

Dihydroartemisinin Induces Apoptosis in Human Bladder Cancer Cell Lines Through Reactive Oxygen Species, Mitochondrial Membrane Potential, and Cytochrome C Pathway

Abstract

Background: Dihydroartemisinin (DHA) is a semisynthetic derivative of artemisinin and has antiproliferative effect. However, such effects of DHA have not yet been revealed for bladder cancer cells. **Methods:** We used as bladder cancer cell lines to examine the effect of DHA on the cell viability, cell apoptosis, and monitoring of mitochondrial membrane potential ($\Delta\Psi_m$) changes. Furthermore, the effect of DHA on the reactive oxygen species (ROS) production and cytochrome c release were also detected. We employed MTT assay to investigate the cell proliferation effect of DHA on the EJ-138 and HTB-9 human bladder cancer cells. Annexin/PI staining, caspase-3 activity assay, Bcl-2/Bax protein expression, mitochondrial membrane potential assay, cytochrome c release, and ROS analysis were used for apoptosis detection. **Results:** DHA significantly reduced cell viability in a dose-dependent manner. Cytotoxicity of DHA was suppressed by N-acetylcysteine. The growth inhibition effect of DHA was related to the induction of cell apoptosis, which were manifested by annexin V-FITC staining, activation of caspase-3. DHA also increased ROS generation, cytochrome c release, and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in cells. In addition, the downregulation of regulatory protein Bcl-2 and upregulation of Bax protein by DHA were also observed. **Conclusions:** These findings demonstrated that DHA induces apoptosis through mitochondrial signaling pathway. These suggest that DHA may be a potential agent for induction of apoptosis in human bladder cancer cells.

Keywords: Apoptosis, artemisinins, reactive oxygen species, urinary bladder neoplasms

Introduction

Bladder cancer is one of the most common type of the urinary tract cancers, affecting men and women.^[1] It is the fourth most common cancer in men and seventh in women.^[2] The incidence rate of bladder cancer increased significantly in the last years with highest rates in the USA and Europe. Lowest rates were recorded in many African and Asian countries.^[3] About 70%–85% of patients with bladder cancers have superficial and noninvasive tumors at the time of diagnosis.^[3] The surgery of transurethral resection is the most common treatment for these patients.^[4] However, recurrence rate of tumor is high after the surgery.^[5-7] Some adjuvant chemotherapies are used to prevent the tumor recurrence. However, use of these drugs is limited due to severe side effects such as hemorrhagic cystitis.^[8] Therefore, novel strategies are needed for addressing and eliminating bladder cancer cells with less complication.

Currently, the new therapies have been directed toward identifying antiproliferative agents which induce apoptosis in bladder cancer cells.^[9] Plant Secondary Metabolites, as natural products, may cause apoptosis in cancer cells.^[10] Artemisinin is an organic compound isolated from the plant *Artemisia annua* L. and widely used as an effective anti-malaria medication. Moreover, artemisinin showed some effective cytotoxic and growth arrest features on human cancers.^[11,12] Dihydroartemisinin (DHA) is a semisynthetic derivative of artemisinin which has similar anti-malaria and anticancer effects.^[13-16] Recent studies in anticancer activities of DHA have illustrated the involvement of several cellular processes and signaling pathways including cell cycle and apoptosis. For examples, DHA could arrest cell cycle at G2/M phase^[17] and significantly induces apoptosis through downregulation of cyclin D1 and p38 kinase,^[18] inhibition of nuclear factor- κ B^[19]

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and MEK/ERK.^[20] Furthermore, it appears DHA leads to overproduction of reactive oxygen species (ROS) which activates mitochondrial-dependent apoptotic pathways.^[21] Gao *et al.*^[22] reported that similar DHA-related pathways induce cell apoptosis in hepatocellular carcinoma.

However, the effects of DHA have not yet been elucidated in bladder cancer cells, completely. Jun *et al.* in 2013 investigated some features of DHA in T24 bladder cancer cell lines.^[23] However, in Jun *et al.*'s study, evaluation of cell viability, measurement of ROS and mitochondrial membrane potential ($\Delta\Psi_m$), and cytochrome c expression levels have not been performed. In this study, the effects of DHA in the induction of apoptosis were evaluated in bladder cancer cell lines EJ-138 and HTB-9. *In vitro* assessment of cell viability, apoptosis, and fluctuation of mitochondrial membrane potential ($\Delta\Psi_m$) have been determined. Furthermore, the effect of DHA on the ROS production and cytochrome c release were also detected.

In the present study, our results show that DHA induces apoptosis in bladder cancer cell lines, EJ-138 and HTB-9. The underlying mechanism of DHA-induced cytotoxicity may be ROS overproduction which accompanied with mitochondrial dysfunction. These results demonstrated that DHA induced apoptosis through intrinsic classical apoptotic pathway.

Methods

Materials

DHA (Cat: A2679) was purchased from LKT-Laboratory, Inc., Minnesota, USA. MTT (Cat: M5655), dimethyl sulfoxide (DMSO) (Cat: PHR1309), JC-1 probe (Cat: T4069), N-acetylcysteine (NAC) (Cat: 1009005) were from Sigma-Aldrich Munich, Germany. Annexin V-FITC/PI apoptosis detection kit (Cat: 4830-01-K), Caspase-3 Colorimetric Assay kit (Cat: BF3100) were purchased from R & D System. Marker Gene™ Live Cell Fluorescent ROS Detection Kit (Cat: M1049), Antibodies against Bcl2 (sc-7382), Bax (sc-13156), cytochrome c (Cat: Sc-13561), and also horseradish peroxidase secondary antibodies (sc-358923) were purchased from Santa Cruz Biotechnology Co.

Cell culture

The human bladder cancer cell lines, EJ-138 (C429) and HTB-9 (C450), were purchased from National Cell Bank of Iran. The cells were cultured with standard protocol in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. The cell culture flasks were maintained at 37°C in a humidified atmosphere incubator containing 5% CO/95% air gas phase.

Evaluation of EJ-138 and HTB-9 viability by MTT assay

The procedure was carried out by a previously detailed method.^[24] Briefly, 5×10^3 cells/well were plated into a 96-well plate. The RPMI-1640 medium in each well was then switched with media containing various concentrations of DHA (0.1–200 µM) in the absence or presence of 1 mmol/L NAC for 48 h. 20 µl of MTT (Cat: M5655, from Sigma-Aldrich Munich, Germany) solution (5 mg/ml in PBS) was added to each well and the cells were incubated for another 4 h at 37°C. The supernatants were then removed and 200 µl of DMSO (Cat: PHR1309 from Sigma-Aldrich Munich, Germany) was added to each well and plate was continuously shake for 10 min. Finally, the absorbance of each well was measured at 570 nm using a Synergy HT Multi-Mode Microplate Reader.

Flow cytometric cell apoptosis assay

We used annexin V-FITC/PI apoptosis detection kit (Cat: 4830-01-K from R and D system) to evaluate early stages of apoptosis according to the previous protocol.^[25] In brief, cells seeded to a density of 5×10^5 per well in six-well plate and treated with DHA (1–100 µM) in the absence or presence of 1 mmol/L NAC for 48 h. The treated and untreated cells were analyzed by a fluorescence-activated cell sorter flow cytometer (BD Biosciences, San Jose, USA).

Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

Mitochondrial membrane potential ($\Delta\Psi_m$) is related to cells capacity to generate ATP by oxidative phosphorylation. Therefore, the $\Delta\Psi_m$ could be an important indicator of cell health or injury. We used JC-1 probe (Cat: T4069 from Sigma-Aldrich Munich, Germany) to evaluate $\Delta\Psi_m$ as previously described.^[24] Briefly, EJ-138 and HTB-9 cells were treated with DHA (0.1–100 µM) in the absence or presence of 1 mmol/L NAC for 48 h. Culture medium of cells was replacing with HEPES buffer (40 mM, pH 7.4) containing 2.5 mM JC-1 for 30 min at 37°C. Finally, sequential fluorescence of each well was measured at two excitation/emission wavelength pairs, 490/540 and 540/590 nm, using a Synergy HT Multi-Mode Microplate Reader.

Measurement of reactive oxygen species

ROS levels were evaluated using Marker Gene™ Live Cell Fluorescent ROS Detection Kit (Cat: M1049) according to the manufacturer's instructions. Briefly, cells were plated to a density of 25×10^3 per well in 96-well plate and incubated with different concentrations of DHA in the absence or presence of 1 mmol/L NAC for 48 h. After treatment, cell culture was replaced with HBSS buffer containing 2',7'-dichlorofluorescein diacetate (DCF) (20 µM) at 37°C for 30 min in the dark. After washing with HBSS, DCF-fluorescence intensities were recorded at 485 nm (excitation) and 528 nm (emission) using a Synergy HT Multi-Mode Microplate Reader.

Caspase-3 activity assay

A quantitative enzymatic cellular caspase-3 activity was performed by Caspase-3 Colorimetric Assay kit (Cat: BF3100 from R & D system) according to the manufacturer's instructions. After DHA treatment for 48 h, cells were washed with ice-cold PBS, lysed, centrifuged for 10 min at $10,000 \times g$. Same volume of cell lysate was added to 50 μ l of reaction buffer and 5 μ l of specific Caspase-3 substrate (DEVD-pNA) and incubated at 37°C for 1 h. Absorbance was measured at 405 nm using a Synergy HT Multi-Mode Microplate Reader.

Protein extraction and immunoblotting assays

EJ-138 and HTB-9 Cells were plated into 25 cm² tissue culture flasks and treated with DHA (0.1–100 μ M) containing media for 48 h. According to the previous protocols,^[25] immobilized proteins were detected using mouse monoclonal antibody against Bcl2 (1:1000) (sc-7382), Bax (1:1000) (sc-13156), and cytochrome c (1:1000) (Cat: Sc-13561) (all from Santa Cruz Biotechnology Co.) overnight at 4°C, and washed three times. Membranes were incubated with horseradish peroxidase secondary antibodies (1:5000) (sc-358923) for 2 h at room temperature. Then, specific antigen-antibody complexes were visualized using ECL detection reagent according to the manufacturer's instructions.

Statistical analysis

Nonparametric one-way analysis of variance was performed with the Dunnett's test, using the software Statistical Package for the Social Sciences (Statistical Package ver. 18.0; SPSS Inc, Chicago, IL, USA). Each experiment was carried out in triplicate and repeated 3–4 times independently. $P < 0.05$ was considered significant. All data were presented as means \pm standard deviation.

Results

Dihydroartemisinin inhibits proliferation of bladder cancer cell lines

The effect of DHA on cell viability was performed by MTT assay. As shown in Figure 1, incubation of EJ-138 and HTB-9 cell lines with different concentrations of DHA in the presence and absence of NAC (1 mM) for 48 h resulted in a dose-dependent reduction in the cell viability as compared with control. In both cell lines [Figure 1a], a significant inhibitory effect was observed at 1 μ M ($P < 0.05$ versus control group) with maximum trend at 200 μ M ($P < 0.01$ versus control group). However, after cotreatment of DHA and NAC, the inhibitory effects of DHA on the EJ-138 [Figure 1b] and HTB-9 [Figure 1c] cell lines growth were significantly reversed by NAC (1 mmol/L). The results demonstrate that NAC increased resistance to cytotoxicity challenge with DHA.

Apoptotic-dependent cell death in bladder cancer cells are positively correlated with increased concentration of dihydroartemisinin

To determine whether growth inhibitory effect of DHA in EJ-138 and HTB-9 cells were related to cell apoptosis, the effect of DHA on cell apoptosis was evaluated by annexin-V and PI double staining. EJ-138 and HTB-9 cells treated with different concentrations of DHA in the presence and absence of NAC (1 mM) for 48 h and analyzed by flow cytometry. As shown in Figure 2a and b, DHA in a dose-dependent manner significantly ($P < 0.01$) increase the susceptibility of these cell lines to apoptosis. These effects were dose-dependent, and an increased trend in the percentages of apoptosis was detected after treatment with higher concentrations. As expected, NAC significantly ($P < 0.05$) reduces DHA-induced apoptosis in EJ-138 [Figure 2a] and HTB-9 cells [Figure 2b].

Dihydroartemisinin increases mitochondrial permeability of EJ-138 and HTB-9 cells by Collapse of $\Delta\Psi_m$

Depletion of $\Delta\Psi_m$ impairs mitochondria function and leads to the release of cytochrome c into the cytoplasm, which initiates apoptosis cascade.^[26] We measured the dynamics of $\Delta\Psi_m$ by monitoring of red/green fluorescence ratio of the potential-sensitive dye JC-1, a cationic dye which exhibits potential-dependent accumulation in mitochondria, in DHA-treated EJ-138 and HTB-9 cells under confocal microscope. The results showed that DHA treatment was associated with a significant drop in $\Delta\Psi_m$ in a time and dose-dependent manner [Figure 3a-c]. Interestingly, NAC effectively attenuates any decline of $\Delta\Psi_m$ in DHA treated cells [Figure 3a and b].

Dihydroartemisinin sensitizes bladder cancer cells to reactive oxygen species-induced cell apoptosis

To explore the ROS-inducing potential of DHA in EJ-138 and HTB-9 cells, we evaluated the levels of ROS after 48 h treatment with different concentrations of DHA in the presence and absence of NAC (1 mM). The results indicated that DHA promoted ROS production in these cells in a dose-dependent manner [Figure 3d and e]. However, the DHA-dependent ROS generation levels decreased dramatically in the presence of NAC.

Apoptotic effect of dihydroartemisinin mediated through caspase-3 activation

Since caspase-3 may involve in the mitochondrion-initiated apoptosis,^[27] we evaluated the potential contribution of caspase-3 in the DHA-induced apoptosis. We found that caspase-3 was activated significantly in both cell types after stimulation with DHA ($P < 0.01$) [Figure 4]. This effect was time dependent and dose dependent and observed after 48 h treatment. To support the notion that caspase-3 activation is the main mechanism of DHA-induced apoptosis, a caspase

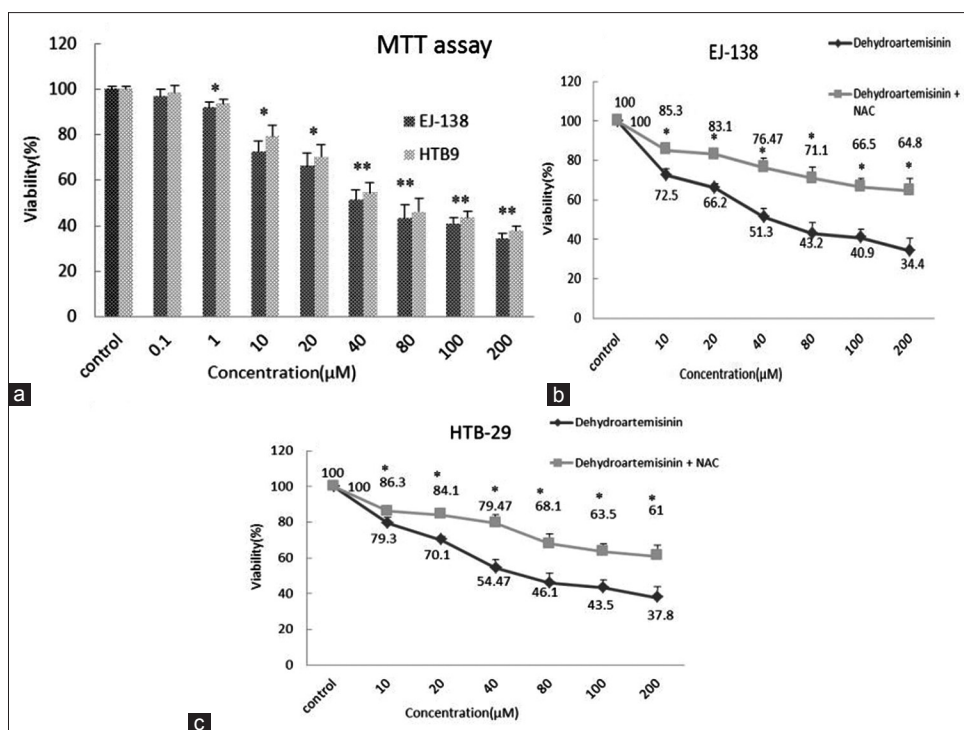


Figure 1: Cell viability assay by using quantitative MTT exclusion. Cells incubated with different concentrations (0.1–200 μM) of dihydroartemisinin for 48 h and proliferation was assessed by MTT. Dihydroartemisinin reduced cell proliferation in EJ-138 and HTB-9 (a) cells in a dose-dependent manner. Dihydroartemisinin suppressed cell growths were significantly blocked by N-acetylcysteine in the EJ-138 (b) and HTB-9 (c) cell lines

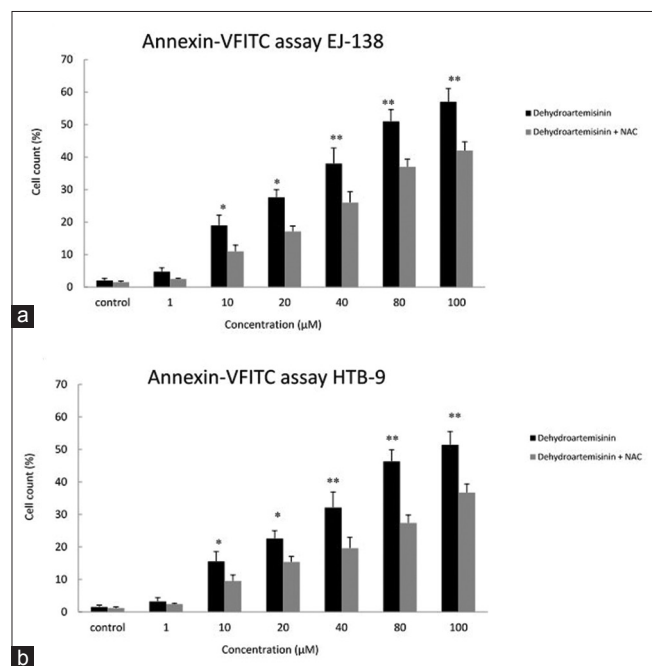


Figure 2: Dihydroartemisinin induced apoptosis in the EJ-138 (a) and HTB-9 (b) cell lines. Cells treated with different concentrations (1–100 μM) of dihydroartemisinin in the presence and absence of N-acetylcysteine (1 mM) for 48 h, and analyzed by flow-cytometry using annexin-V and PI double staining

inhibitor, z-VADfmk, was added to the culture medium. This result suggests that the activated caspase 3 in the cells was significantly attenuated by pretreatment of the cells with this inhibitor (data not shown). We demonstrated

unambiguously that the apoptosis in the DHA-treated EJ-138 and HTB-9 cells is intermediated by caspase-3 activation.

Dihydroartemisinin regulates cytochrome c, Bax and Bcl-2 expression levels to promote apoptosis in bladder cancer cells

Cytochrome C, Bax and Bcl-2 expression levels have been evaluated in DHA-treated cells. These factors involved in mitochondrial mediated apoptotic events. As demonstrated by western blot analysis in Figure 5, the antiapoptotic Bcl-2 protein was noticeably downregulated in response to DHA treatment; in contrast, cytochrome c and pro-apoptotic Bax proteins were strikingly increased. These effects were correlated with increased concentration of DHA. Collectively, the observations indicate that the EJ-138 [Figure 5a] and HTB-9 [Figure 5b] bladder cancer cells became sensitive to DHA-induced apoptosis through release of cytochrome c and regulation of Bcl-2 family proteins.

Discussion

Structurally, DHA, is one of the main active and more water-soluble metabolites of artemisinin derivatives extracted from *A. annua* and a sesquiterpene lactone peroxide with endoperoxide moiety.^[28] Recent studies promised that DHA can be a new potential therapeutic compound in cancer treatment.^[13-16] It has shown that DHA selectively inhibits the growth of many cancer cells types,

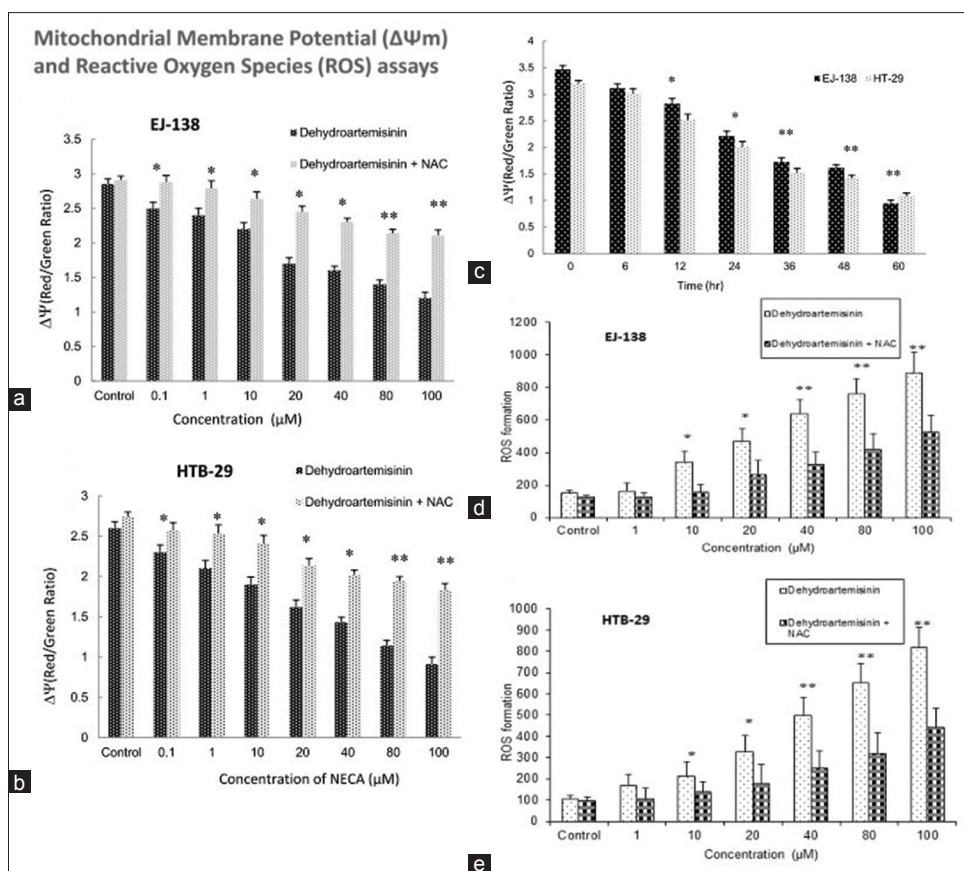


Figure 3: Dihydroartemisinin-dependent depletion of $\Delta\Psi_m$ (a-c) and increased reactive oxygen species generation (d and e). Following treatment with different concentration (0.1–100 μM) of dihydroartemisinin in the presence and absence of N-acetylcysteine (1 mM) for 48 h, cells were loaded with JC-1 dye and fluorescence ratio measured. (c) Effect of time in $\Delta\Psi_m$, cells were treated with 20 μM of dihydroartemisinin in a time intervals between 0-60 h. Following treatment, EJ-138 (d) and HTB-9 (e) cells were loaded with dichlorofluorescein diacetate and fluorescence was measured

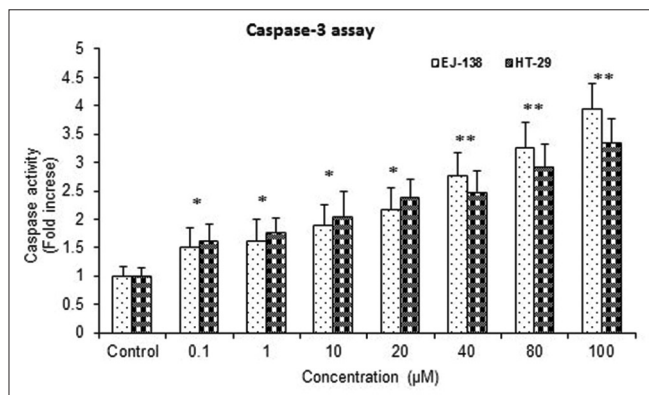


Figure 4: Dihydroartemisinin induce apoptosis through caspase-3 activation. Cells incubated with different concentration (0.1–100 μM) of dihydroartemisinin for 48 h and the enzymatic activity was measured by a quantitative colorimetric caspase-3 activity assay. The activity of caspase-3 is increased in a concentration-dependent manner

such as leukemia,^[29] pancreas,^[30] breast^[31] and prostate^[32] cancers. However, effectiveness and the molecular mechanisms of DHA-mediated cytotoxicity in bladder cancer cells remain unclear. Therefore, in the current *in vitro* study, we evaluated the effects of DHA on the apoptosis induction in EJ-138 and HTB-9 bladder cancer cells. Furthermore, we intend to explore the potential

mechanisms of DHA-mediated cytotoxicity in these cells.

Here we indicated that DHA inhibited cell proliferation and induced apoptosis in a concentration-dependent manner in EJ-138 and HTB-9 cells [Figure 1a-c]. Recently, some artemisinin derivatives showed potential anticancer effects on RT112 bladder cancer cells,^[33] but there is no further information about molecular mechanisms of this cytotoxicity. Our recent results showed that DHA initiated intrinsic or mitochondrial-dependent apoptotic pathway. Probably, these antiproliferative and apoptotic features of DHA mediated by disturbed antioxidant-oxidant balance and lead to ROS overproduction. As confirmed by our results [Figure 3d and e], this imbalance can be reversed by NAC in DHA-treated EJ-138 and HTB-9 cell lines. It is suggested that the cleavage of the endoperoxide moiety of DHA by heme iron cause ROS generation through Fenton reaction.^[26,34] In addition, DHA-mediated DNA damage and alkylation of proteins like histidine-rich protein (42 kDa), pfATP6 and the endoplasmic reticulum calcium ATPase may involve in cytotoxicity.^[35] Actually, ROS overproduction in plasmodium falciparum infected red blood cells by artemisinin, and its most potent derivative (DHA) is the main mechanism of antimalarial effects.^[12] Our results showed that DHA-induced ROS formation in EJ-138 and

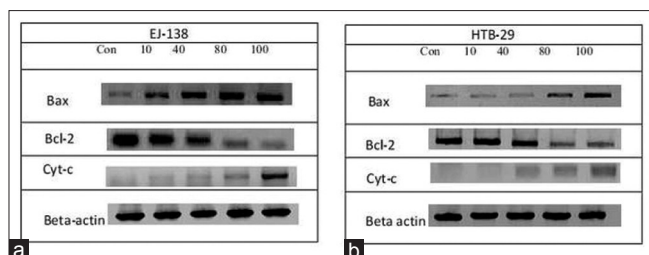


Figure 5: Dihydroartemisinin upregulates Bax and cytochrome c and downregulates Bcl-2 to promote apoptosis in EJ-138 (a) and HTB-9 (b) cell lines. Cells were treated with the indicated concentrations of dihydroartemisinin for 48 h and then the expression levels of proteins analyzed by western blotting

HTB-9 are parallel with other studies,^[36-40] indicating main mechanism of DHA-mediated cytotoxicity in cancer cells to be ROS over-production.

Our annexin-V staining results showed that apoptosis may be main type of cell death induced by DHA, as confirmed by other studies.^[37,38] The collapse of mitochondrial membrane potential ($\Delta\Psi_m$) and depletion of ATP are the direct consequences of ROS on mitochondria which preceding release intermembrane space proteins, such as cytochrome c and activating caspase proteases cascade.^[41] According to our results, DHA causes significant depletion of $\Delta\Psi_m$ in EJ-138 and HTB-9 cells [Figure 3a-c]. These observations are confirmed with other studies,^[37,38] which altogether indicating that ROS-mediated $\Delta\Psi_m$ depletion and mitochondrial dysfunction are crucial in DHA-induced apoptosis in bladder cancer cells. Along with $\Delta\Psi_m$ depletion, we also demonstrated that DHA-induced activation of caspase-3 increased cytochrome c release [Figures 4 and 5]. This finding showed that the intrinsic mitochondria pathway contributed in DHA-induced apoptosis in EJ-138 and HTB-9 cells. According to other studies, mitochondria-dependent apoptosis pathway seems to be main DHA-induced apoptotic pathway in different cells.^[42] The ratio of antiapoptotic protein, Bcl-2, and proapoptotic proteins, and Bax determines tendency to cell apoptosis.^[43] In the present study, treatment of cells with DHA reduced expression of the Bcl-2 and increased expression of the Bax protein [Figure 5]. These results suggested that the Bcl-2 and Bax are involved in the apoptosis induced by DHA. These observations are in agreement with the study of Lu *et al.*^[21] that reported DHA induces cell apoptosis in colorectal cancer cells by increasing the ratio of Bax/Bcl-2. It seems artemisinin induced Bax-independent apoptosis in nonsmall cell lung cancer cells. It seems $\Delta\Psi_m$ did not play any significant role on artemisinin-induced cytotoxicity. Interestingly, these apoptotic effects of artemisinin were not prevented by NAC,^[44] which indicates DHA and artemisinin engage different pathways to induce apoptosis.

Conclusions

This study showed that DHA has antiproliferative and

apoptotic activity against EJ-138 and HTB-9 bladder cancer cells through ROS generation. The present result showed that DHA may consider as new therapeutic opportunity in bladder cancer. DHA, also, may increase efficacy of other chemotherapy agents.^[36] Therefore, we believe that DHA might be a promising natural compound in cancer cell apoptosis. Our results may help to complete clear molecular mechanisms of DHA-induced apoptosis signaling pathway in bladder cancer cells.

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Conflicts of interest

There are no conflicts of interest.

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