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ORIGINAL ARTICLE

Cardioprotective effect of citicoline against arsenic trioxide-induced injury in H9C2 cell line

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ABSTRACT

Cardiotoxicity is one of the most serious adverse effects of exposure to arsenic. It has been shown that cytidine 5'-diphosphocholine or citicoline exerts a variety of biological functions, including anti-inflammatory and antioxidant activities. The aim of current study was to investigate the effect of citicoline on arsenic trioxide (ATO)-induced cardiotoxicity in H9C2 cells. H9C2 cardiac cells exposed to ATO for 24 h to induce cytotoxicity. The cells were pre-incubated with citicoline for 72 h prior to the exposure to ATO. The effect of citicoline on cell viability was investigated by MTT method. Malondialdehyde (MDA), total thiol groups, and total antioxidant power (TAP) levels were also evaluated. The results indicated that citicoline could effectively enhanced cell viability of H9C2 cells exposed to ATO. Treatment with ATO increased MDA levels, while pretreatment with citicoline reduced lipid peroxidation. Moreover, it could enhance total thiol contents and TAP levels. Taken together, our data suggest that citicoline has protective effects against ATO-induced oxidative stress in H9C2 cells, that could be attributed to inhibition of oxidative stress and its antioxidant effects.

KEY WORDS: Arsenic trioxide; cardiotoxicity; citicoline; oxidative stress; H9C2 cells

Introduction

Arsenic, one of the most toxic elements, can be found in water, air and soil. It is one of the global health problems that affect the function of the many organs and tissues. Arsenic can cause rapid poisoning and death. It has been known that arsenic causes different cancer types in humans. Arsenic exposure correlated with cardiovascular and neurodegenerative diseases (Ratnaike, 2003). Although arsenic has been known as a poison, previous studies have demonstrated that arsenic trioxide (ATO) has an inhibitory effect on the propagation of various types of cancer including acute promyelocytic leukemia (APL), breast, skin, lung, kidney and gastric cancers (Méndez-Gómez *et al.*, 2008). ATO is a well-known anti-leukemic drug used for the treatment of newly diagnosed and relapsed APL (Kwong and Todd, 1997). However, the clinical use of ATO is limited due to its cardiac toxicity including T wave changes, QT prolongation, torsade de pointes, and sudden cardiac death (Wu *et al.*, 2001; Pei *et al.*, 2013).

It has been reported that ATO-induced oxidative stress associated with increased free radical production in the cells (Flora, 2011). There is a large body of evidence that suggest induction of reactive oxygen species (ROS) plays an important role in arsenic-induced toxicity (Basu *et al.*, 2005). Previous studies have demonstrated that arsenic causes oxidative stress in myocardial cells, which is associated with an increase in ROS production and inhibition of antioxidant capacity (Kumazaki *et al.*, 2011; Vineetha *et al.*, 2014; Soumya *et al.*, 2015). Therefore, antioxidant drugs and ROS adsorbents can reduce the toxicity of clinical use of ATO (Harris and Shi, 2003). Cytidine 5'-diphosphocholine or citicoline is an endogenous compound and a conciliator in the synthesis of membrane phospholipids such as phosphatidylcholine. Citicoline increases some brain neurotransmitters including dopamine, serotonin, norepinephrine and also involved in biosynthesis of acetylcholine, providing choline (Martinet *et al.*, 1979). Citicoline is used in acute stages

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of brain strokes and cerebrovascular damage. It has been used for the treatment of heart and cerebral ischemia. Citicoline is an intermediate product in the production of phosphatidylcholine, and it appears to stimulate biosynthesis of phospholipids and reduce inflammation, thereby stabilizing the cell membrane. In vivo studies suggest that citicoline has protective effects under oxidative stress conditions in cardiac ischemia (González-Pacheco *et al.*, 2014). The mechanisms underlying the protective effects of citicoline included increasing glutathione synthesis, preserving cardiolipin (an exclusive inner mitochondrial membrane component) and sphingomyelin, maintaining the arachidonic acid content, attenuating lipid peroxidation and restoring Na^+/K^+ ATPase activity (Secades and Frontera, 1995; Fioravanti and Yanagi, 2005). In vitro studies have showed that citicoline has vascular protective effects and induces angiogenesis (Krupinski *et al.*, 2012). But, it has not been identified whether citicoline has protective effects on ATO-induced toxicity in H9C2 cardiac muscle cells. Therefore, the main purpose of this study is to investigate the protective effect of citicoline on ATO-induced cytotoxicity in H9C2 cells.

Materials and methods

Cell culture

H9C2 cells (Pasteur institute, Iran) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), and 5% CO_2 atmosphere at 37 °C. Cells were used for experiments during the exponential growth phase.

Analysis of cell viability

The effects of citicoline on cell viability were determined by the 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay. This assay is based on the reduction of soluble tetrazolium, a yellow salt, into insoluble purple formazan by viable cells. Briefly, about 1×10^4 cells were cultured at 100 µl per well, using of 96-well plates. H9C2 cells were incubated with different concentrations of citicoline (50, 100, and 200 µM) for 72 h. Then, cells were treated with ATO for 24 h. After incubation times, MTT (Sigma Aldrich, USA) was added to each well and incubated at 37 °C for 4 h. Then, the culture medium was removed and 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm wavelength with a microplate reader (Bio-Tek ELX800, WA, USA).

Measurement of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) assay is the most generally used method in the detection of lipid peroxidation products in the cells. Malondialdehyde (MDA) is an end product of lipid peroxidation. Thiobarbituric acid (TBA, Sigma Aldrich, USA) can react with MDA (Sigma Aldrich, USA) to carry out a pink color. Briefly, 500 µL of sample was added to 2.5 mL of trichloroacetic acid (TCA, Sigma Aldrich, USA) (20% (w/v)) and centrifuged at 3500 g for 10 minutes. Afterward, 2 mL of TBA (0.2% in 2 M sodium sulfate), and 2.5 mL of sulfuric acid (0.05 M) were added and incubated for 30 minutes in boiling water bath. Then, the absorbance was measured at 532 nm using a microplate reader.

Measurement of total thiol molecules

5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma Aldrich, USA) is used to investigate the concentration of thiol groups. The samples were mixed with DTNB (10 mM), EDTA buffer (20 mM, pH=8.2), and tris(hydroxymethyl)aminomethane. Afterward, the test tubes were then centrifuged for 10 minutes at 5000×g at room temperature. DTNB creates a yellow complex with thiol molecules. The absorbance was measured at 412 nm in the plate reader.

Measurement of total antioxidant power (TAP)

TAP is measured by ferric reducing antioxidant power (FRAP) assay. The FRAP assay is an antioxidant capacity assay that measures the antioxidant potential in samples through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). In this method, acetate buffer was added to ferric chloride ($FeCl_3$), 2,4,6-Tripyridyl-s-triazine (TPTZ, Sigma Aldrich, USA) and acetic acid. The sample was mixed with the FRAP solution. The absorbance was read using a microplate reader at 593 nm.

Statistical analysis

The results were expressed as mean ± SD. Comparing the variation between groups was analyzed by one-way analysis of variance (ANOVA) and followed by Tukey's post hoc test to determine significant difference between groups. A difference of $p<0.05$ was considered significant.

Results

Effect of ATO on cell viability

H9C2 cells were treated with various concentrations of ATO (5–50 µM) for 24 h. The results showed that the viability of H9C2 cells was significantly decreased with increasing arsenic concentrations. Concentration of 10 µM was chosen for the next study ($p<0.001$) (Figure 1).

Citicoline protects H9C2 cells against ATO-induced cytotoxicity

H9C2 cells were pre-treated with different concentrations of citicoline for 72 h and then exposed to ATO for 24 h. As shown in Figure 2, our results showed that citicoline is able to decrease ATO-induced cytotoxicity in H9C2 cells. Concentrations of 50 and 100 µM of citicoline were selected for subsequent experiments ($p<0.01$, and $p<0.001$ respectively).

Citicoline attenuates ATO-induced increase in MDA levels

The results as shown in Figure 3 indicated that ATO markedly enhanced the MDA levels compared with the

control group. However, we observed that citicoline (50 and 100 μM) significantly reduced MDA levels compared with the ATO group (p<0.01, and p<0.001 respectively).

Citicoline attenuates ATO-induced decrease in total thiol molecules

As shown in Figure 4, treatment with ATO significantly decreased levels of total thiol molecules in H9C2 cells compared with the control group. Whereas, pretreatment with citicoline (100 μM) increased the total thiol molecules in H9C2 cells when compared with the ATO group (p<0.05).

Effect of citicoline on the total antioxidant power (TAP)

Effects of citicoline on TAP levels were studied by FRAP assay. As shown in Figure 5, treatment of H9C2 cells with ATO decreased TAP levels. However, pretreatment of H9C2 cells with citicoline (100 μM) effectively enhanced TAP levels compared with the ATO group (p<0.05).

Discussion

The aim of the present study was to evaluate the effects of citicoline on ATO-induced injury in H9C2 cells. Our findings indicated that citicoline exhibits protective effects on oxidative damage induced by ATO in cardiac H9C2 cells.

ATO has been used as a medical treatment in traditional Chinese medicine for more than 2,000 years (Waxman and Anderson, 2001). Although arsenic has been known as a poison, it has a potential role in the treatment of syphilis, APL, and various types of cancer (Hoonjan *et al.*, 2018). The clinical use of ATO is limited due to its severe cardiac toxicity effects. Aquaglyceroporins are recognized as a subfamily of aquaporin proteins that facilitate the movement of water and other small solutes. They are involved in arsenic uptake by the heart cells and play an essential role in arsenic accumulation and toxicity (Liu *et al.*, 2004; Zhao *et al.*, 2008).

Recent reports have indicated that chronic exposure to arsenic induces oxidative stress and DNA damage in the peripheral blood polymorphonuclear cells, resulting in increased apoptosis of these cells (Pei *et al.*, 2013). The cytotoxicity induced by arsenic is related to DNA fragmentation, increased intracellular calcium, and changes in cardiac ion channels (Das *et al.*, 2010; Zhang *et al.*, 2017). Arsenic exposure in cells has been shown to increase lipid peroxidation and oxidative stress (Sampayo-Reyes *et al.*, 2010). Oxidative stress is a state of imbalance between production of oxidants and their elimination by antioxidant systems with increased accumulation of ROS in cells and tissues (Méndez-Gómez *et al.*, 2008). These overwhelming amounts of ROS can damage cellular proteins, lipids, nucleic acids which ultimately results in cell death (Foyer and Noctor, 2005). Studies have been shown that ATO increased ROS formation, including the superoxide, peroxyl and hydroxyl radicals. Overproduction of ROS exacerbates oxidative stress which promotes permeability of the mitochondrial membrane. ROS can induce

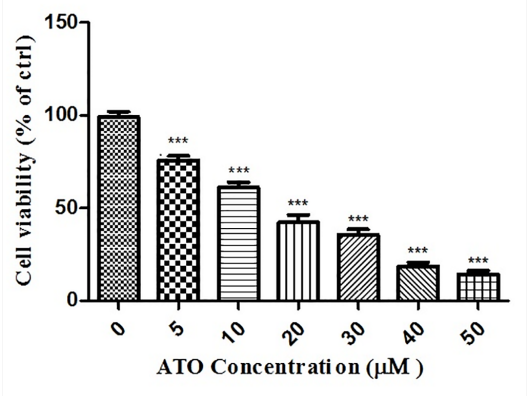

Figure 1. The effect of arsenic trioxide (ATO) (5–50 μM) on H9C2 cell viability was measured by MTT assay. Results are reported in mean ± SD (n=5). $^{***}p$<0.001 compared with the control group.

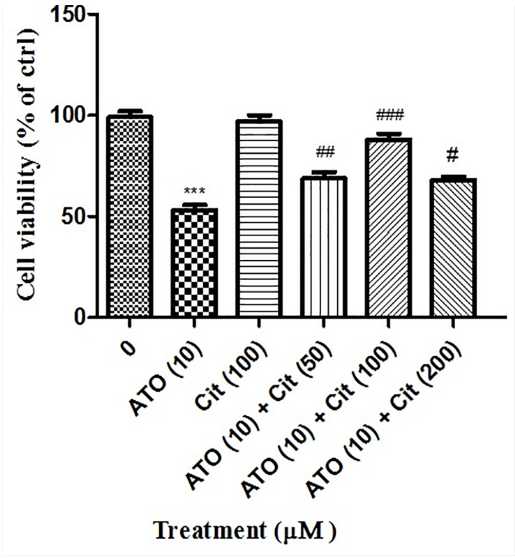

Figure 2. Protective effects of citicoline (50–200 μM) on ATO-induced toxicity in H9C2 cells. Cell viability was measured by MTT assay. Results are reported as mean ± SD (n=5). $^{***}p$<0.001 compared with the control group. $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 compared with the ATO group.

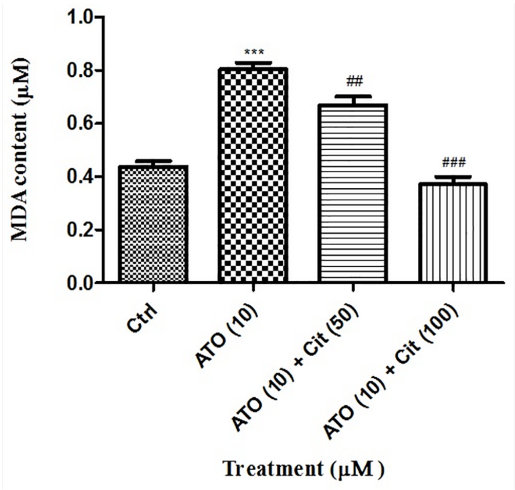

Figure 3. Effects of citicoline on increased lipid peroxidation induced by ATO toxicity. Lipid peroxidation levels were measured by TBARS test. Results are reported as mean ± SD (n=3). $^{***}p$<0.001 compared with the control group. $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 were compared with the ATO group.

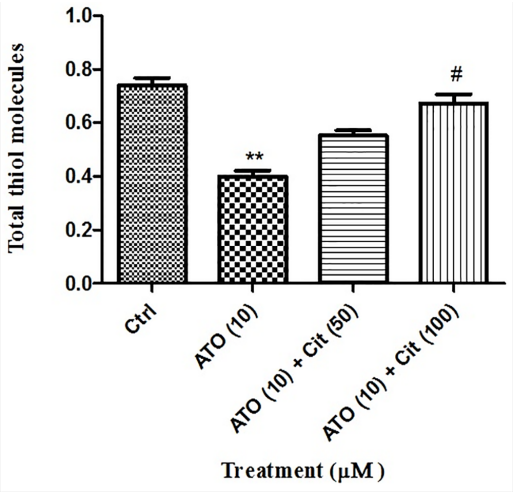

Figure 4. Effects of citicoline on reduction of thiol molecules due to ATO toxicity. Results are reported as mean ± SD (n=3). $^{**}p<0.01$ compared with the control group. $^{\#}p<0.05$ was compared with the ATO group.

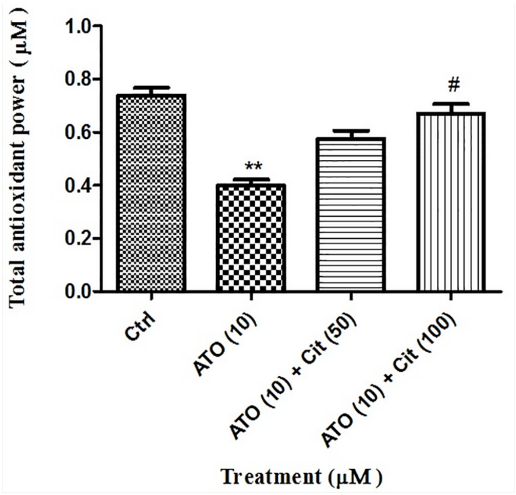

Figure 5. Effects of citicoline on decreasing total antioxidant power due to ATO toxicity. Total antioxidant power was measured by FRAP test. Results are reported as mean ± SD (n=3). $^{**}p<0.01$ compared with the control group and $^{\#}p<0.05$ compared with the ATO group.

release of cytochrome *c* and increase caspase-3 activity, leading to cell death (Aune and Pogue, 1989; Meier *et al.*, 1989; Shaughnessy *et al.*, 1989; Szatrowski and Nathan, 1991).

In the present study, we found that citicoline exerts cytoprotective effect against ATO-induced loss in cell viability in H9C2 cells. Consistent with our results, a study has shown that citicoline improved cell viability and showed protective effect against 6-hydroxydopamine -induced toxicity in SH-SY5Y neuroblastoma cells (Barrachina *et al.*, 2003). MDA is one of the end products of lipid peroxidation. Our data demonstrated that ATO enhanced the MDA levels in H9C2 cells, while citicoline decreased MDA levels compared with the ATO group. Our finding is in accordance with the current research showing that citicoline is a neuroprotective drug and can reduce MDA levels in the patients of head trauma (Salehpour *et al.*, 2015).

Antioxidant enzymes include catalase (CAT), superoxide dismutase (SOD), and glutathione are the important components in the scavenging system of ROS (Reiter, 1998). Results demonstrated that citicoline effectively enhanced the TAP levels compared with the ATO group. Our results are consistent with a previous report indicating that citicoline effectively enhanced the levels of SOD activity and GSH content after transient cerebral ischemia (Qian *et al.*, 2014).

In accordance with our findings, other studies have shown that citicoline exerts its protective and antioxidant effects against cell death by promoting phospholipid resynthesis and reducing the progression of ischemic cell damage with inhibiting glutamate release (Putignano *et al.*, 2012).

Thiol compounds have an antioxidant role due to the presence of sulfhydryl free groups in their structures and protect the cells against the damage of free radicals. Our results indicated that H9C2 cells treated with ATO showed a marked decrease in total thiol levels. However, pre-treatment with citicoline significantly increased total thiol levels under ATO toxicity condition. These findings are in agreement with recent studies showing that citicoline treatment enhances glutathione reductase activity after cerebral ischemia in rats (Adibhatla *et al.*, 2003).

In summary, the results of this study suggested the protective role of citicoline against ATO-induced cell injury and apoptosis in H9C2 cells, which could be linked with inhibition of oxidative stress and its antioxidant effects.

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Conflict of interest. *The authors have no conflict of interests.*

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