

G-CSF Administration Attenuates Brain Injury in Rats Following Carbon Monoxide Poisoning via Different Mechanisms

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ABSTRACT: Acute severe carbon monoxide (CO) poisoning induces hypoxia that leads to cardiovascular and nervous systems disturbances. Different complex mechanisms lead to CO neurotoxicity including lipid peroxidation, inflammatory and immune-mediated reactions, myelin degeneration and finally neuronal apoptosis and necrosis. Granulocyte colony-stimulating factor (G-CSF) is considered to be a novel neuroprotective agent. In this study, we evaluated the efficacy of G-CSF therapy on CO neurotoxicity in rats with acute CO poisoning. Rats were exposed to 3000 ppm CO in air (0.3%) for 1 h, and then different doses (50, 100, and 150 $\mu\text{g}/\text{kg}$) of G-CSF or normal saline were administered intraperitoneally. Water content of brain as an indicator for total edema and blood brain barrier integrity (Evans blue extravasation) were evaluated. Malondialdehyde was determined in order to evaluate the effect of G-CSF on CO-

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induced lipid peroxidation in brain tissues. Also, the effect of G-CSF on myeloperoxidase activity in the brain tissue was evaluated. The effect of G-CSF administration on induced apoptosis in the brain was measured using TUNEL method. To evaluate the level of MBP, STAT3 and pSTAT3 and HO-1 proteins and the effect of G-CSF on these proteins Western blotting was carried out. G-CSF reduced water content of the edematous poisoned brains (100 µg/kg) and BBB permeability (100 and 150 µg/kg) ($P < 0.05$). G-CSF (150 µg/kg) reduced the MDA level in the brain tissues ($P < 0.05$ as compared to CO poisoned animals). G-CSF did not decrease the MPO activity after CO poisoning in any doses. G-CSF significantly reduced the number of apoptotic neurons and Caspase 3 protein levels in the brain. Western blotting results showed that G-CSF treatment enhanced expression of HO-1 and MBP, STAT3 and pSTAT3 proteins in the brain tissues. Based on our results, a single dose of G-CSF immediately after CO poisoning significantly attenuates CO neurotoxicity via different mechanisms. © 2015 Wiley Periodicals, Inc. *Environ Toxicol* 00: 000–000, 2015.

Keywords: carbon monoxide poisoning; granulocyte colony stimulating factor; myelin basic protein; neurotoxicity; rat; apoptosis; malondialdehyde; myeloperoxidase

INTRODUCTION

Carbon monoxide (CO) is a colorless and odorless gas with lower density than air. It is a highly toxic gas. High incidence of CO poisoning occurs mainly during the winter season. CO poisoning leads to high morbidity and mortality (Hampson, 2005; Gulati et al., 2009; Weaver, 2009). Organs sensitive to oxygen decline or depletion such as brain and heart are the main affected organs in CO poisoning (Prockop and Chichkova, 2007). Acute CO poisoning accompanied by severe cerebral hypoxia may cause reversible and short-term or delayed neurological deficits (Bateman, 2007). Hours to days after poisoning several mechanisms are activated that lead to neural tissue damage and delayed neurological sequels in the CNS (Brunssen et al., 2003). Increased oxidative stress following increased hemeoxygenase-1 (HO-1) protein and mitochondrial dysfunction due to CO binding to cytochrome C oxidase causes excessive production of reactive oxygen species (ROS) and direct cellular injury (Weaver, 2009). Events such as enhanced adhesion of neutrophils to the vascular lining and neutrophil aggregation and consequent release of myeloperoxidase lead to activation of inflammatory process and production of more ROS (Thom et al., 2001; Weaver, 2009). Apoptosis, necrosis, white matter demyelination, lipid peroxidation, cytotoxic/vasogenic edema and inflammation are considered to be involved in CO neurotoxicity pathogenesis (Han et al., 2007; Guan et al., 2009; Wang et al., 2009).

Agents with ability to inhibit or modulate these processes could be considered as potential treatments for CO poisoning. Granulocyte colony-stimulating factor (G-CSF) is a 19.6 KD glycoprotein and hematopoietic growth factor which is considered as a novel neuroprotective agent (Schneider et al., 2005). In various animal models of brain damage like stroke (Schabitz et al., 2003), brain hemorrhage (Park et al., 2005), Alzheimer (Sanchez-Ramos et al., 2009), Parkinson (Cao et al., 2006), traumatic brain injury (Sheibani et al., 2004) and neonatal hypoxia/ischemia (Yata et al., 2007) G-CSF could reduce infarct size, neuronal apoptosis, edema and inflammation (Gibson et al., 2005). Anti-apoptotic effect of G-CSF is mediated through the activation

of the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway (Gibson et al., 2005; Solaroglu et al., 2006a; Solaroglu et al., 2006b; Yata et al., 2007). Also, G-CSF administration improves sensorimotor deficits recovery. G-CSF is able to mobilize hematopoietic stem cells to the injured region of the brain and provoke them to differentiate to neural cells (Solaroglu et al., 2007). Several clinical trials have showed G-CSF's safety and beneficial effects in stroke patients (Shyu et al., 2006). With regard to these pieces of evidence, in this project the effect of G-CSF administration on CO neurotoxicity in rats and its possible mechanisms were evaluated.

MATERIALS AND METHODS

Chemicals

CO cylinder was obtained from Arad Gas Company (0.3% CO/air), Iran. Recombinant human G-CSF was purchased from Pooyesh Darou, Iran.

In Situ Cell Death Detection Kit, POD was from Roche Co. (Cat. N: 11684817910) STAT3, pSTAT3 (Tyr 705) and Caspas 3 antibodies were purchased from Cell Signaling. Glucose Transporter GLUT3, Myelin Basic Protein (MBP), beta Actin and Rabbit IgG secondary antibodies were provided by Abcam. HO-1 (H-105) antibody was from Santa Cruz. All other chemicals had analytical grade from commercial companies.

Animals

Male Wistar rats weighing 200–250 g (purchased from Bu Ali Research Center, Mashhad, Iran) were kept under the same laboratory conditions with free access to food and water, temperature 25°C and 12-h light/dark cycles throughout the study. All experimental protocols were approved by the Animal Care Committee of Mashhad University of Medical Sciences.

CO Intoxication and G-CSF Treatment Protocol

CO exposure was performed in a 12-L airtight chamber. The flow rate of CO/air mixture and CO concentration were controlled by a CO analyzer (Carbon Monoxide Analyzer model 707, TPI Co, South Korea). Rats were placed in the cage and exposed to 3000 ppm CO for 60 min to induce CO intoxication. All rats gradually lost consciousness during CO exposure in the cage. After CO exposure rats were immediately administered either normal saline or different doses of G-CSF (50, 100, or 150 $\mu\text{g}/\text{kg}$). The control group was exposed to room air in the same chamber with no treatment.

Mortality Rates in Different Groups of CO-Poisoned Rats

Twenty-four hours after CO intoxication, survival rates of CO-poisoned rats were recorded. In order to evaluate the effect of G-CSF on mortality rate, rats were pretreated 1 h before CO exposure with 50, 100, or 150 $\mu\text{g}/\text{kg}$ G-CSF.

Brain Water Content (Cytotoxic Edema)

Twenty-four hours after CO exposure rats were decapitated. The entire brain was removed and weighed immediately and then placed in a laboratory oven (105°C) for slow evaporation for 24 h. After this time brains were weighed again. The water content (%) was calculated as follow:

$$\% \text{Water content} = (\text{wet weight} - \text{dry weight}) / (\text{wet weight}) \times 100\% \text{ (Guan et al., 2009).}$$

Vasogenic Edema

In order to assess the integrity of the blood-brain barrier (BBB) quantitatively, Evans blue (EB) extravasation technique was performed (Belayev et al., 1996). EB (2%, 2 mL/kg) was injected to rats via the tail vein immediately after CO poisoning. Rats were anaesthetized with sodium pentobarbital 24 h after EB injection. Transcardial perfusion with normal saline through the left ventricle was performed until a colorless perfusion fluid comes out from the vena cava. Brain samples were removed, weighed and placed in 50% TCA solution. Samples were homogenated and centrifuged at $12,000 \times g$ for 15 min, then the extracted dye was diluted with ethanol (1:3) and its fluorescence ($\lambda_{\text{excitation}} = 620 \text{ nm}$ and $\lambda_{\text{emission}} = 680 \text{ nm}$) was evaluated using a spectrofluorimeter (infinite M200; TECAN, Grödig, Austria). Based on a linear standard curve (external standards in the same solvent 100–4000 ng/mL) the total EB content was determined and expressed as EB per gram of wet tissue.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

This spectrophotometer method was performed to determine malondialdehyde (MDA) content as a standard and conventional product for analyzing lipid peroxidation (Draper and Hadley, 1990). 24 h after poisoning and treatment, brain samples were removed, weighed and stored at -80°C until assay. Briefly, brain tissues were homogenized in cold 1.15% potassium chloride to make a 10% homogenate. 0.5 mL of the 10% homogenate was mixed with 3 mL phosphoric acid 1% w/v and 1 mL TBA solution and boiled for 45 min at 95°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 vortexed. The absorbance of the supernatant was measured at 532 nm. The amount of MDA was expressed as nmol per gram of wet tissue.

Brain Myeloperoxidase (MPO) Activity Assay

Twenty-four hours after poisoning and treatments, the brains were removed and kept in -80 until the test day. Brain homogenates were prepared in lysis buffer containing 5 mM EDTA, 10 mM Tris, Glycerine 10%, 1 mM PMSF, and leupeptin. Analysis was performed using a rat myeloperoxidase/MPO ELISA kit (HK105, Hycult Biotech, Netherlands) according to the manufacturer's instructions.

Tunnel Test

Tunnel test can detect fragmented DNA in the nucleus during apoptosis. This method was performed using In Situ Cell Death Detection Kit, POD (Roche CO, USA). Rat brains were removed and fixed in formalin for 24–48 h one day after poisoning and treatments. Paraffin fixed coronal sections processed for deparaffinization and hydration. To block endogenous tissue peroxidase, samples were incubated in H_2O_2 methanol solution for 10 min. Samples were incubated with proteinase K for 30 min. Samples were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase enzyme for 60 min in a moisture condition. Following blocking with BSA for 20 min, samples were incubated with the converter and chromogen DAB. In the final step, hematoxylin staining was performed for counter staining. For each sample, 3 slides were prepared and 10 visual scopes of each slide were studied. The mean number of apoptotic cells as dark brown condensed cells in each field of view was reported ($\times 400$).

Western Blot Analysis

The relative levels of STAT3 and pSTAT3 (Tyr 705), MBP and HO-1, 24 h after poisoning and G-CSF administration (the best selected dose according the rest of the results), was evaluated by Western blot analysis. Brain tissues were homogenized in a lysis buffer containing 50 mM Tris (pH

7.4), 2 mM EDTA, 2 mM EGTA, 2 mM NaF, 1 mM Na_3VO_4 , 10 mM B-glycerophosphate, 10 mM 2-mercaptoethanol, sodium desoxycholate, and 1 μL protease and phosphatase inhibitor cocktail and 1 mM phenylmethanesulfonyl. Total protein concentrations were determined using Bradford protein assay kit (Thermo, Pierce Biotechnology, USA) using BSA as standard (Bradford, 1976). Proteins were separated on a 12% SDS-PAGE under reducing conditions at a constant voltage (120 v) for 1:45 h. The separated proteins were electro-transferred to the polyvinylidene difluoride (PVDF, Bio Rad) membrane. Membranes were incubated in 5% non-fat dry milk in TBST buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.5% Tween 20) for 1 h at room temperature to block non-specific binding sites. After blocking, the membranes were incubated overnight at 4°C with primary antibodies against STAT3 (1/1000) and pSTAT3 (Tyr 705) (1/1000), MBP (1/1000), HO-1(1/200) and β -actin (1/1000) or GluT3 (1/10,000). The blots were then incubated for 1 h at room temperature with a HRP conjugated secondary antibody (1:3000). Protein bands were visualized using the enhanced chemiluminescence method (ECL detection kit, Pierce Biotech, Inc.) and Alliance 4.7 Gel Doc (UVTEC, Cambridge). Band intensities were quantified using UV BAND image analysis software (UVTEC, Cambridge).

Statistical Analysis

Data were expressed as means \pm SEM and analyzed with SPSS software (Window version 13.0; SPSS Inc.). One-way analysis of Variance (ANOVA) was used to calculate differences between means. Results were considered statistically significant when P -value were 0.05 or less.

RESULTS

CO Poisoning Induction

All rats lost consciousness within 40 min after exposure to CO in the cage. We measured serum level of carboxyhemoglobin (COHb) immediately after CO exposure to confirm the CO poisoning. Results showed that the serum COHb level was approximately 60% in the CO-poisoned rats. These findings showed that CO poisoning has been successfully induced.

Rate of Mortality after Poisoning and the Effect of G-CSF on Mortality Rate

During induction of CO intoxication and up to 24 h after poisoning, the rate of mortality in the CO-poisoned group was 35% ($n = 20$). Pretreatment with 100 and 150 $\mu\text{g}/\text{kg}$ G-CSF reduced the mortality rate to 20% ($n = 10$). Pretreatment with 50 $\mu\text{g}/\text{kg}$ G-CSF did not affect mortality rate (35%, $n = 10$).

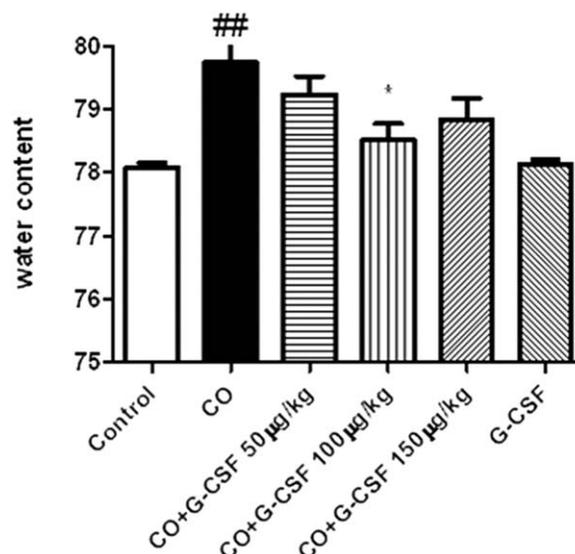


Fig. 1. Percentage of brain water content values reflects brain edema and are expressed as means \pm SEM. $n = 5$ for each group. ## $P < 0.01$ vs. control. * $P < 0.05$ vs. CO exposed rats.

The Effect of G-CSF on Cytotoxic and Vasogenic Edema

Determination of tissue water and Evans blue contents 24 h after CO poisoning showed that brain edema, both cytotoxic and vasogenic, has been occurred in CO poisoned rats. Water content was increased from 78.08% in the control rats to 79.75% in the CO-exposed group ($P < 0.01$) (Fig. 1). G-CSF administration (100 $\mu\text{g}/\text{kg}$) significantly reduced cytotoxic edema (78.52% vs. 79.75% in CO poisoned rats, $P < 0.05$) (Fig. 1).

G-CSF also attenuated vasogenic edema. Tissue Evans blue content was higher in the CO poisoned group as compared with the control ($P < 0.001$). G-CSF at doses of 100 and 150 $\mu\text{g}/\text{kg}$ significantly reduced Evans blue extravasation induced by CO poisoning ($P < 0.05$) (Fig. 2).

Anti-Oxidant Activity of G-CSF in CO Intoxication

Determination of malondialdehyde (MDA) levels, a marker of oxidative stress, revealed that CO poisoning raised MDA level (170.6 ± 3.5 as compared with 92.2 ± 7.9 in the control group, $P < 0.001$). Rats who received 100 and 150 $\mu\text{g}/\text{kg}$ G-CSF had significantly less MDA levels than CO poisoned rats ($P < 0.05$) (Fig. 3).

The Effect of G-CSF on MPO Activity in the Rat Brain

In order to assess whether CO poisoning and G-CSF administration have any effect on the infiltration of PMN leukocytes in the brain, MPO activity was measured in the rat brains after 24 h. The MPO activity significantly increased

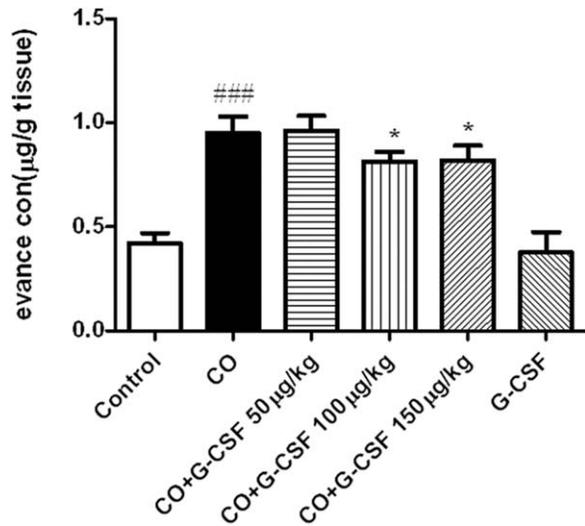


Fig. 2. The effect of G-CSF administration on BBB permeability in CO exposed rats. Results are expressed as means \pm SEM. $n=5$ for each group. $\#P < 0.001$ vs. control. $*P < 0.05$ vs. CO exposed rats.

from 1.02 ± 0.23 ng/mg tissue in the control group to 3.6 ± 0.24 ng/mg tissue in the CO poisoned rats. G-CSF could not prevent this increase at any dose (Fig. 4).

Anti-Apoptotic Effect of G-CSF in CO Poisoning

Tunnel staining showed there was a significant increase in the number of apoptotic cells in the CO-poisoned group compared to the control group ($P < 0.05$). G-CSF treatment (150 µg/kg) significantly reduced the number of apoptotic cells ($P < 0.05$) [Fig. 5(A,B)].

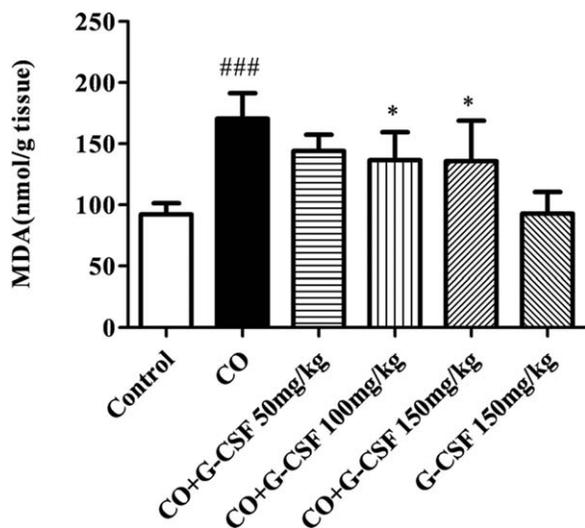


Fig. 3. MDA levels in the brain of CO poisoned rats and the effect of G-CSF administration. Results are expressed as means \pm SEM. $n=7$ for each group. $\#P < 0.001$ vs control. $*P < 0.05$ vs. CO exposed rats.

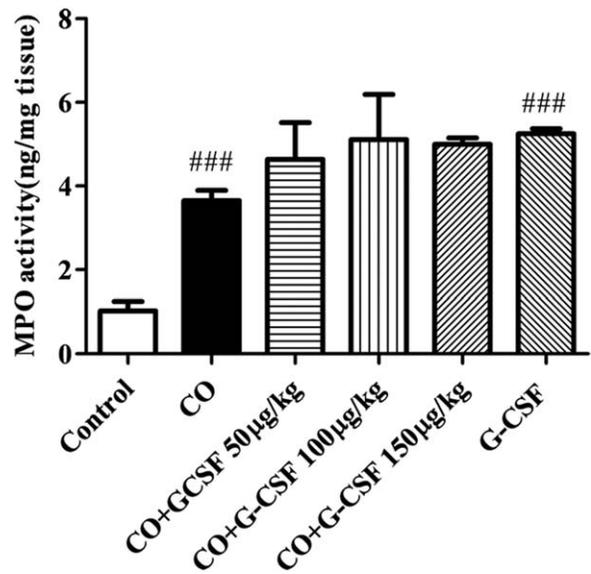


Fig. 4. Changes of MPO activity of the brain tissue after acute CO poisoning and the effect of G-CSF administration. Samples were collected at 24 h following CO exposure. Results are expressed as means \pm SEM. $n=6$ for each group. $\#P < 0.001$ vs. control.

Western Blot Analysis

The Effect of G-CSF on Protein Levels of STAT3 and pSTAT3 (Tyr 705)

The effect of G-CSF (150 µg/kg) on the expression of STAT3 and pSTAT3 was investigated using Western blot analysis. Immunoblots of STAT3 and pSTAT3 were detected at 79/86 kDa. There was significant difference in the intensities of both STAT3 and pSTAT3 between the poisoned and G-CSF groups at 24 h post-poisoning ($P < 0.05$) (Fig. 6).

The Effect of G-CSF on Levels of the HO-1 Protein

The expression of protein HO-1 after poisoning and the effect of G-CSF (150 µg/kg) administration were investigated using Western blot analysis. HO-1 bands were detected at 32 kDa. Relative expression of this protein was significantly higher in the CO-poisoned group as compared with the control ($P < 0.05$). G-CSF significantly enhanced this increase as compared with the CO-poisoned rats ($P < 0.05$) (Fig. 7).

The Effect of G-CSF on Levels of MBP Protein

Western blot analysis showed the expression of MBP protein which is one of the most important proteins in the brain involved in myelin synthesis. MBP expression was significantly decreased after CO intoxication ($P < 0.01$) as compared to the healthy rats. G-CSF administration (150 µg/kg) ameliorated the induced demyelination. The expression of

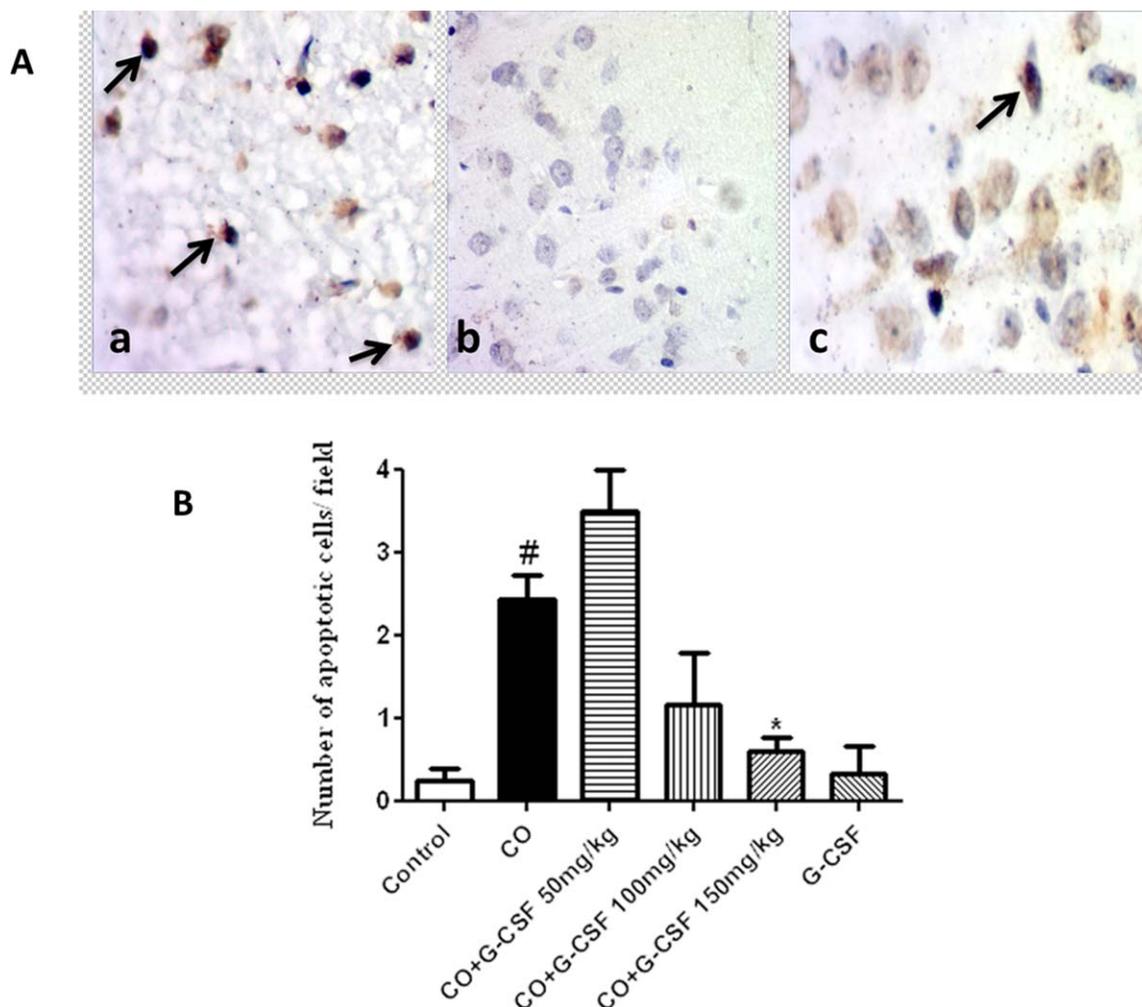


Fig. 5. A: Photomicrograph of TUNEL staining in the cortical region of midbrain in different experimental groups. There are some scattered TUNEL-positive cells in the cortical neurons of CO-poisoned rats (a), control group showing absence of TUNEL labeled cells in the cortical neurons (b) and rare positively labeled cells were detected in the cortical neurons of G-CSF treated group (150 µg/kg) (c), $\times 400$. B: Number of TUNEL-positive cells in the brain tissue of CO-poisoned rats and the effect of G-CSF administration. Results are expressed as means \pm SEM. $n = 3$ for every group. # $P < 0.05$ vs. control.* $P < 0.05$ vs. CO exposed rats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MBP was significantly higher in G-CSF-treated groups compared to the CO poisoned ones ($P < 0.001$) (Fig. 8).

The Effect of G-CSF on Cleaved Caspase 3 Levels

Relative expression of cleaved Caspase 3 was enhanced in the brain tissues of the poisoned rats. G-CSF (150 µg/kg) reduced this increase significantly as compared with poisoned group ($P < 0.05$) (Fig. 9).

DISCUSSION

Since the main mechanism of CO poisoning is tissue hypoxia, hypoxia-sensitive organs such as the heart and the brain are damaged more seriously than other organs. Clinical

and animal studies have shown serious CNS injuries following CO poisoning. According to recent animal studies on CO poisoning, rats are the preferred animal model. CO poisoning is induced under 3000 ppm CO for 60 min (Bunc et al., 2006; Guan et al., 2009). In a pilot study, we showed that COHb levels were reduced to 60% under these conditions. In another study, we have shown that examining brain tissue slides of CO-poisoned rats showed histopathological changes induced by CO poisoning (Ghorbani et al., 2013) indicating an efficient CO intoxication.

G-CSF is a hematopoietic factor with neuroprotective effects. It is currently under investigation for phase II clinical trial in the treatment of cerebrovascular accidents (Solaroglu et al., 2007). In a study performed by Schnittger in 2004, it was shown that acute CO poisoning is associated with distinct increase in blood neutrophil counts and G-CSF

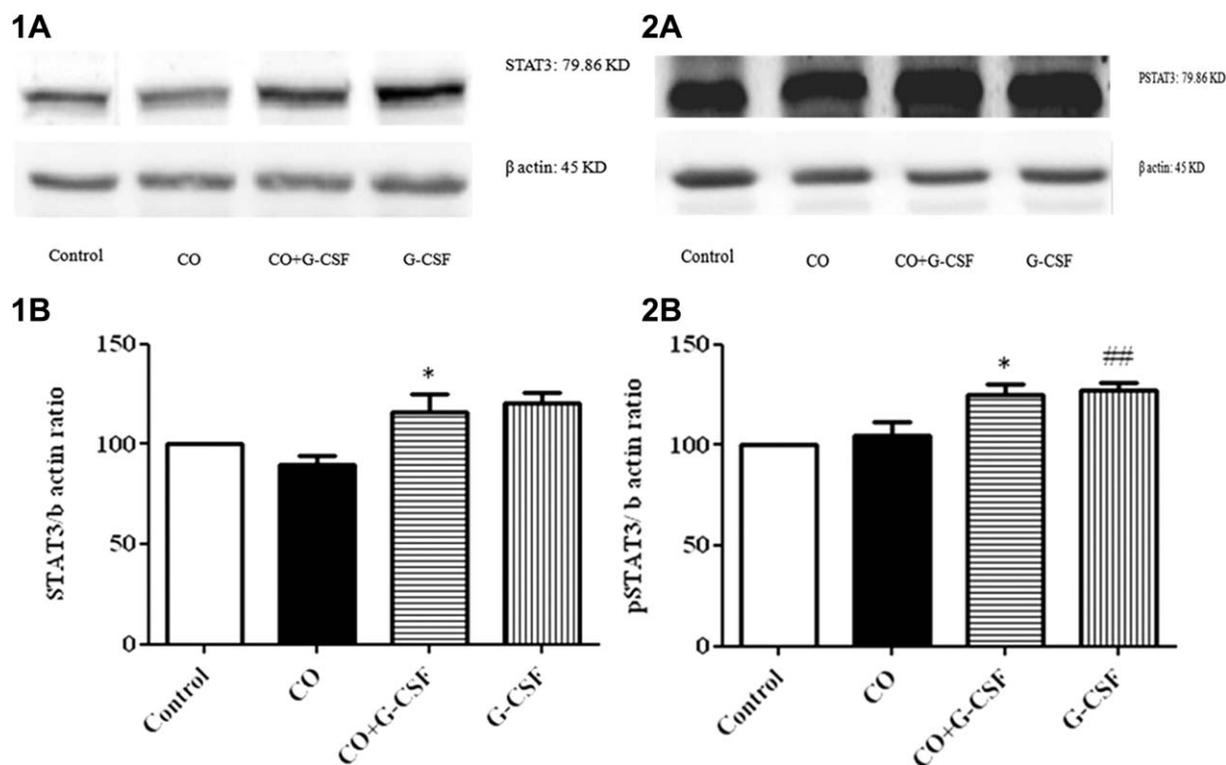


Fig. 6. Western blot analysis of STAT3 and pSTAT3 protein levels in the brain of rats 24 h after CO exposure. Bands intensities were normalized to β -actin in the same sample. G-CSF administration enhanced relative expression of these two proteins. Data are means \pm SEM; group sizes were $n = 6$ in each case; ### $P < 0.05$ vs. control.* $P < 0.05$ vs. CO exposure.

plasma concentrations. Treatment with hyperbaric oxygen (HBO₂), as an effective treatment for acute CO poisoning, caused more increase in G-CSF plasma level and also modulated neutrophil generation of H₂O₂ (Schnittger et al., 2004). These findings suggest that G-CSF may play a major role in HBO₂ effect.

Pathophysiological cascade mechanisms of CO poisoning starts with COHb formation and consequent hypoxemia. Brain hypoxia leads to oxidative stress and cell death by induction of necrosis and apoptosis (Weaver, 2009). Increasing levels of cytosolic heme and the HO-1 protein results in inflammation and intracellular oxidative stress (Guan et al., 2009). Other events are aggregation of platelet-to-neutrophil, neutrophil degranulation and subsequent release of MPO, proteases and ROS (Thom et al., 2006). Therefore, oxidative stress, lipid peroxidation and apoptosis are the main events in CO poisoning pathogenesis. Lipid peroxidation products such as MDA may form adduct with MBP and alter its structure (Thom et al., 2006). Stimulation of lymphocytic immunologic response activates microglia and induces neuropathologic effects (Thom et al., 2004; Weaver 2009). In this study, we attempted to investigate different stages mentioned above including expression of HO-1 and MBP, apoptosis, MPO activity, lipid peroxidation and edema.

Results showed that CO poisoning caused apoptosis, lipid peroxidation, brain edema and induced significant enhance-

ment in the MPO enzyme activity and expression of HO-1 and MBP proteins. G-CSF has been able to ameliorate these steps in the brain injury cascade activated following CO poisoning. Also, G-CSF administration reduced the rate of mortality, as an important end point outcome in CO poisoning. Other studies have also proved neuroprotective effect of G-CSF by reduction of mortality rate following stroke and other neurodegenerative disorders (Gibson et al., 2005; Yata et al., 2007; Pitzer et al., 2010).

Brain inflammatory response is one of the important stages involved in CO neurotoxicity. Concentrations of inflammatory factors such as TNF- α , IL-1 β , and IL-6 have been reported to be higher in the intoxicated brains (Sun et al., 2011). Cytotoxic edema, as a result of inflammation occurs after CO poisoning. CT scan findings of people with severe CO intoxication who have died immediately or with delay, confirm the occurrence of severe cerebral edema (Guan et al., 2009; Weaver, 2009). Astrocytes which are important cell type within the CNS and have a critical role in BBB function and integrity maintenance are affected and disrupted after CO intoxication. Vasogenic edema occurs as a result of BBB integrity impairment (Yardan et al., 2009). In this study, we have found a clear brain edema and BBB disruption 24 h after CO poisoning that was ameliorated by administration of 100 μ g/kg G-CSF. G-CSF has anti-inflammatory properties and its ability to modulate

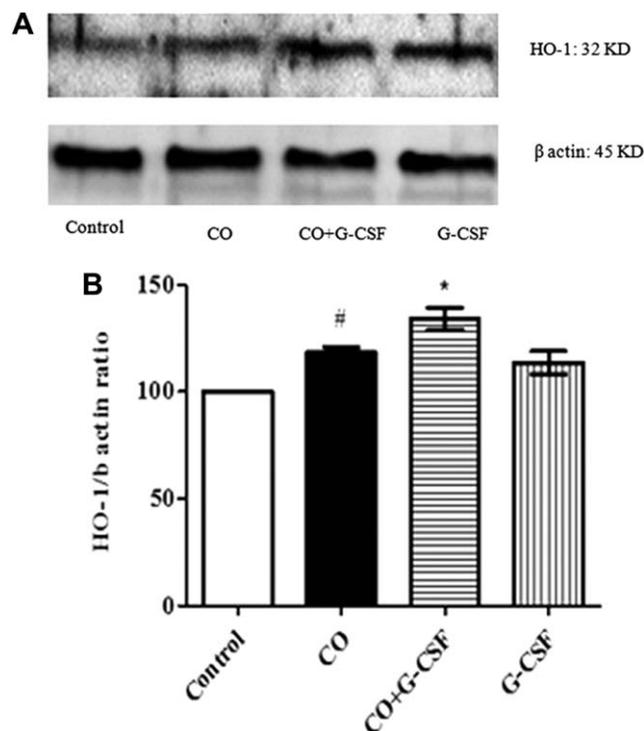


Fig. 7. Western blot analysis of HO-1 protein levels in the brain of rats 24 h after CO exposure. Bands intensities were normalized against β -actin in the same sample. CO exposure increased HO-1 levels; G-CSF produced a further significant increase in relative expression of HO-1. Data are means \pm SEM; group sizes were $n = 6$ in each case; # $P < 0.05$ vs. control. * $P < 0.05$ vs. CO exposure.

inflammatory responses has been well studied. G-CSF exhibits its anti-inflammatory effect by inhibition of T-cells migration and infiltration to injured brain and consequent reduction of TNF- α and INF- γ . As a result in different experimental brain injury models, G-CSF has suppressed brain edema efficiently (Zavala et al., 2002; Gibson et al., 2005). Lee et al. (2005) showed that after an induction of transient focal ischemia in rats, G-CSF has improved brain function and suppressed the inflammatory infiltration and the BBB disruption (Lee et al., 2005). Zang (2009) in a model of intracerebral hemorrhage in rats showed that G-CSF administration had a proliferative effect on astrocytes as it was able to stimulate astrocytes and increase glial fibrillary acidic protein in the brain tissues (Zhang et al., 2009). It is possible to explain the BBB improvement after G-CSF administration by its beneficial effects on astrocytes.

The inducible HO-1 acts as an important cellular defense mechanism against oxidative stress, apoptosis, inflammation and cellular injury. It was shown that HO-1 was increased after acute CO poisoning. HO-1 plays an essential protective role in CO neurotoxicity due to its anti-apoptotic and anti-inflammatory effects (Guanet al., 2009). In our study, the induction of HO-1 was observed after CO exposure. G-CSF

was capable of enhancing this expression. CNS lipid peroxidation and apoptosis, two major events following exposure to CO can upregulate HO-1 protein expression in the brain. Proper induction of HO-1 expression could exert cytoprotective effects against various injuries like oxidative stress and apoptosis. Different studies has been performed and shown G-CSF ability to induce HO-1 as a protective agent. Wei et al. (2011) demonstrated that G-CSF exerted its beneficial effects by inducing the HO-1 protein while administration of an inhibitor of HO-1 reversed G-CSF's protective effects in a mouse model of renal injury (Wei et al., 2011). Stimulation or inhibition of JAK2/STAT3 pathway, the main signaling pathway of G-CSF leads to an increase or a reduction in HO-1 expression (Shih et al., 2010). This might explain the G-CSF anti-inflammatory and antioxidant properties.

Lipid peroxidation in the CNS is an important process activated by CO exposure. The main product of lipid peroxidation, MDA, makes adduct with MBP, a key protein in the brain and affects its structure and function. Demyelination and lipid peroxidation are observed after CO exposure (Thom et al., 2004; Guan et al., 2009). The level of MBP expression in the brain tissue can indicate the degree of demyelination in the white matter. We have shown in another study that by staining brain tissue slides with loxol fast blue, CO poisoning can cause myelin fiber disarrangement and demyelinated sequelae (Ghorbani et al., 2013).

G-CSF administration reduced MDA brain content and lipid peroxidation as well as facilitated myelin regeneration. Anti-oxidant effect of G-CSF has been demonstrated in some studies including spinal cord injury and brain ischemia (Sanli et al., 2010). Also, G-CSF has shown a therapeutic role in ischemic vascular diseases via protecting endothelial cells against oxidative stress (Kojima et al., 2011). The G-CSF ability in CNS remyelination has been confirmed in other studies. In a model of experimental autoimmune encephalomyelitis, Zavala showed that treatment with G-CSF displayed restricted demyelination, reduced T cells recruitment in the CNS, and very distinct autoimmune inflammation (Zavala et al., 2002). In addition, this effect has been established in a spinal cord injury (Pitzer et al., 2010).

MPO activity is augmented in the brain after CO exposure. This is due to neutrophils degradation following perivascular effects of exogenous CO (Thom et al., 2006). Blood MPO elevation has been demonstrated in human and animal models of CO poisoning. Immunohistochemical studies confirmed co-localization of MPO and nitrotyrosin in the brain microvasculature (Thom et al., 2006). There are controversial reports regarding the effect of G-CSF on MPO activity or concentration. It has been shown that MPO activity was reduced in an animal model of spinal cord injury but not in a model of transient brain ischemia. However, G-CSF administration had no significant effect on MPO activity (Chu-fung, 2007). In a recent study, administration of G-CSF to preterm sheep with hypoxic-ischemic brain injury

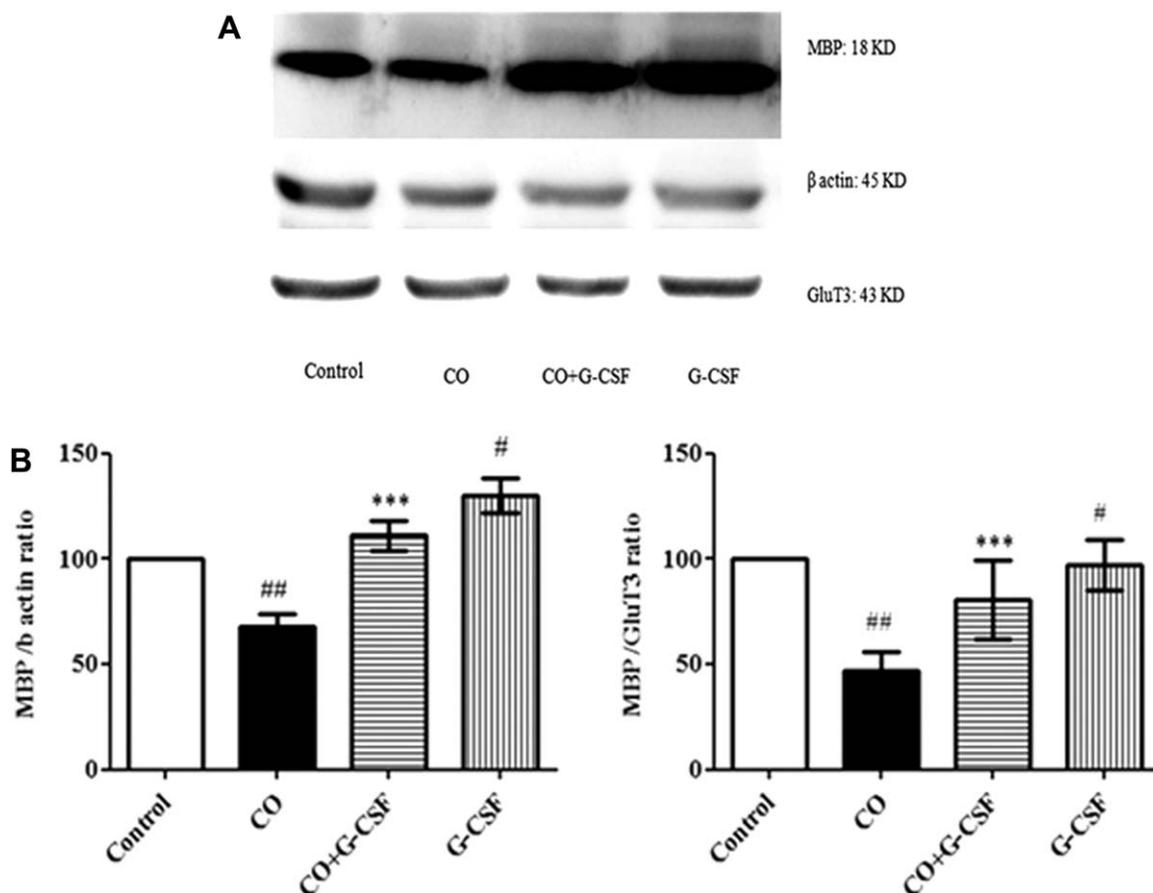


Fig. 8. Western blot analysis of MBP protein levels in the brain of rats 24 h after CO exposure. Bands intensities were normalized against β -actin or GluT3 in the same sample. CO exposure decreased MBP relative expression. G-CSF administration enhanced relative expression of MBP. Data are means \pm SEM; group sizes were $n = 6$ in each group; ## $P < 0.01$ and # $P < 0.05$ vs. control.* $P < 0.001$ vs. CO exposure.

did not influence enhanced invasion of neutrophils and MPO positive cell numbers (Jellema et al., 2013).

G-CSF specifically stimulates the neutrophilic lineage of granulocytes. Therefore, neutrophilia increases the turnover of neutrophils and subsequent MPO activity is expected. G-CSF may exert its anti-inflammatory effects by interaction with monocytes and macrophages (Solaroglu et al., 2006a).

G-CSF as a growth factor is expected to show anti-apoptotic effects. Exogenous CO can induce apoptosis in the nervous system following hypoxic injury (Piantadosi et al., 1997). Brvar (2010) reported that 31% of neuronal cells underwent apoptosis in the brain tissue when investigating the time-dependent protective effect of hyperbaric oxygen on CO neurotoxicity (Brvar et al., 2010). Following hypoxia-ischemia, excitatory amino acid release leads to intracellular Ca^{2+} accumulation and higher ROS production (Banasiak et al., 2000). Apoptosis would occur via mitochondria mediated or external pathways.

G-CSF ameliorated CO-induced brain injury and this may occur in part by inhibition of apoptotic cell death. We

assessed its anti-apoptotic effect. G-CSF decreased tunnel positive cells and relative Caspase 3 expression. Also G-CSF treatment associated with increased expression of STAT3 and pSTAT3. Binding of G-CSF to its receptors activates several intracellular signaling cascades including the Janus kinase, signal transducers and activators of transcription (STAT), phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Schabitz et al., 2003; Jung et al., 2006). Since protein levels of STAT3 and pSTAT3 are affected after G-CSF administration in CO poisoned rats, one may conclude that the JAK2/STAT3 pathway takes part in the anti-apoptotic effect of G-CSF.

In summary, this study has provided evidence that G-CSF treatment could prevent brain injury following CO exposure. Neuroprotection was associated with reductions in the levels of an oxidative stress marker, MDA, inflammation and the nerve cell apoptosis. Moreover, G-CSF stimulated HO-1 enzyme expression and myelin regeneration. BBB reconstruction and CNS remyelination may be due to the

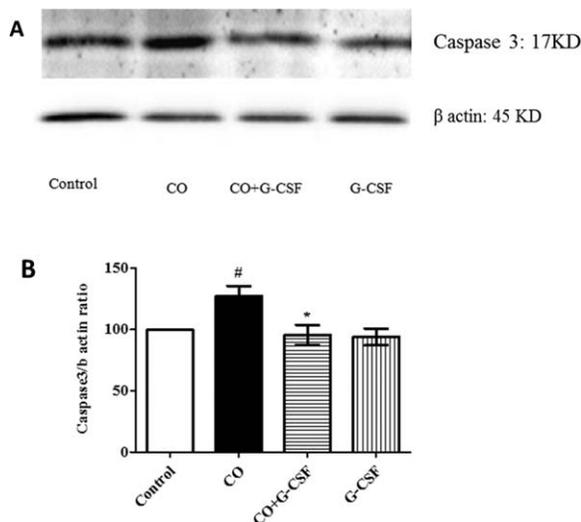


Fig. 9. Western blot analysis of Caspase 3 protein levels in the brain of rats 24 h after CO exposure. Bands intensities were normalized against β -actin in the same sample. CO exposure increased cleaved Caspase 3 relative expression. G-CSF administration decreased relative expression of cleaved Caspase 3 significantly. Data are means \pm SEM; group sizes were $n = 6$ in each group; $\#P < 0.05$ vs. control. $*P < 0.05$ vs. CO exposure.

proliferative effect of this anti-apoptotic agent on astrocytes. G-CSF' anti-oxidant and anti-inflammatory effects could be explained by its inducible effect on HO-1.

Further studies are required to exploit the G-CSF's potential effects in clinical CO poisonings.

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