Effects of hyperbaric oxygen on Notch signaling pathway after severe carbon monoxide poisoning in mice

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Abstract

Demyelination of the cerebral white matter is the most common pathological change after carbon monoxide (CO) poisoning. Notch signaling, the mechanism underlying the differentiation of astrocytes and oligodendrocytes, is critical to remyelination of the white matter after brain lesion. The purpose of this work was to determine the effects of hyperbaric oxygen (HBO) on Notch signaling pathway after CO poisoning for the explanation of the protective effects of HBO on CO-poisoning-related cerebral white matter demyelination. The male C57 BL/6 mice with severe CO poisoning were treated by HBO. And HBO therapy shortened the escape latency and improved the body mass after CO poisoning. HBO therapy also significantly suppressed protein and mRNA levels of Notch1 and Hes5 after CO poisoning. Our findings suggested that HBO could suppress the activation of Notch signaling pathway after CO poisoning, which is the mechanism underlying the neuroprotection of HBO on demyelination after severe CO poisoning.

Key words: carbon monoxide poisoning; carboxyhemoglobin; demyelination; Hes5; hyperbaric oxygen; Notch signaling pathway; oligodendrocyte precursor cell; oligodendrocyte; remyelination; white matter

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Introduction

In China, the overall crude poisoning mortality was 5.9 per 100,000 people in 2016.[1] And the poisoning mortality by harmful gases and vapors was 1.2 and 0.7 per 100,000 people in males and females, respectively, among which carbon monoxide (CO) was the most common environmental poison.1 More than 16,000 CO-poisoned patients were treated in North America hyperbaric chambers from 1992 to 2002.[2] In the USA, CO poisoning occurs 50,000 times annually, resulting in 1000 to 2000 deaths.[3] CO poisoning can cause damage to the brain, heart, lung, liver and other organs.[4] Brain damage is also clinically known as a neurologic and neuropsychiatric sequela secondary to CO poisoning, the typical manifestations of which include: consciousness disorders, dementia, mental symptoms, increased muscle tension and paralysis agitans.[5-7] The above abnormalities may persist after acute CO poisoning, or may go through a period of apparent recovery before recrudesce.[8] It is currently believed that demyelination of the cerebral white matter is the most common pathological change.[9,10] Douglas and Haldane[11] first proposed the laws of combination of hemoglobin with CO and oxygen in 1912. Haldane[12] proposed that oxygen either at high concentration or pressure could be used as an antagonist to CO poisoning in 1917. Since then, the hypoxia mechanism which originated in the carboxyhemoglobin (COHb) theory has been widely accepted, and meanwhile the therapeutic administration of oxygen has been thought to be more reasonable. A variety types of oxygen therapy including hyperbaric oxygen (HBO),[13] normobaric oxygen[14,15] and high flow nasal cannula oxygen therapy,[16] are the most effective methods of treating CO poisoning. Their underlying mechanisms involve hastening COHb dissociation, restoring oxygen-carrying capacity of blood and alleviating cell hypoxia.[17,18] Our previous study found that HBO could improve CO poisoning related cerebral white matter demyelination and impairment of activities of daily living.[19]

Signaling pathways which could affect remyelination include Notch,[20,21] Wnt/β-catenin,[22,23] and bone morphological protein signaling pathways.[24,25] Among them, Notch signaling is one of the most widely studied pathways. In adult mammals, Notch signaling not only plays a pivotal role in the regulation of neural stem cells[26] but also in the differentiation of astrocytes and oligodendrocytes.[27] The mechanisms regulating the differentiation of oligodendroycte precursor cells into mature oligodendrocytes are critical to remyelination after brain injury.[28,29] Activation of Notch signaling could inhibit the differentiation of neural stem cells into neurons and oligodendrocytes, while promoting its differentiation into astrocytes.[30] It is also reported that the activation of Notch signaling is closely related to the pathological process of brain ischemia reperfusion injury.[31-33] Besides relief of hypoxia, whether HBO could promote brain remyelination after CO poisoning through Notch signaling, is not yet investigated. This study was intended to explore the effects of HBO on

Notch signaling in severe CO-poisoned mice.

Materials and Methods

Animals

All experimental protocols were approved on March 13, 2013 by the Experimentation Ethics Committee of the Sixth Medical Center, Chinese PLA General Hospital in Beijing, China (approval No. 201303). Healthy male C57 BL/6 clean mice (n = 25) weighing 20–25 g, 8 weeks old, were obtained from the Laboratory Animal Centre of PLA Academy of Military Medical Sciences in Beijing, China. All experiments were designed and reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.[34]

Experimental protocol

Preparation of CO poisoning model

As previously published reported,[35,36] severe CO poisoning was performed in a homemade 44-L plexiglas poison box. Mice inhaled 2000 ppm CO for 20 minutes. After that, the 2000 ppm CO gas was continuously injected from the inlet at a speed of 5 L/min for 20 minutes, and the outlet was opened at the same time. Then 4000 ppm CO was given for up to 20 minutes and 5000 ppm CO was given for 8–10 minutes. Mice were removed out of the poison box and breathed room air.

Verification of CO poisoning model

The experimental animals were randomly divided into six groups with three mice in each group. Mice in the control group were given no intervention. Immediately after CO modeling, the living mice were randomly divided into 0 hour after poisoning group, 0.5 hours after poisoning group, 1 hour after poisoning group, 2 hours after poisoning group and 3 hours after poisoning group. After being exposed to fresh air, the mice in each group were anesthetized with 10% chloral hydrate at the corresponding time point. About 0.3 mL of blood was taken from the open heart and the content of COHb was detected by blood gas analyzer (RAPIDPoint 500, Siemens, Erlangen, Germany).

Because there was a mortality rate of 20% to 30% in the process of model preparing and few mice still died after termination of CO exposure,[37] the mice were grouped after successful modeling. For backup, another 2 to 4 animals were reserved at the same time.

Experiment grouping and process

The experimental animals were randomly divided into three groups with five mice in each group. Sham group: the mice were placed in the poison box and fresh air was ventilated continuously for the same duration as the poison exposure time. CO group: the mice inhaled CO to prepare the poisoning model, and then breathed fresh air. CO + HBO group: HBO therapy was given to the mice after CO modeling.

HBO therapy: the mice were placed into the animal chamber, which was purged with pure oxygen for 10 minutes to ensure that the oxygen fraction in the chamber was > 95%. The pressure was then steadily increased to 2.5 ATA (1 ATA = 101,325 kPa) for the first session and maintained for 60 minutes. Next, the pressure was steadily decreased to normal pressure. The time of compression or decompression was 10 minutes separately. The first session of HBO therapy was given to the mice 3 hours after CO modeling. At the same time point of the first session, one session of HBO therapy daily was given at the following 2nd and 3rd days. And the total sessions of HBO therapy were three. The process of the second and third sessions was the same to the first time except the maintained pressure was 2.0 ATA.

The changes in body mass of mice in each group were monitored before the experiment and on the seventh day after CO exposure. The mice in each group were anesthetized on the 9th day after exposure, and the brain tissue was taken for relevant detections.

Morris water maze

A training of Morris water maze (Zhongshidichuang Sci Tech, Beijing, China) was performed on the 3rd to 5th days after CO exposure, and a testing was performed on the 6th day.

Navigation task: Each mouse was carried out four times a day and entered the water from four different directions each time. The escape latency was recorded. The average of the four incubation periods was recorded as the day's score, and the last day was taken as the final score.

Western blot

The brain tissue samples were homogenized and centrifuged at 12,000 × g for 10 minutes at 4°C. Supernatants were collected, and protein concentrations were determined with a bicinchoninic acid kit (Jianchen Biological Institute, Nanjing, China). The protein samples were separated using 10–15% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After being blocked with 5% non-fat dry milk in Tris-buffered saline for 2 hours, the membranes were incubated overnight at 4°C with primary antibodies for Notch1 (rabbit, 1:1000, Abcam, Cambridge, UK, Cat# ab52627, RRID: AB_881725), Hes5 (rabbit, 1:500, Abcam, Cat# ab25374, RRID: AB_448776) and tubulin (mouse, 1:10,000, Abcam, Cat# ab7291, RRID: AB_2241126), separately. After incubation, the membranes were washed with Tris-buffered saline with Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000, ZSGB-BIO, Beijing, China, Cat# ZB-5301) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000, ZSGB-BIO, Cat# ZB-2305) for 2 hours at room temperature. Antigen-antibody complexes were detected using an enhanced chemiluminescence plus chemiluminescence reagent kit, and the membrane was exposed to X-ray film for detection. Band densities were quantified using Quantity One software version 6.0 (Bio-Rad Laboratories, Hercules, CA, USA).

Reverse transcription-polymerase chain reaction detection

Total RNA was extracted from brain tissue, and 2 μL of RNA sample was taken for determination of concentration and purity. The extracted RNA was treated with DNase and complementary DNA was synthesized by reverse transcription kit. The primers of *Notch1* and *Hes5* used are shown in **Table 1**. Glyceraldehyde 3-phosphate dehydrogenase was used as internal reference. The reaction conditions were pre-denatured

Table 1: Polymerase chain reaction primer sequence

Gene of interest	GenBank number	Primer sequence (5′–3′)
GAPDH	NM_001289726.1	F: CCA TCA CCA TCT TCC AGG AGC GAG
		R: CAC AGT CTT CTG GGT GGC AGT GAT
Notch-1	NM_008714.3	F: CGG TGA ACA ATG TGG ATG CT
		R: ACT TTG GCA GTC TCA TAG CT
Hes-5	NM_010419.4	F: AAG TAC CGT GGC GGT GGA GAT GC
		R: CGC TGG AAG TGG TAA AGC AGC TT

Note: F: Forward; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; R: reverse.

3 minutes at 95°C, denatured at 94°C for 30 seconds, annealed at 60°C for 30 seconds, extended for 30 seconds at 72°C. After 35 cycles, it was extended at 72°C for 10 minutes. There were three secondary holes in each sample. The mRNA transcription level in the sham group was set as 1, and the ratio of mRNA transcription level to the sham group in CO and HBO groups was calculated.

Statistical analysis

All values were analyzed using SPSS software version 21.0 (IBM SPSS Inc., Chicago, IL, USA). The values of COHb were presented as the mean ± standard deviation (SD). Non-parametric test, namely Kruskal-Wallis *H* test, was adopted for the data if not satisfying the condition of homogeneity of variance. Mann-Whitney *U* test was used for pairwise comparison. Other data were expressed as the mean ± SD and one-way analysis of variance was carried out. The Student-Newman-Keuls was used for the equal variances assumed, and the Games-Howell was used for the equal variances not assumed when pairwise comparison. A *P*-value < 0.05 was considered to indicate statistical significance.

Results

The general condition of mice after CO poisoning

When inhaling 2000 ppm CO gas for 10–15 minutes, the mice gradually behaved from irritability to depression, presenting with reduced physical activity, shortness of breath, and hair erect. When inhaling 4000 ppm CO gas for 5–10 minutes, all mice lost consciousness with wheezing intensified. Cherry red could be seen obviously around the mouths, noses and toes, and some mice even showed myotonia or convulsions. When inhaling 5000 ppm CO, mice began to die around 8 minutes and all mice died at 14–15 minutes. Once the process of poisoning is stopped, the surviving mice regained consciousness after inhalation of fresh air for 45–50 minutes, while the physical activity and foraging behavior increased significantly in 1–2 hours, and the general condition of the mice returned to the pre-poisoning state in 2–3 hours.

COHb content in CO poisoning models

There were 25 mice in total. Three mice were selected for the control group according to the random number table, and the remaining twenty-two mice were selected for CO poisoning model. Five mice which died immediately after termination of poisoning were excluded, and the remaining seventeen mice were randomly divided into five different time groups (0, 0.5, 1, 2 or 3 hours after poisoning). There were three mice in each group, and the last remaining two mice were as backup. No more mice died after termination of CO exposure. The mortality rate for poisoning model was 23% (5/22).

There was a statistically significant change in COHb level among the different groups (Kruskal-Wallis H test, $\chi^2 = 16.279$, $P = 0.006$). Immediately after termination of CO poisoning, compared with pre-poisoning state, the COHb level in mice was suddenly increased to 75.97% ($P < 0.05$), and the COHb level was decreased rapidly after breathing fresh air, decreased by about half to 30.70% at 1 hour ($P < 0.05$). COHb level was 6.93% at 2 hours after poisoning which was not significantly different from the ratio of pre-poisoning mice ($P > 0.05$), and COHb decreased to 0.13% at 3 hours after poisoning ($P > 0.05$; **Figure 1**).

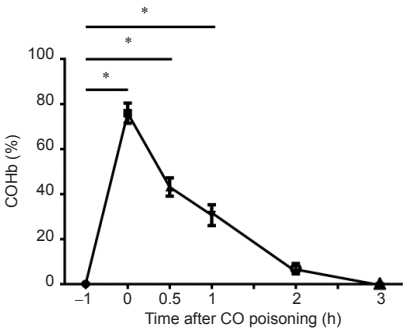

Figure 1: COHb content in CO poisoning models.
Note: Data are expressed as mean ± SD ($n = 3$). *$P < 0.05$ (Kruskal-Wallis *H* test). CO: Carbon monoxide; COHb: carboxyhemoglobin.

HBO shortens the escape latency after CO poisoning

There were 22 mice in total. Five mice were selected for the sham group according to the random number table, and the remaining seventeen mice were prepared for CO poisoning model. Four mice which died immediately after termination of poisoning were excluded, and the remaining thirteen mice were randomly divided into CO and CO + HBO groups. There were five mice in each group, and the last remaining three mice were used as backup. No more mice died after termination of CO exposure. The mortality rate for experiment grouping was 24% (4/17).The swimming path for each group is shown in **Figure 2A**. The average escape latency among each group was statistically significant ($P < 0.05$). Among them, the escape latency was significantly longer in CO group compared with sham group ($P < 0.05$), and the escape latency was significantly shorter in CO + HBO group compared with CO group ($P < 0.05$; **Figure 2B**).

HBO improves the body weight after CO poisoning

There was a significant difference in the increase of body mass among groups ($P < 0.05$). The increase of body mass in CO group was significantly lower than that in sham group ($P < 0.05$), while the increase in CO + HBO group was significantly higher than that in CO group ($P < 0.05$; **Figure 3**).

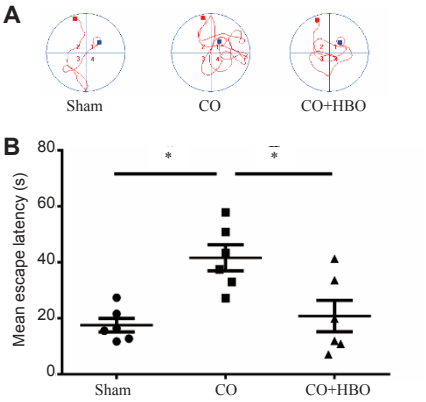

Figure 2: Effect of HBO on the spatial learning and memory ability changes after CO poisoning.
Note: (A) The swimming path during Morris water maze for each group. (B) The escape latency in CO group was significantly longer compared with that in sham group, and it was significantly shorter in CO + HBO group compared with that in CO group. Data are expressed as mean ± SD (n = 5). *P < 0.05 (one-way analysis of variance followed by Student-Newman-Keuls test). CO: Carbon monoxide; HBO: hyperbaric oxygen.

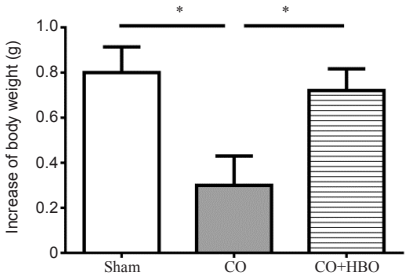

Figure 3: Effect of HBO on the change of body weight after CO poisoning.
Note: Data are expressed as mean ± SD (n = 5). *P < 0.05 (one-way analysis of variance followed by Student-Newman-Keuls test). CO: Carbon monoxide; HBO: hyperbaric oxygen.

HBO suppresses the protein expression of Notch1 and Hes5 after CO poisoning

The levels of the Notch1 and its downstream Hes5 protein in brain tissue among groups were significantly different (P < 0.05). Among them, CO poisoning led to a significant increase in the above protein levels compared with sham group (P < 0.05), and HBO could significantly downregulate the increase of both protein levels (P < 0.05; **Figure 4**).

HBO suppresses the mRNA expression of *Notch1* and *Hes5* after poisoning

The levels of the *Notch1* and *Hes5* mRNA in brain tissue were significantly different among groups (P < 0.05). CO poisoning led to a significant increase in the expression levels of the two genes compared with sham group (P < 0.05), while HBO could significantly downregulate the levels of both mRNA (P < 0.05; **Figure 5**).

Discussion

To date, the criteria of severity classifications after CO poisoning reached no overall consensus. The loss of consciousness, neurological deficits, or COHb > 25% may work as markers of serious cases.[38] Death due to CO poisoning is identified by values of COHb > 50% in postmortem blood.[39] Our previous

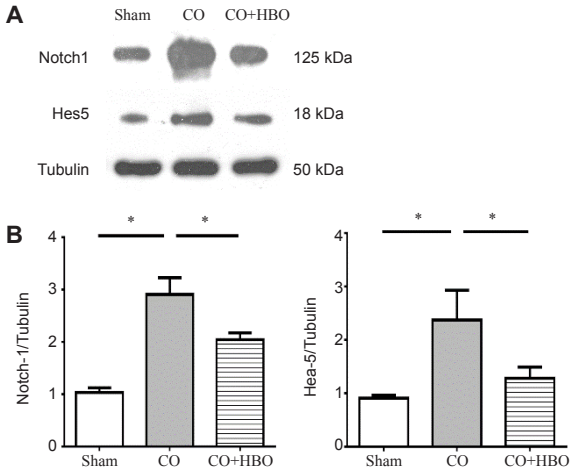

Figure 4: Effect of HBO on the Notch1 and Hes5 protein expression in brain tissue after CO poisoning.
Note: Data are expressed as mean ± SD (n = 5). *P < 0.05 (one-way analysis of variance followed by Student-Newman-Keuls test). CO: Carbon monoxide; HBO: hyperbaric oxygen.

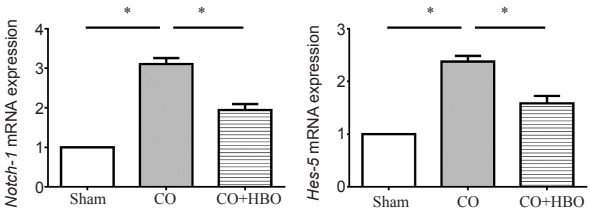

Figure 5: Effect of HBO on the *Notch1* and *Hes5* mRNA expression in brain tissue after CO poisoning.
Note: Data are expressed as mean ± SD (n = 5). *P < 0.05 (Mann-Whitney U test). CO: Carbon monoxide; HBO: hyperbaric oxygen.

data indicated that patients with neurologic sequelae were also those being more severe poisoned at acute stage.[40] Therefore, we wished to establish a severe CO poisoning model based on loss of consciousness, neurological deficits and high COHb at acute stage. Although some scholars have once successfully established poisoning models through abdominal injection of CO,[41,42] models established through inhalation of CO are more commonly applied now.[35,36,43] In our present study, severe CO poisoning model (which is closer to death and a better simulation of real poisoning) was established by a combination of dynamic and static inhalation method in mice. The results suggested that HBO could suppress the activation of Notch signaling pathway, which is the mechanism underlying the neuroprotection of HBO on demyelination after severe CO poisoning.

The success of animal model can be confirmed in the following four aspects. First of all, after poisoning all mice appeared to have a long-term coma in the acute stage, which was consistent with the clinical characteristics of severe CO poisoning. Second, the mortality rate of poisoning was stable at about 20% to 30% in both pre-experiments and formal experiments, indicating the severity of the poisoning model was well controlled. Second, the levels of COHb in all poisoned mice reached more than 70%, which was far higher than the clinical diagnosis standard of severe CO poisoning.[38] Third, spatial learning and memory abilities were decreased after CO

poisoning in the subacute stage, suggesting a brain injury may occur. Finally, the monitoring of body mass could reflect the change of the general condition after poisoning. After 7 days of observation, the mouse body mass in CO group increased by the smallest amount, indicating a poor general condition. This change may be related to the decline of mental state, intelligence level, and feeding ability, in line with the clinical characteristics of severe CO poisoning induced brain injury.

The Notch pathway is an evolutionarily conserved signaling network, which is fundamental in regulating developmental processes in invertebrates and vertebrates through short-range communication between cells.[44,45] Notch is activated by a unique process that includes ligand binding and multistep proteolytic processing.[46] In mammals, there are four Notch receptors (Notch 1–4) and five kinds of Notch ligands (Delta-like 1, 3, 4, Jag-1 and Jag-2).[47,48] Canonical Notch signaling is initiated by γ-secretase-mediated cleavage of the Notch receptor, leading to the release of the active intra-cellular domain of Notch that associates with a DNA binding protein, resulting in the activation of downstream targets *Hes1*/*Hes5* genes.[49,50]

Our results showed both the Notch1 receptor and its downstream target gene *Hes5* in mouse brain tissue increased significantly on the 9th day after CO poisoning. The possible explanation could be that the Notch signaling was activated by CO poisoning, which inhibited the differentiation of neural stem cells into oligodendrocytes, thus affecting myelin regeneration. The effect was achieved through the target gene *Hes5*. The Notch effectors Hes1 and Hes5 function in at least two ways: first, to act as transcriptional repressors to directly inhibit myelin genes transcription,[51] and second, to form heterodimers with other pro-myelinating basic helix-loop-helix factors to sequester their activity.[52,53] Moreover, our findings indicate that HBO could probably promote remyelination by inhibiting the activation of the Notch signaling and its downstream target gene *Hes5*. In an experimental autoimmune encephalomyelitis model, a classical model to study demyelination, the activation of the Notch pathway has been shown to inhibit oligodendrocyte precursor cells differentiation, hamper their ability to produce myelin and eventually cause glial scarring.[54,55] These findings are consistent with results in our previous *in vitro* study[56]: both the oligodendroycte precursor cells and oligodendrocytes in the brain of rats were damaged to a certain extent following CO poisoning.[54]

Sex differences have been found previously in ischemic stroke mortality.[57] In our previous study,[40] we also found that sex differences may affect the severity of poisoning and prognosis after carbon monoxide poisoning and females have an advantage over their male spouses, particularly in premenopausal couples. To date, most of our understanding of CO poisoning originates in male rodents. One reason could be that the study results may be probably influenced by the menstrual cycle stage in female rodents. Very few studies are available for female rodents, which may indicate a future research direction.

In summary, Notch signaling pathway can be activated by CO poisoning. And HBO can treat CO poisoning by suppressing Notch signaling pathway. Here are the shortcomings of this study: First, Notch signaling is a dynamic process, and we only detected the value at the 9-day time point after CO exposure; and secondly, whether Notch signaling plays a leading role in the process of white matter demyelination after CO poisoning, which needs further investigation.

Author contributions
Study design and data analysis: HJH, DFF & ZHY; manuscript writing and figures preparation: HJH & QS; data collection and study guidance: QS. All authors read and approved the final manuscript.
Conflicts of interest
The authors have no conflicts of interests to declare.

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