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Protective effect of erythropoietin on myocardial apoptosis in rats exposed to carbon monoxide

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ABSTRACT

Aims: Cardiac complications are common in carbon monoxide (CO) poisoning and associated with high morbidity and mortality. We have previously shown that erythropoietin (EPO) could reduce CO-induced cardiac ischemia in rat. In the current study, the anti-apoptotic effect of EPO during CO cardiotoxicity was investigated in order to elucidate the mechanism of EPO anti-ischemic action.
Main methods: Wistar rats were exposed to CO (250, 1000 and 3000 ppm). EPO (5000 IU/kg) was administered to all groups by intraperitoneal injection at the end of CO exposure period. TUNEL and caspase-3 activity levels were assessed to investigate the effects of CO exposure and subsequent EPO administration on myocardial apoptosis. The changes of mitochondrial membrane potential (MMP) were also assessed with sensitive lipophilic dye JC-1 by flow cytometry. The roles of Bcl2 and Bax in EPO protective effect were investigated by Western blotting.
Key findings: Myocardial apoptosis was observed following CO exposure. Moreover, mitochondrial membrane depolarization and significant reduction in Bcl2/Bax ratio were shown following CO poisoning especially at 3000 ppm. On the other hand, EPO administration could effectively suppress apoptosis in myocardial cells. Also, EPO significantly prevented the CO-induced depolarization of MMP ($p < 0.001$) and preserved Bcl2/Bax ratio ($p < 0.01$).
Significance: EPO reduces myocardial injury due to CO intoxication. Thus EPO could be suggested as a possible candidate for the management of CO cardiotoxicity with clinical applications.
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1. Introduction

Carbon monoxide (CO), although necessary for cell signaling, could lead to significant health problems after exposure to high concentrations or even chronic low exposure. CO sources vary from second hand smoking to vehicular exhaust, industrial emissions, poorly ventilated gas heaters contributing to air pollution [1]. Acute CO poisoning in relative high concentrations is a potentially life-threatening condition. Cardiac and nervous tissues are mainly affected from CO. Cardiotoxicity and myocardial injury occur due to moderate and severe CO exposure [2, 3], with the most common manifestations being tachycardia, ischemia, dysrhythmia, infarction and cardiac arrest in severe cases [4–6]. Ischemic electrocardiographic (ECG) changes have been reported following CO poisoning even in patients with few cardiac risk factors [7, 8]. The long term mortality from myocardial disorders in CO poisoning is twice that of patients with no cardiac involvement and three times that of negative controls [3]. Recently myocardium involvement in CO exposure has been identified as CO related cardiomyopathy [7]. A link between ambient CO air pollution and heart failure in the elderly has been found. Burnett et al. reported that the rise in ambient air CO levels correlated with exacerbations in hospital admission for heart failure [8].

While CO heart effects range from mild to myocardial fibrosis and contractile dysfunction, with the main pathophysiologic process considered being hypoxic damage, a number of cardiospecific mechanisms at cellular or subcellular level are implicated to myocardial injury. CO

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acts directly to myocardial mitochondria through impairment of mitochondria respiratory chain at the cytochrome C oxidase level [4–5]. In cultures of endothelial cells exposed to increasing CO concentrations, apoptosis resulted with concurrent caspase-1 rise. On the other hand, addition of a caspase-1 inhibitor reduced endothelial cell death [9]. On a tissue level, apoptotic cell death due to CO intoxication has been described in nervous tissues [10], however there are limited data concerning CO-induced cardiac apoptosis.

Recently, erythropoietin (EPO), a hematopoietic cytokine, has been investigated extensively as an anti-ischemic and tissue-protective agent [11, 12] with cardioprotective action [13–16] that is unrelated to its erythropoietic property [14]. In cardiac ischemia/reperfusion studies, EPO administration improved ventricular and cardiac hemodynamic functions and led to reduction of infarct size, inhibition of myocardial apoptosis and normalization of hypertrophic index [14, 17]. *In vivo* and *in vitro* studies have shown that the anti-apoptotic mechanism has a main role in EPO tissue protective effect [15, 18].

Acute or chronic CO exposure, that potentially aggravates chronic diseases, the link with myocardial apoptosis and the EPO ability to reverse or inhibit myocardial apoptosis pose intriguing pathophysiological questions with major therapeutic implications for acute and chronic diseases. Our previous findings showed that EPO administration could reduce ischemic ECG changes from severe CO poisoning in rat [19]. More specifically, ST segment elevation and depression, T wave inversion and first-degree AV block were observed in rats following moderate to severe CO exposure. Ischemic ECG changes reduced significantly in EPO-treated animals. To elucidate the mechanism of this effect, we hereby studied myocardial apoptosis in Wistar male rats exposed to CO and the effect of consequent EPO administration. *In situ* myocardial apoptosis was monitored with TUNEL test, myocardial caspase-3 activity and myocardial mitochondria membrane potential was measured, along with Bcl/Bax protein ratio, involved in the regulation of myocardial apoptosis in the rat myocardium.

2. Methods

2.1. Animal

Wistar male rats, weighing 200–250 g were housed in the Animal Center of Buali Research Institute. They were kept under standard conditions (21–23 °C temperature, 12 h/12 h light/dark cycle) with free access to food and water. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 85-23, Revised 1996) and in conformity with EU Directive 2010/63/EU for animal experiments. The study was approved by the Animal Care Committee of Mashhad University of Medical Sciences.

2.2. Experimental groups and study design

The animals were intoxicated by three different CO concentrations; 250 ppm, 1000 ppm and 3000 ppm. These three different concentrations and times of CO exposure were chosen on the basis of previous studies to induce severe (3000 ppm/40 min), moderate (1000 ppm CO/40 min) and mild (250 ppm/90 min) CO poisoning in rat, respectively [20–22]. The animals were placed in a 12L airtight plexiglass container with entrance and exit taps. CO was flowed to the container at a constant flow for different times based on the relevant animal group (Table 1). The CO concentration was monitored continuously with a CO analyzer (model 707 carbon monoxide analyzer, TPI, Korea). At the end of CO exposure, recombinant human EPO (rhEPO, Pooyesh Draou Co, Iran; 5000 IU/kg) was injected intraperitoneally [18]. EPO dosing scheme was decided based on previous published studies [13, 14, 18]. In all groups, the animals were exposed to ambient air for 2 h after CO exposure. The animals' heart was removed after anesthesia induction by Ketamin/Xylazin mixture (100/10 mg/kg). Animal groups are described in Table 1.

2.3. Toxicological analysis

Blood samples were taken from the abdominal aorta of animals and heparinized. Carboxyhemoglobin levels were assessed with a spectrophotometer calibrated for rat blood (Jasco, Japan).

2.4. Determination of caspase-3 activity in the heart

Activity of caspase-3 in the heart was measured by using a casapase-3/CPP32 fluorometric assay kit (Biovision, USA). In brief, the heart tissues were homogenized in ice-cold lysis buffer, centrifuged at 10,000*g* at 4 °C for 10 min, supernatant collected and equal volume of tissue lysate of each sample was incubated with reaction buffer containing 50 μM caspase-3 substrate (DEVD-AFC) at 37 °C for 2 h. The yellow-green fluorescence, emitted from cleavage of substrate at 505 nm was measured with spectrofluorometer (Jasco, Japan). Fold increase in caspase-3 was determined by comparing the results of the samples with the control. Protein concentrations were determined by the bicinchoninic acid method (Sigma Aldrich, USA).

2.5. In situ detection of apoptosis (TUNEL)

To determine myocardial apoptosis, in situ cell death detection kit (Roche, Germany) was used for the TdT-mediated dUTP-biotin nick end labeling (TUNEL) test. Hearts were removed after reoxygenation, washed in cold PBS and fixed in formaldehyde 10%. Fixed tissues were then embedded in paraffin blocks and specimens at 5 μm thickness were prepared on poly-L-lysine-coated slides. Specimens were dewaxed by heating up to 80 °C for 20 min in an oven, rehydrated in serial alcohol dilutions, digested with proteinase K (20 μg/ml) and then washed with PBS. The slides were then incubated with terminal deoxynucleotidyl transferase (TdT) reaction mixture for 1 h at 37 °C in a humidified chamber. The nonspecific bindings were avoided by incubation with BSA 3% in PBS for 20 min. Then, the slides were incubated with anti-fluorescein antibody conjugated with peroxidase for 30 min at 37 °C. By adding diaminobenzidine solution as a substrate, the apoptotic cells were visualized as brown-colored cells. The slides were counterstained with hematoxylin, mounted and viewed in a light microscope (Olympus, Japan). Apoptosis index was assessed from 50

Table 1
CO intoxication and EPO administration in different animal groups (5 animals in each group).

	Group	CO exposure time-schedule	EPO administration
1	3000 ppm CO	1000 ppm (20 min) followed by 3000 ppm (40 min)	NO
2	1000 ppm CO	1000 ppm (40 min)	NO
3	250 ppm CO	250 ppm (90 min)	NO
4	3000 ppm CO + EPO	1000 ppm (20 min) followed by 3000 ppm (40 min)	5000 IU/kg (immediately after intoxication)
5	1000 ppm CO + EPO	1000 ppm (40 min)	5000 IU/kg (immediately after intoxication)
6	250 ppm CO + EPO	250 ppm (90 min)	5000 IU/kg (immediately after intoxication)
7	EPO	NO	5000 IU/kg
8	Control	NO	NO

randomly microscopic fields for each case. Assay was performed in a blind manner.

2.6. Mitochondria isolation

Mitochondria were isolated from fresh rat's heart using a commercial mitochondria isolation kit (Sigma-Aldrich Chemie, USA). Briefly, the fresh rat heart was washed twice by two volumes of extraction buffer (10 mM HEPES, 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA, pH 7.5). The heart was cut into small portions and suspended with 10 volumes of extraction buffer containing 0.25 mg/ml trypsin, incubated on ice for 3 min and spinned. The pellet was resuspended in extraction buffer containing trypsin and incubated for 20 min on ice, added 10 mg/ml albumin, then spinned again and after washing by extraction buffer, the sample was hemogenated using a pestle and glass tube, then the homogenate was centrifuged at 600*g* for 5 min at 4 °C. The supernatant was collected and centrifuged at 11,000*g* for 10 min at 4 °C. Isolated mitochondria were pelleted and resuspended in respiratory buffer (10 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM ATP, 0.08 ADP, 5 mM sodium succinate, 2 mM K2HPO4, 1 mM DTT). Mitochondrial protein was estimated by the BCA method in accordance with the manufacturer's instructions.

2.7. Detection of mitochondrial membrane potential (MMP) ($\Delta\Psi_m$)

MMP was evaluated by measuring the fluorescence change of 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1) in a flow cytometer. JC-1, a lipophilic fluorescent cation, sensitively incorporates into the mitochondrial membrane where it can form aggregates due to the physiological membrane potential of mitochondria. JC-1 aggregation leads to a shift from green to orange fluorescence. Isolated mitochondria were incubated with JC-1 (0.2 μg/ml) at room temperature in dark for 7 min. Changes in JC-1 signals were analyzed in a flow cytometer (FACSCalibur, Becton Dickinson, USA). For each experiment data from 20,000 mitochondria were recorded (gated on FSC-SSC parameter). Fluorescent changes were calculated at FL-1 for JC-1. Total depolarization of the $\Delta\Psi_m$ by valinomycin (0.5 μg/ml) was used as positive control.

2.8. Western blot

Heart tissues were lysed at 4 °C in 50 mM Tris buffer containing 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM sodium orto-vanadate, 10 mM glycerophosphate, 10 mM 2-ME, sodium deoxycholate, PMSF and protease inhibitor. The lysate was centrifuged at 10,000*g* at 4 °C and the supernatant was used as cellular proteins. Protein content of supernatant was determined by Bradford protein assay (thermo, USA). Subsequently, an equal protein amount of each sample (50 μg) was separated on a 12% SDS-PAGE and blotted to PVDF membrane. The non-specific protein binding was avoided by treating the membranes by 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 3 h at room temperature. Membranes were incubated with monoclonal antibodies for anti-Bcl2 (1:1000; Abcam, USA) and anti-Bax (1:1000; Abcam, USA) for 3 h at room temperature, washed three times with PBS buffer containing 0.1% Tween-20 and then incubated with the secondary antibody conjugated to horseradish peroxidase (anti-mouse IgG antibody, 1:20,000; Abcam, USA) and detected with an enhanced chemiluminescence system (ECL, Thermo, USA). The density of each protein band was determined using a gel-doc system (Alliance, Germany) and analyzed by Uvitec software (UK).

2.9. Statistical analysis

Data analysis was performed using SPSS version 11.5. Individual groups were assessed with one-way ANOVA and Tukey post hoc test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Carboxyhemoglobin (COHb) levels after CO intoxication

Carboxyhemoglobin values are shown in Table 2. The COHb level is a biochemical marker of CO poisoning severity. High level of COHb was shown in 3000 ppm CO group.

3.2. EPO decreased CO-induced myocardial apoptosis

Apoptosis cell death is expected in cardiac ischemia. In addition, ischemic injury occurs due to CO intoxication. In this study, to evaluate whether the effect of CO and EPO administration on myocardial apoptosis, in situ assay of DNA fragmentation was performed and activity of caspase-3 was measured.

As TUNEL results showed, intoxication by 3000 ppm and 1000 ppm CO significantly increased TUNEL-positive cells to 17.6% ($p < 0.001$) and 11.11% ($p < 0.001$) respectively compared with the control (1.4%). The increase in positive stained cells in the 250 ppm CO group was not significant in comparison to the control (from 1.4% to 2.4%). EPO administration after 3000 ppm CO intoxication significantly reduced the percentage of apoptotic cells to 11.11% (3000 ppm CO + EPO vs. 3000 ppm CO, $p < 0.001$), also EPO administration after 1000 ppm of CO decreased apoptotic index significantly to 6% in comparison to the untreated 1000 ppm CO group ($p < 0.01$) (Fig. 1).

Caspase-3 is a key factor which implicated as a downstream caspase in the apoptosis pathway. The effect of EPO on CO-induced apoptosis was further investigated by evaluation of caspase-3 activity in all groups. As shown in Fig. 2, intoxication by 3000 ppm CO significantly increased caspase-3 activity to 9.1 fold when compared with the control group ($p < 0.001$). EPO treatment effectively prevented the increase of caspase-3 activity (3000 ppm CO + EPO vs. 3000 ppm CO, $p < 0.001$) which indicated its inhibitory effect on caspase-3. No significant increase in caspase-3 activity was shown following intoxication by 1000 and 250 ppm of CO in comparison with the control (Fig. 2).

3.3. EPO decreased CO-induced MMP disruption

MMP is necessary for cell viability as MMP disruption results in apoptosis. To determine whether CO and EPO influence MMP, the CO exposure groups of 3000 ppm, with or without EPO administration, where the most detrimental effects were observed, were chosen. Isolated mitochondria from heart tissues were incubated with JC-1 as a MMP sensitive dye. Since the uptake of the cationic carbocyanine dye (JC-1) into the mitochondrial matrix depends on membrane potential, JC-1 concentrates in the matrix of healthy mitochondria to form J-aggregates and emits red/orange fluorescence in contrast to JC-1 monomer which emits green fluorescence. MMP was measured in the 3000 ppm CO and also the EPO receiving group. The results were compared with the controls (Figs. 3, 4). The control group exhibited a normal pattern of mitochondria with high fluorescence intensity in FL-1. Intoxication by 3000 ppm CO significantly decreased fluorescence (3000 ppm vs. control, $p < 0.001$). There was no significant difference between the 3000 ppm CO and the valinomycin positive control group. EPO treatment resulted in a significant increase in the fluorescence intensity as compared to the untreated-3000 ppm CO group (3000 ppm CO + EPO vs. 3000 ppm CO, $p < 0.001$).

Table 2
Blood carboxyhemoglobin levels after CO poisoning in animals.

Groups	Mean ± SD	Range (%)
3000 ppm groups	70 ± 8	60–76
1000 ppm groups	31 ± 11	19–46
250 ppm groups	10 ± 5	13–10
Control group	1 ± 0.9	0.7–1.5

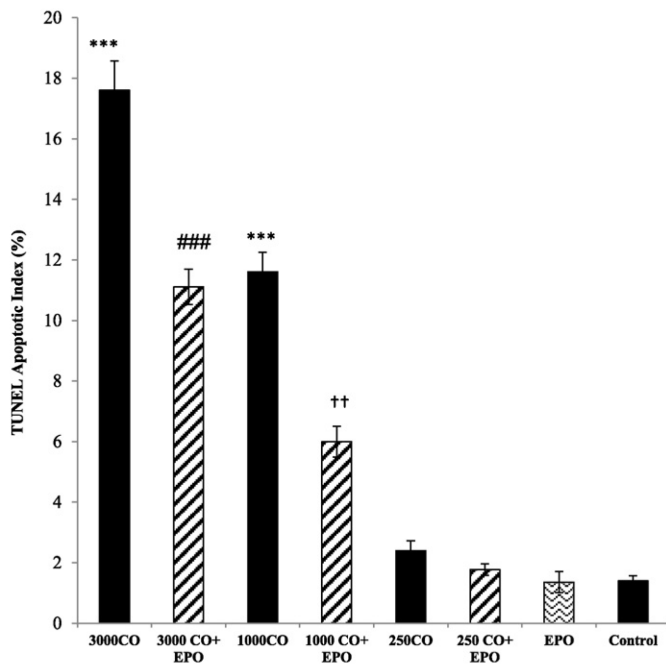

Fig. 1. Quantitative analysis of TUNEL staining. Apoptotic index represents TUNEL positive nuclei. Apoptotic index was significantly higher in the 3000 and 1000 ppm CO groups in comparison with the control ($^{***}p < 0.001$ vs. control), EPO administration following CO intoxication significantly reduced TUNEL positive cells in these groups ($^{###}p < 0.001$ & $^{††}p < 0.01$, vs. untreated group). Values are expressed as mean ± SEM.

3.4. Western blot results

Bcl2 family has roles in regulation of apoptosis and mitochondrial membrane potential. To address whether CO intoxication and EPO protective role also influence the Bcl2 family proteins, the immunoblotting of Bax and Bcl2 were assessed at the 3000 ppm CO group, where the most cardiotoxic CO effects were noticed, and at 3000 ppm CO + EPO group compared to control groups (Fig. 5). Our results showed that 3000 ppm CO poisoning decreased Bcl2/Bax ratio significantly in compared with the control ($p < 0.01$), while EPO significantly preserved this ratio (3000 ppm CO + EPO vs. 3000 ppm CO, $p < 0.01$).

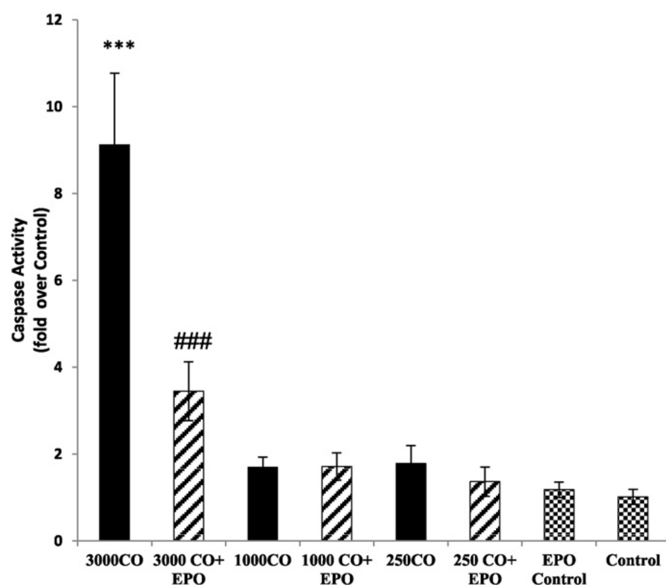

Fig. 2. Effect of EPO and CO on caspase-3 activity. Caspase-3 activity increased significantly by 3000 ppm CO compared to the control ($^{***}p < 0.001$, vs. control). This was reduced by EPO treatment ($^{###}p < 0.001$, vs. 3000 ppm CO group). Values represent mean ± SEM.

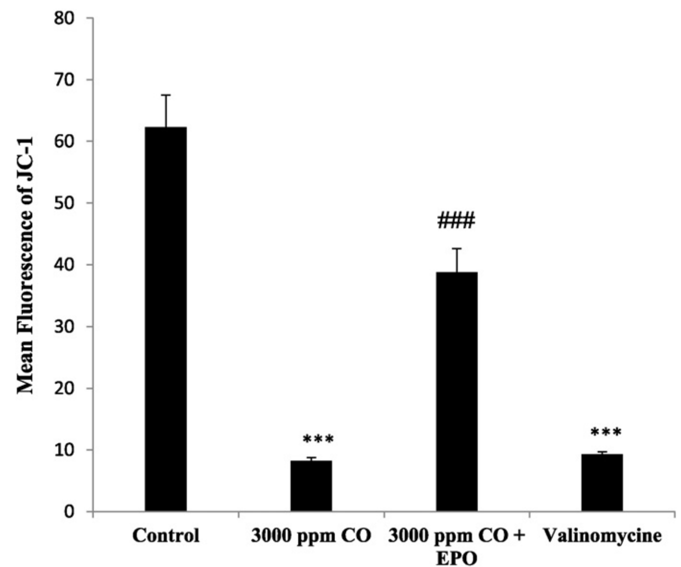

Fig. 3. Quantitative analysis of CO poisoning and EPO effect on mitochondria membrane potential ($\Delta\Psi_m$), 3000 ppm CO intoxication resulted in decreased red fluorescence intensity (8.25 ± 0.5 vs. 62.32 ± 5.2, $^{***}p < 0.001$). EPO treatment effectively improved fluorescence intensity in FL1 compared to the untreated group ($^{###}p < 0.001$, vs. 3000 ppm CO group). For the positive control group, isolated mitochondria were treated with valinomycin. Results are mean ± SEM.

4. Discussion

Acute or chronic exposure to CO confers an increased risk for the cardiovascular system ranging from cardiac arrhythmias to myocardial injury, fibrosis and cardiomyopathy [5, 10]. Exposure to higher concentrations of exogenous CO, acutely or chronically via air pollution can exacerbate outcomes of cardiovascular disease [23], despite the fact that at very low concentrations of CO (nanomoles), especially by using carbon monoxide-releasing molecules (CORMs), anti-apoptotic effects are observed. Recent findings on animal models suggest that chronic CO exposure via atmospheric air pollution promotes cardiac hypertrophy, elevates basal heart rate and leads to impaired contractility and spontaneous arrhythmias [24]. On the other hand, while apoptosis is a mechanism for eliminating redundant cells, in the same time is a key factor in the pathogenesis of heart diseases, including heart failure. Other studies have documented apoptosis occurrence in the nervous tissue due to CO poisoning [10, 25]. However, there is little data on the role of apoptosis in CO-induced cardiac injury.

The results of the present study revealed the occurrence of myocardial apoptosis following exposure to 1000 ppm and even more significantly to 3000 ppm of CO in rats. However there was no statistically significant difference in apoptosis occurrence between the 250 ppm CO group and the control.

The induction of apoptosis is associated with the activation of aspartate specific cysteine proteases, including caspace-3. In this aspect mitochondria may play an important role in apoptosis by releasing cytochrome *c* and activating caspase-9, which activates caspase-3, the molecule responsible for DNA cleavage. Caspase-3, as a common component of apoptotic signaling, mediates both mitochondria dependent and death receptor-dependent apoptosis pathways [26]. CO exposure in the present study at 3000 ppm led to a 9 fold increase of caspase-3 levels compared to controls. It is interesting though that CO exposure to 1000 ppm was able to significantly increase TUNEL-positive cells at 11.11% of rat myocardium, while no significant caspase-3 elevation was noticed, possibly indicating the difference in sensitivities between the two methods and different time course of DNA cleavage and

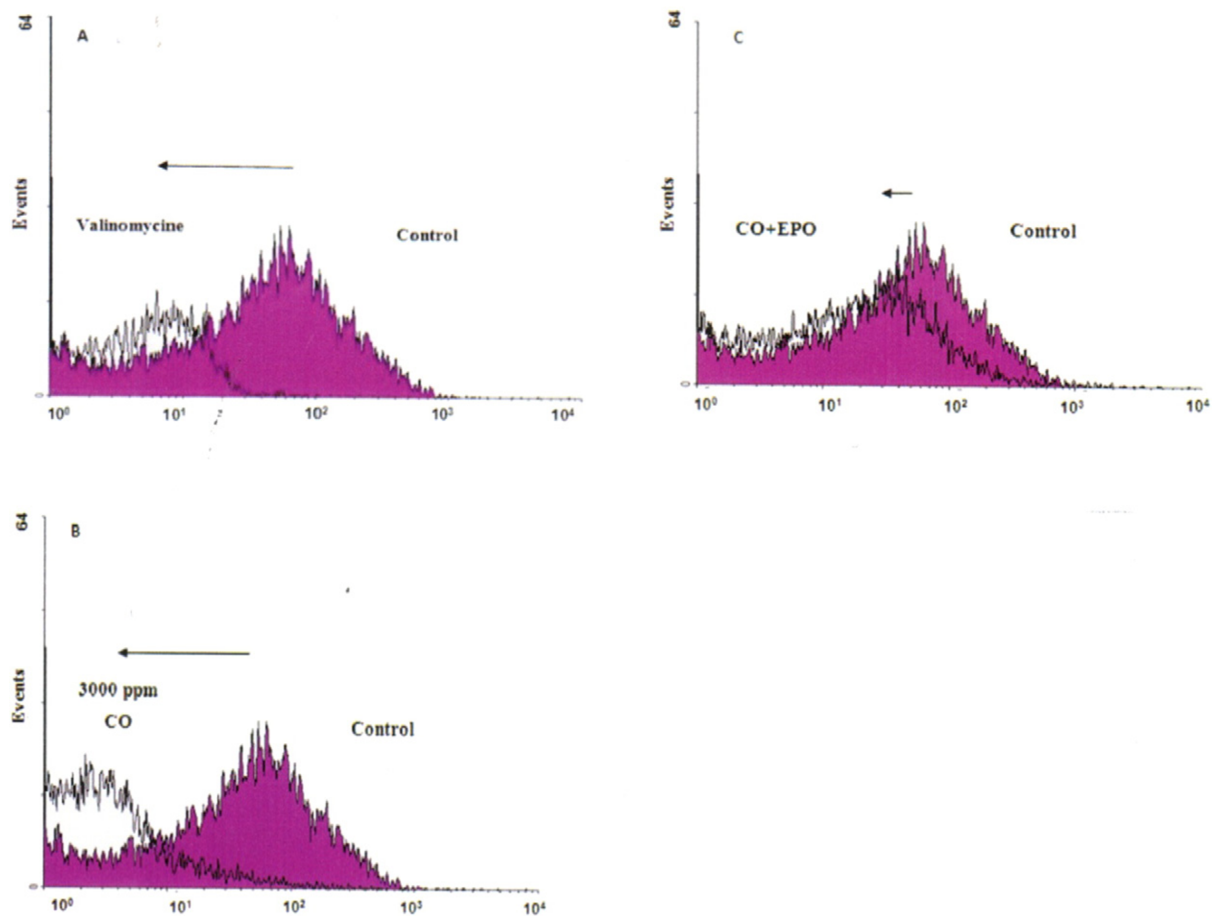

Fig. 4. Histogram of mitochondria membrane potential using JC-1 fluorescence intensity in FL1. Effect of (A) valinomycin (as positive control), intoxication by 3000 ppm CO (B) and EPO treatment (C) on changes from control of median fluorescence.

changes in caspase-3 activity. Caspase-3 activity is increased in the early stages of apoptosis, while DNA cleavage takes place later in the process.

During CO poisoning, tissue hypoxia, impaired cellular respiration and consequently stress responses such as production of reactive oxygen species (ROS) lead to cell injury resulting in neuronal and cardiac insults [25]. It is well known that CO is cardio-toxic and myocardial ischemia occurs as a result of sub lethal acute CO intoxication [6, 27]. According to our previous study, EPO suppressed ischemic changes demonstrated in electrocardiogram recordings which was induced by CO poisoning in rat [19]. It is well known that apoptosis can occur during hypoxic/ischemic conditions [28]. Since anti-apoptotic effects play a role in the EPO anti-ischemic property [12], the EPO effect on CO-induced myocardial apoptosis was studied. The results of the present study demonstrated that administration of 5000 IU/kg EPO after CO intoxication, effectively reduced TUNEL-positive cells and caspase-3 activity as compared to the control. Cai et al., who examined the effect of 5000 IU/kg EPO 24 h prior to cardiac ischemia in rat, have shown the EPO cardio-protective effect as well as an improvement of the heart function. Their results indicated reduction in number of TUNEL-positive cells and caspase-3 activity [29]. According to *in vivo* studies, EPO administration at the onset or after ischemia could reduce apoptosis and improve cardiac function [30, 31]. Moon and colleagues have pointed out that a single dose (3000 IU/kg) of EPO was enough for protection against cardiac ischemia compared to repeated doses in rat. In addition, they have described that EPO administration even after permanent ligation still had cardio protective effect [32]. Their results have shown that EPO continued to reduce myocardial apoptosis 1, 4 and 8 weeks after infarction. Such reduction reached 50% at 24 h, while 8 weeks after EPO administration still accounted for 15–25% compared to the controls. In the present study, an EPO protective effect on CO-induced myocardial injury was evident through its anti-apoptosis property.

Mitochondrial injury occurs during apoptosis that include mitochondrial membrane depolarization, loss of mitochondrial oxidative phosphorylation and release of cytochrome *c*. However, some studies have described that loss of the MMP may be an early event in the apoptosis pathway [33, 34]. The results of the present study showed that CO intoxication induced mitochondrial membrane depolarization significantly in the myocardial cells, while EPO administration increased MMP to 4.7 fold compared to the control. It's believed that EPO antioxidant property has implicated to its anti-apoptotic and protective effects [35]. Since the role of oxidative stress in CO poisoning, the EPO protective effect on CO cardiotoxicity in this study may be related to its antioxidant property. It has also been suggested that EPO may exert a direct protective effect at least through anti-apoptotic and anti-degenerative activity independent of its erythropoietic effect [36].

Apoptosis is regulated by various apoptosis-related proteins. Bcl-2 protein acts as an anti-death factor, preventing the release of cytochrome *c* and other apoptogenic factors from mitochondria [37] while activation of Bcl2 family proteins is considered to be an upstream regulator of the MMP. On the other hand, Bax proteins reduce MMP and thereby cause cytochrome *c* release and caspase activation, which leads to apoptosis. Our results showed that EPO improved Bcl2/Bax ratio which was reduced by CO exposure at 3000 ppm.

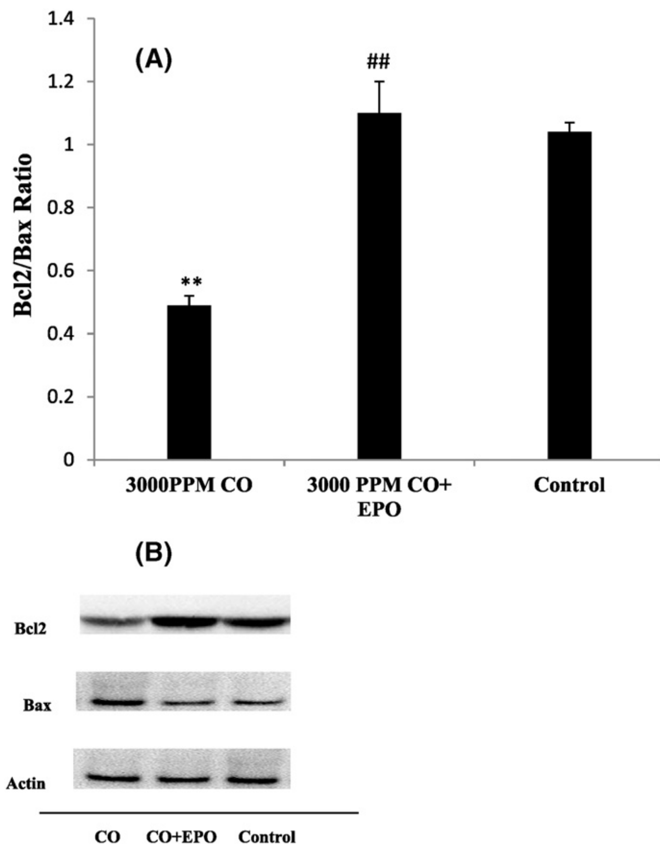

Fig. 5. Western blot analysis of Bcl2 and Bax in response to CO toxicity and EPO treatment A) represent quantitative analysis of Bcl2 and Bax expression, the Bcl2/Bax ratio was significantly reduced in the 3000 ppm CO group compared to the control (**$p < 0.01$, vs. control). However, this ratio was significantly preserved by EPO treatment (##$p < 0.01$, vs. 3000 ppm CO group). Data are expressed as mean ± SEM. B) Effect of EPO and CO on Bcl2 and Bax expression compared to the control.

5. Conclusion

Since cardiotoxicity is a major consequence of CO exposure leading to high morbidity and mortality, investigation of effective treatment is important in managing of CO poisoning. Our findings showed that EPO reduced myocardial injury due to CO intoxication which could be suggested as a possible candidate for the management of CO poisoning cardiotoxicity. Although, more studies are needed to further substantiate these data for clinical applications.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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