

Hydrogen sulfide improves neural function in rats following cardiopulmonary resuscitation

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Abstract. The alleviation of brain injury is a key issue following cardiopulmonary resuscitation (CPR). Hydrogen sulfide (H₂S) is hypothesized to be involved in the pathophysiological process of ischemia-reperfusion injury, and exerts a protective effect on neurons. The aim of the present study was to investigate the effects of H₂S on neural functions following cardiac arrest (CA) in rats. A total of 60 rats were allocated at random into three groups. CA was induced to establish the model and CPR was performed after 6 min. Subsequently, sodium hydrosulfide (NaHS), hydroxylamine or saline was administered to the rats. Serum levels of H₂S, neuron-specific enolase (NSE) and S100 β were determined following CPR. In addition, neurological deficit scoring (NDS), the beam walking test (BWT), prehensile traction test and Morris water maze experiment were conducted. Neuronal apoptosis rates were detected in the hippocampal region following sacrifice. After CPR, as the H₂S levels increased or decreased, the serum NSE and S100 β concentrations decreased or increased, respectively (P<0.05). The NDS results of the NaHS group were improved compared with those of the hydroxylamine group at 24 h after CPR (P<0.05). In the Morris water maze experiment, BWT and prehensile traction test the animals in

the NaHS group performed best and rats in the hydroxylamine group performed worst. At day 7, the apoptotic index and the expression of caspase-3 were reduced in the hippocampal CA1 region, while the expression of Bcl-2 increased in the NaHS group; and results of the hydroxylamine group were in contrast. Therefore, the results of the present study indicate that H₂S is able to improve neural function in rats following CPR.

Introduction

Brain damage following cardiac arrest (CA) is typically the result of ischemic or hypoxic injury in vulnerable areas of the brain, such as the hippocampus, cortex and thalamus, and triggers a series of pathophysiological processes following CA/cardiopulmonary resuscitation (CPR). The necrosis and apoptosis of numerous nerve cells leads to various neuronal dysfunctions, including anterograde amnesia, learning difficulties, emotional and social behavioral changes, depression, and potentially coma, persistent vegetative state and mortality. Thus, brain damage has been considered to be a major sign of post-CA syndrome, which often increases the mortality rate in addition to the effects caused by the primary diseases that induce CA (1). Effective attenuation of brain injury is one of the key aims of CPR.

As the third gaseous signaling molecule *in vivo*, hydrogen sulfide (H₂S) has a relatively small molecular mass, which allows it to traverse the cell membrane freely without requiring a receptor. Previous studies have indicated that H₂S is involved in the pathophysiological process of ischemia-reperfusion injury and shock, and exerts protective effects on neurons (2-7). H₂S has been demonstrated to increase the expression of γ -aminobutyric acid B receptor subunits 1 and 2, and subsequently reduce excitotoxic injury (8,9). H₂S mediates signals between neuronal cells and astrocytes by increasing the influx of Ca²⁺ in order to maintain calcium homeostasis and regulate synaptic activity (10). Furthermore, H₂S is known to exert protective effects on neurons via its antioxidative functions (11). However, the effects of H₂S on brain injury after CA are not clear and the results of previous studies appear contradictory. For example, the administration of the H₂S donor sodium

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sulfide (Na_2S) 1 min prior to CPR has been demonstrated to significantly improve the survival rate and neurological function of rats at 24 h (12) or 10 days after CPR (13). Furthermore, Derwall *et al* observed that high-dose Na_2S significantly reduces microglial activation in striatal areas, although this did not translate into improved neurological outcome in a porcine model of prolonged CA (14). Knapp *et al* reported that Na_2S therapy is associated with a temporary beneficial effect on neurological outcome (3 days after CPR) (15). Therefore, the present study aimed to clarify the modulation of H_2S levels in the serum of rats and to evaluate the effects of treatment with the H_2S donor sodium hydrosulfide (NaHS) or hydroxylamine, a cystathionine- β -synthase (CBS) inhibitor, on brain injury following CA. These effects were evaluated by examining biomarkers of brain injury, neurologic deficit scoring (NDS) and the neural behavior after CPR. CBS is able to catalyze the synthesis of H_2S from L-cysteine in mammalian central nervous system tissues (16) and it was hypothesized that hydroxylamine application may reduce the levels of H_2S .

Materials and methods

Subjects and groups. All animal procedures were approved and conducted in accordance with the Animal Ethics Committee of Sun Yat-Sen University (Guangzhou, China) and the UK Home Office Animals (Scientific Procedures) Act 1986. All procedures conformed to Directive 2010/63/EU of the European Parliament. Animal experiments were conducted in the Assisted Circulation Key Laboratory of Ministry of Health at Sun Yat-Sen University. Animals were provided by the Laboratory Animal Center of Sun Yat-Sen University.

A total of 60 healthy male Sprague-Dawley rats, aged 10–12 months old and weighing 450–550 g, were allocated at random into the NaHS, hydroxylamine or routine CPR control groups ($n=20$ per group). The further 40 rats were used as spare rats to supplement the rats if any died prior to the end point of observation. A further 100 male rats of the same weight and brood were prepared as blood donors.

Induction of the CA model. The rat model of CA was established as described in our previous study (17). Briefly, the rats were anesthetized via a bolus intraperitoneal injection of 10% chloral hydrate (3 ml/kg). The degree of anesthesia was evaluated by measurement of muscular tone, corneal reflex and pain reflex. Tracheal intubation was performed through the mouth and cardiac monitoring was performed by electrocardiography using limb leads (V3404; SurgiVet, Inc., Waukesha, WI, USA). A 24G close-vein indwelling needle (BD Intima II™ Vein™; Suzhou Becton Dickinson Medical Devices Co., Ltd., Suzhou, China) was inserted into the right femoral vein to establish a transfusion passage. A 22G close-vein indwelling needle was inserted into the right femoral artery. A three-way cock valve was connected to the remaining arterial needle. One end was connected to an injector with heparin, and the other end was connected to the BL-420E Biological Data Acquisition and Analysis system (Chengdu TME Technology Co., Ltd., Chengdu, China). After the chest skin of the rats was shaved, two disposable acupuncture needles (30G HuanQiu; Suzhou Acupuncture Supplies Co., Ltd., Suzhou, China) were transcutaneously inserted into the epicardium between the fourth rib

of the left sternal border and the third rib of the right sternal border. The stimulator electrode of the BL-420E system was then connected, which was used to supply direct and constant electrical stimulation of the epicardium with crude current, continuous single stimulation, a delay of 100 msec, a wave width of 1 msec, a frequency of 50 Hz, an initial intensity of 1–2 mA and a stimulation duration of 3 min. CA was defined as follows (18): i) Systolic arterial pressure quickly reduced to <25 mmHg after electrical stimulation; ii) the arterial pulse wave from blood pressure monitoring disappeared after electrical stimulation; and iii) the electrocardiographic wave displayed on the cardiac monitor indicated ventricular fibrillation, pulseless electrical activity or asystole following the cessation of electrical stimulation.

CPR. CPR was performed with the Utstein style (18) following a 6-min period without any intervention. External chest compression was performed using an external chest compression machine for small animals, developed by the Key Laboratory of Assisted Circulation of Sun Yat-Sen University, at a rate of 200 compressions/min, with equal compression-relaxation, and depth of compression to 1/3 of the anteroposterior chest diameter. Intermittent positive pressure ventilation was performed using a Model 683 Small Animal Ventilator (Harvard Apparatus, Inc., Holliston, MA, USA) with the initial parameters as follows: Set frequency, 70 times/min; tidal volume, 0.65 ml/100 g; and inspired oxygen concentration, 21%. Parameters for the next respirator were adjusted according to blood gas analysis. Adrenalin (2 $\mu\text{g}/100$ g) was immediately administered to the rats at the initiation of CPR and was readministered at 3 min intervals as required. The administration of liquid during CPR was limited to <2 ml. Defibrillation was performed with direct-current single-phase (defibrillation energy, 5 J) if the electrocardiogram displayed ventricular fibrillation at 1 min after CPR. If the defibrillation failed, CPR was repeated and defibrillations were repeated at 1 min after CPR. Spontaneous circulation was restored (18) if supraventricular cardiac rhythm was restored; the average arterial pressure was >60 mmHg and was maintained for ≥ 10 min. If the spontaneous circulation of the rats was not restored after 10 min with the above treatment, CPR was considered to have failed.

Treatments following resuscitation. Rats that exhibited successful restoration of spontaneous circulation (ROSC) were monitored using an electrocardiogram and for hemodynamics for 4 h. During this period, rats with weak autonomous respirations were mechanically ventilated. The respiratory condition of each rat was evaluated every 15 min to determine whether further mechanical ventilation was required. Mechanical ventilation was stopped at 4 h, after all tubes were removed and wounds were sutured. Each rat was fed in a separate cage. All animal experiments were conducted on a thermostatic table (Suzhou Liying Experimental Co., Ltd., Suzhou, China), which maintained the rat body temperature at $37.5\pm 0.2^\circ\text{C}$. A heat lamp (Suzhou Liying Experimental Co., Ltd.) was additionally used for rats with a body temperature of $<36.5^\circ\text{C}$. Immediately following ROSC, NaHS (14 $\mu\text{mol}/\text{kg}/\text{day}$, diluted in 1.5 ml normal saline; Sigma-Aldrich Trading Co. Ltd, Shanghai, China) was injected into the rats via the femoral

vein and then every subsequent 8 h intraperitoneally (three equal 0.5-ml doses/day) in the NaHS group. Hydroxylamine solution (40 μ mol/kg/day, diluted in 1.5 ml normal saline; Sigma-Aldrich Trading Co. Ltd) was administered to the rats in the hydroxylamine group in the same manner as that used for NaHS. The control group received routine CPR, and an equal quantity of saline as a control in the same manner as that used the first two groups. The end point of observation was 7 days. Animals with failed CA induction, or animals that died prior to the end point of observation, were excluded from the final data analysis.

Neurological deficit scoring (NDS). NDS was performed prior to CA and at 4, 12, 24, 72 and 168 h after CPR, as previously described (19).

Sample collection. After NDS at each specified experimental time point (prior to CA and 4, 12, 24, 72 and 168 h after), 2 ml blood was obtained from the femoral vein. A 1-ml volume of this blood sample was sealed in an EP tube containing coagulant and deproteinization reagent for H₂S measurement. The remaining 1 ml blood was collected for the examination of neuron-specific enolase (NSE) and S100 β . Samples were centrifuged at 2,504 \times g for 15 min at 4°C, within 30 min of collection. The sealed EP tube with serum was numbered and stored at -80°C. An equal volume of blood from donor mice was transfused immediately into the experimental mice following blood collection, in order to avoid unstable blood circulation and its influence on the experiment results. Serum levels of H₂S were measured using the deproteinization method (20) and the levels of NSE and S100 β were measured using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA).

Morris water maze, beam walking and prehensile traction tests. As described in previous studies (21,22), the Morris water maze test consisted of a hidden platform test, probe test and visible platform test. Animals were subjected to the Morris water maze test individually 1 day prior to CA and 3, 5 and 7 days after CPR. The hidden platform test was performed in duplicate, once in the morning and once in the afternoon, with the average number as the result for each day of testing. The water maze apparatus (Zhenghua Biotech Co., Ltd., Hebei, China) consisted of a circular glass pool of 150 cm diameter and a movable glass platform 12 cm in diameter and 35 cm in height. A video camera was positioned on a platform that was 210 cm above the bottom of the pool. The wall of the maze was painted black and the water was dyed black using ink. The pool was filled with water to the 36-cm mark and maintained at 24 \pm 1°C. The pool was divided arbitrarily into four equally sized quadrants. The platform was submerged in the middle of zone I such that its surface was 1 cm below the water surface and 20 cm away from the wall of the maze. The hidden platform test measured rat behavioral change by quantifying escape latency and swimming distance. The probe test was conducted 7 days after CPR, subsequent to the hidden platform test. After removing the platform, the animals were left to swim for 120 sec. During this period, the number of instances the animal passed each quadrant, as well as the swimming was recorded by a video tracking system. In the visible platform test, which

was performed after the probe test, escape latency and swimming distance were recorded.

The beam walking test (BWT) and the prehensile traction test were conducted 1 day prior to CA and 3, 5 and 7 days after CPR, following established methods (23,24).

Preparation of samples for apoptosis analyses. Following the endpoint of the neural behavioral tests, the animals in each group were divided at random into two subgroups for the analysis of apoptosis by either immunohistological examination by TUNEL assay, or by western blot analysis. The brains of rats that were to undergo immunohistochemical (IHC) examination were fixed via transcardial perfusion of the rats with 4% paraformaldehyde for 20-30 min prior to decapitation and removal of the brain. Brain tissue was then fixed in 4% paraformaldehyde. Sections of brain tissue 3-4 mm in size were excised between the optic chiasm and optic nerve, embedded in paraffin and sectioned for IHC examination. Hippocampal volume was measured by stereological analysis after hematoxylin and eosin staining (25). Rats that were to undergo western blot analysis were sacrificed by cervical dislocation. The brains were removed immediately, and the hippocampal region was isolated and rapidly frozen using liquid nitrogen. Samples were stored at -80°C prior to detection of cysteine-containing aspartate-specific protease-3 (caspase-3) and B-cell leukemia/lymphoma 2 (Bcl-2) protein expression levels.

TUNEL assay for apoptosis analysis. Apoptosis assays were performed using a TUNEL assay (TUNEL Cell Apoptosis Detection kit; Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China). Cells exhibiting characteristics of apoptosis, combined with brown nuclear staining, were considered to be positive for apoptosis. Expression of caspase-3 and Bcl-2 in the hippocampal CA1 region was examined using streptavidin biotin peroxidase complex immunohistochemical staining kits (Wuhan Boster Bio-Engineering Ltd. Co., Wuhan, China). Cells displaying dark brown staining were considered to be positive.

Western blot for apoptosis analysis. The expression levels of caspase-3 and Bcl-2 in the hippocampal CA1 region were additionally examined using western blot analysis, according to a previously reported method (26). The primary antibodies included polyclonal rabbit anti-active caspase-3 antibody (1:1,000; #9664; Cell Signaling Technology, Inc., Danvers, MA, USA) and polyclonal rabbit anti-Bcl-2 antibody (1:50; ab7973; Abcam, Inc., Cambridge, MA, USA). The secondary antibody was goat-anti-rabbit IgG (1:5,000; SC-2004; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h. Semi-quantitative image analysis was performed using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). Five optical microscope fields (DVM6; Leica Microsystems GmbH, Berlin, Germany) were selected at random from the same region of each tissue section and the apoptotic index (AI) was calculated as a percentage of total apoptotic cells. For caspase-3 and Bcl-2 expression, integrated optical density (IOD) was determined for each field. Average IOD from the five fields was calculated as the mean IOD for the region. Western blot analysis results were analyzed following

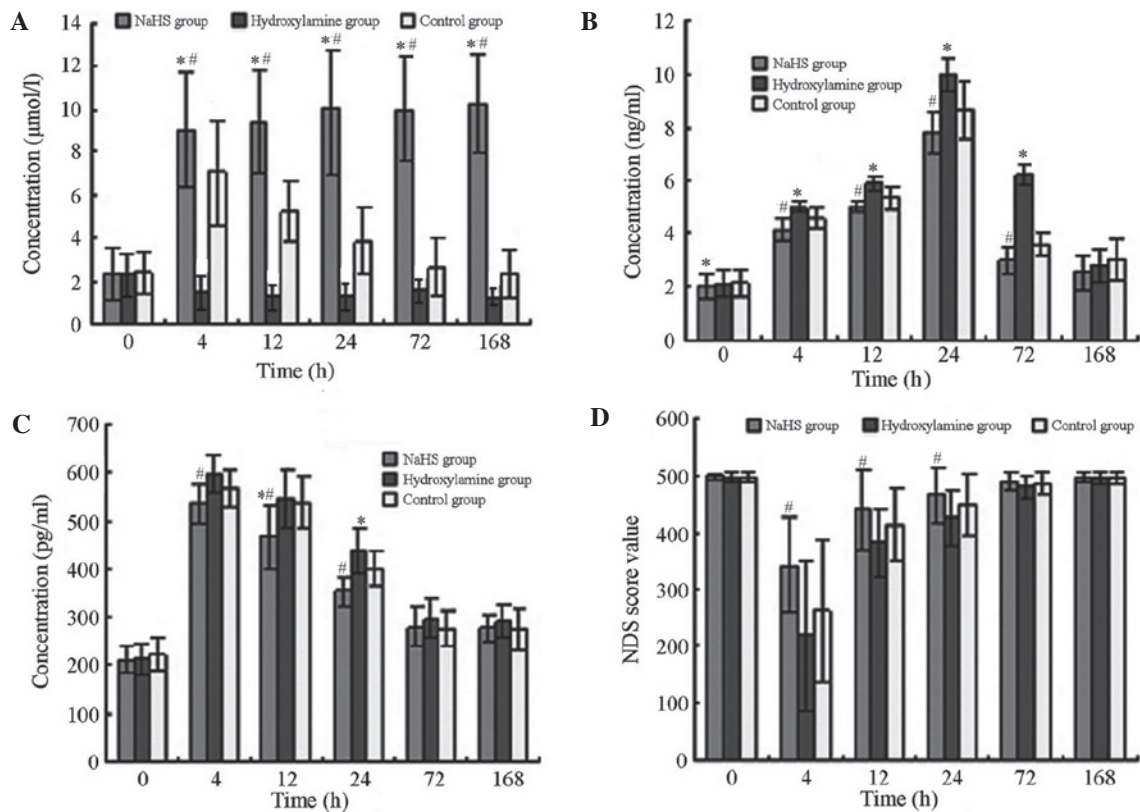


Figure 1. Comparison of serum concentrations of (A) H_2S , (B) NSE, (C) S100 β and (D) NDS at different time points before and after CPR. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. hydroxylamine group. NaHS, sodium hydrosulfide; H_2S , hydrogen sulfide; NSE, neuron-specific enolase; NDS, neurological deficit scoring; CPR, cardiopulmonary resuscitation; CA, cardiac arrest.

scanning of the blots. Quantity One image processing software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to semi-quantitatively calculate the luminance ratio of each protein band.

Statistical analysis. Statistical analyses were performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Categorical data were organized into contingency tables. Quantitative data are expressed as the mean \pm standard deviation. Pair-wise comparisons between groups were performed using the Student-Newman-Keuls method. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CA model. In the NaHS group, the establishment of the CA model was attempted in 31 Sprague-Dawley rats with a success rate of 93.5% (29/31) and an ROSC rate of 93.1% (27/29). Seven rats died after ROSC prior to the end point of observation (7/27, 25.9%). In the hydroxylamine group, establishment of the CA model was attempted in 35 rats with a success rate of 91.4% (32/35) and an ROSC rate of 93.8% (30/32). A total of 10 rats died after ROSC prior to the end point of observation, resulting in a mortality rate of 33.3% (10/30). In the control group, CA was successfully induced in a total of 32 rats (97.0%, 32/33). The ROSC rate was 90.6% (29/32) and 9 rats died after ROSC but prior to the end point of observation, resulting in a mortality rate of 31.0% (9/29). No significant differences were observed in the rates of successful

CA model induction, ROSC and mortality following ROSC among the three groups ($P > 0.05$).

Comparison of serum H_2S , NSE and S100 β concentrations and NDS after CPR. As presented in Fig. 1, the differences in serum H_2S , NSE and S100 β concentrations and NDS before and after CPR were statistically significant ($F = 223.496$, 111.667, 30.194 and 7.318; $P < 0.001$, < 0.001 , < 0.001 and 0.001, respectively). In the control group, the H_2S concentration gradually increased following CA, peaking at 4 h after CPR, then reduced over time. The H_2S concentration in the NaHS group increased after CPR while the H_2S concentration in the hydroxylamine group reduced after CPR. Significant differences were detected in H_2S levels among the three groups at each time point ($P < 0.05$). Serum concentrations of NSE and S100 β in all animals gradually increased and peaked respectively at 24 and 4 h, respectively, after CPR prior to declining. The peak values of the two parameters were lowest in the NaHS group and highest in the hydroxylamine group. NSE and S100 β concentrations in the NaHS group were significantly reduced compared with those in the hydroxylamine group at 4, 12, 24 and 72 h after CA/CPR ($P < 0.05$), but not at 168 h. Following CA/CPR, NDS values reduced significantly, with the lowest values observed at 4 h, followed by a trend of gradual increase. The range of NDS decline was the lowest in the NaHS group and was largest in the hydroxylamine group following CPR. NDS values from the NaHS group were significantly higher compared with those in the hydroxylamine group at 4, 12 and 24 h after CA/CPR ($P < 0.05$).

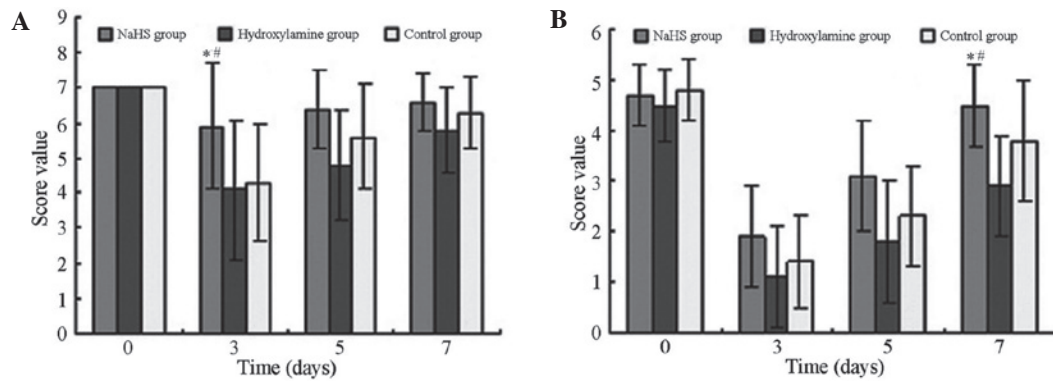


Figure 2. Comparison of scores from the (A) beam walking and (B) prehensile traction tests. *P<0.05 vs. control group; #P<0.05 vs. hydroxylamine group. NaHS, sodium hydrosulfide.

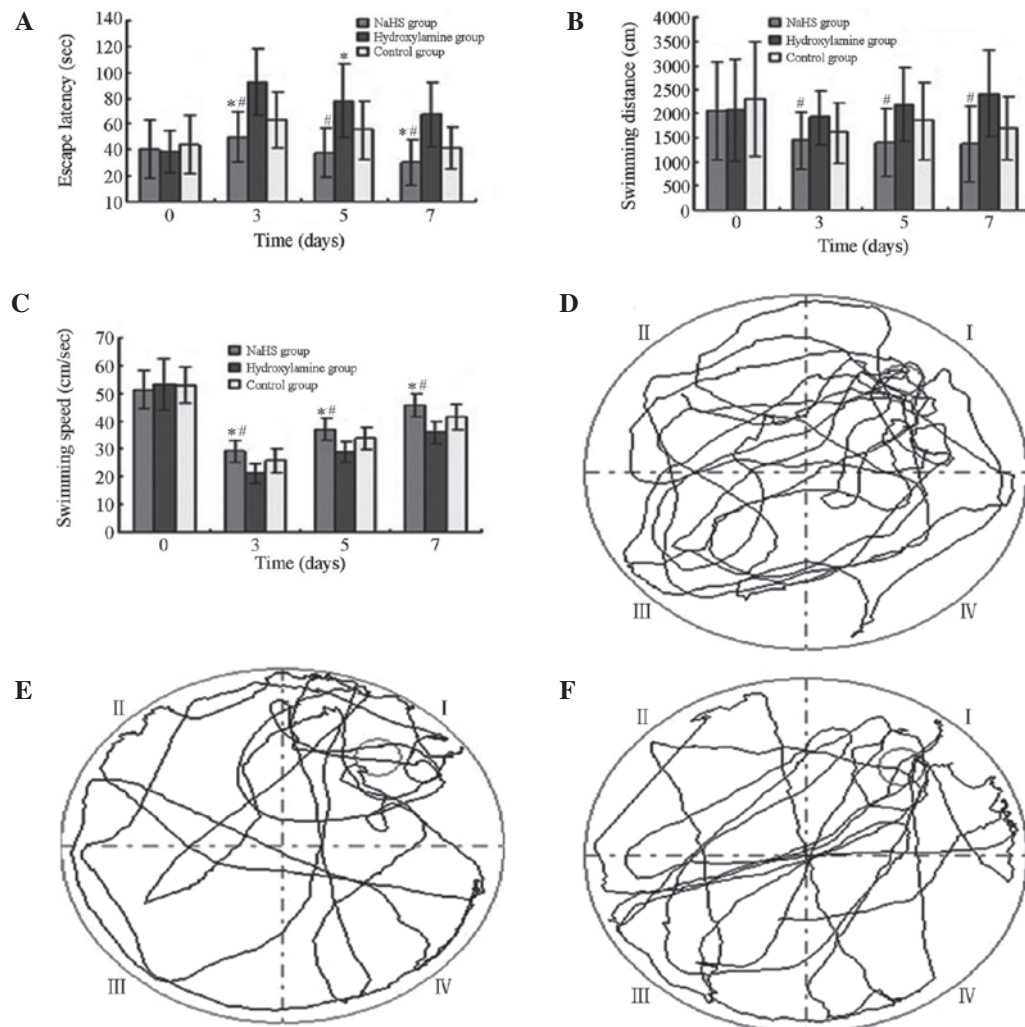


Figure 3. Morris water maze results comparison. (A) Escape latency, (B) swimming distance and (C) swimming speed in the hidden platform test. Swim paths of representative rats from the (D) NaHS, (E) hydroxylamine and (F) control groups in the probe test. *P<0.05 vs. control group; #P<0.05 vs. hydroxylamine group. At any time point after CPR, the NaHS group had the shortest escape latency and swimming distance, and the quickest swimming speed, whereas the hydroxylamine group had the longest escape latency and swimming distance, and the slowest swimming speed. NaHS, sodium hydrosulfide; CPR, cardiopulmonary resuscitation.

BWT and the prehensile traction test. As presented in Fig. 2 the scores of the beam walking and prehensile traction tests suggest significant differences among the three groups ($F=5.503$ and 3.246 ; $P=0.007$ and 0.046 , respectively). After

CA/CPR, the scores in all groups declined and reached the lowest value at 3 days. Among the three groups, scores in the hydroxylamine group exhibited the most marked decline at 3 days; however, the scores subsequently recovered. At all

Table I. Comparison of results from the Morris water maze probe test and visible platform test after cardiopulmonary resuscitation (mean \pm standard deviation).

Groups	Probe test		Visible platform test	
	Distance (cm)	Platform passing (n)	Escape latency (sec)	Swimming speed (cm/sec)
NaHS	5,329.6 \pm 540.0 ^{a,b}	7.6 \pm 2.0 ^{a,b}	5.8 \pm 2.3	44.7 \pm 4.3 ^{a,b}
Hydroxylamine	4,070.4 \pm 500.9	3.4 \pm 1.9	6.4 \pm 2.5	36.4 \pm 3.9
Control	4,835.8 \pm 700.7	5.1 \pm 2.2	5.3 \pm 2.4	40.9 \pm 5.1

^aP<0.05 vs. control group; ^bP<0.05 vs. hydroxylamine group. NaHS, sodium hydrosulfide.

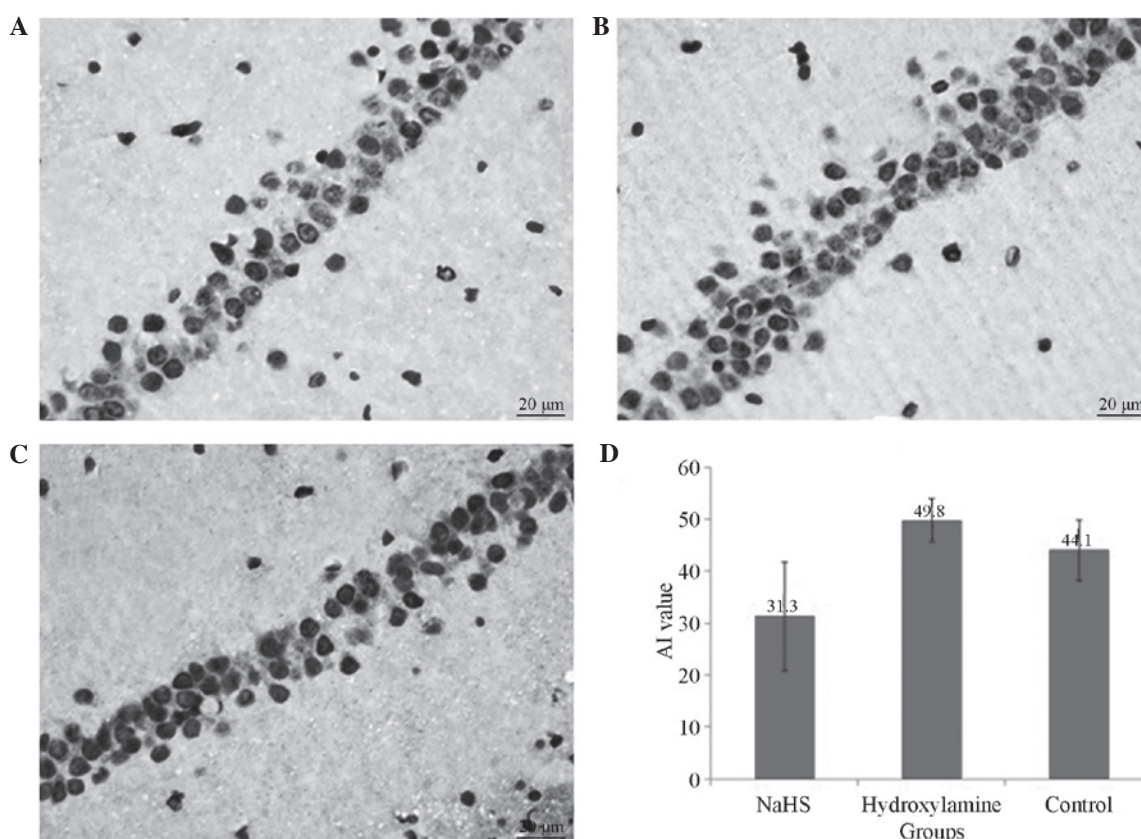


Figure 4. Comparison of apoptotic analysis in the rat hippocampal CA1 region at 7 days after cardiopulmonary resuscitation (CPR; TUNEL assay; magnification, x400). Representative images from the (A) NaHS, (B) hydroxylamine and (C) control groups. (D) Significant difference in the AI of neurons in the rat hippocampal CA1 region was observed among the three experimental groups ($F=8.269$, $P=0.002$). NaHS, sodium hydrosulfide; AI, apoptotic index.

time points after CPR conduction, the NaHS group exhibited the highest score, whereas the hydroxylamine group presented significantly reduced scores ($P<0.05$).

Morris water maze test. As displayed in Fig. 3, in the hidden platform test, there were significant differences in escape latency, swimming distance and speed among the three groups ($F=12.289$, 3.712 and 17.441 ; $P=0.000$, 0.031 and <0.001 , respectively). At all time points after CPR, the NaHS group exhibited the shortest escape latency and swimming distance, and highest swimming speed, whereas the hydroxylamine group displayed the longest escape latency and swimming distance, and lowest swimming speed. As shown in Table I, in the probe test, the swimming distance and number of pass-

ings of the platform differed significantly among the groups ($P<0.05$). The NaHS group exhibited the longest swimming distance and the highest number of passings of the platform, whereas the hydroxylamine group had the shortest swimming distance and the least number of passings of the platform. In the visible platform test, no significant differences were observed in escape latency among the three groups ($F=1.131$, $P=0.330$).

Apoptosis of neurons in the hippocampal region. All rats that underwent CA presented with numerous TUNEL-positive pyramidal neurons in the CA1 region of the hippocampus at 7 days after CPR. Significantly reduced numbers of TUNEL positive neurons were observed in the NaHS group compared

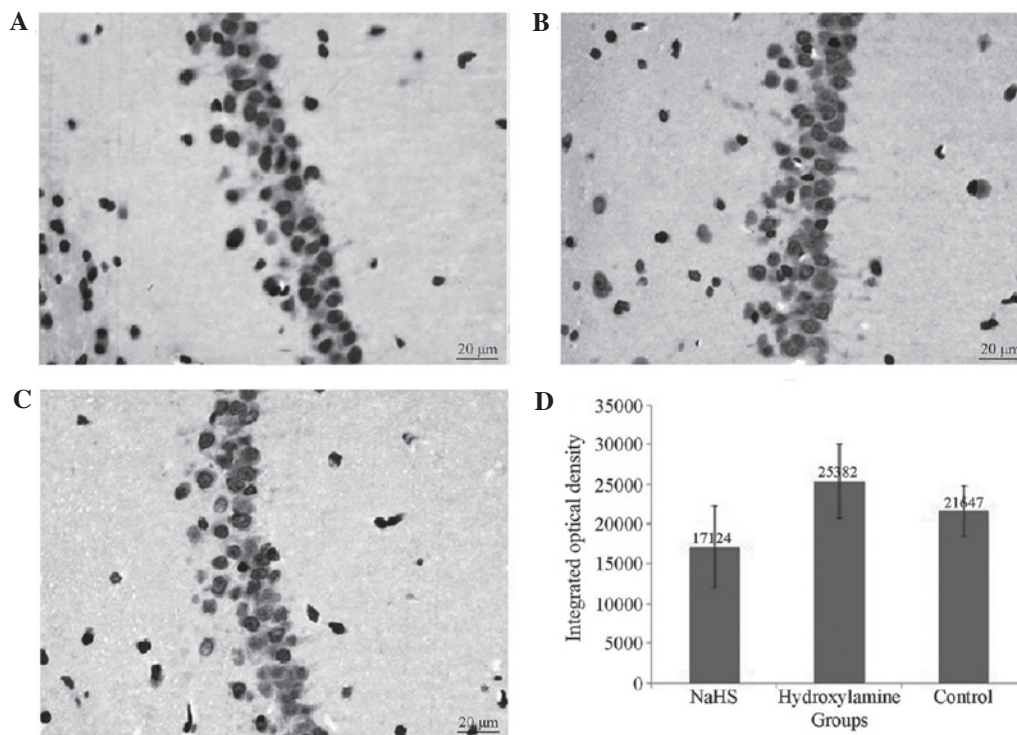


Figure 5. Comparison of caspase-3 expression by immunohistochemical analysis in the rat CA1 region at 7 days after CPR (streptoavidin biotinperoxidase complex; magnification, x400). Images from the (A) NaHS group, (B) hydroxylamine group and (C) control group. (D) Comparison of the integrated optical density of caspase-3 expression in the rat hippocampal CA1 region among the three experimental groups ($F=8.774$, $P=0.001$). NaHS, sodium hydrosulfide; CPR, cardiopulmonary resuscitation.

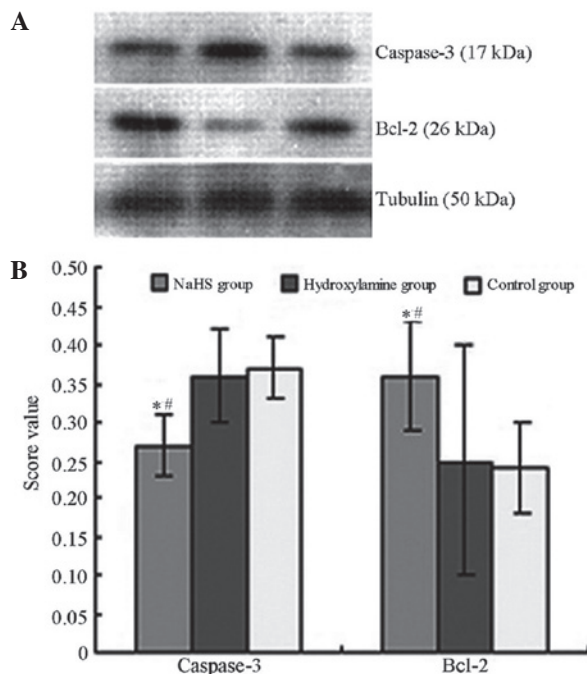


Figure 6. Western blot analysis of hippocampal caspase-3 and Bcl-2 protein expression among groups. (A) Western blot images. (B) Semi-quantitative comparison of caspase-3/tubulin and Bcl-2/T=tubulin luminance ratio among groups ($F=12.514$ and 4.411 ; $P=0.000$ and 0.022 , respectively). * $P<0.05$ vs. control group; # $P<0.05$ vs. hydroxylamine group. NaHS, sodium hydrosulfide.

with the hydroxylamine group (Fig. 4). Concomitantly, significantly reduced caspase-3 expression levels (Figs. 5 and 6) and increased Bcl-2 (Figs. 6 and 7) expression levels were observed

in the NaHS group compared with the hydroxylamine and control groups. No significant differences were detected in the expression levels of caspase-3 and Bcl-2 between the hydroxylamine and control groups.

Stereological analysis of the hippocampus. Stereological analyses detected no significant differences in the volume of the hippocampus among the groups at 7 days after CPR ($P>0.05$; Fig. 8).

Discussion

The results of the present study indicate that the serum H_2S concentration in rats of the NaHS group after CPR was significantly elevated, whereas the serum H_2S concentration in rats treated with hydroxylamine was reduced, suggesting that exogenous treatments are able to alter H_2S levels *in vivo*. Furthermore, NaHS treatment attenuates neuronal injury and improves neural functional performance, whereas hydroxylamine exaggerates neuronal injury and exacerbates learning and memory problems. NaHS treatment markedly increases Bcl-2 expression levels and reduces caspase-3 expression. The present results suggest that H_2S may possess potential therapeutic value for brain injury following CA.

NSE is a specific enzyme located primarily in brain neurons and neuroendocrine cells, and is involved in glycolysis. Following CA/CPR, NSE may be released from ischemically injured neurons and traverse the blood brain barrier into the circulation; therefore, serum NSE levels reflect the severity of brain damage after CA and indicate disease outcome (27,28).

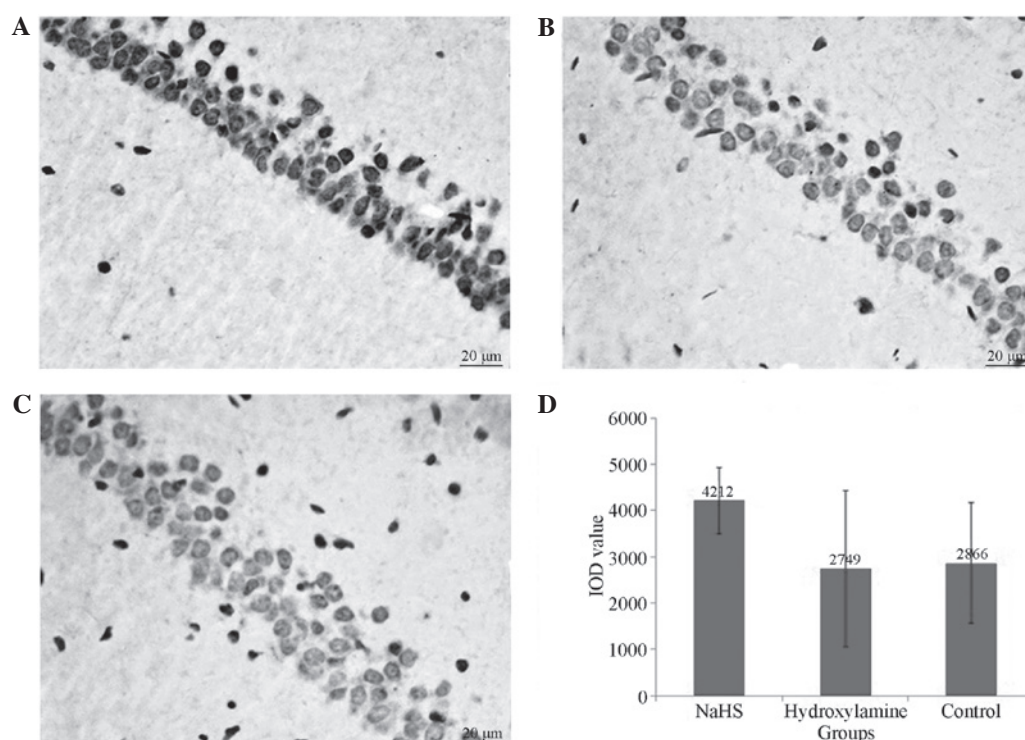


Figure 7. Comparison of Bcl-2 expression by immunohistochemical analysis in the rat CA1 region at 7 days after CPR (streptavidin biotin peroxidase complex; magnification, x400). Representative images from the (A) NaHS, (B) hydroxylamine and (C) control groups. (D) Comparison of integrated optical density (IOD) of Bcl-2 expression in hippocampal CA1 region of rats among the three experimental groups ($F=6.858$, $P=0.016$). Different lowercase letters indicate statistically significant difference ($P<0.05$). NaHS, sodium hydrosulfide; CPR, cardiopulmonary resuscitation.

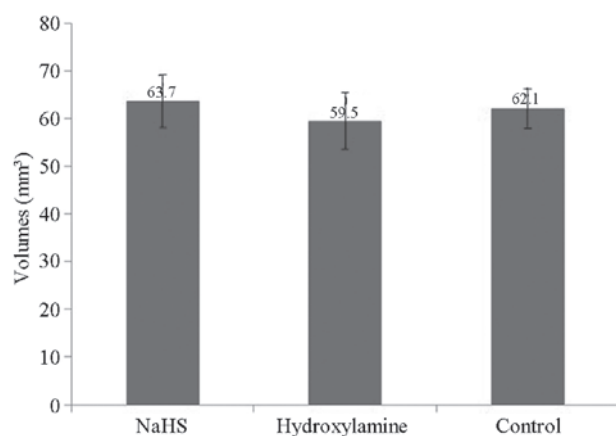


Figure 8. Comparison of hippocampal volume in the three groups ($F=1.627$, $P=0.215$). NaHS, sodium hydrosulfide.

The calcium binding protein S100 β is an axonal growth factor that is predominantly produced and secreted by glial cells, and is widely expressed in nervous tissue. Serum S100 β levels are used as biomarkers for disease progression after CA/CPR and neurological prognosis (29) due to their regularity of alteration and marked correlation with the severity of the injury (30). In the present study, the serum concentrations of NSE and S100 β gradually increased and peaked at 24 or 4 h, respectively, after CA/CPR. In addition, serum NSE and S100 β concentrations reduced or increased according to the H₂S levels in serum, suggesting that H₂S therapy may be able to reduce rat brain damage following CA to certain extent. Compared with NSE and S100 β , NDS is able to more directly indicate the extent

of cerebral function damage after CPR, and to determine the effectiveness of treatment (19,31,32). Therefore, NDS was also evaluated in the present study. The NDS score reduced significantly in rats following CA/CPR. However, the NDS score of NaHS-treated rats was higher than that of rats treated with hydroxylamine at 24 h after CPR, indicating a protective effect against brain damage after CA.

Neural behavior is a crucial function of the body, which controls and coordinates the body's normal activities, in addition to being influenced and regulated by the state of other organ systems. Following CPR, the body may not display clear signs and symptoms of the neurological state, but patients may present with abnormal memory, sensory, motor and cognitive functions. Therefore, neurobehavioral examination is frequently a key index for evaluating brain functional status and prognosis after CPR (33-35). The Morris water maze experiment is a classical neurobehavioral test, which includes a hidden platform test, space exploration test and visible platform test. The Morris water maze is designed to allow animals to learn to search for a hidden platform under water, and by analyzing the time and path taken to search for the platform, animal memory function may be assessed. The Morris water maze allows the effective detection of rat spatial learning and memory abilities, which involves the medial temporal lobe limbic region including the hippocampus (36). In the present study, the spatial learning and memory ability of the rats reduced following CA, but subsequently recovered to a certain extent. In the hidden platform test, the escape latency of the NaHS group was lower than that of the control group at every time point after CPR; however, in the hydroxylamine group the escape latency and swimming distance increased after CPR. In

the space exploration experiments, the NaHS group displayed a significant increase in the number of passings of the platform, while the hydroxylamine group exhibited a significant reduction. This indicated that improving body levels of H₂S led to improved rat spatial learning and memory ability after CPR, and reducing H₂S levels may result in delayed recovery of spatial learning and memorizing abilities.

The BWT is used to detect whether the established operant conditioning reflex is impaired after injury in rats. The BWT is frequently used to assess whether there are impairments in the functions of dynamic balance, motor coordination, learning and memorizing abilities (37,38). The prehensile traction test is an index for evaluating rat forepaw gripping power, muscle strength, balance and motor function (24,39,40). In the present study, the scores for the beam walking and prehensile traction testing reduced significantly in the rats after CA, and increased gradually over the 7 days after CPR. A comparison of the results in these three groups showed that the performances of the NaHS-treated rats were the most improved, while the hydroxylamine group displayed the worst performance. This indicated that increasing H₂S levels improved the balance, coordination and muscle strength of rats after CPR, and reducing H₂S levels delayed the recovery of motor ability.

The results of the neural behavior and function analysis are consistent with previous studies, in which Knapp *et al* reported that the administration of Na₂S reduces sensorimotor deficits 72 h after CA/CPR in rats (15) and Kida *et al* found that Na₂S improves neurological function at 96 h after CA and CPR (13). In contrast to previous studies involving rodents, Derwall *et al* observed in a porcine model that high-dose Na₂S (1 mg/kg) bolus followed by infusion at 1 mg/kg/h for 2 h did not improve neurological outcomes (14). This may be attributable to the hypothesis that a lower concentration of H₂S exerts a protective effect on cells while higher levels of H₂S exposure lead to cytotoxicity (41). In the present study, 14 μ mol/kg/day NaHS was intraperitoneally administered, and it has previously been confirmed that the protective effect against neuronal injury conferred by NaHS is dose-dependent (42). In addition to its function as an inhibitor of CBS, hydroxylamine can be metabolized to NO (43), which may be neurotoxic and contributes to neuronal damage (44).

In the present study, levels of neuronal apoptosis in the hippocampus were assessed at 7 days after CPR, and it was observed that in response to the elevated levels of serum H₂S in the NaHS group, the apoptotic index of pyramidal neurons in the hippocampal CA1 region and the expression level of the pro-apoptotic protein caspase-3 appeared to reduce, while the expression of the anti-apoptotic protein Bcl-2 increased. By contrast, in response to the reduced levels of serum H₂S in the hydroxylamine group, the apoptosis index of pyramidal neurons and caspase-3 expression levels in the CA1 region increased, whereas Bcl-2 expression levels decreased. This result suggests that increased endogenous H₂S levels may inhibit neuronal apoptosis, whereas inhibiting endogenous H₂S production may promote apoptosis. However, this is contradictory to the study by Knapp *et al*, in which the administration of Na₂S failed to significantly reduce the number of TUNEL-positive cells and caspase activity in the CA1 region of the hippocampus 7 days after CPR (15). This may be attributable to the difference in potentials of the H₂S donors that

were used and the short infusion time (0.5 mg/kg, 1 min prior to the initiation of CPR, followed by a continuous infusion of Na₂S for 6 h, 1 mg/kg/h) in the previous study.

In conclusion, the *in vivo* modulation of H₂S levels may influence the occurrence and development of brain damage in rats following CA. After ROSC, elevating the H₂S levels via a therapeutic intervention may improve neural function damage in rats that have undergone CPR.

The present study has certain limitations. i) It was observed that certain doses of NaHS appeared to reduce the biochemical markers of nerve injury, improve nervous function defect score, improve neurobehavioral symptoms and reduce neuronal apoptosis in hippocampal tissue. However, these indices in the hydroxylamine group exhibited adverse changes to various degrees. No significant differences were observed between the hydroxylamine and saline control groups when multiple level comparisons were performed. ii) Theoretically, as cellular apoptosis increases, hippocampal volume is expected to exhibit a certain degree of atrophy; however, no differences in hippocampal volume were observed among the groups in the current study. iii) There were no obvious increases in mortality rate following ROSC, which is the primary index for evaluating the effectiveness of CPR. As for the reason for this, as only a single dose level was used in this experiment, it is hypothesized that there may be an optimal effective dose range of H₂S. iv) Body temperature, heart rate, respiratory rate and blood pressure were measured during the experiment. However, all animals were placed on a thermostatic table 4 h after ROSC and the body temperature was maintained at 37.5 \pm 0.2°C. From 5 h after ROSC to the end point of observation, a heat lamp was additionally used for the rats with poor status and a body temperature <36.5°C. Heart rate and the respiratory rate are directly associated with body temperature. Furthermore, adrenalin affects heart rate and blood pressure in the early stages following ROSC. Blood pressure measurements may differ significantly between these two methods. In addition, mechanical ventilation may effect the respiratory rate. Thus, although the physiological indices were maintained as consistently as possible during the experiment, the above factors were not excluded. Thus, the systemic detection and comparison of certain physiological indices were not performed. The differences between these indices may have affected the analysis of the experimental results. v) The scale of the present study was relatively small, which may have resulted in experimental bias.

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