Erythropoietin (EPO) plays a critical role in the development of the nervous system. In this study, the effects of EPO in carbon monoxide (CO) neurotoxicity were examined. Rats were exposed to 3000 ppm CO for 1 h and then different doses of EPO were administrated intraperitoneally. After 24 h, glial fibrillary acidic protein (GFAP) levels in the serum were determined and water content of brain and the extravasation of a tracer (Evans blue) were measured. Brain lipid peroxidation, myeloperoxidase activity Myelin basic protein (MBP) and BAX/BcL2 protein relative expressions were determined. Cation exchange chromatography was used to evaluate MBP alterations. Seven days after exposure, pathological assessment was performed after Klüver–Barrera staining. EPO reduced malondialdehyde levels at all doses (2500, 5000 and 10,000 u/kg). Lower doses of EPO (625, 1250, 2500 u/kg) significantly decreased the elevated serum levels of GFAP. EPO could not reduce the water content of the edematous poisoned brains. However, at 5000 and 10,000 u/kg it protected the blood brain barrier against integrity loss as a result of CO. EPO could significantly decrease the MPO activity. CO-mediated oxidative stress caused chemical alterations in MBP and EPO could partially prevent these biochemical changes. Fewer vacuoles and demyelinated fibers were found in the EPO-treated animals. EPO (5000 u/kg) could restore the MBP density. CO increased brain BAX/Bcl-2 ratio 38.78%. EPO reduced it 38.86%. These results reveal that EPO could relatively prevent different pathways of neurotoxicity by CO poisoning and thus has the potential to be used as a novel approach to manage this poisoning.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Carbon monoxide (CO) is an odorless, colorless, nonirritating and highly toxic gas that is produced by incomplete combustion of fossil fuels (Thom and Keim, 1989). Unintentional CO exposure accounts for an estimated 400 deaths in our country (Iran) each year. It leads to various adverse effects that range from neuro-behavioral and cardiovascular abnormalities at lower levels to unconsciousness and finally death after acute exposure to higher concentrations. The main mechanism of CO poisoning is hypoxia due to increased carboxyhemoglobin (CH-Hb) concentration in blood. The most cases of mortality happen in the confined places especially in the cold season(s) of the year. CO poisoning has also mechanisms of toxicity beyond the generation of CO-Hb. CO impairs mitochondrial function and then contributes to hypoxia after binding to heme protein in the structure of cytochrome c oxidase.
Hypoxia increases excitatory amino acid and nitrite levels in the brain (Piantadosi, 1996). Brain hypoxia leads to oxidative stress, necrosis, apoptosis, then inflammation and injury (Piantadosi et al., 1997). Furthermore, CO exposure results in intracellular oxidative stress and then neurological damage by increasing cytotoxic heme oxygenase-1 levels (Crone et al., 2004). Moreover, CO binds to platelet heme proteins, and leads to nitric oxide (NO) release. Overload of NO produces peroxynitrite (Thom et al., 2006a) and impairs the mitochondrial function, which contributes to hypoxia (Thom et al., 2008). Effects of CO are not limited to the phase immediately after exposure. A delayed neurological syndrome (DNS) has also been reported. This may include a variety of neurologic or psychiatric symptoms (Thom and Keim, 1989; Choi, 1983; Garland and Pearce, 1967; Min, 1986; Myers et al., 1985).

Several mechanisms have been suggested for DNS. Soluble CO in the plasma induces platelet-to-neutrophil aggregation and neutrophil degranulation by release or induction of myeloperoxidase and other proteases (Robach et al., 2013). These proteases interact with xanthine dehydrogenase in endothelial cells and modify it to xanthine oxidase. This enzyme produces reactive oxygen species leading to oxidative stress and lipid per-oxidation (Thom et al., 2006a; Lee et al., 2005). Lipid per-oxidation products attach to myelin basic protein (MBP), trigger a lymphocytic immunologic response, increase microglia activity and then cause neurological defects (Thom et al., 2008).

Erythropoietin (EPO) is a hematopoietic cytokine which has an important role in the development of the nervous system. EPO crosses the integral blood–brain barrier at a very slow rate. EPO transition may occur via receptor mediated transport systems and extracellular pathways (Banks et al., 2004). EPO demonstrates considerable neuroprotection in in vitro and invivo models. EPO receptors are expressed on neurons, astrocytes and oligodendrocytes (Benes, 2000; Bernaudin et al., 2000; Genc et al., 2006; Siren et al., 2001). It acts as a neurotrophic factor and can induce neurogenesis (Sakanaka et al., 1998; Shingo et al., 2001). EPO reduces inflammatory mediators (Agnello et al., 2002; Sattler et al., 2004), therefore, it has been suggested for several clinical indications such as stroke, multiple sclerosis, schizophrenia, retinopathy, Parkinson’s disease, epilepsy, brain trauma and spinal cord injury (Rabie and Marti, 2008). In several hypoxic adaptation processes, EPO expression is regulated by the transcription of two hypoxia-inducible factors, HIF-1 and HIF-2 (Wenger, 2000). Erythropoietin enhances oligodendrogenesis and neurological development after neonatal hypoxic/ischemic brain injury (Iwai et al., 2010). It also increases myelin gene expression in CG4 oligodendrocyte cells (Cervellini et al., 2013).

It is reported that Erythropoietin supports oligodendrogenesis and myelin repair following lyssolecithin-induced injury in spinal cord (Cho et al., 2012).

Erythropoietin and its derivatives can improve ventricular and subventricular zone-derived neurogenesis and oligodendrogenesis (Kaneko et al., 2013). EPO demonstrates an immunomodulatory effect in the treatment of experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN), in animal models of human multiple sclerosis and Guillain-Barré syndrome, respectively (Shin et al., 2012).

There is a case report of using EPO in the management of DNS associated with CO poisoning in two patients (Li et al., 2009). After EPO administration, the patients memory and calculation ability were markedly improved, and they were able to carry out a normal conversation with only mild impairment in their memory. In a randomized, prospective study of 103 patients with CO poisoning, National Institutes of Health Stroke Scale (NIHSS as a neurological scale) score improved significantly and S-100B levels significantly decreased in patients after early administration of EPO. At 30 days, patients in the EPO group had a superior Barthel index and fewer patients had delayed neurologic sequelae (Pang et al., 2013). In our previous study, we detected two brain-derived biomarkers ($S100B$, and MBP) in serum 24 h after CO poisoning in rats (Shahsavand et al., 2012). We found out EPO reduces $S100B$ in serum after CO poisoning. Accordingly, the purpose of this study is to evaluate the mechanisms that EPO could antagonize CO neurotoxicity in a rat model.

2. Materials and methods

2.1. CO poisoning and treatments

Male Wistar rats with average body weight of $250 \pm 10\, g$, average age of $103 \pm 2\, days$, were obtained from the Animal Breeding Unit of Mashhad University of Medical Sciences. All animals were raised under the same laboratory conditions of temperature (25 °C) and lighting (12:12 h light:dark cycle) and with free access to standard laboratory chow and tap water. All experimental procedures involving animals were approved by the Animal Care Committee of Mashhad University of Medical Sciences. Experimental animal groups were as the following: (Thom and Keim, 1989) CO-exposure group; rats were placed in a 16-L Plexiglas chamber, which was ventilated with a mixture of 3000 ppm CO (CO cylinder from Arad Gas Co. Iran.) in air at a flow rate of 4 l/min for 60 min (Guan et al., 2009; Alonso et al., 2003) control group: rats were treated with air by the same method and then treated by normal saline (NS) (Rooney et al., 2010) and treatment groups; rats were treated with intraperitoneal (IP) injections of recombinant human EPO (PD Poitien-B from PooyeshDarou Co. Iran.) in doses of 2500, 5000, 10,000 u/kg immediately after CO exposure. Group sizes were $n=5$ in each case. The animals were randomly assigned to each group. In some experiments lower doses of EPO were used. Animals were decapitated to minimize the intermediating factors. Rats were sacrificed 24 h after CO exposure for all experiments except histological determinations. Rat brains were removed, flash frozen in liquid nitrogen and then stored at −80 °C until assayed.

2.2. Brain water content and survival rates of CO-poisoned rats

The entire brain was removed and weighed immediately. The tissue was then placed in a laboratory oven (110 °C) for evaporation (24 h) and weighed again. The water content (%) was calculated as wet weight − dry weight/wet weight × 100% (Pan et al., 2009). Survival rates of CO-poisoned rats were recorded 24 h after CO exposure.

2.3. Western blot analysis

The expressions of MBP and BAX/Bcl-2 were examined by Western blot analysis. Brain tissues were prepared as described previously (Meng et al., 2009) with slight modifications. Frozen tissue (200 mg) was homogenized in 1 ml cold homogenizing buffer (50 mM Tris–Base, pH 7.4,10 mM NaF, 0.5% sodium deoxycholate, 2 mM EGTA, 10 mM β-glycerophosphate 1 mM Na$_3$VO$_4$ containing 1 mM phenylmethylsulfonylfluoride) and protease inhibitor cocktail. Tissue homogenates were centrifuged at 9500 × g at 4 °C for 15 min to clear the cellular debris. Supernatants were mixed with the 5 × loading buffer (250 mM Tris–HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, 5% glycerol and 5% β-mercaptoethanol) and boiled for 10 min. Protein concentrations were determined using the BSA protein assay kit. Samples containing 12 µg proteins were separated on a SDS-PAGE (12%) resolving gel under reducing conditions and electro-transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking in 5% non-
fat milk in TBST (0.5% Tween 20, 137 mM NaCl, 20 mM Tris—HCl pH 7.5) overnight at 4 °C, the membranes were incubated for 3 h at room temperature with primary antibodies against MBP, BAX, Bcl-2, glucose transporter 3 (Glut3) and β-actin (diluted 1:1000, Abcam, UK). Blots were washed 3 times with TBST then incubated with a HRP-conjugated secondary antibody (diluted 1:2000, Abcam, UK) for 2 h at room temperature. Protein bands were visualized using the enhanced chemiluminescence system (ECL, Pierce, Thermo Fisher Scientific, USA), with Alliance 4.7Chem i& Fl uorescence GelDoc System (UVi tec, Cambridge, UK). Signals were analyzed and quantified using UV band software.

2.4. Histochemical staining

For analysis of neuronal survival, rats that survived for seven days were killed by decapitation. Brains were removed and fixed in 10% formaldehyde before paraffin embedding and sectioning. Serial sections (5 μm) cut (corresponding to bregma – 3.3 cm according to a histology atlas (Herman et al., 1986) and Klüver–Barrera staining method (Luxol Fast Blue). All the samples were analyzed by two independent investigator pathologists blinded to the study. Myelinated areas of both sides in three sections per animal were stained with Luxol Fast Blue. The severity of white matter changes was graded as 1 (normal), grade 2 (disarrangement of the nerve fibers), grade 3 (formation of marked vacuoles), or grade 4 (disappearance of myelinated fibers). Scoring was performed according to a previous study with a slight modification (Zhang et al., 2011).

2.5. Evaluation of blood brain barrier integrity

Animals received an IP injection of a tracer (Evans blue: 1 ml of 1% solution in saline) just after CO exposure (Koyama et al., 2007). Extravasations of Evans blue due to blood brain barrier damage were determined using a fluorometric method (Laas et al., 1983). Briefly, the brain was quickly removed, homogenized in 1.5 ml of 50% trichloroacetic acid (w/v), and then centrifuged at 15000× g for 20 min. The extracted dye was diluted with ethanol (1:3), and its fluorescence was calculated using a fluorescence spectrophotometer (ShimadzuRF-540, Japan, λex: 620 nm and λem: 650 nm). Calculations were based on the external standard (62.5–500 ng/ml) in the same solvent. The data were expressed in terms of Evans blue μg/wet tissue weight (g) (Laas et al., 1983).

2.6. Evaluation of serum GFAP level after exposure

GFAP is an astrocyte intermediate filament protein. GFAP concentration in CSF is high in traumatic brain injury, normal pressure hydrocephalus, dementia, and stroke (Vos et al., 2004). The released GFAP from the injured brain can be measured in peripheral blood (Stochetti et al., 2007, 2008). Compared to previously studied biomarkers after CO poisoning, GFAP is brain-specific and not normally presented in blood (Pelinka et al., 2004). In a preliminary assay, lower doses of EPO were effective as neuroprotective against GFAP rise due to CO poisoning. Thus, rats which were treated with lower doses of EPO (625, 1250, 2500 μg/kg) after CO exposure were analyzed. Blood samples were taken from the jugular vein just before and 24 h after exposure and processed to serum by centrifuging at 15000× g for 20 min, and then stored at –20 °C until use. For all groups, GFAP levels were calculated with a commercial immuno enzymatic colorimetric assay kit (Cusabio kit, Wuhan, China). In each animal, serum GFAP level detected as the difference between, before and after experiment, biomarker levels.

2.7. Measurement of lipid peroxidation level in the brain

Malondialdehyde (MDA) levels, last products of lipid peroxidation, were measured in the brain. MDA reacts with thiobarbituric acid (TBA) as a TBA-reactive substance to produce a red color complex with high absorbance at 535 nm. We followed a published method with some modifications (Hosseinzadeh et al., 2007). Briefly, 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 ml of tissue homogenate then the mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml of n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 900× g for 20 min. The upper layer was transferred to another clean tube and its absorbance was measured at 532 nm. 1, 1, 3, 3-tetramethoxyxpropane was used as the standard for MDA.

2.8. MPO activity in the brain

Rats were sacrificed 24 h after CO exposure. The entire brain was removed, weighed immediately and then flash frozen until assayed. MPO activity was calculated with a commercial immuno enzymatic colorimetric assay kit according to the instructions of the manufacturer (Hycult Biotech, Canton, USA).

2.9. Tissue preparation for cation exchange chromatography

Brain tissue was processed for column chromatography as outlined previously with some modifications (Thom et al., 2006b). Briefly, the brains were frozen in liquid nitrogen, homogenized in 5 ml chloroform:methanol (2:1 v/v), and then diluted with chloroform:methanol to obtain a ratio of 1 g brain to 19 ml ice cold chloroform:methanol “one volume”. After centrifugation (2000× g for 5 min) and two washes in one volume of fresh chloroform:methanol, the residue was washed once in ice-cold acetone. After centrifugation (2000× g for 5 min) and three washes with 10 ml water, the residue was resuspended in 10 volumes of 0.03 N HCl. The suspension was incubated for 1 h at 4 °C while stirring.

**Fig. 1.** Effect of Erythropoietin (EPO) on brain water content following carbon monoxide (CO) poisoning. Brain water content was significantly increased in CO exposed rats (3000 ppm for 60 min) as compared with the control animals 24 h after poisoning. EPO was administrated immediately after poisoning. EPO even at the highest dose (10,000 μg/kg) could not decrease the brain water content as a marker of edema 24 h after poisoning. Values are mean ± SEM. Group sizes were n = 5 in each case. **p < 0.01 vs. control animals.**
centrifuged at 44,000×g, and was freeze dried overnight. The freeze dried sample was dissolved in 500 μl 0.1 M acetic acid and applied to a carboxymethylcellulose column equilibrated with 0.1 M acetic acid. Proteins were eluted using a linear gradient of sodium chloride (0–0.75 M) in 0.1 M acetic acid and 500 μl fractions were collected. Protein concentrations in the fractions were determined by measuring absorbance at 280 nm. Fractions of pooled proteins were freeze dried and stored at −80 °C until they were analyzed by Western blot and ELISA (to compare the MDA-MBP adduct levels) assays. To measure the MDA-MBP adduct levels in the fractions with ELISA, resuspended fractions (void volume and fraction 5) from cation exchange chromatography were applied to 96-well microtiter immunoassay plates that were coated by anti-MBP (Cusabio ELISA kit) and incubated for 2 h at 37 °C. Separately, for each sample total protein amount was assessed using BSA protein assay kit. The antigen solution was removed; secondary goat-anti rabbit antibody conjugated to HRP-avidin antibody, wells were washed three times with washing solution. After incubation with the rabbit anti-MDA antibody, wells were washed three times with washing solution. Secondary goat-anti rabbit antibody conjugated to HRP-avidin (100 μl, 1:120,000) was added to the wells and incubated for 3 h at 37 °C. Plates were again rinsed three times with washing solution. 100 μl TMB (3, 3′, 5, 5′ tetra methyl benzidine) was added to each well and incubated for 1 h at 37 °C. Finally, 50 μl stop solution was added and the absorbance of each well was measured at 450 nm in 30 min.

### 2.10. Statistical analysis

Data were expressed as means ± SEM then analyzed with SPSS software (version 11.5). Differences between group means were analyzed using one-way analysis of variance followed by Tukey–Kramer multi-comparison test. Results were considered statistically significant when p was less than 0.05. For survival rate Chi-Square test was used. Results were considered statistically significant when two sided p value was less than 0.05.

### 2.11. Effect of EPO on survival rate

Control rats remained viable during the observation phase (12/12; 100%). However, exposure to CO led to some degree of mortality after 24 h, with only 4/12 (33.33%) surviving rate. In contrast, 10/12 (83.33%), 12/12 (100%) and 12/12 (100%) of animals treated with EPO 2500 u/kg, 5000 u/kg and 10,000 u/kg survived, respectively. Statistical analysis indicated that the improvement in 24 h survival rate was statistically significant (p < 0.01) and (p < 0.001) for increasing doses of EPO treatments, respectively (Table 1).

### 2.12. Effect of EPO on histological scoring in Klüver–Barrera stained brains

Seven days after CO exposure, Klüver–Barrera staining showed increased number of vacuoles and demyelinated fibers in the CO-exposed rats (p < 0.001). However, remarkably fewer vacuoles and demyelinated fibers were found in the EPO treated group. As shown in Table 2, there was a significant reduction in the grading score compared to the CO-exposed group with doses of 2500, 5000 and 10,000 u/kg of EPO (p < 0.05, p < 0.01 and p < 0.01, respectively).

### 2.13. Histochemical scoring in Klüver–Barrera stained brains

<table>
<thead>
<tr>
<th>Group</th>
<th>Control CO-exposed to 3000 ppm for 60 min</th>
<th>CO exposure and treated with EPO 2500 u/kg</th>
<th>CO exposure and treated with EPO 5000 u/kg</th>
<th>CO exposure and treated with EPO 10,000 u/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klüver Barrera</td>
<td>3.83 ± 0.408***</td>
<td>2.5 ± 0.836#</td>
<td>2.33 ± 1.03##</td>
<td>2.16 ± 0.75##</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, group sizes were n = 5 in each case. 
***p < 0.001 vs. control.
#p < 0.05 and ##p < 0.01 vs. CO-exposed rats.
Fig. 2. Western blot analysis of myelin basic protein (MBP) and BAX/Bcl-2 protein levels in carbon monoxide (CO)-exposed rats. Blots were quantitated by scanning densitometry; (left) MBP band intensities were normalized to β-actin and glucose transporter 3 (Glut3) in the same sample. BAX/Bcl-2 band intensities were normalized to β-actin in the same sample. Data are means ± SEM. Group sizes were n = 5 in each case. *p < 0.05 and ***p < 0.001 vs. control animals; ##p < 0.01 and ###p < 0.001 vs. CO exposed animals.

Fig. 3. Mean Evans blue dye (mg)/tissue weigh (g) at 24 h after carbon monoxide (CO) poisoning. CO (3000 ppm for 60 min) significantly raised Evans blue levels in rats in comparison to the control 24 h after poisoning. All animals received an IP injection of Evans blue (1 ml, 1% solution in saline) just after CO exposure, and then EPO was administrated. EPO administration could significantly protect the blood brain barrier after poisoning. Values are mean ± SEM. Group sizes were n = 5 in each case. ***p < 0.001 vs control animals; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. CO exposed animals.

Fig. 4. Mean serum glial fibrillary acidic protein (GFAP) level at 24 h after carbon monoxide (CO) poisoning. CO (3000 ppm for 60 min) significantly raised the serum GFAP level in rats in comparison with control animals 24 h after poisoning. EPO was administrated immediately after poisoning. EPO administration could significantly decrease the serum GFAP elevation after poisoning but not in a dose dependent manner. Values are mean ± SEM. Group sizes were n = 5 in each case. ***p < 0.001 vs control animals; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. CO exposed animals.
3.5. Effect of EPO on blood brain barrier integrity and GFAP release into serum

The blood brain barrier permeability to Evans blue was evaluated 24 h after CO poisoning. After CO exposure more than 4 fold increase of Evans blue in the wet tissue was detected in CO-exposed samples compared to the control (0.917 ± 0.087 µg/g and 0.228 ± 0.072 µg/g of wet tissue in CO-exposed and control samples, respectively, p < 0.001) (Fig. 3). This clearly indicates extrava-sations of Evans blue due to CO poisoning which suggests a major disruption in the blood brain barrier. EPO could protect the blood brain barrier from the disruption as a result of CO poisoning at 5000 and 10,000 u/kg by 30.2% (p < 0.05) and 39.2% (p < 0.01), respectively. The mean serum GFAP level in CO-exposed rats was significantly increased as compared with the control group (7.67-fold higher) 24 h after CO poisoning (Fig. 4). EPO administration even in lower doses (625, 1250, 2500 u/kg) could decrease the serum GFAP elevation after CO poisoning by 77.47% (p < 0.001), 77.24% (p < 0.01) and 85.28% (p < 0.001), respectively.

3.6. Effect of EPO on lipid peroxidation

Free radical formation which leads to lipid peroxidation was measured as tissue MDA level. There was an increase (182.1%) in the brain MDA level following CO poisoning as compared with the control group (3.466 vs. 1.661 nmol/mg protein tissue, p < 0.001) (Fig. 5). EPO treatment resulted in a significant and dose-dependent decrease in the free radical-mediated lipid peroxidation starting from the 2500 u/kg dose as showed by a reduction in the MDA levels (p < 0.05). In EPO treated groups with doses of 625 and 1250 u/kg, MDA levels were 3.302 and 3.257 nmol/mg tissue protein content (p < 0.05 as compared with the CO-exposed group), respectively. Therefore, EPO at these doses could not decrease the MDA level after poisoning. However, at higher doses (2500, 5000 and 10,000 u/kg) EPO could reduce MDA levels to 2.212, 1.667 and 1.884 nmol/mg tissue protein content, respectively (p < 0.05 as compared with the CO-exposed group).

3.7. Effect of EPO on MPO activity in the brain

The MPO activity in brain tissues after CO poisoning was evaluated using immune-enzymatic colorimetric assay. There was approximately 3 fold increase (382.6%) in the brain tissue following CO exposure as compared with the control group (3.980 vs. 1.04 ng/mg tissue, p < 0.001) (Fig. 6). EPO treatment resulted in a significant and dose-dependent decrease in the MPO activity starting from the dose 2500 u/kg. In comparison to the CO-exposed group, EPO at doses of 2500, 5000 and 10,000 u/kg could reduce MPO activity to 2.566 (p < 0.01), 2.162 (p < 0.001) and 1.112 (p < 0.001) ng/mg tissue, respectively.

3.8. Effect of EPO on MBP alterations

To find whether EPO altered CO-induced changes in MBP, cation-exchange column chromatography was performed (Fig. 7). Acid-soluble proteins from brain homogenates were fractionated. Five peaks were resolved in control samples, while proteins eluted from brains of rats 24 h after CO exposure exhibited only one peak and the majority of the proteins were eluted in the void volume. The pattern of protein elution from CO-exposed rats subsequently treated with EPO was not entirely normal, but showed an intermediate pattern between samples from control and the CO-exposed rats. As an additional probe for alterations in protein structure, MBP contents of pooled fractions were analyzed using Western blotting. MBP content was markedly reduced in samples from rats 24 h after CO exposure. MBP contents were restored in rats who were treated with EPO (5000 u/kg) following CO exposure.

![Fig. 5](image1.png)

**Fig. 5.** Effect of erythropoietin (EPO) on lipid peroxidation following carbon monoxide (CO) poisoning. Malondialdehyde (MDA) levels were measured in 10% homogenates of brain from poisoned rats with CO (3,000 ppm for 60 min). EPO was administrated immediately after poisoning. Values are mean ± SEM. Group sizes were n = 5 in each case. ***p < 0.001 vs. control animals; #p < 0.05 and ###p < 0.001 vs. CO exposed animals.

![Fig. 6](image2.png)

**Fig. 6.** Mean myeloperoxidase activity at 24 h after carbon monoxide (CO) poisoning. Malondialdehyde (MDA) levels were measured in 10% homogenates of brain from poisoned rats with CO (3,000 ppm for 60 min). EPO was administrated immediately after poisoning. EPO administration could significantly decrease the MPO brain activity elevation after poisoning and in a dose dependent manner. Values are mean ± SEM. Group sizes were n = 5 in each case. ***p < 0.001 vs. control animals; #p < 0.05, ##p < 0.01 and ###p < 0.00 vs. CO exposed animals.
This indicated that although the pattern of MBP in EPO (5000 u/kg)-treated animals was not totally normal, MBP content was preserved.

4. Discussion

In this study, we demonstrated that EPO could inhibit CO...
neurotoxicity cascade as it is shown in Fig. 8. EPO could increase the survival rate after CO poisoning. Moreover, it was shown that CO poisoning could cause a major GFAP rise in the serum which is probably due to astrocyte injury and hypoxia. Previous studies showed that EPO can cross to central nervous system only when it was administered in suprapharmacological doses (2000–5000 u/kg per dose) (Brines et al., 2000). However, our data showed that EPO has some neuroprotective effects at lower doses. This was manifested by the reduction of GFAP released from the brain into the serum by EPO at a dose of 625 u/kg. This could be due to integrity loss of BBB after CO poisoning that let a big molecule such as EPO to cross into the brain. It has been reported that EPO could directly decrease the risk of astrocyte swelling after stroke and other brain damages (Gunnarson et al., 2009). On the other hand, hypoxia-ischemia elevates astrocyte water uptake. EPO antagonizes mGlu R activity in astrocytes and interferes with the frequency of water channel aquaporin 4 (AQP4) oscillations to decrease water permeability (Gunnarson et al., 2009). This is the probable mechanism that EPO could prevent the astrocyte damage and GFAP release subsequently. These effects are immediate in contrast to other neuroprotective effects of EPO that depend on gene activation.

It is reported that MDA levels increased remarkably in the cerebral cortex 24 h after CO exposure (Wang et al., 2009). We found that this increase could be relieved by doses of EPO higher than 2500 u/kg. EPO reduces lipid peroxidation by both decreasing NO synthesis and XO activity after brain injury in a model of closed head injury in rats (Öztürk et al., 2008). EPO also reduces nitrite and nitrate (as an index of NO formation) in the hippocampus as observed after ischemia (Calapai et al., 2000). They mentioned that NO synthesis and XO activity increment are two mechanisms of toxicity after CO poisoning. Therefore, EPO could cause tissue MDA reduction by affecting NO synthesis and XO activity. Our results showed that EPO could significantly decrease the MPO activity after CO poisoning in a dose dependent manner. As we mentioned before, increased MPO activity can cause lipid peroxidation. Reduction of MDA by EPO, as shown in Fig. 6, could be in some part, the mechanism of tissue protection by EPO. We have reported that EPO could increase the hemoglobin level even 24 h after administration (Shahsavand et al., 2012). Therefore, the neuroprotective effects of EPO could be in some part, due to a rise in hemoglobin and thus better oxygen delivery.

On the other side, EPO could not reduce the edema that was produced 24 h after CO exposure. It is known that EPO can cause an “encephalopathic like syndrome” at high doses (Delanty and Needell, 1998). Thus, we examined the possibility to induce edema with high doses of EPO. However, even at these doses, EPO did not induce edema after 24 h. Then, we evaluated the EPO effect on blood brain barrier integrity after CO poisoning. It was demonstrated that EPO could protect the blood brain barrier from damages that lead to vascular deficits. EPO has been observed to present direct protection for the blood brain barrier. EPO pretreatment in rats reduced the total duration of seizure that induced by pentylenetetrazol and the blood brain barrier permeability (Üzüm et al., 2006). Also, it could protect the blood brain barrier against vascular endothelial growth factor (VEGF)-induced permeability in an in vitro model of bovine blood brain barrier (Martínez-Estrada et al., 2003). Depending on the mechanisms, brain edema theoretically is classified into two categories: cytotoxic and vasogenic edema. Vasogenic edema is caused by disruption of the blood brain barrier. In some studies, the total brain edema and vascular permeability (as a result of vasogenic brain edema) were determined by evaluating the water content and Evans blue extravasations into the brain tissue, respectively. Cortical extracellular brain edema (vasogenic edema) is a kind of extracellular edema that produced by an endothelial capillary alteration, commonly known as an enhancement in the capillary permeability or “open blood–brain barrier”. It permits large molecules like Evans blue to pass the blood brain barrier. It is thought that the combined brain edema consists of both brain edema types, cellular and extracellular in variable ratios; that can be produced simultaneously from the beginning or can appear consecutively (Iencean, 2003). All together our findings have shown that CO poisoning may cause a degree of combined brain edema and EPO was only able to reduce the extracellular edema. The charge pattern of MBP based on cation exchange chromatography in the EPO-treated group was in-between the control and that observed from untreated CO-exposed rats. Klüver-Barrera staining and Western blot analysis also supported these results concerning MBP. We found some increase in BAX/Bcl-2 ratio as a sign of early apoptotic events (Wenzel et al., 2004).

5. Conclusion

It is concluded that EPO can relatively protect the brain against CO-induced neurotoxicity through different pathways. Therefore, EPO could be introduced as a possible clinical management for CO poisoning. However, in this regard it is necessary to further examination to explore other aspects of this remarkable effect and also clinical pharmacokinetics of systemically administered EPO under condition of CO poisoning should be studied.

Funding

This work was supported by a grant (No. 88479) from the Research Council of Mashhad University of Medical Sciences.

Acknowledgment

This research was part of a Ph.D. thesis. The authors would like to thank Dr. Sadr Nabavi for his comments in the pathological assessment.

Transparency document

Transparent document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2015.09.015.

References


Kabir, S.A. Moallem et al. / Food and Chemical Toxicology 86 (2015) 56–64


