

# Magnesium sulfate ameliorates carbon monoxide-induced cerebral injury in male rats

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**Abstract.** Carbon monoxide (CO) has been shown to induce several cardiovascular abnormalities, as well as necrosis, apoptosis and oxidative stress in the brain. Magnesium sulfate (MS) has been shown to have beneficial activities against hypoxia in the brain. In the present study, the possible protective effects of MS against CO-induced cerebral ischemia were investigated. For this purpose, 25 male Wistar rats were exposed to 3,000 ppm CO for 1 h. The animals were divided into 5 groups (n=5 in each group) as follows: The negative control group (not exposed to CO), the positive control group (CO exposed and treated with normal saline), and 3 groups of CO-exposed rats

treated with MS (75, 150 and 300 mg/kg/day) administered intraperitoneally for 5 consecutive days. On the 5th day, the animals were sacrificed and the brains were harvested for the evaluation of necrosis, apoptosis and oxidative stress. Histopathological evaluation revealed that MS reduced the number and intensity of necrotic insults. The Bax/Bcl2 ratio and malondialdehyde (MDA) levels were significantly decreased in a dose-dependent manner in the MS-treated rats compared to the positive control group, while a significant dose-dependent increase in Akt expression, a pro-survival protein, was observed. In addition, MS administration reduced pro-apoptotic indice levels, ameliorated histological insults, favorably modulated oxidative status and increased Akt expression levels, indicating a possible neuroprotective effect in the case of CO poisoning. On the whole, the findings of this study indicate that MS may prove to be useful in protecting against CO-induced cerebral injury.

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## Introduction

Carbon monoxide (CO) is a toxic gas produced by the incomplete combustion of fossil fuels (1,2). It is a cause of significant morbidity and mortality worldwide with no specific antidote. Although both normobaric and hyperbaric oxygen are used as a common treatment, neurological sequelae are common in survivors of CO poisoning (3,4). In the USA, CO poisoning accounts for 50,000 referrals to emergency departments and causes 334 deaths annually (5). In Iran, the improper use of gasoline and natural gas appliances cause a significant number of CO poisoning cases (6). Reports from different parts of Iran

have shown that the mortality and morbidity rates were higher compared to other parts of the world (6). The majority of cases of CO poisoning occur in the colder months of the year due to the use of fossil fuels in heating appliances (7,8). Therefore, CO poisoning is regarded as one of the most challenging cases of poisoning in Iranian health system. The pathophysiology of CO poisoning centers on the production of carboxyhemoglobin, which reduces the oxyhemoglobin concentration and consequently diminishes tissue oxygen delivery (9,10). Since CO affinity to hemoglobin is approximately 230-270-fold higher than that of O<sub>2</sub> to hemoglobin, even at low CO concentrations, the carboxyhemoglobin concentration becomes sufficiently high to induce toxicity (9). The clinical manifestations of CO poisoning are non-specific (i.e., headaches, fatigue, confusion, nausea, dizziness, visual problems, chest pain, shortness of breath, loss of consciousness and seizures) and they are principally associated with the deleterious effects of normobaric on the brain and heart (11,12).

Magnesium sulfate (MS) is used for the treatment of several conditions, including eclampsia, pre-eclampsia and the prevention of torsade de pointes (13). It has attracted the interest of scientists due to its protective properties against cerebral ischemia/reperfusion (I/R) (14-16), as it has been shown to reduce brain cell necrosis, apoptosis and oxidative stress levels (17-21). Moreover, MS is inexpensive, widely available, is simple to administer and lacks severe adverse drug reactions for common uses in the treatment of pre-eclampsia, eclampsia and torsade de pointes (22,23).

B-cell lymphoma-2 (Bcl2) controls mitochondrial membrane permeability in order to impede apoptotic signal transduction, whereas Bcl2-associated-X protein (Bax), as a pro-apoptotic factor, disrupts mitochondrial membrane potential and induces caspase-3 activation, leading to irreversible apoptosis (24). As shown by recent literature, the Bax/Bcl2 ratio alone stands as an index of cell apoptosis or survival (25-28). In addition, in our previous studies using a model of CO poisoning, a clear connection between apoptosis and the Bax/Bcl2 ratio was proven by TUNEL assay and caspase activity measurements (29). Moreover, Akt is regarded as a pro-survival factor (30,31), whose activation induces phosphorylation at different sites. Activated Akt influences a number of factors involved in apoptosis, either by transcription regulation or direct phosphorylation, yielding favorable effects against ischemia-induced apoptosis. Thus, chemicals capable of inducing Akt expression/activity may be used in the treatment of I/R injury (2,32-35).

Considering the importance of CO poisoning and with regard to the promising properties of MS, in the present study, we examined the effects of MS on CO-induced cerebral injury in rats.

## Materials and methods

**Animals.** In the present study, 25 male Wistar rats (8-10 weeks old; weight, 200-250 g), were obtained from the Animal House of Zabol University of Medical Sciences (Zabol, Iran). The animals were kept under standard conditions (at 25°C with a 12 h/12 h light/dark cycle) and were allowed access to food and water ad libitum. The present study was approved by the Ethics Committee of Zabol University of Medical

Sciences (approval no. ZBMU.1.REC.1394.112). All animals were treated in accordance with the guidelines for the Care And Use Of Laboratory Animals prepared by the Animal Research Ethics Committee of Zabol University of Medical Sciences and in conformity with EU Directive 2010/63/EU for animal experiments. The animals were randomly divided into 5 groups namely, the intact group (rats that were not exposed to CO), the control (rats that were exposed to CO and received normal saline) and 3 MS-treated groups (rats that were exposed to CO and received MS 75, 150 and 300 mg/kg). CO poisoning was induced by exposing the animals to CO at 3,000 ppm for 60 min, as previously described (36). Immediately following the exposure period, the first dose of MS (or normal saline for the control group) was administered intraperitoneally (i.p.) and the next 4 doses were administered on the next 4 consecutive days on a daily basis (a total of 5 doses of MS).

**Chemicals.** Protein kinase B (Akt; cat. no. 4685S; dilution, 1/1,000), Bcl2-associated-X (cat. no. 2772S; dilution, 1/1,000), Bcl2 (cat. no. 2876S; dilution, 1/1,000) and anti- $\beta$ -actin (cat. no. 4967S; dilution, 1/1,000) antibodies and secondary rabbit antibody (anti-rabbit IgG, HRP-linked; cat. no. 7074S) were all purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The Coomassie (Bradford) Protein Assay kit was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A CO capsule (99.999% purity) was obtained from Darman Gas (Tehran, Iran). Thiobarbituric acid was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and MS was purchased from Pasteur Institute (Tehran, Iran).

**Study design and treatments.** For CO poisoning induction, the rats were placed in a 12-liter airtight Plexiglas container which was connected via polyethylene glycol (PEG) tubes to oxygen and CO capsules. The CO concentration was continuously monitored by a CO analyzer (TPI 707 Carbon Monoxide Analyzer; TPI Korea Co., Anyang, Korea) and had a constant level of 3,000 $\pm$ 100 ppm for 1 h. Subsequently, the animals were exposed to ambient air and MS was injected (i.p.) at 3 doses (75, 150 and 300 mg/kg). On the 5th day, at 2 h after the final injection, the animals were anesthetized by an intraperitoneal administration of ketamine (90 mg/kg) and xylazine (10 mg/kg) and sacrificed. The brain samples were then collected and harvested for further evaluation. Moreover, for western blot analysis and malondialdehyde (MDA) assay, the harvested samples were preserved in cryotubes and stored at -80°C, as previously described (37).

**Carboxyhemoglobin level assessment.** Within 30 min following exposure CO, blood samples were obtained from the tail of the animals. The serum carboxyhemoglobin concentration was measured using a spectrophotometer calibrated for rat blood (Jenway 6305; Bibby Scientific Ltd., Staffordshire, UK), to ensure the induction of CO poisoning (38).

**Histopathological examinations.** For histopathological evaluation, serial brain sections (5- $\mu$ m-thick; corresponding to bregma -3.3 cm) according to a histological atlas (39) were obtained. The samples were placed in microtubes containing 10% formalin for fixation and 24 h later, they were sent to the

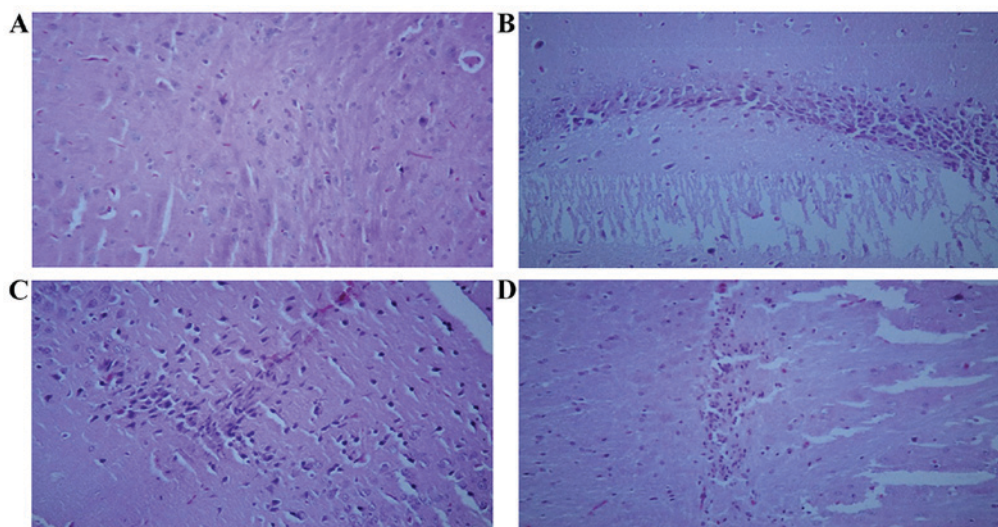


Figure 1. Representative images of (A) intact brain and histological insults observed in whole-brain samples following exposure to CO at 3,000 ppm, including (B) mild insults (dispersed necrotic cells and/or lymphatic infiltration), (C) moderate insults (bifocal necrotic areas) and (D) severe insults (>2 necrotic foci) (magnification, x40). CO, carbon monoxide; MS, magnesium sulfate.

Pathology Department of Amiralmomenin Hospital (Zabol, Iran). Following H&E staining, pathological insults were evaluated based on the severity of the injury, by a pathologist who was blinded to the grouping and treatments. The findings were categorized into 3 grades of mild (dispersed necrotic cells and/or lymphatic infiltration) (Fig. 1B), moderate (necrotic unifocal and/or bifocal area) (Fig. 1C), and severe (more than two necrotic areas) (Fig. 1D) insults.

**Bax, Bcl2 and Akt protein expression assessment.** For the determination of Akt, Bax, Bcl2 and  $\beta$ -actin expression, western blot analysis was performed. For this purpose, first, approximately 200 mg of harvested whole brain samples which were kept at  $-80^{\circ}\text{C}$ , were weighed, homogenized using a mechanical homogenizer, sonicated and centrifuged using a refrigerated centrifuge at  $10,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. The supernatants were then collected, the protein contents were measured using the Coomassie (Bradford) Protein Assay kit which was purchased from Thermo Fisher Scientific, Inc. and samples were placed in a hot bath (boiling water) for the denaturation of proteins.

In order to determine the total Akt, Bax, Bcl2 and  $\beta$ -actin levels, 5–10  $\mu\text{l}$  of supernatant was loaded into 12% SDS page wells and proteins were separated using gel electrophoresis (Bio-Rad power supply, 120 v for 1.5 h; Bio-Rad, Hercules, CA, USA). At the end of the electrophoresis period, proteins were transferred (Bio-Rad power supply, 350 mA, 25–45 min; Bio-Rad) to a PVDF membrane using transfer buffer (25 mM Tris, 1.2 mM glycine, and 20% methanol; pH 8.0). The membrane was washed 3 times (each time for 5 min) with Tris-buffered saline (TBS). 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  $4^{\circ}\text{C}$ , the membranes were incubated with the primary antibodies for 1 h at room temperature on a rocker. The membrane was then washed 5 times (each time for 5 min) in washing buffer, in order to remove any unbound conjugate proteins. The samples were then treated with the secondary antibody for 1 h at room temperature, washed thoroughly with

TBST and visualized by means of 500–1,000  $\mu\text{l}$  of enhanced chemiluminescence (Pierce, Rockford, IL, USA) to visualize the blots using Syngene ChemiDoc (Syngene, Frederick, MD, USA). Eventually, blot analysis was carried out using GeneTools software.

**Thiobarbituric acid reactive substances (TBARS) assay.** MDA is a product of lipid peroxidation that can be measured by spectrophotometric methods (40,41). In this study, the brain samples (200 mg) were homogenized in cold 1.15% potassium chloride to yield a 10% homogenate. Subsequently, 0.5 ml of the 10% homogenate was mixed with 3 ml of phosphoric acid 1% w/v, boiled for 45 min at  $95^{\circ}\text{C}$  and centrifuged at  $12,000 \times g$  for 10 min. After cooling to room temperature, 4 ml n-butanol was added and the reaction mixture was vortexed. The absorbance of the supernatant was measured at 532 nm (40) using a spectrophotometer (Jenway 6305; Bibby Scientific Ltd.) and the level of MDA was expressed as nmol per gram of wet tissue.

**Statistical analysis.** Data were analyzed using SPSS version 16 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey's post hoc test, was used for data analysis.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Carboxyhemoglobin concentration and the effect of MS on CO-induced brain histological insults.** The mean blood carboxyhemoglobin concentration was  $70 \pm 8\%$  in the CO-exposed rats. Brain histopathological evaluations revealed that MS treatment decreased the number and intensity of brain insults in the CO-poisoned rats. As shown in Fig. 1, brain samples were stained with H&E and brain insults were categorized as mild (dispersed necrotic cells and/or lymphocytic infiltration), moderate (necrotic cells with low foci) and severe (multi foci necrosis). As shown in Table I, in the control



Table I. Histological findings in intact rats and in rats exposed to CO at 3,000 ppm for 1 h and treated with normal saline (control) and MS at 75, 150 and 300 mg/kg.

Treatment	No injury	Mild	Moderate	Severe
Intact	5/5	0/5	0/5	0/5
Normal saline	0/5	2/5	1/5	2/5
MS 75 mg/kg	1/5	1/5	2/5	1/5
MS 150 mg/kg	3/5	0/5	1/5	1/5
MS 300 mg/kg	3/5	1/5	1/5	0/5

<sup>a</sup>'n/m' indicates that 'n' rats out of 'm' rats (which is 5 in each group) had the specific grade of histopathological insults. CO, carbon monoxide; MS, magnesium sulfate.

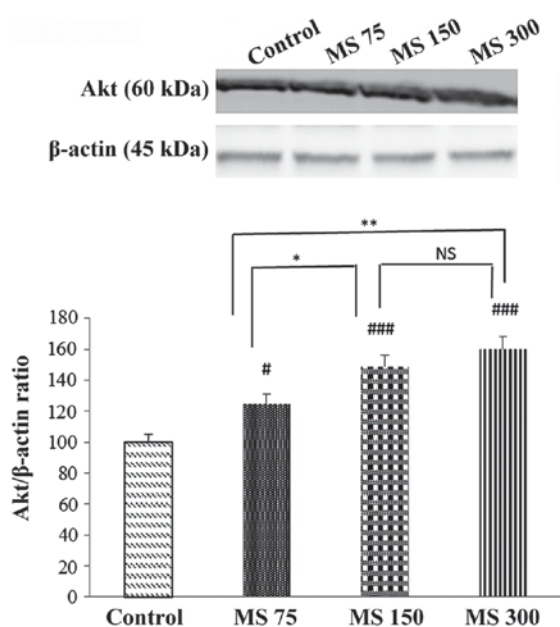


Figure 2. Effects of MS at 75, 150 and 300 mg/kg administered for 5 consecutive days, on Akt protein expression levels in CO-poisoned rats. Bars represent the means  $\pm$  SEM. <sup>#</sup> $P < 0.05$  and <sup>###</sup> $P < 0.001$  indicate significant differences between the control and treatment groups. <sup>\*</sup> $P < 0.05$  and <sup>\*\*</sup> $P < 0.01$  indicate significant differences among the MS-treated groups.  $\beta$ -actin was used as the endogenous control. The control group rats were CO-poisoned animals treated with normal saline. NS, not significant. MS, magnesium sulfate; CO, carbon monoxide.

group (normal saline-treated), 2 out of the 5 animals exhibited mild insults, 1 out of 5 had moderate insults and 2 out of 5 had severe insults, whereas in the group treated with MS at 300 mg/kg, 1 out of 5 had mild and moderate insults and no animal showed severe insults. Furthermore, in the animals treated with MS at 75 and 150 mg/kg, the number and severity of insults were decreased. Taken together, these results demonstrated that as compared to the control animals, all MS doses reduced CO-induced damage to the brain tissues (Table I).

**Effects of MS on Akt protein levels in CO-poisoned rats.** As depicted in Fig. 2, the expression levels of Akt, as a pro-survival protein, significantly increased following treatment with MS as compared to the control group (normal saline-treated rats).

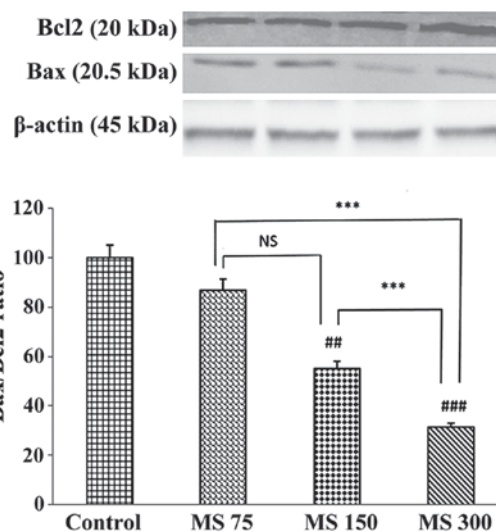


Figure 3. Effects of the 5-day administration of MS (75, 150 and 300 mg/kg/day) on Bax/Bcl2 proteins ratio in the brains of rats. Bars represent the means  $\pm$  SEM values. All groups were compared to the normal saline-treated control group (one-way analysis of variance followed by Tukey's post-hoc test was used for statistical analysis). <sup>\*\*\*</sup> $P < 0.001$  indicates statistically significant differences among the MS-treated groups. <sup>##</sup> $P < 0.01$  and <sup>###</sup> $P < 0.001$  indicate significant differences between the control and MS-treated groups.  $\beta$ -actin was used as the endogenous control. The control group rats were CO-poisoned animals treated with normal saline. NS, not significant; MS, magnesium sulfate; CO, carbon monoxide; Bcl2, B-cell lymphoma-2; Bax, Bcl2-associated-X protein.

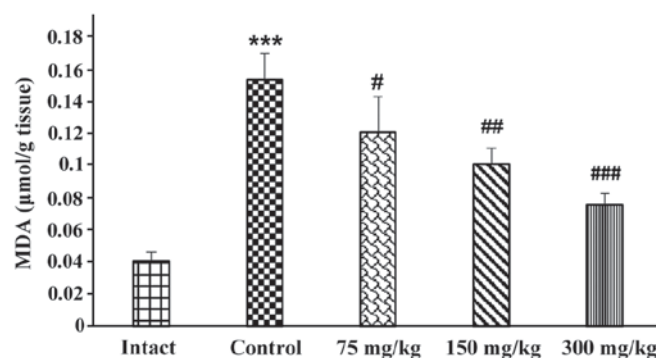


Figure 4. Effect of 5-day MS administration on MDA levels in CO-poisoned rats. Results are expressed as the means  $\pm$  SEM of each group. <sup>\*\*\*</sup> $P < 0.001$  indicates significant difference between the intact and control group. <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$  and <sup>###</sup> $P < 0.001$  indicate significant differences between the control and treatment groups. The control group rats were CO-poisoned animals treated with normal saline. MS, magnesium sulfate; CO, carbon monoxide; MDA, malondialdehyde.

In addition, significant differences were observed between the MS 300 and MS 75 ( $P < 0.01$ ), and between the MS 150 and MS 75 ( $P < 0.05$ ) groups. Furthermore, the differences between MS treatment at 150 and 300 mg and the control groups were significant (for both cases  $P < 0.001$ ).

**Effect of MS on Bax/Bcl2 protein ratio in brain cells following treatment with MS in CO-poisoned rats.** Compared to the control group, in the MS treatment groups, Bax protein expression levels were decreased, while Bcl2 (an anti-apoptotic protein) expression levels were increased (Fig. 3). It was evident

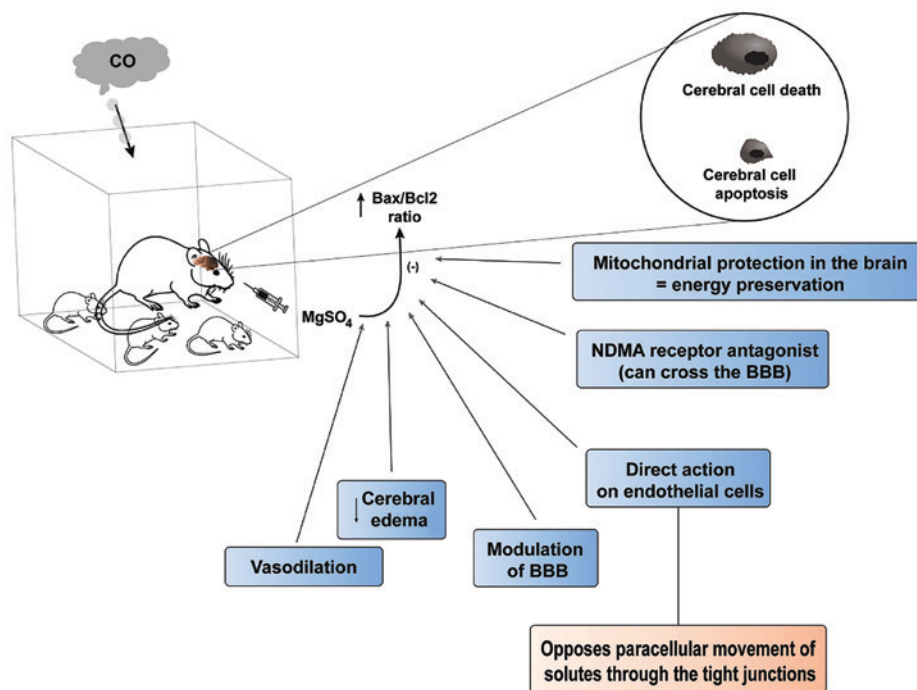


Figure 5. A schematic presentation of the present study along with the underlying mechanisms suggested for the neuroprotective effects of magnesium sulfate (61-65). MgSO<sub>4</sub>, magnesium sulfate; NDMA, N-ditrosodimethylamine; BBB, blood-brain barrier; CO, carbon monoxide; Bcl2, B-cell lymphoma-2; Bax, Bcl2-associated-X protein.

that following treatment with MS at 150 and 300 mg/kg, the Bax/Bcl2 ratio was decreased in comparison to the control group ( $P<0.01$  and  $P<0.001$ , respectively). Based on our data, MS was able to decrease the Bax/Bcl2 apoptotic index in brain cells following CO poisoning.

**TBARS assay.** The present study demonstrated that oxidative stress was increased following CO poisoning compared to the control ( $P<0.001$ ). MS treatment (75, 150 and 300 mg/kg) dose-dependently decreased the oxidative stress levels in rats in comparison to the control group ( $P<0.05$ ,  $P<0.01$  and  $P<0.001$ , respectively) (Fig. 4).

## Discussion

The neuro-protective properties of MS make it a potential candidate for the alleviation of the deleterious effects of cerebral I/R injury (17). Since CO induces damage by inducing hypoxia, tissues with a greater O<sub>2</sub> consumption, including the heart and brain, are more vulnerable to the effects of CO poisoning (9,42). CO poisoning intensity depends on a number of factors, including CO levels during exposure and the exposure period (9). Furthermore, CO poisoning has acute and delayed consequences (1,39,43). In the current study, we demonstrated that MS decreased brain cell necrosis, apoptosis and oxidative stress, while it increased pro-survival Akt protein levels in a dose-dependent manner. Previously, we reported several neuroprotective and cardioprotective substances, which may be used to decrease CO poisoning consequences, in animal models (2,33,36,37,42,44-46). More specifically, in a recent study from our group, MS administration was found to exert positive effects against the cardiotoxicity of CO in rats (33). Magnesium dilates blood

vessels and lowers the heart rate. However, the early administration of magnesium in high-risk patients has been shown to have no effect on mortality (47).

Moreover, CO poisoning induces cerebral hypoxia that may lead to infarction and necrosis (9). In previous studies, the potent effects of MS in decreasing the infarct size in animal models were observed (14,17). Marinov *et al* demonstrated that the intra-arterial administration of a single dose of MS 90 mg/kg reduced the cerebral infarct size in animals submitted to reversible middle cerebral artery occlusion (17). Consistent with this, the results of this study revealed that MS (at 75, 150 and 300 mg/kg) decreased the number and intensity of cerebral insults in a dose-dependent manner in an animal model of CO poisoning.

It is known that Akt protein plays a key role in cell survival by inhibiting apoptosis (48). Mechanistically, PI3kinase/Akt (also known as protein kinase B) pathway activation is considered neuroprotective in the case of cerebral I/R (48-50). In the present study, it was found that MS increased brain Akt protein expression levels and decreased apoptosis in the context of CO poisoning. This finding is consistent with the observations of Yu *et al*, indicating that the PI3kinase/Akt pathway activation is critically important for brain cell survival in case of cerebral ischemia through decreasing apoptosis (50).

In addition, the present study demonstrated that MS treatment decreased the Bax/Bcl2 ratio, an apoptotic index, in brain tissues post-CO poisoning. Bax is a mitochondrial pro-apoptotic protein, the expression of which increases during the activation of the intrinsic apoptotic pathway and leads to mitochondrial injury (51). At the same time, Bcl2 is an anti-apoptotic protein which is produced in order to counteract pro-apoptotic signals and protect from mitochondrial injuries (52). The Bax/Bcl2 ratio is considered a measure of cell

susceptibility to apoptosis (53) and in the current study, this ratio was found to be significantly decreased following MS administration in a dose-dependent manner. Ravishankar *et al* demonstrated that cerebral ischemia increased apoptosis via increasing pro-apoptotic Bax and decreasing anti-apoptotic Bcl2 expression in an animal model of cerebral hypoxia (54). The results of this study are in agreement with the data reported by several previous studies on MS effects in animal models of cerebral I/R injury and clinical studies on global cerebral ischemia associated with cardiac arrest and cardiac surgery which showed MS neuro-protective and cerebral anti-apoptotic properties (15,21,55).

Oxidative stress is able to damage cell components (e.g., proteins, DNA and lipids) and organelles (56) and has been shown to be related with various pathological conditions (57,58); increased oxidative stress leads to neuronal death by damaging brain cellular lipids, proteins and nucleic acids and induces apoptosis through the transcription of the pro-apoptotic BID and BAD factors (59). Yavuz *et al* demonstrated that a single dose of MS reduced brain oxidative stress following CO poisoning and that the intraperitoneal administration of MS at 100 mg/kg was sufficient to significantly decrease lipid peroxidation (60). The findings of this study (for a brief summary, see Fig. 5) are in agreement with that study as MS treatment reduced lipid peroxidation at all 3 doses (75, 150 and 300 mg/kg) and proved that lipid peroxidation co-exists with apoptotic induction in CO poisoning and that brain cell Akt pathway activation favorably modulates the Bax/Bcl2 apoptotic index downstream (60).

In conclusion, the current study demonstrated that MS administration decreased the deleterious effects of CO poisoning on the brain by decreasing neuronal necrosis and apoptosis and reducing oxidative stress, while increasing Akt expression in brain cells.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

GB and RR and MH conceived and designed, and supervised the study. JS, MS, KTa and SB collected and analyzed the data. HJ performed the histopathological analysis. KTs, AOD, AAM, MFW, AT and DAS interpreted the data and prepared the manuscript. All authors have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zabol University of Medical Sciences, Zabol, Iran (approval no. ZBMU.1.REC.1394.112). All the animals were treated in accordance with the guidelines for care and use of laboratory animals prepared by the Animal Research Ethics Committee of Zabol University of Medical Sciences and in conformity with EU Directive 2010/63/EU for animal experiments.

## Patient consent for publication

Not applicable.

## Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. All the remaining authors have no competing interests to disclose.

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