



Simvastatin attenuates delayed encephalopathy induced by carbon monoxide poisoning in rats by regulating oxidative stress, inflammation and NF- κ B pathway

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Abstract

Background Carbon monoxide (CO) poisoning is a leading cause of morbidity and mortality worldwide. The delayed encephalopathy occurs a period after poisoning in patients without effective treatment. Simvastatin (Sim), a lipid-lowering drug, was reported to exert endothelial protective effects and inhibit inflammatory response. This research focused on the effects of Sim on delayed encephalopathy caused by CO poisoning.

Objective The acute CO poisoning model was established by exposing rats to 2500 ppm CO gas for 40 min, then 3000 ppm for 30 min or until they lost consciousness. Rats in the treatment group were given Sim (20 mg/kg/day, ig.). The behavioral tests included the Morris water maze test and shuttle box. The pathological changes were evaluated by H and E staining. The inflammatory mediators were analyzed by ELISA. The expression levels of eNOS, iNOS and the NF- κ B-related proteins were analyzed by Western blot.

Results The results showed that Sim could alleviate CO-induced behavioral disorders and the hippocampal nerve cells apoptosis. Sim administration reversed the effects of CO on oxidative stress-related molecules. Sim could also inhibit the production of the inflammatory mediators induced by CO. The level of eNOS was decreased after CO exposure, while iNOS was increased. Sim could significantly inhibit the effects of CO. Furthermore, Sim inhibited the phosphorylation of I κ B α (an NF- κ B inhibitory protein), i.e., the activation of NF- κ B, which indicated that Simvastatin reduced the inflammatory response induced by CO poisoning partially through inhibiting the activation of NF- κ B signaling pathway.

Conclusion To sum up, our research indicated that Sim could attenuate the delayed encephalopathy induced by CO poisoning via regulating oxidative stress, inflammation and NF- κ B pathway.

Keywords Simvastatin · Delayed encephalopathy · CO poisoning · Oxidative stress · Inflammation

Introduction

Carbon monoxide (CO) poisoning, a leading cause of morbidity and mortality worldwide, is mainly caused by tissue hypoxia and direct carbon monoxide-mediated damage. Symptoms of acute CO poisoning include headache, nausea and vomiting, dizziness, neuropsychological

impairment, and even confusion, loss of consciousness, or deaths (Weaver 2009). Brain is a major target of CO toxicity and CO can induce different patterns of brain injury in acute and delayed stages (Lo 2007). Delayed neurologic sequela (DNS), a neurological disorder characterized by dementia, psychiatric symptoms, and extrapyramidal symptoms, occurs after a period of “spurious recovery” after poisoning in 3–30% of patients (Thom 1995). However, so far, there are no effective treatment measures for the treatment of DNS after CO poisoning. It is reported that acute brain injury of patients is largely caused by hypoxia. Brain hypoxia causes oxidative stress, necrosis, and apoptosis, which contribute to further inflammation and injury. Researchers found that DNS is also linked to immunological response (Thom et al. 2004), and anti-inflammation therapy plays a crucial role in CO poisoning. Li et al. reported that erythropoietin (EPO)

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could protect against CO poisoning-induced brain damage by inhibiting the TLR4-NF- κ B inflammatory signaling pathway (Pang 2016).

Simvastatin, an HMG-CoA reductase inhibitor, is a lipid-lowering drug by decreasing cholesterol synthesis and increasing low-density lipoprotein (LDL) catabolism (Todd and Goa 1990). In addition to lipid-lowering effects, Simvastatin has been reported to exert endothelial protective effects and inhibit inflammatory response (Musial 2001). Besides, Simvastatin also plays a role in protecting the cardio-cerebrovascular system and promoting nerve regeneration (Amin-Hanjani 2001). For example, Balduini et al. (Balduini 2003) found that Simvastatin prophylactic administration could protect neonatal rats with hypoxic-ischemic brain injury against long-lasting cognitive and morphological consequences by reducing pro-inflammatory mediator's production. Endres (1998) reported that Simvastatin could increase cerebral blood flow, reduce cerebral infarction range and attenuate cerebral injury of cerebral infarct rats by enhancing eNOS activity, while, in eNOS-deficient mice, Simvastatin failed to increase cerebral blood flow and reduce cerebral infarction, which indicated that eNOS is a potent target of Simvastatin effect on cerebral injury. Another research indicated that Simvastatin can down-regulate the expression of iNOS in ischemic brain tissues and inhibit the inflammatory response secondary to acute ischemia, thereby attenuating the brain damages (Hess et al. 2000). These experiments showed the positive effects of Simvastatin on neuroprotection, but there were no reports on the protective effects on CO poisoning. This study focused on the effects of Simvastatin on delayed encephalopathy caused by CO poisoning.

Materials and methods

Animals and treatment

Male Sprague–Dawley (SD) rats (220 ± 20 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Animals were housed for 1 week with free access to food and water. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (2012) and approved by the Animal Ethics Committee of the First Hospital of Jilin University (Approval no. 2019–033). All rats were randomly assigned to four groups ($n = 10$ for each group): sham group (NC group), Simvastatin control group (Sim), CO poisoning group (CO) and Simvastatin treatment group (Sim + CO). The CO poisoning model was established according to the previous report (Thom et al. 2004). Briefly, the rats were exposed to 2500 ppm CO gas for 40 min, then 3000 ppm for 30 min or until they lost consciousness. Then the rats were removed to breathe room

air and regained consciousness. The rats in sham group (non-CO-treated) were permitted to breathe fresh air for the same periods of time. Rats in Sim group were given Simvastatin by intragastrical administration at the dosage of 20 mg/kg/day. Treatment was started after rats regained consciousness and persisted for 14 days.

Behavioral tests

The cognitive function of rats was evaluated by a Morris water maze test in a circular pool according to a previously report (Wang 2019). Briefly, a transparent platform was merged 1.5 cm below the water. Each trial was performed twice a day for four consecutive days. The escape latency, swimming route and swimming time were recorded.

Learning and memory abilities of rats were tested by shuttle box with passive avoidance. Briefly, in the beginning, rats were permitted to move across the shuttle room for 5 min, and behavior trainings twice per day were performed. The parameters were set following previous reports (Wang 2019). The active avoidance response (AAR) time was calculated by the EthoVision XT9 Software Analysis System.

Analysis of oxidative stress-related molecules

The oxidative stress of hippocampal homogenates was characterized by the level of superoxide dismutase (SOD) activity, glutathione (GSH) and methane dicarboxylic aldehyde (MDA) contents, and measured by the enzymatic method according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China).

Western blot

The total protein was extracted from the brain tissue with RIPA lysis buffer and quantified by BCA method. Equal amount of total protein was loaded and separated in SDS-PAGE and transferred onto the PVDF membrane. The PVDF membranes were blocked with 5% skimmed milk at room temperature for 2 h, and then incubated with primary antibodies (eNOS: #32,027; iNOS: #131,205; Phospho-I κ B α : #5209; I κ B α : #4812; purchased from CST, at a dilution of 1: 1000) at 4 °C overnight, and then incubated with HRP-labelled secondary antibodies (HRP-linked anti-rabbit IgG: #7074; purchased from CST, at a dilution of 1: 10,000) at 37 °C for 1 h. The Bio-Rad imaging system was used to analyze the optical density (OD) of each protein. The relative OD values of the target protein were normalized to β -actin.

ELISA

The levels of inflammatory mediators were measured by commercial ELISA kits according to the manufacturer's instructions (R&D Systems).

H and E staining

H and E staining was performed according to standard protocols and was evaluated by two independent pathologists. Briefly, the brain tissue was fixed in 4% paraformaldehyde overnight. After dehydration by gradient alcohol, transparented by xylene, the tissue is embedded in paraffin. Paraffin-embedded brain tissues were sliced into 4- μ m-thick sections and then mounted onto slides, and finally stained with hematoxylin and eosin solution. H and E results were evaluated by two independent pathologists with professional opinion under a light microscope in a double-blind manner.

Luciferase reporter assay

An NF- κ B luciferase reporter was constructed as per the previous report (Zhao 2017). The cells were co-transfected with NF- κ B luciferase reporter and Renilla luciferase-expressing plasmid. The levels of luciferase activity were determined using a luciferase assay system.

Statistical analysis

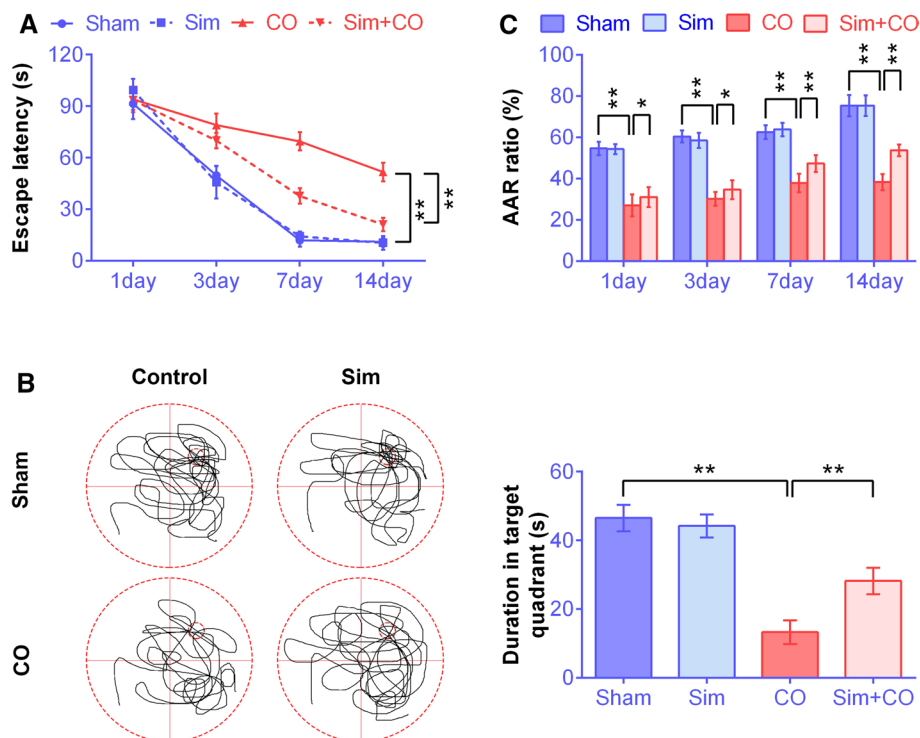
Statistical analysis was performed using SPSS software. All data were shown as mean \pm standard deviation (mean \pm SD). One-way ANOVA was used to determine the statistical difference. p value < 0.05 was considered significant.

Results

Simvastatin alleviated behavioral disorders induced by CO poisoning

The spatial or position learning and memory of rats were evaluated by the Morris water maze test. In the present results (Fig. 1a), the escape latency of rats in the sham and Sim group was rapidly decreased from 90 to 10 s during 7 days and kept at this level for another 7 days, and the escape latency was prolonged from 10 s to about 60 s at the 14th day after CO poisoning ($p < 0.01$ CO. vs. sham). After Sim treatment (Sim + CO), the escape latency was significantly shorter than that of the CO group on the 14th day ($p < 0.01$ vs CO). The duration in the target quadrant was also measured and the results showed that rats in the CO group showed a significant decrease compared with the sham group ($p < 0.01$), and Sim treated poisoning rats (Sim + CO) showed a significant increase compared with the CO group in duration in the target quadrant ($p < 0.01$) (Fig. 1b). A shuttle box test was performed to evaluate the

Fig. 1 Simvastatin alleviated behavioral disorders induced by CO poisoning. **(a)** The mean escape latency reached the hidden platform in a Morris water maze test. **(b)** The active avoidance response (AAR) time calculated by the EthoVision XT9 Software Analysis System. **(c)** Representative swimming tracks of rats. **(d)** The time spent in target quadrant (an indicator of spatial reference memory). * $p < 0.05$ and ** $p < 0.01$



classical conditioned reflex (Fig. 1c). The active avoidance response (AAR) ratio in the CO group was notably decreased compared with sham or Sim-alone group ($p < 0.01$) from 1st day to 14th day. The Sim treatment (Sim + CO) could obviously increase the AAR ratio after the 7th day. This result indicates that CO exposure can induce cognitive activity and simple memory damages in rats, and Sim can significantly improve the cognitive ability of rats with CO poisoning.

Simvastatin alleviates hippocampal nerve cell apoptosis induced by CO poisoning

H&E staining was used to observe the effect of Sim on the hippocampal tissue. As shown in Fig. 2, tissues from the sham or Sim alone treated rats showed normal-appearing neural structures and neurons in the cortex and hippocampus. In the CO group, the neurons in the hippocampal tissues were pyknotic, with enlargement of the intracellular space. Besides, CO-poisoned brain section shows neuronal swelling and nuclear vacuolization. Sim treatment diminishes the brain damage. Sim administration attenuated these histological changes of edema and neuronal necrosis in the hippocampus. The results indicate that Sim alleviates hippocampal neural structure injury and nerve cell apoptosis induced by CO poisoning.

Simvastatin inhibited oxidative injury induced by CO poisoning

To determine the oxidative stress, SOD activity, GSH and MDA contents in the hippocampus were measured. Compared with those in the normal rats, the MDA content (Fig. 3a) was obviously increased after CO exposure ($p < 0.01$), while Sim significantly decreased the production of MDA ($p < 0.01$ vs. CO). The level of SOD activity (Fig. 3b) and the content of GSH (Fig. 3c) were significantly decreased in the hippocampus of CO group ($p < 0.01$ vs. sham). Also, Sim administration induced the increase of SOD and GSH ($p < 0.01$ vs. CO). These results indicate that Sim inhibits oxidative injury induced by CO poisoning.

Simvastatin reduces the inflammatory response induced by CO poisoning

Further, the inflammatory mediators in the hippocampus were analyzed by ELISA (Fig. 4a). The levels of TNF α , IL-1 β and IL-6 were all increased in various degrees, especially IL-6 was increased about eight times compared with sham. Sim treatment inhibited the production of these inflammatory mediators ($p < 0.01$ vs. CO). The expression levels of eNOS and iNOS were analyzed by Western blot. The results (Fig. 4b) showed that the level of eNOS was decreased after CO exposure, while the expression of iNOS was increased. Sim could significantly inhibit the effects of CO ($p < 0.01$ vs. CO). The results show that Sim could inhibit the inflammatory response induced by CO poisoning.

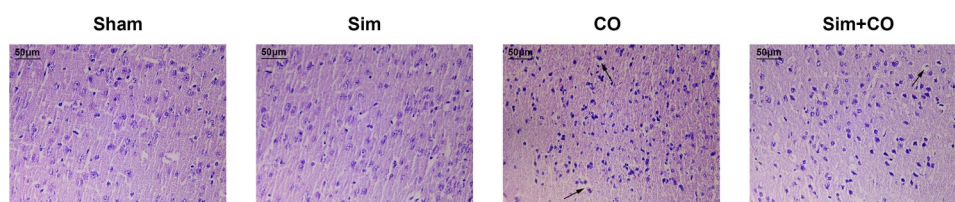


Fig. 2 Simvastatin alleviates hippocampal nerve cells apoptosis induced by CO poisoning. H and E staining of hippocampal tissue. The brain section of control and Sim alone treated rats shows the

normal neurons in cortex and hippocampus. CO-poisoned brain section shows neuronal swelling and nuclear vacuolization (arrows). Sim treatment diminishes the brain damage

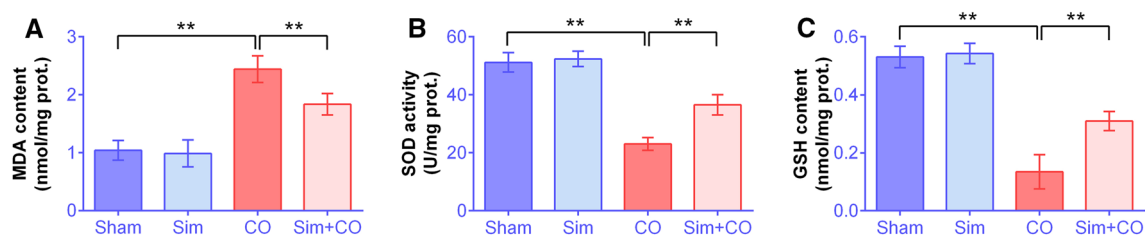


Fig. 3 Simvastatin inhibited oxidative injury induced by CO poisoning. The level of MDA content (a), SOD activity (b) and GSH content (c) measured by enzyme methods. ** $p < 0.01$

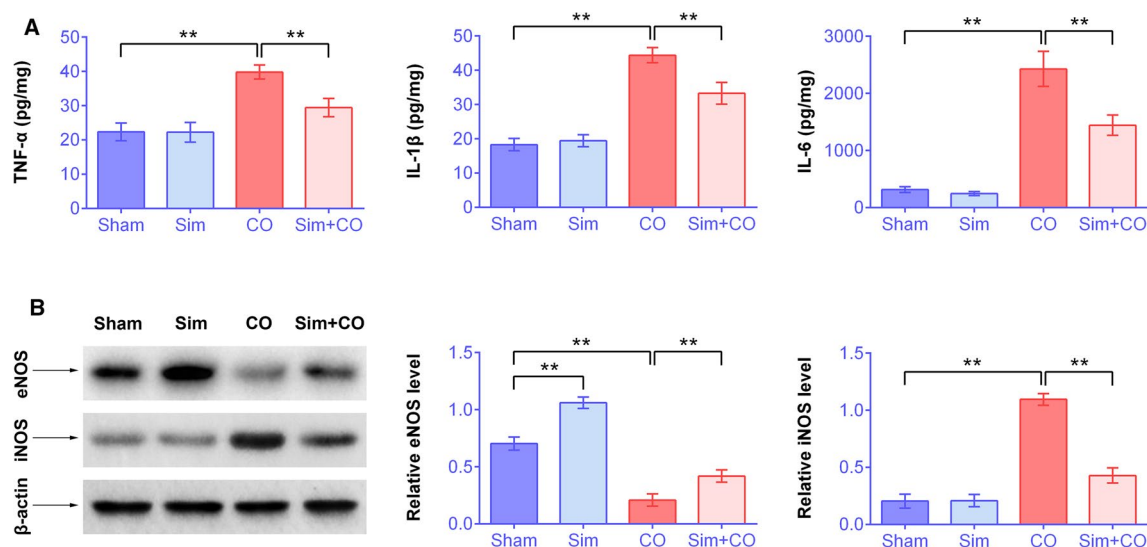


Fig. 4 Simvastatin reduces the inflammatory response induced by CO poisoning. **a** The level of TNF α , IL-1 β and IL-6 in brain tissue measured by ELISA. **b** The expression of eNOS and iNOS in brain tissue measured by Western blot. ** $p < 0.01$

Simvastatin inhibited CO-induced activation of NF- κ B signaling pathway

Since NF- κ B signaling pathway plays a key role in the inflammatory responses and oxidative stress, the activation of NF- κ B was detected by Western blot and luciferase reporter assay. The degradation of I κ B α protein (a NF- κ B inhibitory protein) and the phosphorylation were increased. Sim treatment inhibited the phosphorylation of I κ B α , i.e. the activation of NF- κ B (Fig. 5). The level of phosphorylated p65 (p-p65, NF- κ B subunit) was increased significantly in CO treated rats, which was inhibited by Sim ($p < 0.01$). In addition, the transcriptional activation of NF- κ B was analyzed by luciferase reporter assay. CO promoted the transcriptional activation of NF- κ B and Sim decreased the reporter activity, which was consistent with the above results. Therefore, the inhibitory effect of Sim on the inflammatory response is partially through the NF- κ B signaling pathway.

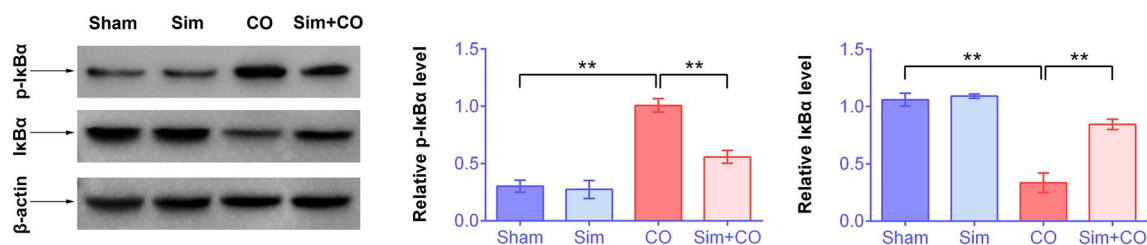


Fig. 5 Simvastatin inhibited CO induced activation of NF- κ B signaling pathway. **a** The expression of I κ B α and p-I κ B α in brain tissues measured by Western blot. **b** The expression of p65 and p-p65 in

Discussion

Carbon monoxide (CO) is one of the most common lethal poisons worldwide. CO poisoning is the leading cause of poisoning-related morbidity and mortality, among which the neurologic sequelae is the most frequent form of morbidity. Hypoxia and cellular theories are confirmed to be the mainly pathophysiologic mechanisms of CO toxicity. CO-induced oxygen delivery damage and mitochondrial oxidative phosphorylation lead to ischemic and anoxic brain injury or the delayed encephalopathy induced by brain lipid peroxidation. Brain injury is caused by or related to oxidative stress, nitrate stress, inflammation, apoptosis, excitotoxicity and so on. Statin, as a kind of HMG-CoA reductase inhibitor used as lipid-lowering drug, was reported to have protective effects on brain injury diseases by inhibiting oxidative stress or inflammation (Brown and Watson 2018). Barone et al. reported

brain tissues measured by Western blot. **c** The transcriptional activity of NF- κ B analyzed by luciferase reporter assay. ** $p < 0.01$

that long-term high-dose atorvastatin administration could inhibit brain oxidative and nitrosative stress in an Alzheimer disease (AD) model, thereby improving the cognitive ability of animals (Barone 2011). Another research demonstrated that low-dose simvastatin largely improved cerebrovascular function and AD symptoms by regulating inflammation and oxidative stress in aged amyloid precursor protein transgenic mice (AD model) (Tong 2009). Previous reports indicated that statins have protective effects on cerebrovascular function and cognitive ability, which was related to inflammation and oxidative stress. Thus, in the present research, we investigated the effects of Simvastatin on CO-induced delayed encephalopathy, and the results showed that Sim indeed alleviated behavioral disorders and the hippocampal nerve cells apoptosis induced by CO poisoning.

In the pathophysiology of CO poisoning, oxidative stress plays a key role. The oxidative stress induced by CO more easily damages organs which are sensitive to hypoxia such as brain, lung, and heart. Immediate and delayed neuronal injury appeared in selective regions of the brain after CO poisoning, which may not be readily explained by tissue hypoxia (Sharma et al. 2009). Another mechanism is related to the production of reactive oxygen species (ROS) in the brain, and the subsequent events of oxidative stress (Piantadosi et al. 1997). In clinical research, eighty-eight patients with acute CO poisoning and 35 healthy adults were enrolled to evaluate the relationship of oxidative stress parameters and intoxications for long-term outcomes in CO poisoning. Results showed that TOS (oxidative stress index) and carboxyhemoglobin (COHb) levels in COP patients were significantly increased compared to the healthy group, and after treatment, both of them were decreased (Kavakli 2010). In rats after acute CO poisoning, neuronal damage was found, and both necrosis and apoptosis contribute to CO poisoning-induced brain cell death (Piantadosi et al. 1997). In addition, oxidative damage was also found in lymphocyte membranes after acute CO poisoning, which was partially mediated by mitochondrial cyclooxygenase inhibition caused by CO (Miró 1999). Moreover, Miró reported that CO could alter the mitochondrial respiratory chain at the cytochrome c oxidase level (Alonso et al. 2003). Nitric oxide (NO) plays vital role in immunity, including NO-mediated oxidative damage in chronic inflammation (Kaur and Halliwell 1994). Excessive NO is produced in a variety of inflammatory diseases. Large amounts of NO, generated primarily by iNOS (inducible nitric oxide synthase, presented in various cell types upon inflammatory stimulation) can be toxic and pro-inflammatory (Guzik et al. 2003). These previous reports demonstrate that oxidative stress damages not only neurons but also other types of cells, such as immune cells. In our research, the oxidative stress was also increased in the CO poisoning rats, and Sim treatment could significantly

attenuate the oxidative stress, which was attributed to the protective effects of Sim on CO poisoning. Another important molecule, nitric oxide (NO), also plays an important role in hydroxyl radical generation induced by CO poisoning in rat. The increase of NO also induced damage in brain tissue (Hara 2007). Our research investigated two main enzymes in the NO system, i.e. endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). We found that in the CO-poisoned rats, eNOS was decreased while iNOS was increased, and Sim could inhibit the tendency of CO poisoning.

Inflammation is another factor that promotes delayed encephalopathy. NF- κ B, an important transcription factor regulating a variety of physiological and pathological processes including inflammation, is up-regulated in a variety of brain injuries (O'Neill and Kaltschmidt 1997). Researchers found that CO poisoning is also linked to inflammatory response, and anti-inflammation therapy plays a crucial role in CO poisoning. Li et al. reported that erythropoietin (EPO) could protect against CO poisoning-induced brain damage, including histological edema and neuronal necrosis, by inhibiting the TLR4–NF- κ B inflammatory signaling pathway (Pang 2016). In our present research, CO induced the activation of NF- κ B in brain tissue, and Sim could inhibit the activation of NF- κ B signaling pathway, as well as the subsequent production of some pro-inflammatory mediators. These results preliminarily elucidated the role of NF- κ B in CO-induced delayed encephalopathy and the protective mechanism of Sim. However, more details of the regulatory mechanism and the effects of Sim still need further research.

To sum up, our research indicated that Simvastatin could attenuate the delayed encephalopathy induced by carbon monoxide poisoning via regulating oxidative stress, inflammation and NF- κ B pathway. This study will provide a new idea for the treatment of CO poisoning.

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Author contributions DHX and LP conceived and designed the experiments; ZC and ZLL analyzed and interpreted the results of the experiments; WL performed the experiments

Compliance with ethical standards

Conflict of interest The authors state that there are no conflicts of interest to disclose.

Ethical approval All animal experiments were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the First hospital of Jilin University (Approval no.2019-033).

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