A novel effective chemical hemin for the treatment of acute carbon monoxide poisoning in mice

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Abstract. There is no effective drug for the therapy of acute carbon monoxide (CO) poisoning. The purpose of the present study was to investigate the potential preventive and therapeutic effects of hemin on an animal model of acute CO poisoning and to provide a potential therapeutic candidate drug. A total of 80 Kunming mice were randomly divided into four groups, namely the air control, acute CO poisoning, hemin-treatment + CO and hemin-pretreatment + CO groups (n=20 each). Furthermore, the mortality rate of mice, blood carboxyhaemoglobin (HbCO) concentration and serum malondialdehyde (MDA) concentration were measured, and pathological changes of the hippocampal area were determined using histochemical staining. The mice with acute CO poisoning had a 50% mortality rate at 1 h, with an increase in blood HbCO, serum MDA levels and pathological impairments of the hippocampus. Furthermore, the mortality rate, blood HbCO and serum MDA levels of mice with pretreatment and treatment of hemin were decreased. Additionally, the pathological changes of the hippocampal area were improved in the hemin-treatment and hemin-pretreatment groups compared with the mice treated with CO. These results suggest that hemin is a novel effective chemical for the prevention and treatment of acute CO poisoning in mice. Therefore, the present study provides a novel method and experimental basis for the application of hemin in treating patients with acute CO poisoning.

Introduction

Carbon monoxide (CO) is one of the most common types of asphyxiant poisoning gas in industrial manufacture and daily life, and both the incidence and mortality rates (2.24/100,000 in Europe in 2005) (1) of acute CO poisoning are the highest among cases of acute gas poisoning (2). Clinically, hyperbaric oxygen chamber therapy is the suggested treatment for acute CO poisoning (3), as high pressure may accelerate the dissociation of carboxyhaemoglobin (HbCO) to increase the discharge of CO (4). However, the therapeutic mechanism and effect of hyperbaric oxygen chamber therapy for treating post-CO poisoning encephalopathy remain unproven (5,6), and the therapy is not always readily available. In addition, dexamethasone and hypertonic glucose dehydration therapy are often used to prevent or treat delayed encephalopathy (7); however these treatments act only as symptomatic therapies or are used to prevent CO poisoning complications. A recent hypothesis indicated that an antioxidant such as caffeic acid phenethyl ester and anti-nitric oxide therapy may be valuable for neuroprotection against CO poisoning (8). Thus far, the majority of research studies (9-11) have focused on the treatment for delayed encephalopathy caused by CO poisoning, whereas few (12) have reported the emergency treatment for CO poisoning.

Hemin is an artificially synthesized chloride of heme, with similar chemical characteristics (13). Furthermore, hemin may increase the oxygen carrying capacity and may compete with HbCO to bind CO to increase the body tolerance to hypoxia. In addition, hemin is an activator of neuroglobin (14). Neuroglobin participates in oxygen transport and storage in neurons, helps increase the intracellular partial pressure of oxygen in neurons, and is important in protecting neurons from hypoxic injury. A previous animal study indicated that hemin is able to induce heme oxygenase-1 activity to show neuroprotection (15). Although there is no current evidence in clinical trials, it was hypothesized that hemin may be able to prevent and treat acute CO poisoning. Due to the lack of effective drug therapies in clinically treating acute CO poisoning and the high mortality rate among CO-poisoned patients, the aim of the present study was to identify novel therapeutic options to reduce the mortality rate of patients with CO poisoning. To test this hypothesis, the potential protective effect of hemin...
on acute CO poisoning was examined in mice. In the present study, an animal model was generated by single intraperitoneal injection of CO to Kunming mice (16). Using preventive and therapeutic injection of hemin, its preventive function and therapeutic effect was studied on acute CO poisoning in the mice model; in particular, the present study attempted to establish whether hemin administration was able to reduce the mortality rate.

**Materials and methods**

**Animals.** A total of 280 Kunming mice (male:female ratio, 1:1), aged 5 weeks, weighing 18-22 g, were obtained from Guangdong Medical Laboratory Animal Centre (Foshan, China). All mice were raised in the Jinan University Medical Pharmacology Laboratory (Guangzhou, China) with controlled temperature (25°C), humidity (50-60%) and 12 h light:dark cycle. Animals were acclimatised with ad libitum access to standard laboratory food and tap water for one week and fasted for 24 h prior to all experiments. The experimental protocol of the present study was approved by the Jinan Medical University Animal Care Committee.

**Chemicals and reagents.** CO (purity >99.90%) was purchased from Guangzhou Wanqiqiti, Ltd. (Guangzhou, China), hemin was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and a commercial Malondialdehyde (MDA) Assay kit (TBA method) was purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Formaldehyde (3.5%) and sodium phosphate were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). All other chemicals were of the highest quality commercially available.

**Determination of CO model and optimal dose of hemin.** A total of 60 Kunming mice, weighing 18-22 g, were divided into six groups (n=10 each) used for acute CO exposure in order to determine the median lethal dose (LD50). A dose of 60 ml/kg was used as the initial exposure dose via intraperitoneal injection. Results from the initial exposure dose (60 ml/kg) were used to select the subsequent doses (90, 135, 202.5, 303.8 and 455.7 ml/kg), and by these up-and-down procedures (17), the mortality and 1-h mortality rate were recorded. The modified Spearman-Karber method (18) was used to calculate LD50 and to identify the lower limit of the calculated LD50 as the administration dose (150 ml/kg).

The optimal dose of hemin used in the present study was based on the results of preliminary experiments. In the preliminary experiment, 100 Kunming mice were divided into five groups (n=20 each), including the air control, CO-poisoning, low (10 mg/kg), moderate (20 mg/kg) and high dose (40 mg/kg) of hemin groups. CO was administered at the LD50 (150 ml/kg) via intraperitoneal injection. Mice exposed to CO were administered intraperitoneally with a dose of hemin (10, 20 or 40 mg/kg) when they started to show symptoms of CO toxicity, such as anxiety and hyperactivity. The air control group was administered intraperitoneally with a dose of air (150 ml/kg) and an equivalent volume of phosphate-buffered saline [PBS; 0.1 M sodium phosphate (pH 7.2), 0.9% saline]. The 1-h mortality rate of mice in the different groups was then recorded.

**Animal grouping and drug treatment.** A total of 80 Kunming mice (18-22 g) were randomly divided into four groups (n=20 each) as follows: i) Air control, mice were injected intraperitoneally with air using the same dosage as LD50 (150 ml/kg) and PBS solvent (40 mg/kg); ii) CO-poisoning group, CO poisoning was induced in mice using the dosage of LD50 by a single intraperitoneal injection (15) and treated with the same dose of PBS solvent; iii) hemin-treatment + CO-poisoning group, mice were injected intraperitoneally with hemin (40 mg/kg) 2 min after CO exposure, when mice began to exhibit symptoms of toxicity; and iv) hemin-pretreatment + CO-poisoning group, hemin (40 mg/kg) was administered to mice 15 min prior to CO exposure.

**Mortality and survival curve.** Physical and behavioral changes in the mice were recorded. These included the time when the skin or mucosa of mice turned to cherry red and the times when the mice exhibited hyperactivity, opisthotonus or fatigue. Mortality at 1 h was calculated as the number of dead mice at 1 h/number of mice in each group. Furthermore, the time of death was recorded and the survival curve plotted.

**Determination of blood HbCO concentration.** HbCO was determined by double-wavelength spectrophotometry (19), which was measured as the resistance of HbCO to reduction by sodium dithionite (Na2S2O4) compared with reduced oxyhaemoglobin. HbCO has peak absorbance at a wavelength of 535 nm, whereas that of oxyhaemoglobin is at 578 nm. Therefore, this quotient was used to determine the HbCO concentration according to the experimental equation:

\[
\text{HbCO}(\%) = \left( \frac{A535}{A578} \right) \times 100\% - 2.68
\]

A535 is absorbance in \( \lambda \) at 535 nm; A578 is absorbance in \( \lambda \) at 578 nm. A total of 10 mice were randomly selected in each group and from each mouse 0.1 ml blood was harvested from the tail 30 min after CO exposure. Blood was collected in a dry Eppendorf tube that was rinsed with heparin in advance. Subsequently, 0.1 ml blood was mixed thoroughly within 10 min due to the instability of the reducing reagent. HbCO concentration could be calculated by the experimental equation.

**Determination of serum MDA concentration.** MDA, which is a parameter of oxidative stress, was measured by the thiorbarbituric acid (TBA) method (20). MDA reacts with TBA to form red-coloured MDA-reactive products with a peak absorbance at a wavelength of 535 nm. Therefore, spectrophotometry was used to determine the serum MDA concentration.

In total, 10 mice in each group were randomly selected, and from each mouse, 0.1 ml blood was collected from the caudal veins at 5 and 30 min after CO exposure. Each blood sample was thoroughly mixed with 0.5 ml normal saline (0.9%) and 0.5 ml TBA (0.5%), and this mixture was heated at 95°C for 40 min. Following cooling to room temperature, the sample
Establishment of an animal model of acute CO poisoning.
Initially, the LD50 of acute CO poisoning was measured using the modified Spearman-Karber method. Six different CO dosages were evaluated according to geometric progression, with a group interval of 1.5 (60.0, 90.0, 135.0, 202.5, 303.8 and 455.7 ml/kg), and administered via a single intraperitoneal injection. The 1-h mortality rate of mice was presented in Table I and the calculated LD50 was 150 ml/kg.

| Dose (ml/kg) | Mice (n) | Mortality (n) | 1-h mortality rate (%) |
|-------------|----------|--------------|------------------------|
| 60.0        | 10       | 0            | 0                      |
| 90.0        | 10       | 1            | 10                     |
| 135.0       | 10       | 4            | 40                     |
| 202.5       | 10       | 6            | 60                     |
| 303.8       | 10       | 9            | 90                     |
| 455.7       | 10       | 10           | 100                    |

LD50 value calculated using the modified Spearman-Karber method was 166 ml/kg (95% confidence intervals: 150 to 169 ml/kg). In the following experiment, the lower limit of LD50 was set as the poisoning dose for animal models, which was 150 ml/kg, intraperitoneally. LD50, median lethal dose.

Determination of the optimal dose of hemin. It was revealed that the mortality rate following administration of a high dose of hemin (40 mg/kg) was markedly lower than that of the low (10 mg/kg) and moderate (20 mg/kg) dosage groups (Table II). Therefore, in the following experiments, 40 mg/kg was used as the optimum therapeutic dose of hemin.

| Group                        | Mice (n) | Mortality (n) | 1-h mortality rate (%) |
|------------------------------|----------|--------------|------------------------|
| Control                      | 20       | 0            | 0                      |
| CO poisoning                 | 20       | 10           | 50.0*                  |
| Low dose of hemin (10 mg/kg) | 20       | 8            | 40.0*                  |
| Moderate dose of hemin (20 mg/kg) | 20       | 7            | 35.0*                  |
| High dose of hemin (40 mg/kg) | 20       | 4            | 20.0*                  |

High dose (40 mg/kg) of intraperitoneal hemin was selected as the optimal therapeutic dose. *P<0.01 vs. control group, bP<0.05, cP<0.01 vs. CO poisoning group. CO, carbon monoxide.

Results

Hemin reduced the mortality rate of CO-poisoned mice. The mortality rate of mice at 1 h was 50.0% in the acute CO-poisoning group; 20.0% in the hemin-treated and 5.0% in the hemin-pretreated groups. Furthermore, the χ^2 test indicated that there was a significant difference in the mortality rate of mice at 1 h between the CO-intoxication and hemin-treated groups (P<0.05), and between the CO-intoxication and hemin-pretreated groups (P<0.01) (Table III).

Hemin alleviated the symptoms of acute CO poisoning. In the control group, no abnormal reaction manifestation was observed. In the CO-poisoning group, following intraperitoneal injection of CO, symptoms of poisoning were evident. At 2 min, the skin and mucosa of mice began to turn cherry red. At 5-7 min, mice became anxious and hyperactive and after 15 min mice exhibited fatigue and weakness of the limbs, and the skin and mucosa turned cherry red as well. In addition, they showed generalized convulsion, and 4 mice exhibited...
opisthotonus. After 30 min, CO-poisoned mice became less active and did not react to tactile stimuli. In the hemin-treated CO-poisoned group, at 2 min after CO injection, mice became anxious and the skin and mucosa started to turn cherry red. However, following injection of hemin, the time when the symptoms of poisoning appeared was postponed. In total, 10 min after intoxication, mice became anxious again and after 45 min the mice became quiet and stayed still together, while 2 mice appeared to be opisthotonus. In the hemin-pretreated CO-poisoned group, at 3 min after CO injection, the mice started to become hyperactive and the skin and mucosa started to turn cherry red. At 14 min, mice exhibited anxiety, while none of the mice exhibited opisthotonus. At 50 min, the mice appeared quiet again.

**Hemin prolonged the survival time of CO-poisoned mice.** The mean mortality time in the CO-intoxication group was 29.4 min (minimum, 16 min; maximum, 42 min), whereas it was increased to a mean of 43.3 min in the hemin-treatment group (minimum, 30 min; maximum, 50 min) and only 1 mouse died at 51.0 min in the hemin-pretreatment group. The mean mortality time of the control group was >1 h; therefore, the survival rates of control groups was 100% (Fig. 1). Furthermore, there was a statistically significant difference between the death time of both the hemin-treatment group and hemin-pretreatment group and that of the CO-poisoning group (P<0.05 and P<0.01, respectively).

**Hemin decreased the level of HbCO of CO-poisoned mice.** The HbCO level of mice 30 min following administration of CO is presented in Fig. 2. The HbCO level of the CO-poisoning group was significantly higher than that of the hemin-treatment group (P<0.01). Additionally, there was a statistically significant difference between the HbCO level of the hemin-pretreatment and that of the CO-poisoned groups (P<0.01).

**Hemin reduced the serum MDA concentration in CO-poisoned mice.** The serum MDA concentration in each group subjected to CO poisoning increased following exposure, and there was no statistically significant difference between the MDA concentration of each group 5 min after intoxication. At 30 min following CO intoxication, a statistically significant difference was observed between the serum MDA concentration of the CO-poisoning group and both the hemin treatment and hemin pretreatment groups (both P<0.01). Furthermore, there was no statistically significant difference between the MDA concentration of the control group at 5 and 30 min (Fig. 3).

**Pathological changes in the hippocampus of mice.** HE staining (Fig. 4) indicated that there was swelling of cells at...
the CA1 region of the hippocampus and dentate gyrus, and that the number of pyramidal cells decreased. Some neurons underwent necrosis and exhibited pyramidal cells that were triangular or polygonal in shape, reduced in size and undergoing karyopyknosis with unclear nucleoli (Fig. 4Ab). In the control group, there were no marked changes to the neurons in any region of the hippocampus in either side of the brain. Furthermore, there was no swelling or necrosis, which indicated that the cells were healthy (Fig. 4Aa). Compared with the CO-poisoning group, the swelling of neurons in the hippocampus of mice in the hemin-treated and hemin-pretreated groups was less evident, the number of cells undergoing necrosis decreased and the cell arrangements for both groups were more orderly (Fig. 4Ac and d). The morphology of cells exhibited unclear cell boundaries with several necrotic cells (Fig. 4Ba). In the control group, there were no evident pathological changes in the nerve cells, and they exhibited aligned nuclei (Fig. 4Ba). Compared with the CO-poisoning group, the hemin-treated and hemin-pretreated groups exhibited clearer boundaries with fewer necrotic cells (Fig. 4Bc and d).

Nissl staining (Fig. 5) indicated that, in the CO-poisoning group, the neurons of the hippocampus CA1 region were swollen and the number of Nissl bodies decreased. Some neurons underwent necrosis with unclear nucleoli and irregular boundaries (Fig. 5Ab). In the control group, there were no marked changes to the neurons in any region of the hippocampus in either side of the brain (Fig. 5Aa). Compared with the CO-poisoning group, the swelling of neurons in the hippocampus of mice in the hemin-treated and hemin-pretreated groups was less evident; there were fewer necrotic cells and the cell arrangements were more orderly (Fig. 5Ac and d). In addition, the morphology of cells was irregular with unclear boundaries accompanied by nuclei undergoing karyopyknosis (Fig. 5Bb). In the control group, there were no evident pathological changes in the nerve cells, which indicated that no swelling or necrosis had occurred (Fig. 5Ba). Compared with the CO-poisoning group, the cell arrangements in the hemin-treated and hemin-pretreated groups were more regular, the number of Nissl bodies markedly decreased and fewer necrotic cells were observed (Fig. 5Bc and d).

Discussion

In clinical settings, the initial management of patients with CO poisoning consists of removing the patient from exposure to the toxic atmosphere and supplying pure oxygen to accelerate the elimination of CO and improve tissue oxygenation (24). The exposure to acute CO poisoning is primarily by inhalation. Therefore, CO poisoning animal models were previously established by inhalation, which may be classified as either dynamic or static exposure (25). Dynamic exposure, which is similar to routine human inhalation, may negate the interference of other non-toxic factors such as asphyxia. However, due to the high cost of requirements and higher demand of hermeticity, the extensive use of this approach is not convenient. Conversely, static exposure is simple, but it is difficult to prevent hypoxia induced by CO₂ and other interferential factors. Therefore, the results may not be accurate.

Previous research has demonstrated that the establishment of an acute CO poisoning model by intraperitoneal injection is a convenient and effective animal model (15). Compared with inhalation exposure, intraperitoneal injection of CO results in a more precise CO exposure dose which is more suitable for scientific research. It has been demonstrated that if the level of HbCO in the blood is >10%, toxic reactions can be observed; at levels of ≥30%, moderate poisoning can be observed and at 50% severe poisoning (26). Furthermore, a single injection may result in HbCO levels rapidly reaching >60%, and maintaining stability at 50% within 6 h. Additionally, a single intraperitoneal injection of CO exhibits the same poisoning mechanism and symptoms as those in the clinical settings (27), even though its exposure route is different from clinical acute CO poisoning. In the present study, a modified Spearman-Karber method was used to determine a single intraperitoneal injection lethal dose that was the lower limit of the median lethal dose (LD50) (28). In the present study, following a single intraperitoneal injection of CO, significant CO poisoning manifestations were observed in mice. Furthermore, the binding ability of haemoglobin to oxygen was significantly reduced resulting in tissue hypoxia and lipid peroxidation, and blood MDA levels increased rapidly and pathological changes in the hippocampal cells were observed. In conclusion, the mouse model of the present
The present study demonstrated that both pretreatment and treatment with hemin to mice with CO poisoning was able to significantly reduce their mortality rate. Furthermore, it is able to reduce toxic injury in mice, which was demonstrated by the prolonged onset of symptoms in mice and a significant decrease in the blood HbCO level. In CO poisoning, hypoxia has a proportional association with the HbCO level (30), and therefore, the present results indicated that the degree of poisoning in the pretreatment and treatment groups has been reduced compared with that in the CO-poisoning group. According to these results, the protective mechanism of hemin against CO poisoning may be due to a reduction in the blood HbCO level, which prevents oxygen transfer. Furthermore, it may be because hemin replaces HbCO into oxyhaemoglobin to expel CO and thus, oxygen may be transported to the tissues by oxyhaemoglobin.

Hemin may also combine with free CO in the bloodstream and thus relieve tissue injury. In addition, a previous animal study provided evidence that hemin is able to induce hemoglobinase-1 (HO-1) activity leading to neuroprotection against acute CO poisoning (15). It is believed that HO-1 exhibits protective effects against exogenous CO toxicity (31) through degradation of heme into biliverdin and free iron that show potential biological effects (32,33). By combining the above evidence with the results of the present study, it is suggested that it is another possible mechanism that involves the production of HO-1 induced by hemin (34) following acute CO exposure. However, further studies could be performed in this field to investigate the underlying molecular mechanisms.

The present study also demonstrated that MDA, the blood oxidative stress indicator, decreased significantly in both the hemin-pretreated and hemin-treated groups compared with in the CO-poisoning model group. It is believed that oxidative stress is essential in CO-induced neuronal damage (35). Furthermore, MDA is a common product of lipid peroxidation, which is able to reflect the systemic lipid peroxidation level and therefore indirectly reflects the degree of free radical attack and cell injury extent (36). Therefore, it is possible to measure oxygen radicals and lipid peroxidation level in the brain tissue via serum MDA content. The observations of the present study were consistent with the hypothesis that reactive oxygen species (ROS) may be associated with the acute toxic effects of CO on the central nervous system (8). Additionally, a sudden burst of ROS during ischemic-reperfusion injury in CO toxicity leads to cellular lipid peroxidation, and thus MDA may increase in acute CO poisoning. The decrease in MDA indicated that the protective effect of hemin for acute CO poisoning injury may be associated with the inhibition of lipid peroxidation, reducing ROS in the brain. Furthermore, in clinical settings, oxygen therapy following CO-induced tissue hypoxia may be followed by ischemic-reperfusion injury in the CNS (37), leading to increased production of ROS such as nitric oxide (38). Therefore, it may be hypothesized that hemin may also be an alternative therapy to prevent secondary injury and thus protect the brain.

Histopathological examinations demonstrated that both hemin pretreatment and the hemin treatment in early stages could not only reduce hippocampus oedema and necrosis, but also the number of abnormal cells and neuronal damage. Since the hippocampal area is important in memory, learning and emotional activities, it is highly sensitive to hypoxia, asphyxia and ischemia because of its high metabolic rate (25). Furthermore, white and gray matter in the brain are sensitive to asphyxia and ischemia because of its high metabolic rate (25). Therefore, it may be hypothesized that hemin may also be an alternative therapy to prevent secondary injury and thus protect the brain.

In conclusion, the present study found that hemin has protective effects of decreasing mortality and relieving hippocampus injury.
oedema in mice with acute CO poisoning. Furthermore, the potential protective mechanisms may include a decrease in the level of HbCO, inhibition of lipid peroxidation and reduction of oxygen free radicals in brain cells. Meanwhile, the present study could provide reliable evidence of animal experiments for the treatment of acute CO poisoning. Finally, applications of hemin for acute CO poisoning may provide a reliable basis for future clinical treatment to gain rescue time and further decrease the mortality rate.

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