Original Article
Protective effects of H2S inhalation on cerebral resuscitation in cardiac arrest rats associated with modulation of inflammatory cytokines and p38MAPK signaling pathways in brain tissue

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Abstract: Objective: The aim of this study was to evaluate the protective effects of hydrogen sulfide (H2S) inhalation on cerebral resuscitation in cardiac arrest rats and its effects on inflammatory cytokines and p38MAPK signaling pathways in brain tissue. Methods: One hundred male Sprague-Dawley rats were randomly divided into 5 groups: sham-operated, cardiac arrest resuscitation, H2S, shallow hypothermia, and H2S + shallow hypothermia. Enzyme-linked immunosorbent assay (ELISA) was used to detect the content of inflammatory cytokines TNF-α, IL-16, and IL-1β in brain tissue. Expression of downstream inflammatory signaling factors JNK, p38MAPK, and ERK5 were determined by Western blotting. Results: Neurological function scores (NFS) in the H2S + shallow hypothermia group were significantly higher than those of the cardiac arrest resuscitation group, H2S group, shallow hypothermia group, and H2S + shallow hypothermia group ($P<0.05$). MDA content in the H2S + shallow hypothermia group was significantly lower than the other 3 groups and SOD levels were significantly higher ($P<0.05$). JNK, p38MAPK, and ERK5 levels in brain tissues were highest in the resuscitated cardiac arrest group ($P<0.05$). Levels of JNK, p38MAPK, and ERK5 in the H2S + hypothermal group were significantly lower than those in the other 3 groups ($P<0.05$). Conclusion: Hydrogen sulfide conveyed substantial neuroprotection in rats after cardiac arrest. Mechanisms may be related to regulation of TNF-α, IL-16, and IL-1β levels in brain tissue, inhibition of activation of p38/MAPK signaling pathways, and downregulation of oxidative stress levels in the body.

Keywords: H2S, cardiac arrest, rat, cerebral protection, inflammatory cytokines, experimental study

Introduction

Cerebral ischemia-reperfusion injury after resuscitation from cardiac arrest can cause clinically irreversible brain damage [1], resulting in different degrees of neurological dysfunction with serious effects on long-term outcomes of patients receiving successful resuscitation. Studies [2, 3] have shown that about 80% of patients with intractable global ischemia and hypoxic injury die within one year of successful self-recovery of cardiocerebral resuscitation. Therefore, in the process of cardiocerebral resuscitation, restoration of cerebral blood flow circulation and oxygen supply should be ensured as early as possible to avoid occurrence of cerebral ischemia-reperfusion injury.

Therapeutic shallow hypothermia has been used clinically in the treatment of cerebral pro-

tection during cardiac arrest [4, 5], inhibiting the production of oxygen free radicals, stabilizing cell biofilm, reducing enzyme activity, and inhibiting inflammatory cells. While hypothermia has clear protective effects on brain tissue, the degree of efficacy has not been ideal. H2S, a gaseous signaling molecule, has many functions, including anti-inflammatory [6], anti-oxidative stress [7], and anti-apoptosis roles. H2S can attenuate impairment of cerebral nerve function caused by ischemia-reperfusion injury, having good prospects for therapeutic application in various diseases, including ischemia-reperfusion brain injury, cerebral vascular dementia, and traumatic brain injuries [8, 9]. While H2S has known roles in brain protection, there have been few reports regarding its potential effects against brain damage after cardiopulmonary resuscitation.

Therefore, this present study assessed the protective effects of H2S combined with shallow hypothermia on cerebral resuscitation in rats undergoing cardiac arrest. Additionally, its effects on inflammatory cytokines and p38/MAPK signaling pathways in rat brain tissue were investigated.

Material and methods

Experimental animals

One hundred healthy clean male SD rats, with a weight of 300 ± 20 grams, were provided by Experimental Animal Center of Chinese Academy of Sciences in Shanghai. These rats were given seven days of adaptive feeding before the experiment. They were given food and water, freely. The litter was changed every other day. Rats with normal food and water, normal activity, good mental states, and no adverse reactions were included in the experiment. A well-replicated cardiac arrest rat model was used for this experimental study. Rats were divided into cages with equal numbers of animals and raised in the teaching animal laboratory. The laboratory was well-ventilated and the environment was free from pollution and natural lighting. Experimental relative humidity was controlled between 40% and 60%. Room temperature was maintained at 22 C~24 C, with a light and dark cycle of 12 hours.

Reagents

Key chemical reagents used included sodium pentobarbital (manufacturer: Sigma USA, batch number: 57-33-0), 4% paraformaldehyde (manufacturer: Beijing Regen Biotech Co., Ltd., batch number: DF0135), isoflurane (manufacturer: Shanghai Abbott Pharmaceuticals Co., Ltd., batch number: Zhunzi H01591), adrenaline (manufacturer: Wuhan Yuancheng Chuangxi Technology Co., Ltd., batch number: 205-750-4), and H2S gas (manufacturer: Chengdu Taiyu Industrial Gas Co., Ltd., lot No.: 7783-06-4).

Major instruments and equipment

Major instruments and other equipment used included an electronic balance (model: MODELDS-671, manufacturer: Dalian Xinghai Electronic Weighing Apparatus Co., Ltd.), digital thermometer (OMRON MC-612), desktop high-speed centrifuge (model: TG16-WS, manufac-

turer: Xiangyi Centrifuge Instrument Co., Ltd.), a variety of animal experimental surgical instruments (manufacturer: Shanghai Yuyan Scientific Instrument Co., Ltd., provided by the school's laboratory), small animal breathing machine (model: DW3000-B, manufacturer: Huaibei Zhenghua Biological Instrument Equipment Co., Ltd.), vein infusion pump (model: LP2000-P2, manufacturer: Beijing Xinyi Medical Technology Co., Ltd.), multi-functional microplate reader (model: SpectraMax i3x, manufacturer: Molecular Devices, USA), electrophoresis apparatus (model: DYY-16D, manufacturer: Shanghai Wanning Precision Scientific Instrument Co., Ltd.), and an anesthetic gas monitor (model: 715, manufacturer: Wuhan Sichuang Creative Electronics Co., Ltd.).

Cardiac arrest model

The rat model of cardiac arrest was established by transesophageal cardiac pacing, according to which cardiopulmonary resuscitation was performed [10]. Cardiac arrest time was 4 minutes. Rats were given intraperitoneal injections of 2% sodium pentobarbital sodium 50 mg/kg. After anesthesia was completed, orotracheal intubation was performed and left femoral arteries and femoral veins were punctured. Next, monitoring of blood pressure and ECG changes was established. After rat vital signs were stabilized, one end of a quadrupole 5F pacing electrode was inserted into the esophagus to a depth of approximately 5 cm. The other end was connected to an electrophysiological stimulator to give continuous and rapid electrical stimulation to induce ventricular fibrillation. When pacing time reached 90 seconds, stimulation was suspended for 2 seconds and ECG was observed. If an automatic transmigration was displayed, pacing was resumed immediately for 30 seconds or until the ECG showed continuous ventricular fibrillation or no EC activity. Typical pacing times were between 90 seconds and 210 seconds. CPR was started 4 minutes after observation of cardiac continuous ventricular fibrillation or no EC activity. Artificial ventilation assistance was provided with a small animal ventilator. The inspired oxygen concentration was set at 100% and oxygen flow rate at 3 L per minute. Ventilation frequency was set to 60 breaths per minute and tidal volume was set to 6 mL/kg. Epinephrine 0.2 g/kg and 5% sodium bicarbonate 0.03 mL/kg were

given repeatedly. At the same time, hearts were given 200 presses per minute, while simultaneously detecting ECG and carotid blood pressure changes. CPR was considered successful when the electrocardiogram reappeared and there was mean arterial pressure (MAP) >60 mmHg, lasting longer than 10 minutes.

Experimental grouping

Using a random number table method, rats were randomly divided into 5 groups, including sham-operated, cardiac arrest resuscitation, H2S, shallow hypothermia, and H2S + shallow hypothermia, with n = 20 in each group. Among these groups, the sham-operated rats only underwent isolation of the middle tail artery and arterial pressure was monitored by puncture and juxtaposition. The left femoral vein was punctured and rehydration and administration pathways were established. No model treatment was performed. Cardiac resuscitation only was performed in the cardiac arrest resuscitation group. Immediately after cardiac resuscitation, rats in the H2S group were given 30% oxygen containing 0.08% H2S, inhaled through the endotracheal tube for 1 hour. In the hypothermia group, rat body surfaces were wiped with ethanol immediately after cardiac resuscitation and the rectal temperature was rapidly reduced within 15 minutes to 32-34 C. At the same time, rectal temperature was maintained at about 34 C for about 4 hours with an ice bag. Temperatures were raised within 2 hours and the rectal temperature rose to 38 C. After successful CPR, the H2S + hypothermal group was given hypothermia therapy while inhaling H2S. The above method was completed for the H2S group as well as the shallow temperature group.

Comparison of survival and neurological function in rats

After cardiopulmonary resuscitation, this study observed and recorded the survival status of each group of rats by observing behaviors such as eating, drinking water, and activity for 24 hours. Survival rates were calculated. Neurological function deficit scores (NDS) were used to evaluate the neurological function of surviving rats in each group. Analysis was performed 24 hours after successful cardiopulmonary resuscitation. With a total of 80 points, higher

scores indicated better recovery of nerve function.

Histopathological observation in rats

After 24 hours of resuscitation, the rats were killed and cortical tissue samples were taken for fixation, dehydration, embedding, and slicing. Pathological changes of brain tissue were observed under light microscope. Neurons, glial cells, and blood vessels were scored for pathology.

Comparison of brain water content and blood-brain barrier permeability

Rats were anesthetized 24 hours after resuscitation and left hemispheres were taken. Wet tissue weight was determined after removing the surrounding tissue. After drying in an 80 C oven for 72 hours, dry weights were obtained and brain water content was calculated.

For blood brain barrier analysis, five rats in each group were taken at 24 hours after resuscitation and 4 mL/kg of 2% Evan's blue (EB) was injected. After 1 hour, 200 mL of physiological saline was perfused under anesthesia. Hippocampal tissue was isolated when the right atrial appendage emanated from the clear fluid. After grinding and centrifugation, the supernatant and ethanol were diluted at a ratio of 1:3. A Muhiskan MK3 microplate reader was used to detect the content of EB. Absorbance was read at 620 nm, a standard curve was drawn, and EB levels were calculated.

Comparison of oxidative stress levels in brain tissue

Twenty-four hours after resuscitation, brain tissues of rats were taken and homogenized. Changes of malondialdehyde (MDA) and superoxide dismutase (SOD) expression in each group were determined by the thiobarbituric acid method, following manufacturer instructions.

Comparison of inflammatory factor levels

Brain tissue was isolated, homogenized, and centrifuged to obtain protein-containing supernatants. ELISA method was used to detect changes of TNF-α, IL-18, and IL-1β expression in brain tissue from each group.

Table 1. Comparison of brain water content and blood-brain barrier permeability

Group	Brain water content (%)	Blood-brain barrier permeability (μg/g)	Pathology score (score)
Control	74.9 ± 1.9	1.47 ± 0.22	0.23 ± 0.13
Cardiac arrest resuscitation	86.7 ± 1.5[①]	4.91 ± 0.57[①]	7.88 ± 1.36[①]
H2S	82.5 ± 1.3[①,②,③]	3.31 ± 0.29[①,②,③]	5.09 ± 0.72[①,②,③]
Shallow hypothermia	81.7 ± 1.6[①,②,③]	3.14 ± 0.17[①,②,③]	4.79 ± 0.55[①,②,③]
H2S + hyperthermia	78.2 ± 1.1[①,②]	2.62 ± 0.27[①,②]	3.04 ± 0.25[①,②]

[①]$P<0.05$, compared with control group. [②]$P<0.05$, compared with cardiac arrest resuscitation group. [③]$P<0.05$, compared with H2S + hyperthermia.
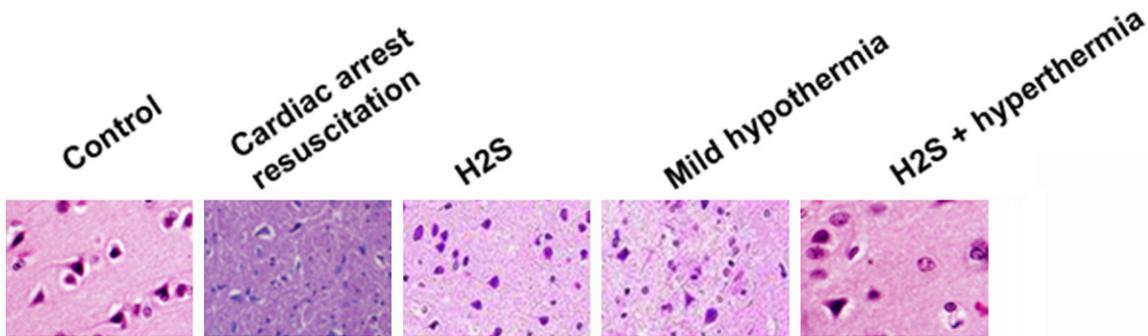
Figure 1. Comparison of pathological conditions of rat brains in each group.

Table 2. Comparison of oxidative stress levels in rats

Group	MDA (nmol/mg)	SOD (nU/mg)
Control	14.57 ± 2.16	125.64 ± 13.25
Cardiac arrest resuscitation	38.92 ± 3.47[①]	19.08 ± 11.34[①,③]
H2S	31.46 ± 3.97[①,②,③]	36.87 ± 6.09[①,②,③]
Shallow hypothermia	30.55 ± 2.31[①,②,③]	41.25 ± 7.13[①,②]
H2S + hyperthermia	21.09 ± 4.22[①,②]	102.65 ± 13.47[①,②]

[①]$P<0.05$, compared with control group. [②]$P<0.05$, compared with cardiac arrest resuscitation group. [③]$P<0.05$, compared with H2S + hyperthermia.

Western-blotting method was used to detect expression of JNK, p38MAPK, and ERK5

Brain tissue was dissected and a whole protein extraction kit was used to extract and quantify total proteins from whole cells, nucleus, and cytoplasm. The amount of whole cell, cytoplasm, and nuclear protein per well was set to 110, 110, and 50 μg, respectively. After loading, SDS-PAGE gels were electrophoresed, transferred to membranes, and blocked. They were then incubated in the appropriate primary and secondary antibodies, separately. After incubation, HRP-DAB substrate color kit was used to display luminescence. Image absorbance (IA) of the band of interest was detected using Image Pro-Plus 6.0 image analysis software. Ratio of the target band IA value/total protein β-actin IA value was taken as the final value of the target protein.

Statistical analysis

SPSS 22.0 statistical software package was used to analyze data. Measured data are expressed as mean ± standard deviation and t-test was used for comparison between the two groups. Chi-squared test was used for categorical variables. One-way analysis of variance was used to compare multiple groups. $P<0.05$ is considered statistically significant.

Results

Comparison of survival and neurological function

At 24 hours after resuscitation, the survival rate of rats in the sham-operated group was 100%. Survival rates of rats in the cardiac arrest resuscitation group, H2S group, shallow hypothermia group, and H2S + shallow hypo-

Table 3. Comparison of levels of inflammatory factors

Group	N	TNF-α	IL-18	IL-1β
Control	20	97.04 ± 18.17	768.36 ± 122.77	0.724 ± 0.018
Cardiac arrest resuscitation	20	148.03 ± 41.92[①]	1183.60 ± 150.19[①]	1.473 ± 0.087[①]
H2S	20	129.64 ± 21.65[①,②,③]	988.68 ± 117.04[①,②,③]	1.236 ± 0.093[①,②,③]
Shallow hypothermia	20	133.87 ± 25.72[①,②,③]	1020.63 ± 134.31[①,②,③]	1.142 ± 0.051[①,②,③]
H2S + hyperthermia	20	112.49 ± 23.42[①,②]	817.32 ± 111.58[①,②]	0.988 ± 0.063[①,②]

[①]$P<0.05$, compared with control group. [②]$P<0.05$, compared with cardiac arrest resuscitation group. [③]$P<0.05$, compared with H2S + hyperthermia.
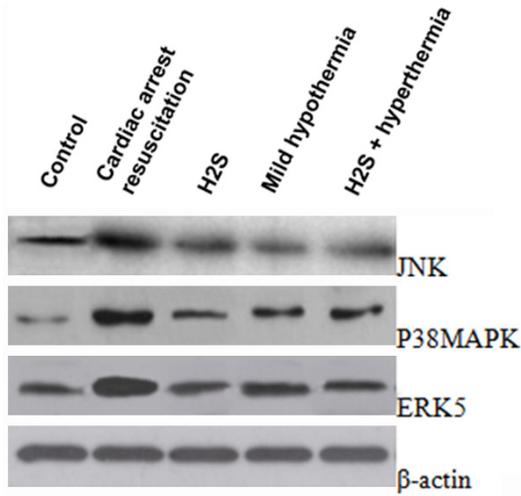
Figure 2. Comparison of JNK, p38MAPK, and ERK5 protein expression in rats.

thermia group were 45.0% (9/20), 70.0% (14/20), 60.0% (12/20), and 85.0% (17/20), respectively. Survival rates of rats in the H2S + shallow hypothermia group were significantly higher than in other model groups (P<0.05). After recovery, median NDS for each group was 80 (80, 80), 37 (33, 46), 57 (53, 65), 61 (56, 69), and 75 (70, 79). Except for the H2S + hypothermia group, NDS scores of other groups were significantly decreased. There were statistically significant differences compared with the sham-operated group (P<0.05). NDS scores in the H2S + hypothermal group were significantly higher than those in the cardiac arrest resuscitation group, H2S group, and shallow hypothermia group (P<0.05).

Comparisons of brain water content and blood-brain barrier permeability

Compared with the sham-operated group, brain water content and blood-brain barrier permeability of the other groups were significantly increased (P<0.05). Compared with the

cardiac arrest resuscitation group, brain water content and blood-brain barrier permeability of rats in the H2S group, shallow hypothermia group, and H2S + hyperthermia group were significantly decreased (P<0.05). In addition, the above indicators decreased more significantly in rats of the H2S + shallow hypothermia group (P<0.05). The above indicators in the H2S + shallow hypothermia group were significantly lower than those in the H2S group and shallow hypothermia group (P<0.05) (**Table 1**).

Comparison of pathological conditions of brain tissue in rats

According to light microscopic analysis, the sham-operated rats showed normal cerebral cortical tissue structure and some cells showed shallow edema. The cardiac arrest recovery group showed changes indicative of severe brain tissue damage, such as brain cells with severe edema, structural abnormalities, and local blood vessels with visible bleeding. The remaining groups had different degrees of brain cell edema, but the structure and the surrounding tissue boundaries were more distinct. Of these, ultrastructural changes of brain cells in the H2S + shallow hypothermia group were the slightest. Histopathological scores in experimental groups were significantly higher than those in the sham-operated group (P<0.05). Compared with the cardiac arrest resuscitation group, histopathological scores of rats in the H2S group, shallow hypothermia group, and H2S + hyperthermia group were significantly lower (P<0.05). The H2S + hypothermia group was the most significantly decreased (P<0.05; Table 1; Figure 1).

Comparison of oxidative stress levels in rats

Compared with the sham-operated group, content of MDA in the brains of the other 4 groups significantly increased while SOD significantly
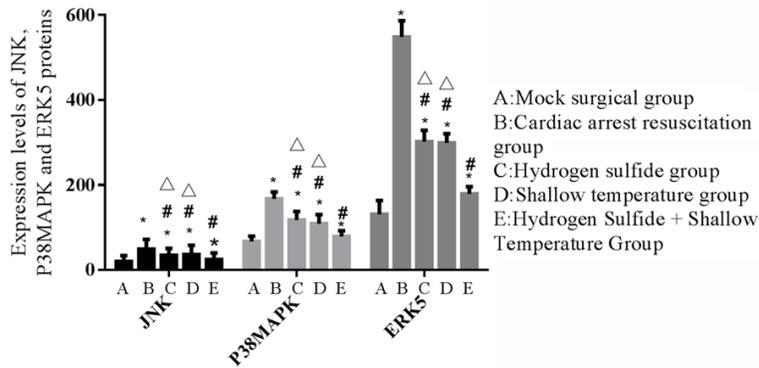
Figure 3. Comparison of expression of JNK, p38MAPK, and ERK5 proteins in different groups of rats. $^*P<0.05$, compared with control group. $^\#P<0.05$, compared with the cardiac arrest resuscitation group. $^\triangle P<0.05$, compared with H2S + hyperthermia group.

decreased ($P<0.05$). MDA content in the resuscitation group was the most significantly increased ($P<0.05$) and SOD was decreased most significantly in this group ($P<0.05$). MDA content in the H2S + shallow hypothermia group was significantly lower than the other 3 groups ($P<0.05$), while SOD was significantly higher (**Table 2**).

Comparison of levels of inflammatory factors

Compared with the sham-operated group, the content of TNF-α, IL-16, and IL-1β in brains of the other four groups increased to varying degrees. Levels of TNF-α, IL-16, and IL-1β in brain tissues of the resuscitation group were significantly increased ($P<0.05$). Levels of TNF-α, IL-16, and IL-1β in the H2S + hypothermal group were significantly lower than those in the other 3 groups ($P<0.05$; **Table 3**).

Expression of JNK, p38MAPK, and ERK5 proteins

Compared with the sham-operated group, the content of JNK, p38MAPK, and ERK5 in brains of the other four groups increased to varying degrees. Levels of JNK, p38MAPK, and ERK5 in brain tissues of the resuscitation group were significantly increased ($P<0.05$). Levels of JNK, p38MAPK, and ERK5 in the H2S + hypothermal group were significantly lower than those in the other 3 experimental groups ($P<0.05$) (**Figures 2** and **3**; **Table 4**).

Discussion

It has been reported [11] that the return of spontaneous circulation (ROSC) rate after on-

site CPR in cardiac arrest patients was less than one-third. The survival rate of patients after arriving at the hospital was less than one-fifth. After resuscitation, they often died due to other complications. Ultimately, less than 5% of patients were successfully discharged. The main cause of death in these CPR patients was ischemia-hypoxic injury of brain cells after ROSC. Therefore, determining how to maintain brain function after CPR injury has become a focus of CPR treatment research. This present study observed the effects of H2S inhalation on brain protection in resuscitated rats with cardiac arrest, investigating effects on inflammatory cytokines and p38MAPK signaling pathways in brain tissue. Results showed that H2S had obvious protective effects on cerebral tissue of rats with induced cardiac arrest. Mechanisms of protection may be related to regulating levels of TNF-α, IL-16, and IL-1β in brain tissue, inhibiting activation of p38MAPK signaling pathways, and downregulating oxidative stress levels.

Studies have shown that oxidative stress [12] and inflammatory responses [13] were key pathophysiological mechanisms leading to cerebral ischemia-reperfusion injury. In the early stage of cerebral ischemia and reperfusion after CPR, oxygen free radical production may be increased [14], leading to increased fluidity of the brain cell membrane and subcellular organelles causing widespread structural damage and impaired cell function. At the same time, cerebral ischemia-reperfusion can lead to Ca^{2+} overload and increased permeability of the cell membrane, further causing cerebral edema and aggravated hypoxic injury. As free radicals increase, they react with polyunsaturated fatty acids, causing lipid peroxidation and MDA production. Therefore, as a product of lipid peroxidation, MDA can reflect the degree of oxidative stress response [15]. An important free radical scavenger, SOD can disproportionate with superoxide anion and reduce production of free radicals [16].

p38/MAPK signaling pathways are involved in the inflammatory response of the body [17].

Table 4. Comparison of JNK, p38MAPK, and ERK5 protein expression in rats

Group	JNK	p38MAPK	ERK5
Control	20.13 ± 14.22	67.31 ± 12.36	131.44 ± 32.45
Cardiac arrest resuscitation	49.15 ± 23.07[①]	167.88 ± 15.62[①,②]	548.09 ± 38.81[①,②]
H2S	34.57 ± 16.72[①,②,③]	117.93 ± 19.91[①,②,③]	302.33 ± 26.51[①,②,③]
Shallow hypothermia	36.77 ± 21.36[①,②,③]	109.48 ± 21.36[①,②,③]	299.42 ± 21.33[①,②,③]
H2S + hyperthermia	24.91 ± 15.44[①,②]	79.33 ± 13.34[①,②]	179.35 ± 16.74[①,②]

[①]$P<0.05$, compared with control group. [②]$P<0.05$, compared with cardiac arrest resuscitation group. [③]$P<0.05$, compared with H2S + hyperthermia.

When the body is stimulated by inflammatory factors (e.g. TNF-α, IL-1β), stress response, lipopolysaccharides, and other factors, p38MAPK signaling pathways can be activated and transferred to the nucleus. This activates downstream transcription factors, such as ERK, to further increase iNOS, ICAM-1, TNF-α, IL-1β, and other inflammatory factor levels that promote the inflammatory reaction process. Studies have shown that [18] p38MAPK can be activated after CPR, aggravating ischemia-reperfusion injury of heart and brain tissue. Studies have also shown that [19, 20], in the pathological process of brain tissue damage, serum TNF-α and IL-1β can be significantly increased, inducing acute inflammatory reactions in brain tissue. Ischemia-reperfusion injury can also activate p38MAPK signaling pathways to activate the body's inflammatory cytokine network, thereby aggravating the brain tissue injury process [21].

H2S is a signaling molecule with a small molecular weight that can rapidly pass through biofilms [22]. Animal experimental studies have shown [23, 24] that administration of H2S inhalation can significantly reduce the degree of global cerebral ischemia-reperfusion injury in rats and improve neurological function. In this study, after successful resuscitation, H2S was inhaled based on hypothermia therapy. Results showed that survival rates and NDS scores of rats in the H2S + shallow hypothermia group were significantly higher than those in other groups. Brain water content and blood-brain barrier permeability were significantly lower in these rats than in other groups. Damage of the ultrastructure of the heart and brain cells was also the slightest. This suggests that the combination of H2S and shallow hypothermia had good protective effects on brain tissue of resuscitated rats after cardiac arrest, significantly

reducing brain damage and improving neurological function. These results were consistent with previous reports. However, the exact mechanisms are not yet clear.

Results of this study showed that, compared with the sham-operated group, MDA content in the other groups significantly increased and SOD significantly decreased (P<0.05). MDA content in the H2S + hypothermal group was significantly lower than that in the other 3 experimental groups (P<0.05), suggesting that H2S combined with hypothermia therapy can significantly inhibit oxidative stress response and reduce the degree of brain tissue damage.

Levels of TNF-α, IL-16, and IL-1β and JNK, p38MAPK, and ERK5 in the H2S + hypothermal group were significantly lower than those in the other 3 experimental groups (P<0.05), demonstrating that H2S combined with hypothermia therapy could significantly reduce levels of TNF-α, IL-16, and IL-1β, inhibit activation of p38MAPK signaling pathways, and reduce inflammatory damage of brain tissue.

In summary, H2S combined with hypothermia therapy led to significant improvement regarding cerebral protection in rats with cardiac arrest. Mechanisms may be related to regulating levels of TNF-α, IL-16, and IL-1β in brain tissue, inhibiting activation of p38MAPK signaling pathways, and downregulating oxidative stress levels of the body.

Disclosure of conflict of interest

None.

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