cytometry, as described in Materials and Methods. The data represent mean±S.D. of three experiments *p<0.05.*

were then isolated to detect the changes of Ca^{+2} concentration. The cells were harvested and washed twice, one half was set aside for apoptosis analysis and the other was re-suspended in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 min and analyzed by flow cytometry (19).

Detection of mitochondrial membrane potential in both HL-60 and WEHI-3 cell lines by flow cytometry. The levels of mitochondrial membrane potential (MMP) of the HL-60 and WEHI-3 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using $DiOC_6$ (4 mol/L). Approximately $5x10^5$ cells/ml cells were

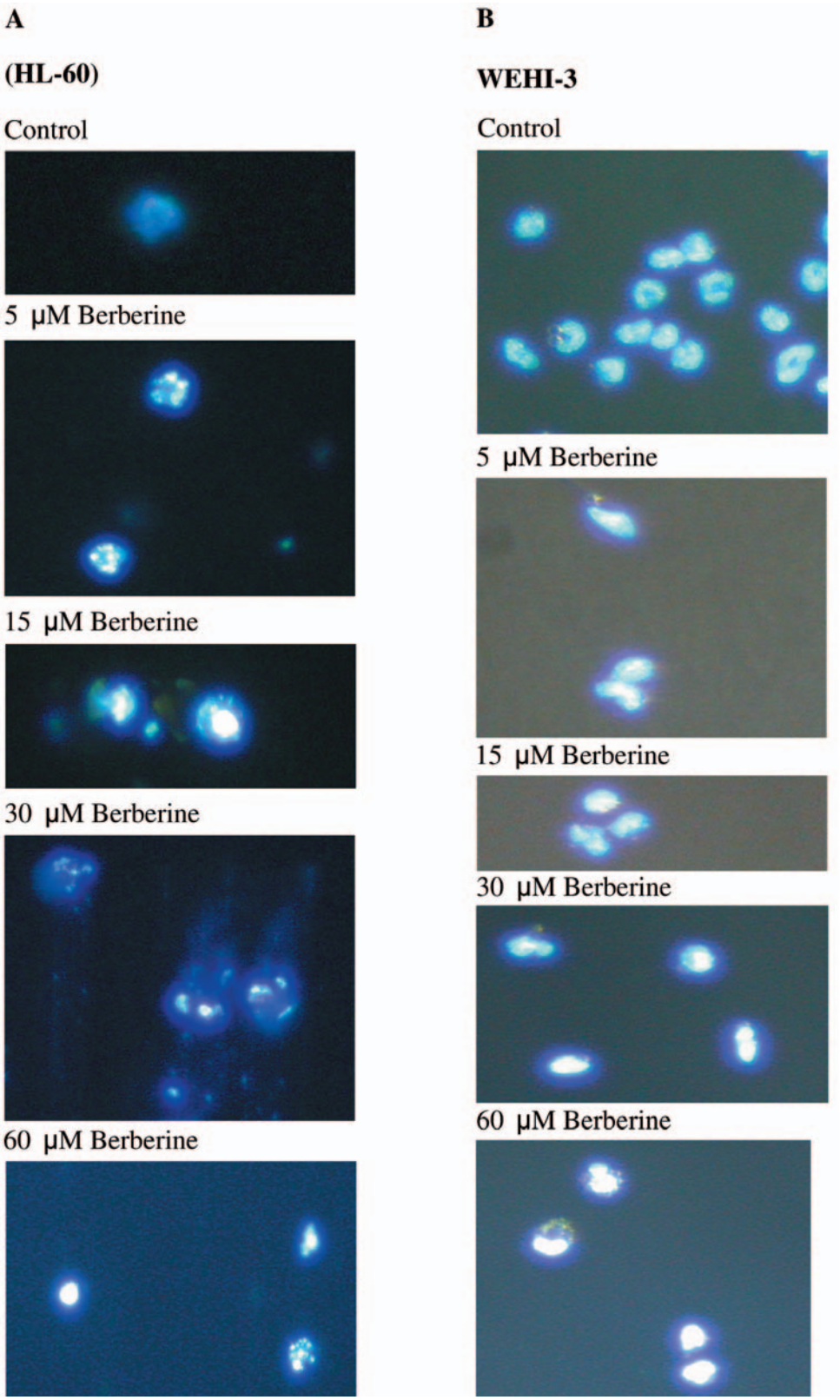

Figure 4. *Berberine-induced apoptosis in HL-60 and WEHI-3 cells as examined by DAPI staining. HL-60 and WEHI-3 cells were incubated with various concentrations of berberine for 48 h. The cells were then harvested and analyzed for apoptosis by DAPI staining (panel A: HL-60; panel B: WEHI-3), as described in Materials and Methods.*

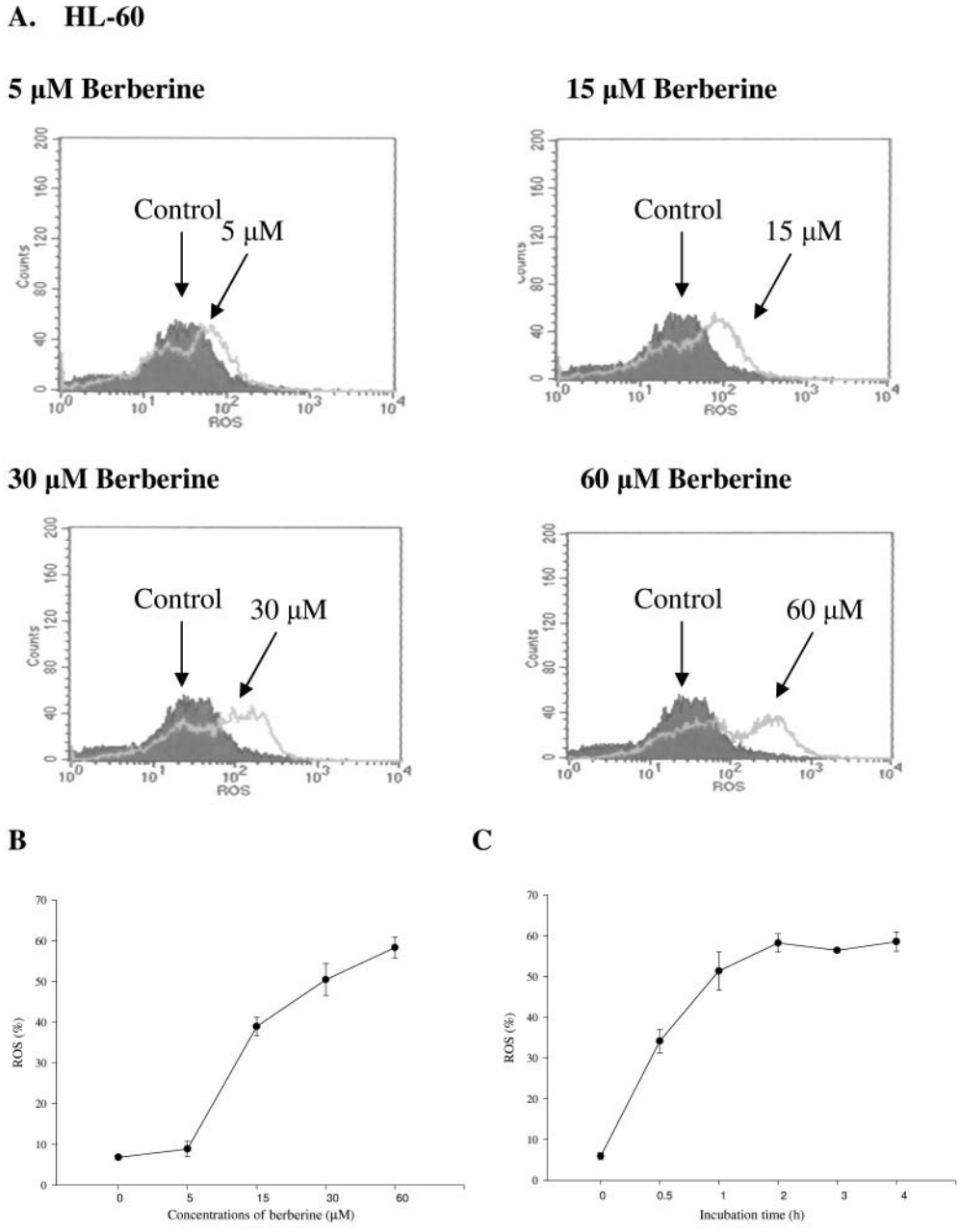

Figure 5. *continued*

treated with or without berberine (0, 5, 15, 30 and 60 μM) for 24 h to detect the changes in the 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 (20).

Caspase-3 activity determination in HL-60 and WEHI-3 cell lines by flow cytometry. Caspase-3 activity in the HL-60 and WEHI-3 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using Phiphiliux green (10 μM) (OncoImmunin, Inc., MD, USA). Approximately $5x10^5$ cells/ml cells were treated with or without berberine (0, 5, 15, 30 and 60 μM) for 24 h to detect caspase-3 activity. The cells were harvested and washed twice, re-suspended in 25 μl of Phiphiliux green, incubated at 37˚C for 60 min and analyzed by flow cytometry (20).

Inhibition of berberine-induced apoptosis by the caspase-3 inhibitor z-DEVD-fmk in HL-60 and WEHI-3 cell lines. In order to examine whether or not caspase-3 activation was involved in apoptosis triggered by berberine, the HL-60 and WEHI-3 cell lines were pretreated with the caspase-3 inhibitor z-DEVD-fmk 3 h prior to

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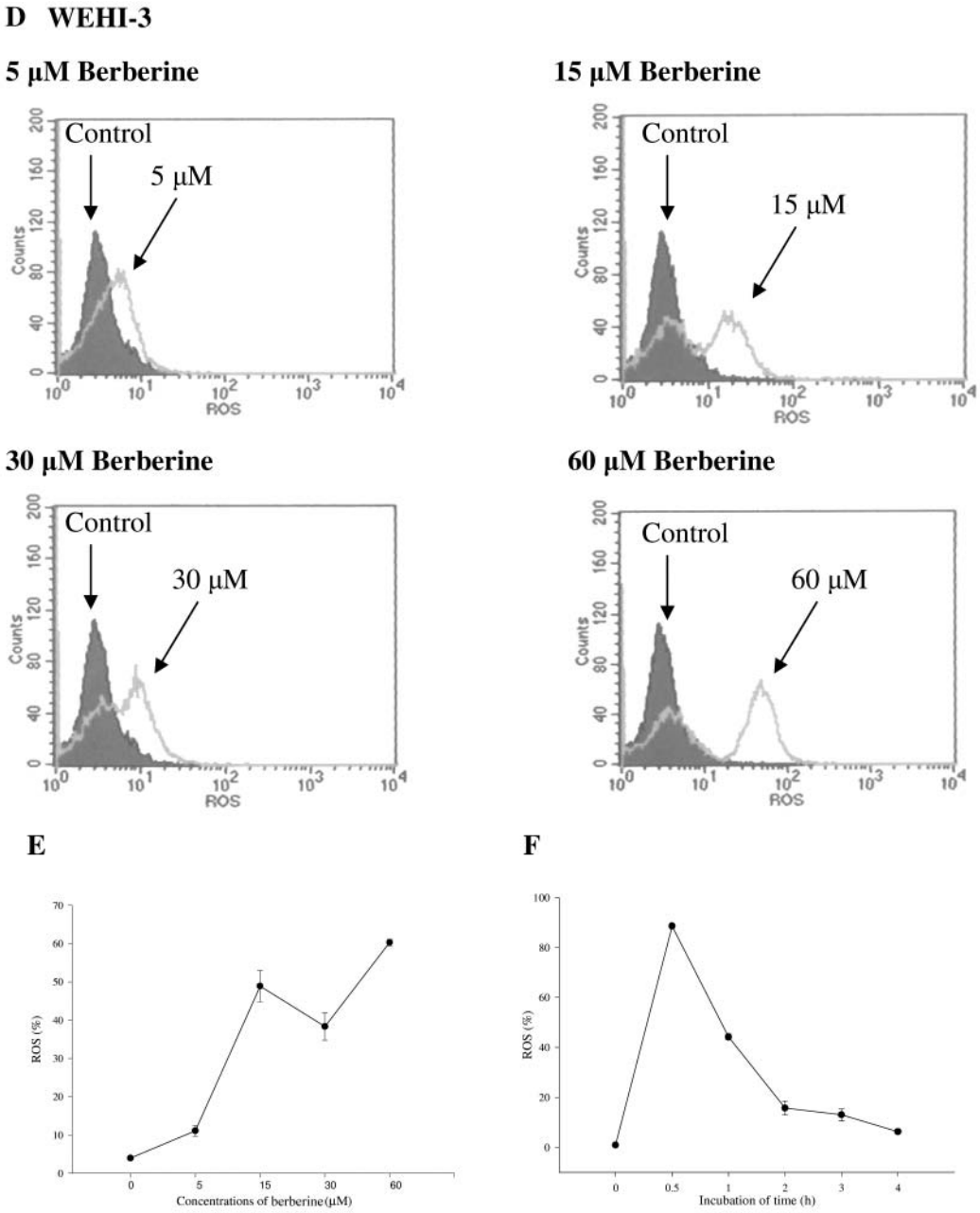

Figure 5. *Berberine affected the production of reactive oxygen species (ROS) in HL-60 and WEHI-3 cells, as examined by flow cytometry. HL-60 or WEHI-3 cells were incubated with various concentrations of berberine for various time-periods. The cells were then harvested and analyzed for ROS by flow cytometry (HL-60: panel A, B and C; WEHI-3: panel D, E and F), as described in Materials and Methods.*

treatment with 30 μM berberine. Apoptosis and caspase-3 activity were then determined, as described above.

Examination of effects of berberine on Fas, Bax, Bcl-2, p53, p21, cytochrome c, caspase-3 in HL-60 and WEHI-3 cells by Western blotting. Approximately $3x10^6$ cells/well (HL-60 or WEHI-3) in 12-well plates were incubated with berberine at 0, 5, 15, 30, and 60 μM for 24 h before being harvested by centrifugation. The protein was extracted as previously described (21). The Fas, p21, p53, Bax, Bcl-2, cytochrome c and caspase-3 levels were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (21)

Statistical analysis. The Student's *t*-test was used to analyze the statistical significance between the berberine-treated and control groups.

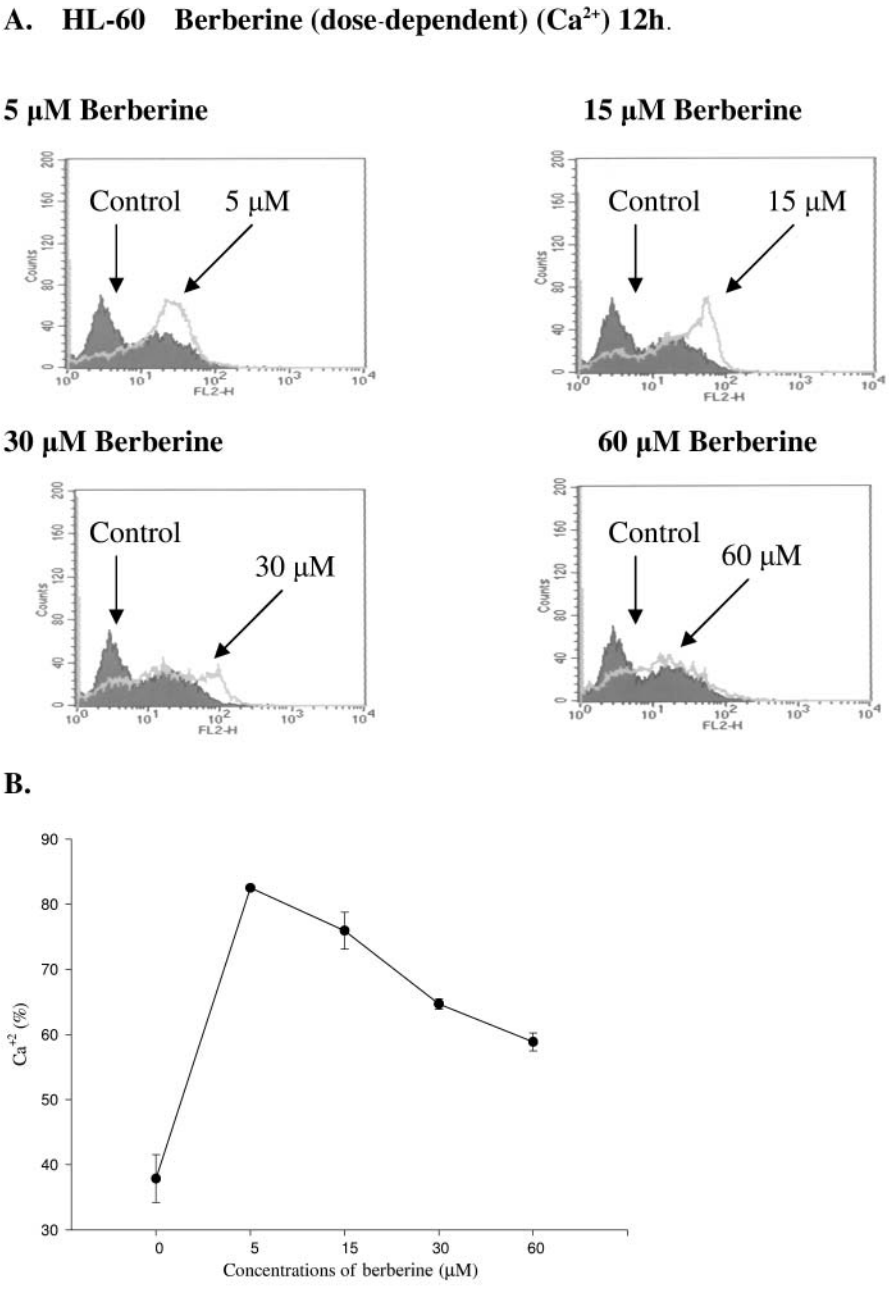

Figure 6. *continued*

Results

Effects of berberine on cell morphology and viability of HL-60 and WEHI-3 cells. In the presence of berberine (0, 5, 15, 30 and 60 μM), the cells were photographed and collected before being stained by propidium iodine and analyzed by flow cytometry. The results indicated that berberine induced cell death of both HL-60 and WEHI-3 cells. Further increasing the concentration of berberine resulted in increased morphological changes (cell death) and a greater decrease in cell viability of both lines (Figure 1A and B; Figure 2A, B, C and D).

Berberine-induced apoptosis in HL-60 and WEHI-3 cells. The flow cytometry results indicated that berberine-treated cells showed a typical pattern of DNA content that reflected the

Figure 6. *continued*

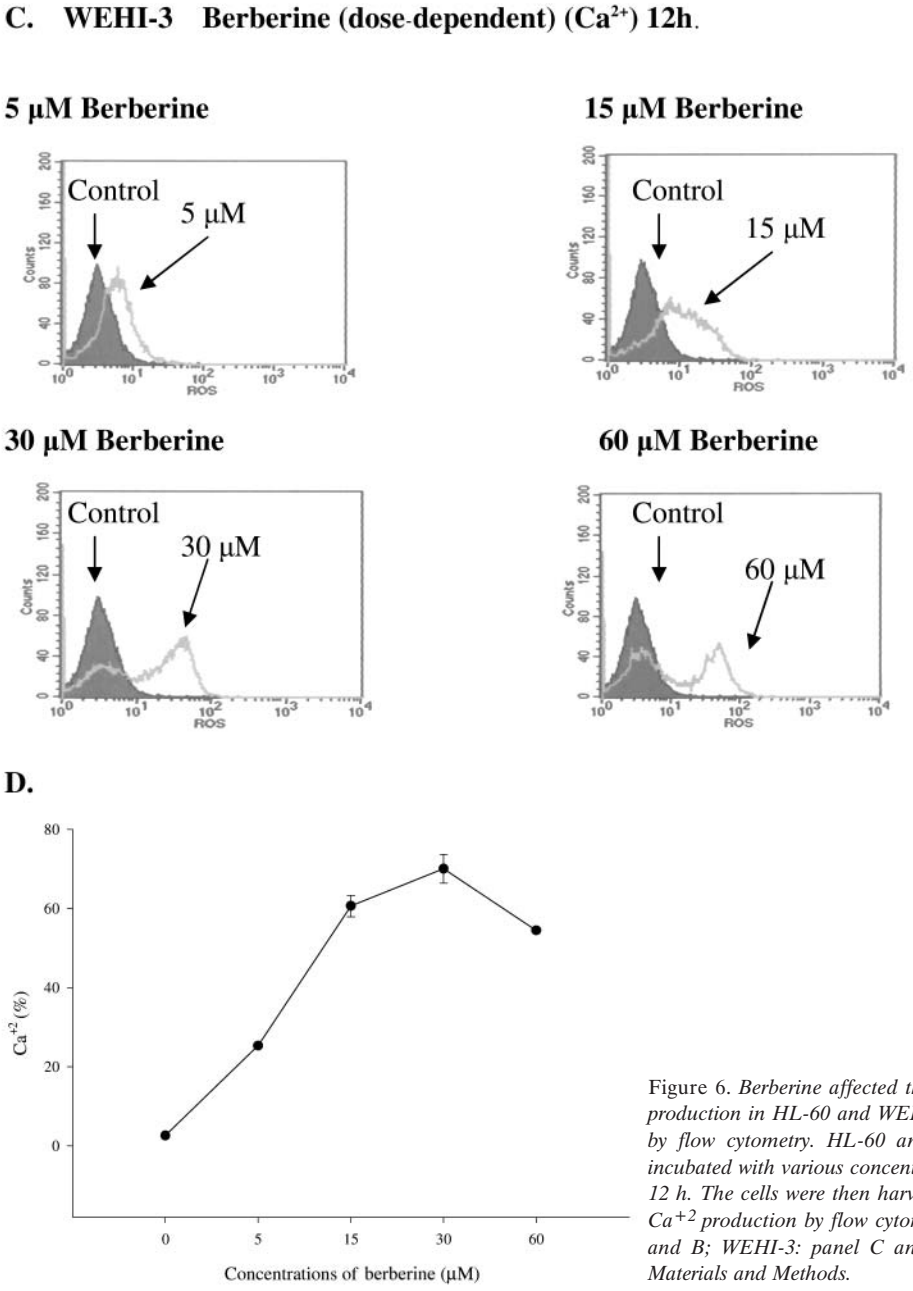

Figure 6. *Berberine affected the production of Ca^{+2} production in HL-60 and WEHI-3 cells, as examined by flow cytometry. HL-60 and WEHI-3 cells were incubated with various concentrations of berberine for 12 h. The cells were then harvested and analyzed for Ca^{+2} production by flow cytometry (HL-60: panel A and B; WEHI-3: panel C and D), as described in Materials and Methods.*

G0/G1-, S- and G2/M-phases of the cell cycle together with a sub-G0/G1- phase (corresponding to apoptotic cells), as shown in Figure 3A and B for HL-60 cells and Figure 3D and E for WEHI-3 cells. A pre-G0/G1 apoptotic peak was very clear after cells had been treated for 48 h. The percentage of apoptotic cells in 30 μM berberine-treated for various time-periods is shown in Figure 3C for HL-60 and Figure 3F for WEHI cells.

Berberine-induced apoptosis as examined by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. The results from both cell lines showed that the percentage of cells stained by DAPI was significantly different between the berberine-treated and control groups. Increasing doses of berberine led to an increase in the DAPI staining in both cell lines examined (Figure 4A and B). Apparently, the effects of berberine-induced apoptosis are dose-dependent.

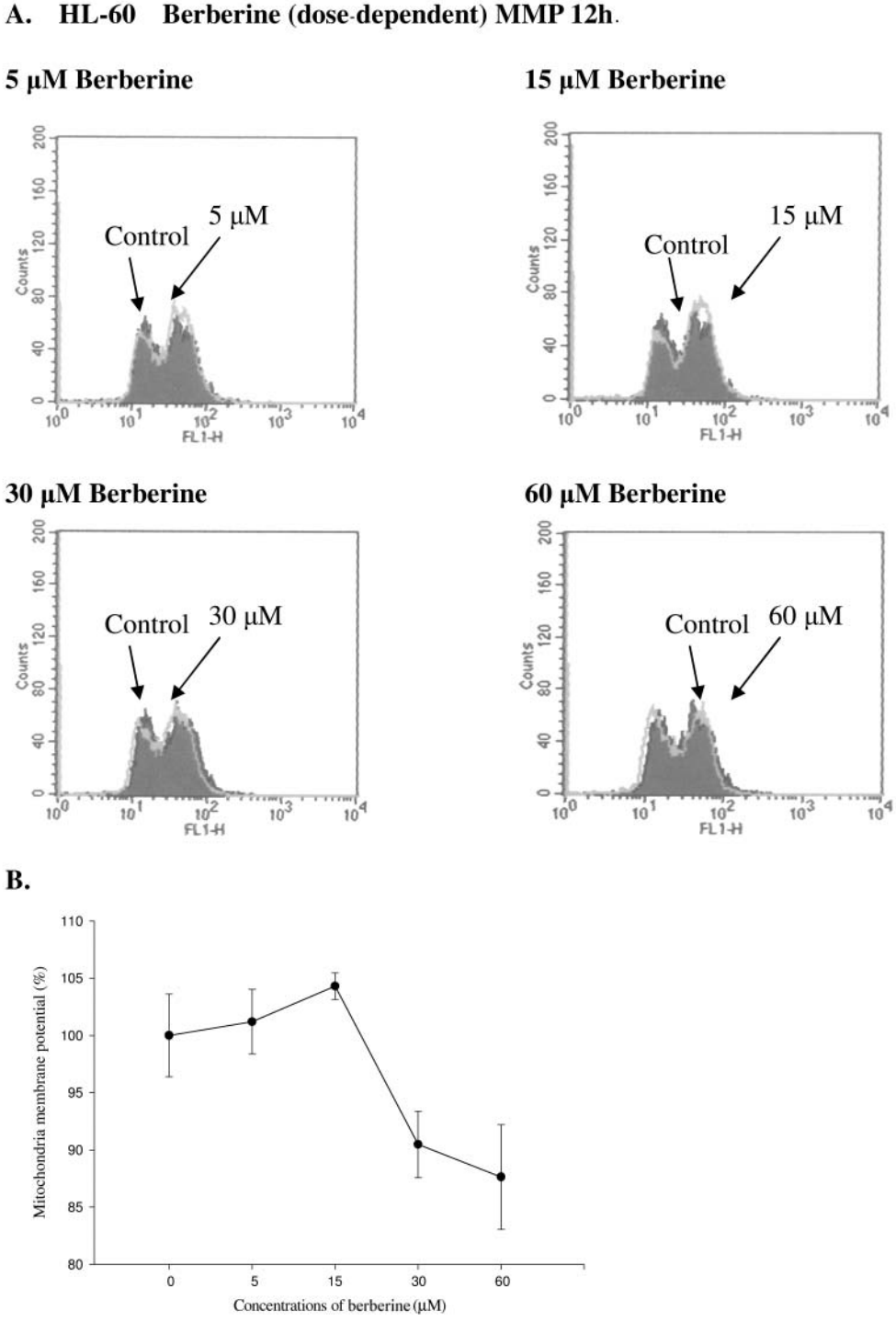

Figure 7. *continued*

Effects of berberine on ROS in HL-60 and WEHI-3 cells. The results from both the cell lines indicated that the percentage of ROS was significantly different between the berberine-treated and the control groups. Increasing the dose of berberine led to an increase in ROS in both cell lines examined (Figure 5A, B and C: HL-60; Figure 5D, E and F: WEHI-3). Thus, it appears that the effects of berberine on the levels of ROS are dose-dependent.

Figure 7. *continued*

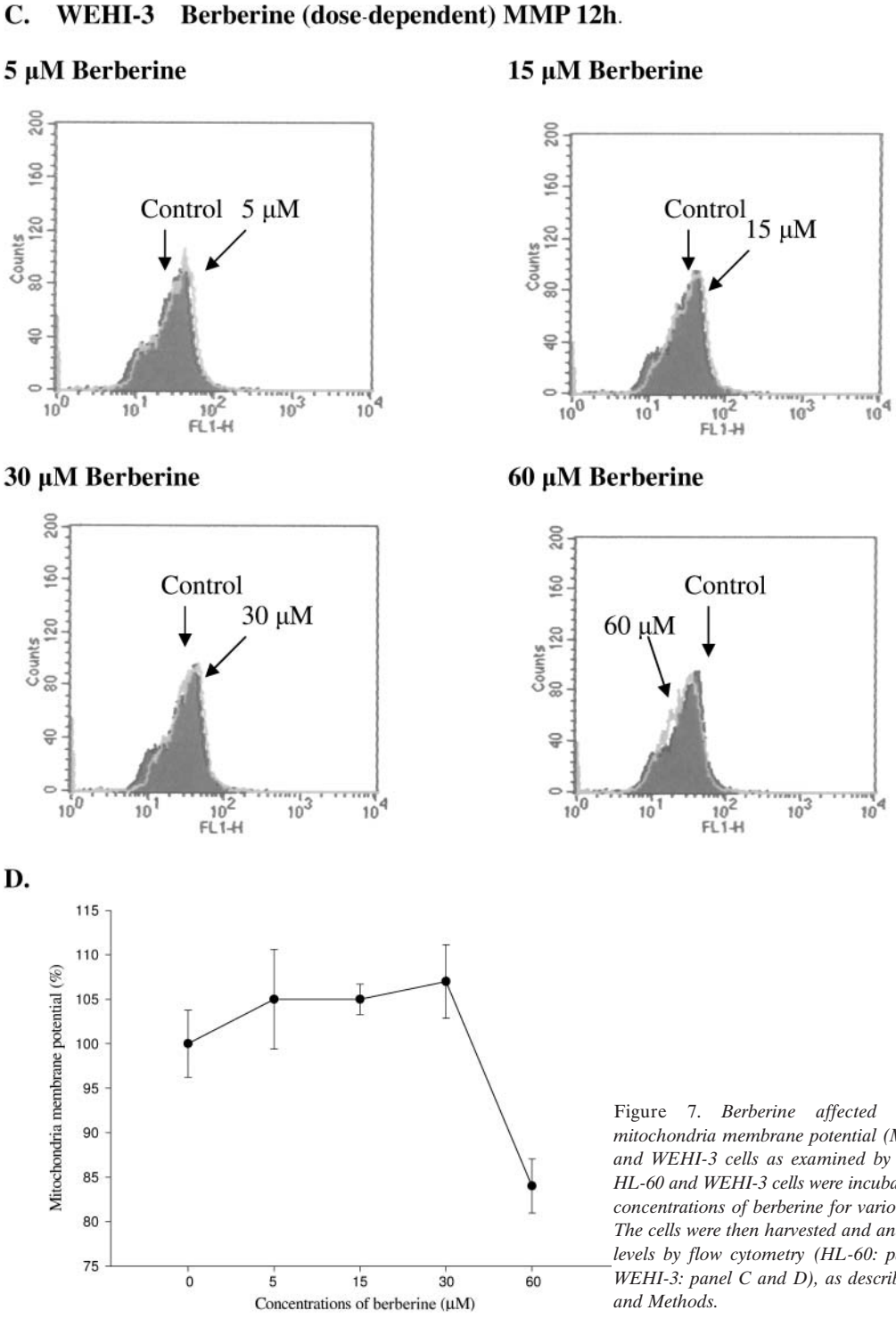

Figure 7. *Berberine affected the levels of mitochondria membrane potential (MMP) in HL-60 and WEHI-3 cells as examined by flow cytometry. HL-60 and WEHI-3 cells were incubated with various concentrations of berberine for various time-periods. The cells were then harvested and analyzed for MMP levels by flow cytometry (HL-60: panel A, and B; WEHI-3: panel C and D), as described in Materials and Methods.*

Effects of berberine on Ca^{+2} in HL-60 and WEHI-3 cells. The results from both cell lines showed that the percentage of Ca^{+2} was significantly different between the berberine-treated and control groups. Increasing the dose of berberine led to an increase in Ca^{+2} concentrations in both cell lines examined (Figure 6A and B: HL-60; Figure 6C and D). The effects of berberine on the levels of Ca^{+2} appear to be dose-dependent.

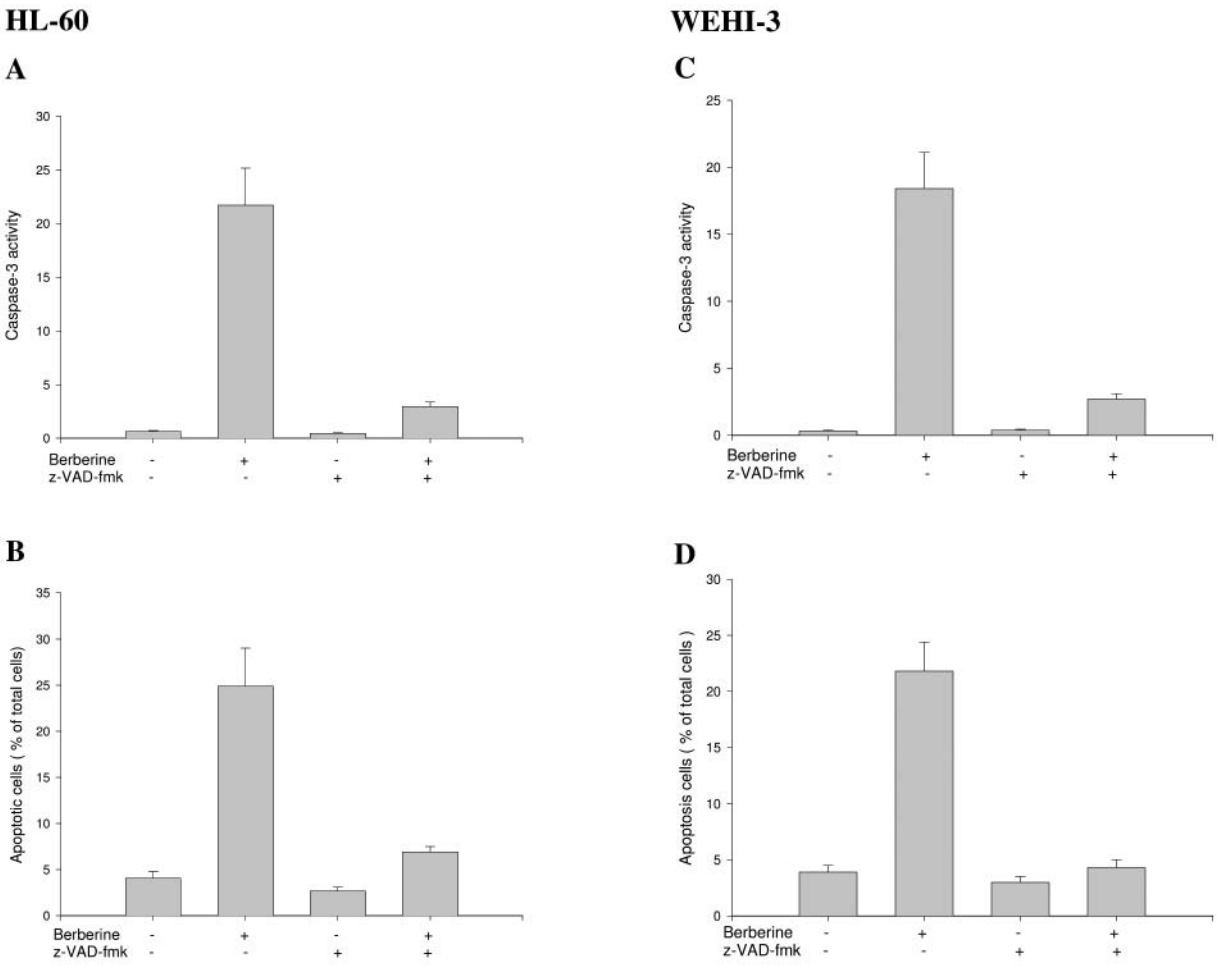

Figure 8. *Effects of berberine on caspase-3 activity and apoptosis in HL-60 and WEHI-3 cells. HL-60 and WEHI-3 cells were incubated with various concentrations of berberine for various time-periods. The cells were then harvested and analyzed for caspase-3 activity (panel A: HL-60; panel C: WEHI-3) and apoptosis (panel B: HL-60; panel D: WEHI-3), as described in Materials and Methods. The data represent mean±S.D. of three experiments *p<0.05.*

Effects of berberine on the mitochondrial membrane potential in HL-60 and WEHI-3 cells. The results from both cell lines show that the percentage of MMP was significantly different between the berberine-treated and control groups. Increasing the dose of berberine led to a decrease in the MMP in both cell lines examined (Figure 7A and B: HL-60; Figure 7C and D). Therefore, the effects of berberine on the levels of MMP are dose- dependent.

Inhibition of berberine-induced caspase-3 activity and apoptosis by the caspase inhibitor z-VAD-fmk in HL-60 and WEHI-3 cells. These experiments detected berberine-induced caspase-3 activity and also examined whether the caspase-3 activation involved in apoptosis was or was not triggered by berberine. The data presented in Figure 8A and C indicate that berberine increased caspase-3 activity, while the caspase inhibitor z-VAD-fmk decreased the caspase-3 activity in both examined cell lines. Hl-60 and/or WEHI-3 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h prior to treatment with berberine. When cotreated with berberine and z-VAD-fmk, inhibition of berberine-mediated caspase-3 activation was accompanied by a marked attenuation of berberine-induced apoptotosis (Figure 8B and D).

Effects of berberine on Bax, Bad, Bcl-2 and cytochrome c levels in HL-60 and WEHI-3 cells in response to varying concentrations of berberine, as studied by Western blotting. The results from Western blotting are presented in Figure 9A, B, C and D for HL-60 cells and Figure 9E, F, G and H for WEHI-3 cells. The data demonstrated that 5-60 μM berberine increased the Bax, Bad and cytochrome levels and decreased Bcl-2 levels in both cell lines examined. These effects of berberine were also dose-dependent.

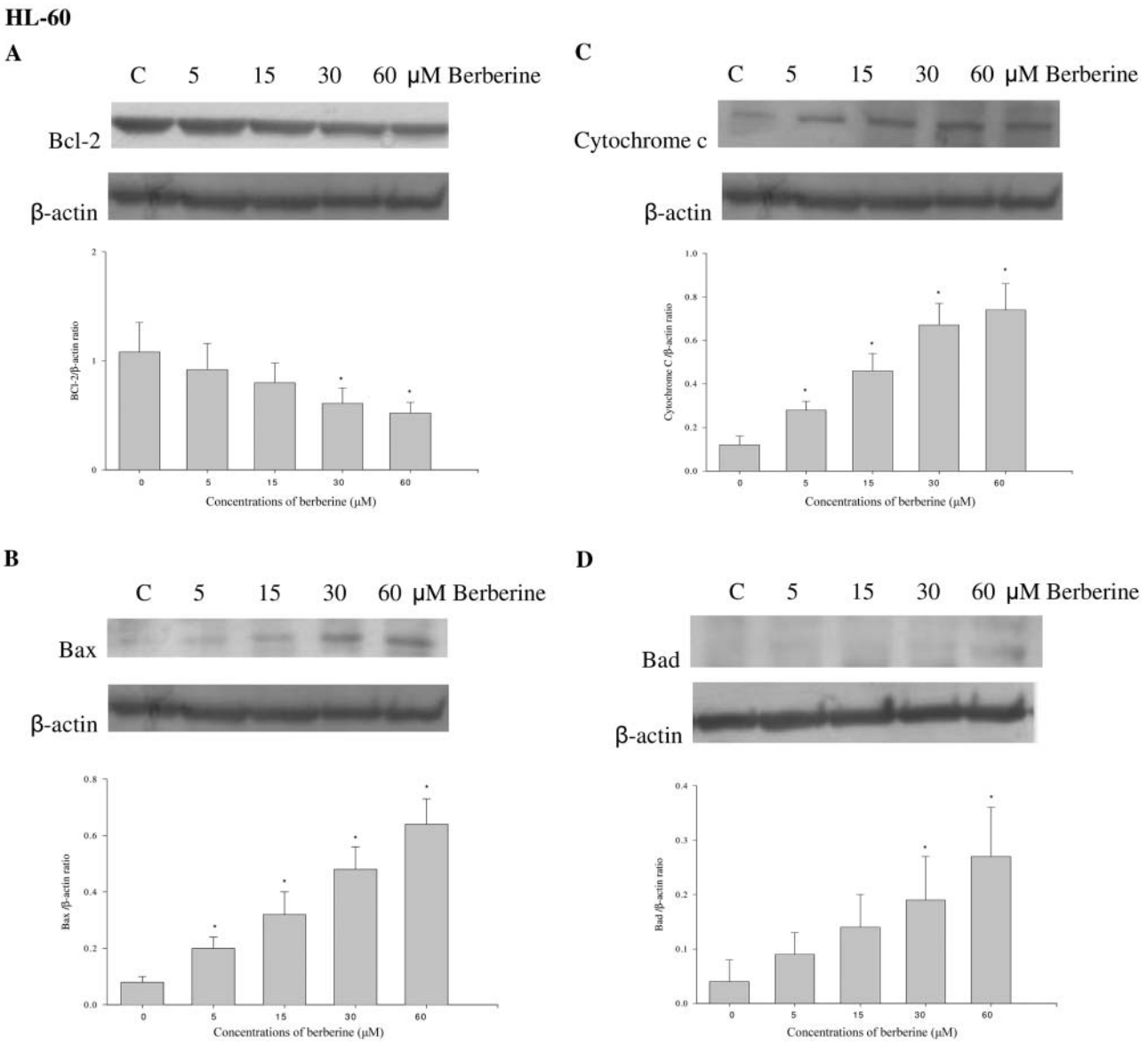

Figure 9. *continued*

Discussion

Although other investigators have already demonstrated that berberine induces apoptosis in HL-60 cells in association with down-regulation of nucleophosmin/B23 and telomerase activity (22), there is still no available information regarding the production of ROS and Ca^{+2} in berberine-treated HL-60 cells, or addressing the effect of berberine on murine WEHI-3 cells. Little is known about the mechanisms by which berberine suppresses growth in these cells. Here, it was found that berberine induced apoptosis in both HL-60 and WEHI-3 cell lines in association with caspase-3 activity and MMP depolarization. Both cell lines, after cotreatment with various concentrations of berberine, showed a marked increase in the caspase-3 activity when compared with the control groups, based on analysis by flow cytometry. Moreover, the fact that the increased expression of caspase-3 activity was associated with a decrease of the levels of MMP suggests that berberine-induced apoptosis is mitochondria-dependent, and that signaling pathways may be involved in regulating caspase-3 activity, since the caspase inhibitor (z-VAD-fmk) led to a decrease in the caspase-3 activity and to apoptosis. A more interesting point is that berberine also

Figure 9. *continued*

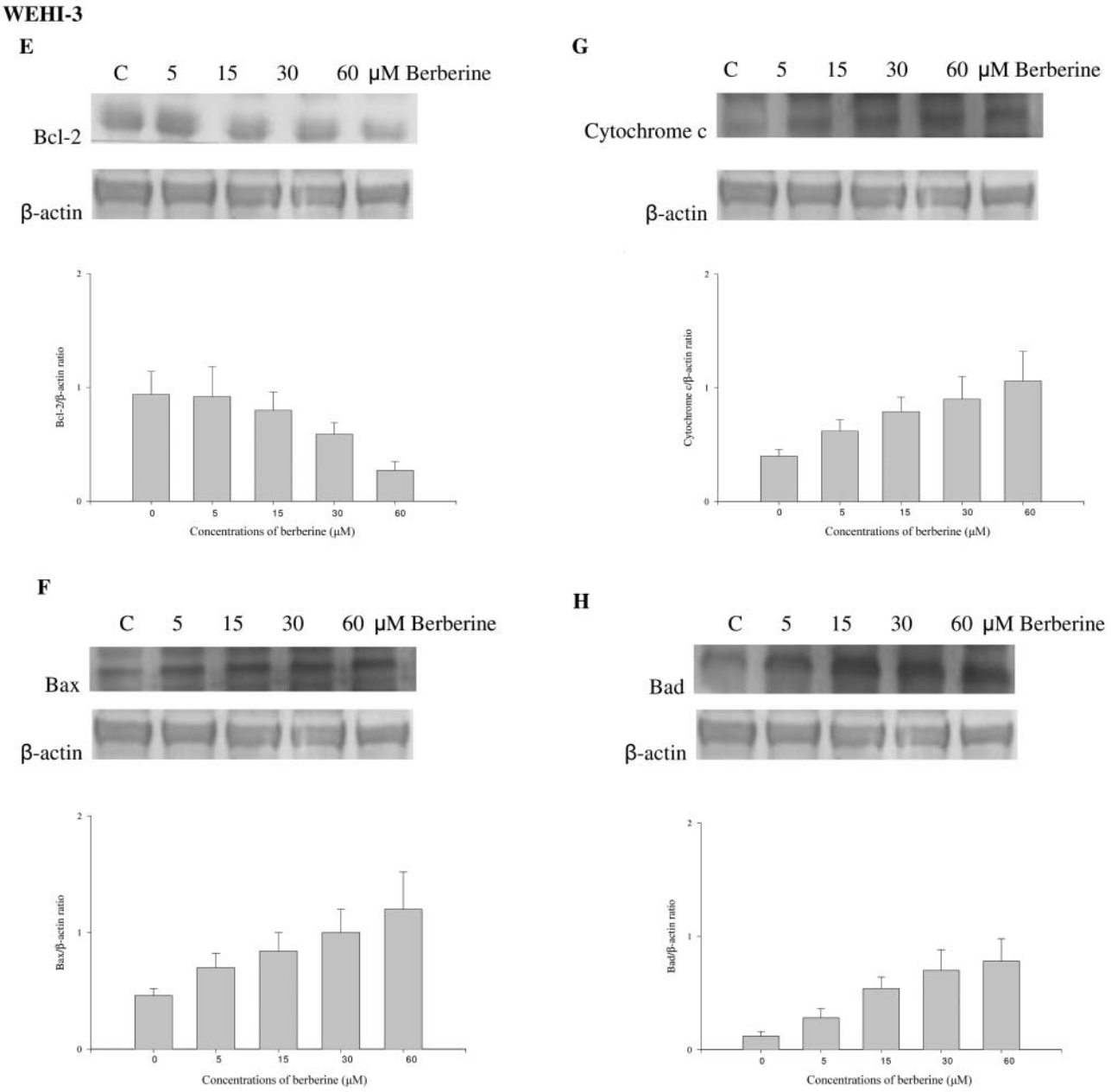

Figure 9. *Representative Western blot showing changes in the levels of Bcl-2, Bax, Bad and cytochrome c in HL-60 and WEHI-3 cells after exposure to berberine. HL-60 or WEHI-3 cells (5x10^6/ml) were treated with 0, 5, 15, 30 and 60 μM berberine for 24 h before the cytosolic fraction and total protein were prepared and determined, as described in Materials and Methods. The evaluation of the Bcl-2, Bax, cytochrome c and Bad levels (panel A, B, C and D for HL-60; panel E, F, G, H for WEHI-3) were carried out by Western blotting, as described in Materials and Methods.*

increased the production of ROS in short periods of time and led to the production of Ca^{+2}.

Apparently, ROS and Ca^{+2} production are also associated with the effects of berberine on apoptosis in both the examined cell lines. High levels of ROS are known to induce not only cell death (23), but also DNA damage and genomic instability (24), which in turn leads to tumorigenesis and its advancement. It was also reported that ROS levels are significantly higher in certain tumor cells when compared with non-tumor cells (25), suggesting that sustained production of ROS may contribute to tumor progression. Although berberine induced ROS early after

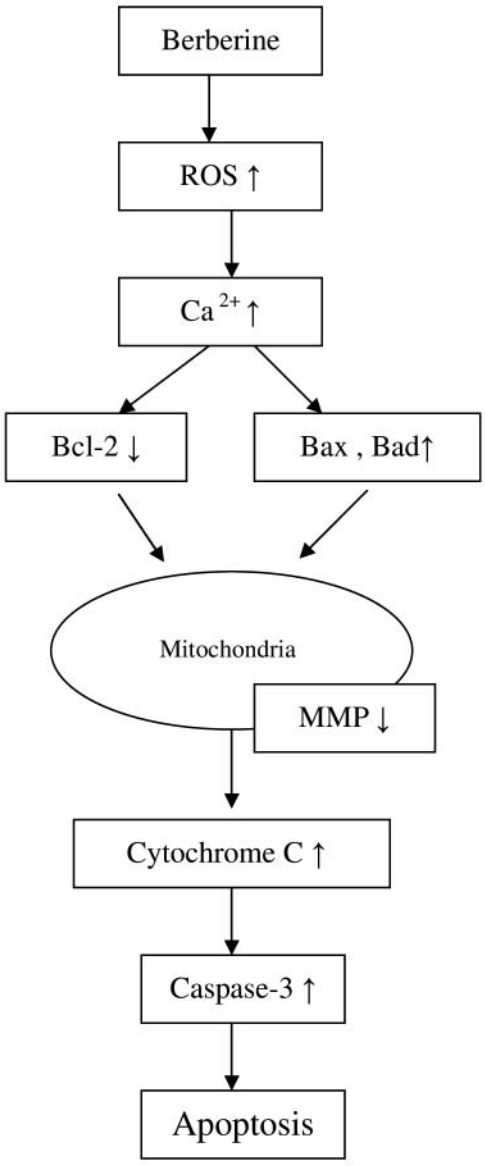

Figure 10. *A proposed model for the berberine mechanism of apoptosis in leukemia cells. Berberine induced ROS and Ca^{+2} production, promoted Bax and Bad production, but decreased the expression of Bcl-2, leading to a decrease in MMP levels, which caused the release of cytochrome c and then led to caspase-3 activity. Finally, apoptosis occurred in both cell lines examined.*

treatment, ROS levels were back to normal 6 hours after treatment. However, it has been reported that ROS is also associated with apoptosis in cancer cells. In addition, low levels of ROS enhance cell proliferation (26, 27). Other studies have shown that antioxidants, which scavenge intracellular ROS, suppress colony formation (28) and proliferation of transformed cells (29).

Our results from flow cytometry analysis also showed that the sub-G1 group occurs in both berberine-treated cell lines, confirmed by DAPI staining. The results of the Comet assay also revealed that berberine induced DNA damage in both examined cell lines in a dose-dependent manner (data not shown). This may be a characteristic of the apoptosis induced by berberine. On the basis of these findings, a working model is presented (Figure 10), by which berberine increased accumulation of Bax and Bad, but decreased the expression of Bcl-2 and led to the depolarization of MMP, thus further enhancing the release of cytochrome c and increasing the activation of caspase-3, finally leading to apoptosis. The results of *in vitro* studies suggest the potential application of berberine in the treatment of leukemia cells.

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