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Morphine Attenuated the Cytotoxicity Induced by Arsenic Trioxide in H9c2 Cardiomyocytes

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Abstract Arsenic trioxide (ATO) is an efficient drug for the treatment of the patients with acute promyelocytic leukemia (APL). Inhibition of proliferation as well as apoptosis, attenuation of migration, and induction of differentiation in tumor cells are the main mechanisms through which ATO acts against APL. Despite advantages of ATO in treatment of some malignancies, certain harmful side effects, such as cardiotoxicity, have been reported. It has been well documented that morphine has antioxidant, anti-apoptotic, and cytoprotective properties and is able to attenuate cytotoxicity. Therefore, in this study, we aimed to investigate the protective effects of morphine against ATO toxicity in H9c2 myocytes using multi-parametric assay including thiazolyl blue tetrazolium bromide (MTT) assay, reactive oxygen species (ROS) generation, caspase 3 activity, nuclear factor kappa B (NF-κB) phosphorylation assay, and expression of apoptotic markers. Our results showed that morphine (1 μM) attenuated cytotoxicity induced by ATO in H9c2 cells. Results of this study suggest that morphine may have protective properties in management of cardiac toxicity in patients who receive ATO as an anti-cancer treatment.

Keywords Arsenic trioxide · Morphine · Cardiomyocyte · Cytotoxicity · H9c2

Introduction

Arsenic trioxide (ATO), as Chinese traditional medicine, is an efficient therapeutic agent used in acute promyelocytic leukemia (APL) and different types of human malignancies including renal cell carcinoma, small-cell lung carcinoma, cervical cancer, hepatocellular carcinoma, breast cancer, fibrosarcoma, and neuroblastoma [1–3]. A growing body of evidence indicates that mechanisms of action of ATO against malignant cells may be related to activation of apoptosis signaling such as pro-caspases [4], down-regulation of anti-apoptotic proteins like Bcl-2 [5], inducing DNA damage [6], mitochondrial membrane potential collapse, and increasing the reactive oxygen species (ROS) formation [7–9]. Moreover, it has been shown that ATO stimulates expression of apoptotic genes such as p53-up-regulated modulator of apoptosis (*PUMA*) and Bcl-2-associated *X* protein (*BAX*) [10, 11]. Our previous study suggested that ATO induces apoptosis in an APL cell line via up-regulation of p73 through suppression of nuclear factor kappa B (NF-κB) which plays a key role in cell survival via increased expression of anti-apoptotic genes such as *survivin* [12–14], *X*-linked inhibitor of apoptosis protein (*X-IAP*), cellular inhibitor of apoptosis proteins (*cIAP1* and *cIAP2*) [15],

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and *BCL2*-like 1 (*BCL-XL*) [16] as well as increasing the lactate dehydrogenase (LDH) leakage [6].

Despite advantages of ATO in treatment of several types of malignancies, certain lethal and toxic side effects at therapeutic doses have been reported such as sudden death [17], hepatotoxicity [18], and also cardiotoxicity, which is manifested by QTc prolongation and T-wave inversion and heart block [19–21]. Also, recent evidence suggests that mitochondrial dysfunction plays a critical role in ATO-mediated cardiotoxicity via increasing ROS production [22, 23]. Therefore, poly-therapy for mitigating the ATO-induced cardiotoxicity seems a necessity based on aforementioned studies. In the current study, we investigated possible protective effects of morphine against ATO-induced cardiotoxicity in H9c2 cardiac muscle cells.

Material and Methods

Cell Line and Treatment

H9c2 cells were purchased from National Cell Bank of Iran (NCBI; Tehran, Iran) and maintained in Dulbecco's modified Eagle's medium (DMEM), containing 0.15 % sodium bicarbonate, 10 % fetal bovine serum (Invitrogen), 0.11 % sodium pyruvate, 0.45 % glucose, 20 μM L-glutamine, 50 μg/ml gentamicin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin, and 25 ng/ml amphotericin B, without phenol red. Cells were grown in 75-cm^2 tissue culture flasks at 37 °C in a 5 % CO_2 humidified incubator. H9c2 cells were treated with 0.5, 1, and 2 μM of ATO (Sina Darou, Tehran, Iran). An appropriate amount of stock solution (0.5 mM in DMEM) of ATO was added to culture medium to obtain the desired concentrations and then incubated for 24, 48, and 72 h. The H9c2 cells were exposed to 1 μM of morphine (Mac Farland & Smith, England) 4 h before ATO treatment and incubated for 24, 48, and 72 h.

Thiazolyl Blue Tetrazolium Bromide Assay

The inhibitory effects of ATO on growth and proliferation of H9c2 cells were investigated by uptake of thiazolyl blue tetrazolium bromide (MTT) by the living cells as described by Momeny et al. [12] in 96-well plates (SPL Lifesciences, Pocheon, Korea) at a concentration of 5000 cells/100 μl/well.

Bromodeoxyuridine Cell Proliferation Assay

The effects of ATO and morphine on DNA synthesis in the H9c2 cells were studied using a colorimetric bromodeoxyuridine (BrdU)-based cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Mannheim, Germany) at 450 nm in different time intervals (24, 48, and 72 h) according to the manufacturer's instructions.

Measurement of ROS Formation

The rate of ROS formation was assayed by fluorescence spectrophotometer (FLX 800, BioTek, USA) using dichlorodihydrofluorescein diacetate (DCFH-DA), a vital fluorescent probe which enters to cells and is hydrolyzed to 2′,7′-dichlorofluorescein ($DCFH_2$). Reaction with ROS forms the highly fluorescent dichlorofluorescin (DCF) which effluxes from the cells. The fluorescence intensity of DCF was measured by fluorescence plate reader at 485-nm excitation and 538-nm emission wave lengths, respectively. Results were communicated as fluorescence percentage of control cells [24].

Caspase 3 Activity Assay

The effects of ATO and morphine on caspase-3 activity in H9c2 cells were measured by colorimetric caspase-3 assay kit (Sigma). This assay is based on hydrolysis of peptide substrate (AC-DEVE-pNA) that is mediated by caspase-3 in a 96-well plate at 37 °C for 2 h. Absorbance of pNA is measured spectrophotometrically at 405-nm wavelength [25].

Cell-Based NF-κB Phosphorylation Measurement

The effects of ATO and morphine on NF-κB activation were assessed by an ELISA assay based on phosphorylated NF-κB form to total ratio at 450 nm (CASE Kit, Super Array Bioscience, Frederick) as described with Janssen and Sen [26].

Analysis of Gene Expression by Real-Time Quantitative PCR

Fast Pure RNA Kit (Takara Bio, Inc., Otsu, Japan) was used to extract total RNA from the cultured cells. Changes in messenger RNA (mRNA) expression of desired genes were evaluated by real-time PCR after reverse transcription of 1 μg RNA from each sample with Prime Script RT Reagent Kit (Takara Bio) according to the manufacturer's instructions. Quantitative real-time RT-PCR was done on a light cycler instrument (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). The PCR assay was performed in a final volume of 20 μl containing 10 μl of SYBR Green master mix, 2 μl of cDNA samples, 0.5 μl of each forward and reverse primers (10 pmol), and 7 μl of nuclease-free water (Qiagen, Hilden, Germany). Thermal cycling environment involved an initial activation step for 30 s at 95 °C followed by 45 cycles including a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve investigation was applied to confirm whether all primers yield a single PCR product. Primers are mentioned in Table 1. Hypoxanthine

Table 1 Primer sequences for real-time RT-PCR experiments

Gene	Forward primer (5′–3′)	Reverse primer (3′–5′)
HPRT	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA
PUMA	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT
BAX	CGAGAGGTCTTTTTCCGAGTG	GTGGGCGTCCCAAAGTAGG
Bcl-2	CGGTGGGGTCATGTGTGTG	CGGTTCAGGTACTCAGTCATCC
Survivin	CCAGATGACGACCCCATAGAG	TTGTTGGTTTCCTTTGCAATTTT
cIAP2	TCCTGGATAGTCTACTAACTGCC	GCTTCTTGCAGAGAGTTTCTGAA
XIAP	ATAGTGCCACGCAGTCTACAA	AGATGGCCTGTCTAAGGCAAA
Bcl-XL	GAGCTGGTGGTTGACTTTCTC	AGATGGCCTGTCTAAGGCAAA
cIAP1	GTCCTCTGCGGAAGTGACTC	GGAAACTGGCGCTGGTATAA

phosphoribosyltransferase1 (HPRT1) was amplified as a normalizer, and fold adjust in expression of each target mRNA relative to HPRT1 was considered based on $2^{-\Delta\Delta Ct}$ relative expression formula [27].

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). All the experiments were carried out in triplicate. For statistical analysis, the Student's *t* test and one-way ANOVA were applied. In order to compare the groups, Dunnett's multiple-comparison test was used. *P* values less than 0.05 were considered significant.

Results

Morphine Antagonizes Inhibitory Effects of ATO on Viability in H9c2 Cells

The activity of complex II was assessed using MTT test after incubating H9c2 cells with different concentrations of ATO (0.5, 1, and 2 μM). Figure 1 shows a significant decrease in the mitochondrial metabolism of MTT to formazan ($p < 0.05$) following treatment with ATO (0.5, 1, and 2 μM) comparing to control group in a concentration-dependent manner. Also, pretreatment with morphine (1 μM) before ATO treatment (4 h) significantly antagonized the inhibitory effects of ATO on viability of H9c2 cells ($P < 0.05$).

Morphine Reverses ATO-Induced ROS (H_2O_2) Formation in H9c2 Cells

As shown in Fig. 2, ATO (0.5–2 μM) induced significant ROS (H_2O_2) formation in comparison with control group in a concentration-dependent manner. A substantial increase in mitochondrial ROS formation was observed in higher concentrations of ATO (2 μM). On the other hand, pretreatment with morphine (1 μM) significantly attenuated ROS production induced by ATO in H9c2 cells ($P < 0.05$).

Morphine Antagonizes Inhibitory Effects of ATO on DNA Synthesis in H9c2 Cells

As shown in Fig. 3, using the BrdU cell incorporation assay to measure proliferation, the incorporation of BrdU in ATO-treated cells apparently affects the subsequent cell growth in H9c2 cells. Our results showed the inhibitory effect of ATO (0.5, 1, and 2 μM) on DNA synthesis in concentration-dependent manner. On the other hand, morphine (1 μM) could antagonize the suppressive effects of ATO in desired concentration on DNA synthesis in H9c2 cells.

Morphine Antagonizes ATO-Induced Caspase-3 Activation

As shown in Fig. 4, activity of caspase-3 enzyme (main mediator of apoptosis) was significantly increased (1.25–1.5-fold comparing to control) in H9c2 cells, following 48 h of exposure with ATO (1 and 2 μM). However, lower concentration of ATO (0.5 μM) did not significantly increase ROS generation compared to control mitochondria ($P > 0.05$). Significant decrease in the caspase-3 activity was observed following the treatment with morphine (1 μM) in the incubation time in all of ATO treated groups ($P < 0.05$), suggesting the role of oxidative stress in mitochondrial permeability transition (MPT)-mediated caspase-3 activation and finally cytochrome c release.

Morphine Antagonizes with ATO-Induced Modulation of Expression of Apoptosis Markers

As shown in Fig. 5, exposure with ATO (2 μM) for 48 h significantly increased expression of apoptotic genes *BAX* and *PUMA* compared to control groups ($P < 0.001$). Furthermore, ATO significantly decreased expression of anti-apoptotic genes *BCL2*, *survivin*, *cIAP1*, *cIAP2*, *XIAP*,

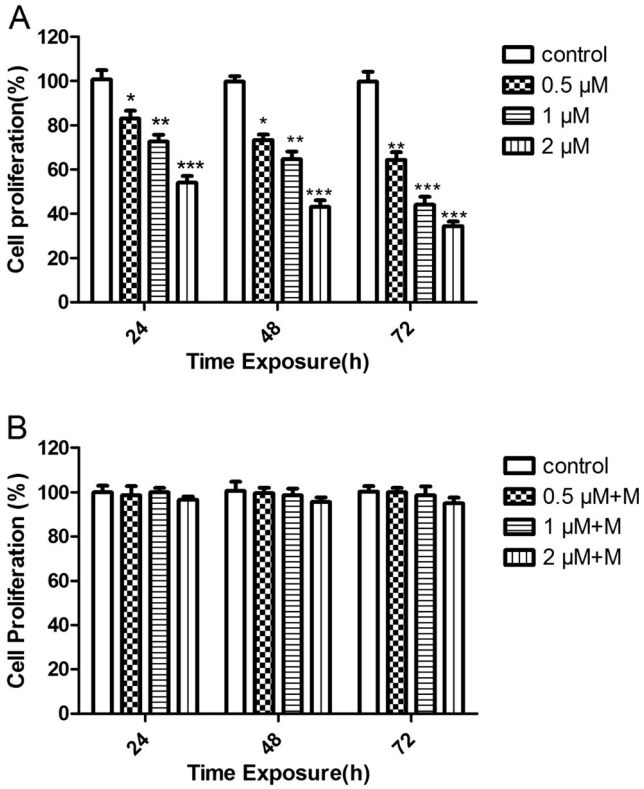

Fig. 1 MTT assay was applied to estimate the proliferative capacity of H9c2 cells over 72 h of treatment with escalating concentrations of ATO (**a**) and ATO plus morphine (1 μM) (**b**). Data are expressed as mean ± SEM and were analyzed by one-way ANOVA and Tukey's post hoc test. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared to control group

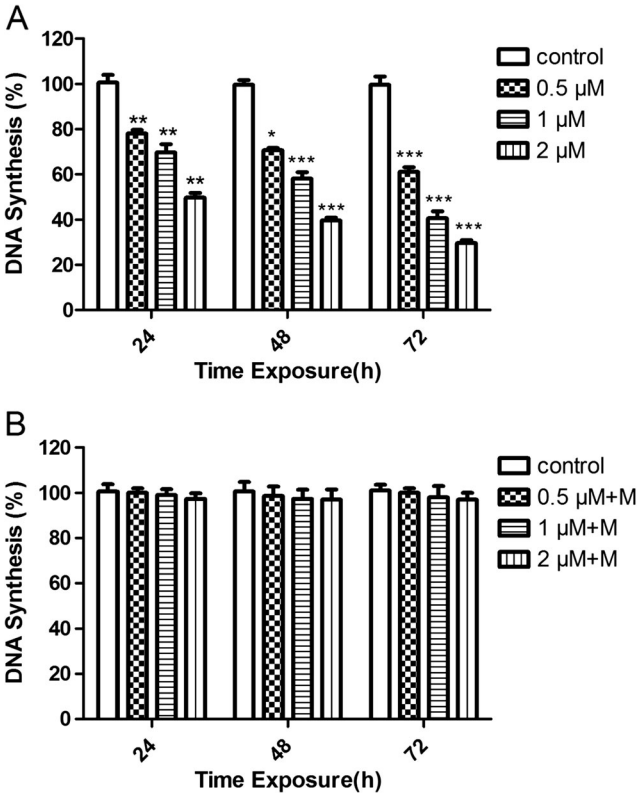

Fig. 3 The effects of enhancing doses of ATO (**a**) and ATO plus morphine (1 μM) (**b**) on DNA synthesis in H9c2 cells over 72 h of treatment. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA and Tukey's post hoc test.*$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared to control group

and *BCL-XL* in comparison with control groups ($P<0.05$ and $P<0.01$). In addition, our results showed that morphine significantly reversed the decrease in expression of apoptotic genes *BAX* and *PUMA* ($P<0.001$) and also increased expression of anti-apoptotic genes *BCL2* and *survivin* in comparison with control group ($P<0.05$ and $P<0.01$, respectively).

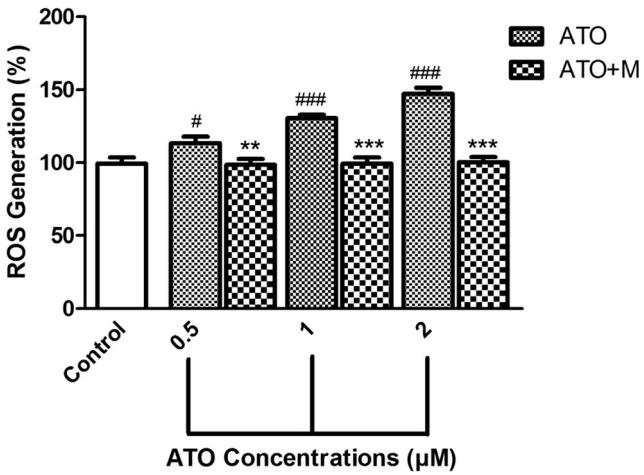

Fig. 2 The level of ROS generation in H9c2 cells exposed to different concentrations of ATO and ATO plus morphine (1 μM) for 48 h. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA and Tukey's post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ compared to control group. **$P<0.01$ and ***$P<0.001$ compared to ATO-related exposed groups

Morphine Reverses ATO-Mediated Suppression of NF-κB Activation in H9c2 Cells

For determining the relationship between inhibition of NF-κB and ATO-induced apoptosis, we used different concentration of ATO (0.5–2 μM) and pretreatment with morphine (1 μM) in H9c2 cell line. As shown in Fig. 6, NF-κB activity significantly decreased following higher concentration of ATO (1 and 2 μM) compared to control group ($P<0.05$). Furthermore, pretreatment with morphine (1 μM) significantly reversed ATO-mediated suppression of NF-κB activation in H9c2 cells after 48 h.

Discussion

Morphine, as an analgesic drug, has been reported to have antioxidant and anti-apoptotic effects including ROS scavenger capacity, inhibition of NADPH oxidase activity, and increasing of glutathione levels in neuronal and heart cells

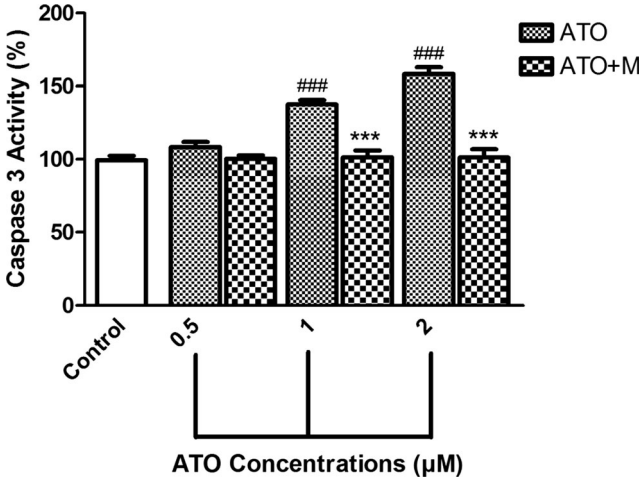

Fig. 4 The caspase 3 activity in H9c2 cells exposed to increasing doses of ATO and ATO plus morphine (1 μM) for 48 h. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA and Tukey's post hoc test. $^{###}P < 0.001$ compared to control group. $^{***}P < 0.001$ compared to ATO-related exposed groups

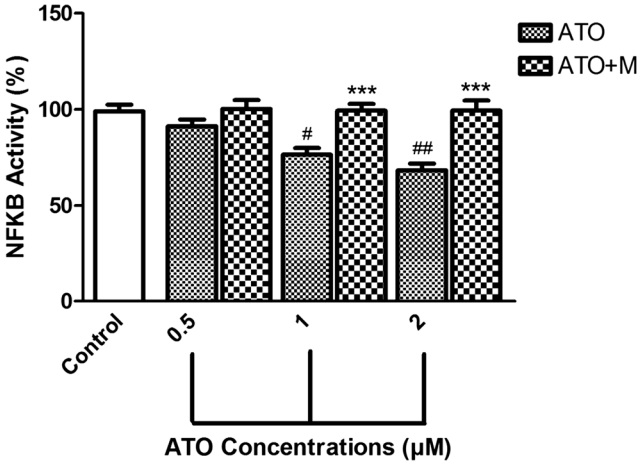

Fig. 6 The NF-κB activity in H9c2 cells exposed to escalating concentrations of ATO and ATO plus morphine (1 μM) for 48 h. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA and Tukey's post hoc test. $^{#}P < 0.05$ and $^{##}P < 0.01$ compared to control group. $^{***}P < 0.001$ compared to ATO-related exposed groups

[28–31]. Based on previous studies, the inhibitory effect of morphine on glutamate-induced astrocyte toxicity is related to intracellular redox reactions which are not prevented by naloxone, suggesting that opioids have receptor-independent mechanisms [32]. Moreover, there are several reports indicating that opioid agonists exert strong protective effects on ischemia-reperfusion conditions in the heart [33]. It has been reported that activation of opioid receptors induces hibernation-like states that increase organ stress resistance via induction of low energy consumption and activation of ATP-sensitive K^+ channels [34, 35]. Besides, morphine induces a preconditioning effect on C8-B4, a microglial cell line via decreasing of LDH release, an indicator of cell toxicity, as well as decreasing the release of TNF-α [35, 36]. Our results

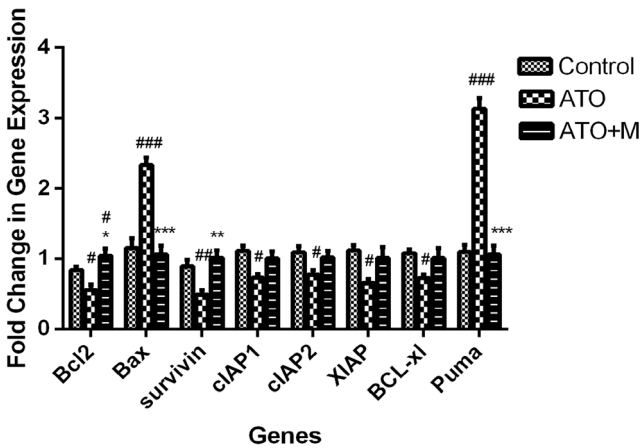

Fig. 5 The expression of apoptotic and anti-apoptotic genes in H9c2 cells exposed to ATO (2 μM) and ATO plus morphine (1 μM) for 48 h. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA and Tukey's post hoc test. $^{*}P < 0.5$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to ATO-related exposed groups. $^{#}P < 0.5$, $^{##}P < 0.01$, and $^{###}P < 0.001$ compared to control groups

are corroborated with evidence indicating that protective role of morphine in ATO-induced oxidative stress is associated with changes in antioxidant activity (GSH level and superoxide dismutase activity) and inhibition of generation of superoxide anion radicals [28, 37].

The concentration ranges of ATO (0.5–2 μM) and morphine (1 μM) were selected on the basis of a series of pilot studies in our laboratory [12, 38, 39]. Although the concentrations of ATO used in this study might seem relatively high, in vitro research works in mechanistic toxicology simulate chronic/low concentration, problems with acute/high-concentration condition in cell lines which cannot be kept alive or operate more than maximum 3–4 weeks following treatment. Obtaining statistically valid results and determination of toxicity mechanisms from in vitro or in vivo test (in small groups) require the administration of relatively large doses so that the effect will occur frequently enough to be detected [40].

Several lines of research have revealed that ATO is used as an anti-tumor drug, especially in treatment of APL; hematopoietic malignancies; and solid tumors such as liver, prostate, breast, and gastric cancers [41–44]. However, there are reported side effects of ATO in heart including T-wave and A-V block [45, 46].

Our data confirmed decreased activity of succinate dehydrogenase (SDH), a constitutive molecule of complex II in mitochondrial electron transfer chain through reduction of MTT to formazan [8]. In addition, it was also found that ATO significantly inhibited mitochondrial complex II activity in a concentration-dependent manner ($P < 0.05$) in H9c2 cells that indicates the disturbance in electron chain transfer. Also, the present data showed that pretreatment with morphine before ATO effectively reduced complex II activity in cardiomyocytes. Evidence suggests that main site of

MTT reduction is mitochondrial complex II and activity of this complex is responsible for most cellular reduction capacity and criteria for evaluation of electron transfer chain [47].

Besides, morphine antagonized the suppressive effects of ATO on DNA synthesis, explaining the increased resistance of cardiomyocytes against ATO-induced apoptosis. In addition, we found a significant increase in ROS generation in H9c2 cells following exposure with ATO (0.5–2 μm). Previous studies show that mitochondrial respiratory chain complex is the main source of ROS production [48] and impairment in any component of respiratory chain could cause mitochondrial dysfunction followed by oxidative stress and cell injury [49], which further causes tissue damage and organ dysfunction.

Also, increasing of ROS production leads to MPT pore opening and release of cytochrome c that triggers caspase-3 activation as the main initiator of apoptotic pathways [50]. Therefore, the caspase-3 activity was measured in H9c2 cells when incubated with ATO and morphine. Similarly, a recent in vitro study on cardiac cells showed that exposure to morphine decreased caspase-3 activation in doxorubicin-induced oxidative stress via mitochondrial dysfunction [51–54]. It has been suggested that ATO causes cardiac toxicity similar to doxorubicin via intrinsic and extrinsic pathways of apoptosis which affected mitochondrial damage, and this study suggested that morphine could reverse ATO-induced cytotoxicity. It seems that the protective effect of morphine against ATO was partly mediated via inhibiting of MPT pore opening [55].

In tumor cells, constitutive NF-κB activity is responsible for proliferation and to protect cells from apoptosis and drug resistance [56]. Previous studies have shown that ATO suppresses NF-κB pathway which has a key role in tumorigenesis via induction of transcriptional level in apoptotic genes such as *PUMA* and *BAX* [10, 11]. In the present study, our data showed that morphine increased the level of NF-κB, suggesting that morphine is able to induce a decrease in expression of apoptotic genes, an increase in the level of anti-apoptotic markers, and and a decrease in caspase-3 activity in ATO-treated cells.

Conclusion

Due to the fact that morphine exerts protective effects on ATO-exposed cardiomyocytes, its utility can be a new strategy for protection and/or management of cardiac toxicity in patients who receive ATO as an anti-cancer treatment.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest to declare regarding the study described in this article and preparation of the article.

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