Therapeutic hypothermia combined with hydrogen sulfide treatment attenuated early blood–brain barrier disruption and brain edema induced by cardiac arrest and resuscitation in rat model

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Abstract

Brain injury remains a major problem in patients suffering cardiac arrest (CA). Disruption of the blood-brain barrier (BBB) is an important factor leading to brain injury. Therapeutic hypothermia is widely accepted to limit neurological impairment. However, the efficacy is incomplete. Hydrogen sulfide (H_2S), a signaling gas molecule, has protective effects after cerebral ischemia/reperfusion injury. This study showed that combination of therapeutic hypothermia and hydrogen sulfide after resuscitation was more beneficial for attenuated BBB disruption and brain edema than that of hypothermia or hydrogen sulfide treatment alone. CA was induced by ventricular fibrillation for 4 minutes. Therapeutic hypothermia was performed by applying alcohol and ice bags to the body surface under anesthesia. We used sodium hydrosulphide (NaHS) as the H_2S donor. Here, we found that global brain ischemia induced by CA and CPR resulted in brain edema and BBB disruption; therapeutic hypothermia or H_2S treatment diminished brain edema, decreased the permeability and preserved the structure of BBB during the early period of CA and resuscitation, and more importantly, improved the neurologic function, increased the 7-day survival rate after resuscitation; the combination of therapeutic hypothermia and H_2S treatment was more beneficial for reducing BBB permeability and brain edema than that of hypothermia or H_2S treatment alone. The beneficial effects were associated with the inhibition of matrix metalloproteinase-9 (MMP-9) expression, attenuated the degradation of the tight junction protein occludin, and subsequently protected the structure of BBB. These findings suggest that combined use of hypothermia and H_2S treatment during resuscitation of cardiac arrest patients could be a potential strategy to improve clinical outcomes and survival rate.

Introduction

Brain injury remains a major problem in patients suffering cardiac arrest [1, 2]. Despite of the advances in cardiopulmonary resuscitation (CPR) methods, the survival rate remains low, and many survivors experience long-term neurological dysfunction [3]. BBB breakdown has been documented in animals with CA, and BBB breakdown is involved in the initiation of transcriptional changes in the neurovascular network that ultimately lead to delayed neuronal dysfunction and cell death [4, 5]. BBB disruption after resuscitation is caused by structural and functional impairment of components of the neurovascular unit, and the tight junctions form a metabolic and physical barrier to restrict the paracellular permeability [6, 7]. Disruption of the structure of BBB leads to extravasation of serum albumin, other macromolecular proteins, and small molecule solutes into extracellular space, resulting in vasogenic brain edema and cell death [8, 9]. MMP-9 is reported to degrade the tight junction complex, leading to BBB leakage, vasogenic brain edema and secondary brain damage [10, 11].

Therapeutic hypothermia is widely accepted as an effective method to improve survival and limit neurological outcomes in patients who achieve return of spontaneous circulation (ROSC) after CA [12]. Models of brain ischemia and trauma have shown that mild to moderate hypothermia protects the BBB and prevents edema formation [13, 14]. Despite that, many studies have proved that a single effective

measure is difficult to achieve the expected recovery effect in clinical practice [14, 15]. Thus, the development of alternative approaches with or without therapeutic hypothermia is an unmet medical need in ameliorating the prognosis of post-CA patients. H_2S has been recognized as the third gaseous signaling molecule, with a relatively small molecular mass, which allows it to traverse the cell membrane freely without requiring a receptor [16]. H_2S has been referred as a neuromodulator and neuroprotectant in the central nervous system, produces anti-oxidant, anti-inflammatory, and anti-apoptotic effects in cerebral ischemia reperfusion injury, and in the field of CPR, the research of H_2S in cerebral resuscitation after CA has gradually increased. [5, 17–19]. Geng et al demonstrated H_2S improved the integrity of BBB, mitigated brain edema; improved neurological outcome and 14-days survival rate in rats after CA and resuscitation [5]. These findings suggest that H_2S could protect the BBB integrity after resuscitation. Therefore, this study will test the hypothesis whether the combination of therapeutic hypothermia and hydrogen sulfide after resuscitation was more beneficial for improving the survival rate and neurological outcome than that of hypothermia or hydrogen sulfide treatment alone, and examine the possible mechanisms for the effects.

Materials And Methods

Animal Preparation

Male Sprague-Dawley rats, weighing 280 to 320 g, 7-8 weeks old, were provided by the Animal Center of Jingling Hospital, Nanjing, China. All rats were housed in controlled room on a 12-h light-dark cycle and fed a standard laboratory diet. This study was approved by the Ethics Committee of Jinling Hospital and was performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health. For experiments, animals were fasted overnight except for free access to water. Animals were anesthetized with intraperitoneal injection of 2% sodium pentobarbital (50 mg/kg) and intubated tracheally with a 14-gauge cannula. Polyethylene catheters (PE-50) were inserted into the left femoral artery and vein and flushed intermittently with saline solution which containing 2.5 IU/ml bovine heparin. The arterial catheter line was connected to a pressure transducer (PT-100, Chengdu Taimeng Software Co.LTD, China) to measure mean aortic pressure (MAP) and the venous catheter was used for medical administration, and the electrocardiogram was recorded by subcutaneous needle electrodes. Core temperature was monitored by a rectal temperature probe (BAT-10, Physitemp Instruments Inc) throughout the experiment to ensure appropriate temperature management.

Cardiac arrest and cardiopulmonary resuscitation

CA and CPR in rats were performed as previously described with some slight modifications [20, 21]. After preparation and subsequent stabilization, cardiac arrest was induced using a 5F pacing catheter, inserted orally into the esophagus of the rats approximately 7 cm in depth. Continuous cardiac pacing was conducted and maintained for 1 min (frequency: 25 Hz; intensity: 25 V; stimulus duration width: 10 ms) to induce cardiac arrest. A stimulation pause was then initiated for a few seconds (1-3 seconds) to observe the change of ECG, as soon as the rhythm reverted spontaneously, an additional 30-second stimulation

was performed immediately until the ventricular fibrillation reappeared and persisted. After 4 minutes of cardiac arrest, CPR was started, including chest compressions performed by the same investigator and ventilation conducted by a volume-controlled small animal ventilator (Beijing zhong shi di chuang science and technology development Co.,LTD, China) (Respiratory parameters: oxygen concentration: 100%, ventilation frequency: 60 breaths/min, tidal volume: 6 ml/kg). After 1 minute of CPR, the animals were counter shocked with a 7-J DC current delivered to the heart through the transoesophageal cardiac pacing electrode. A dose of epinephrine (100 μg/kg) and 5% (w/v) sodium bicarbonate (1.0 ml) were injected via the femoral vein after defibrillation. Additional doses of sodium bicarbonate were administered according to arterial blood gas analysis performed at 10 and 30 minutes after return of spontaneous circulation (ROSC) (the additional required dose was calculated by the formula: bicarbonate (mmol) = (-2.3 - the actual measured value of base excess) * 0.25 * body weight (kg)). ROSC was defined as an organized cardiac rhythm with a MAP > 60 mmHg, which was sustained continuously for at least 5 minutes. If the spontaneous circulation of the rats was not restored after 10 minutes with the above treatment, CPR was considered a failure.

After ROSC, rats were mechanically ventilated and invasively monitored for 6 hours in maintaining the target temperature. Blood samples were drawn for blood gases, glucose, and lactate measurements at baseline and 10 and 30 minutes after ROSC. Rats were then weaned from the ventilator, tracheally extubated, and returned to their cages with easily accessible to food and water. The survival time after CPR was recorded up to 7 days.

Experimental Protocol

The experimental time line is presented in Figure 1. After successful resuscitation, the animals were randomized to one of the four groups: cardiac arrest and resuscitation group (CAR), sodium hydrosulfide group (H_2S), therapeutic hypothermia group (TH), sodium hydrosulfide combined with therapeutic hypothermia group (H_2S+TH). NaHS (Sigma-Aldrich, St. Louis, MO, USA) was freshly diluted in normal saline to the desired concentration (0.3 mg/ml) before administration. The NaHS was infused intravenously with an initial loading dose of 0.5 mg/kg at the start of CPR, followed by a maintenance infusion of NaHS (1.5 $mg \cdot kg^{-1} \cdot h^{-1}$) until 6 hours after ROSC. This dosage was based on a previous study with minor modification [22]. Therapeutic hypothermia was performed as follows: we initiated cooling after ROSC by applying alcohol and ice bags to the body surface under anesthesia. Rectal temperature was measured with a digital thermometer (BAT-10, Physitemp Instruments Inc) and taken as the body temperature, which was reduced to 34°C within 15 minutes of initiating reperfusion. Hypothermia was maintained for 6 hours by exposing the rat to ice bag or a heat lamp, and the distance between the rats and the lamp was adjusted to maintain the target rectal temperature. Hypothermic rats were rewarmed beginning at 6 hours after ROSC at a rate of approximately 1°C/h over 4 hours with a heat lamp until rectal temperature reached 38.0°C. Sham treated animals underwent all procedures except CA and resuscitation, received an equivalent volume of normal saline, and the rectal temperature were maintained at 38.0°C. Throughout the experiment, a total number of 222 rats were used, 5 rats died during the operation, and 17 rats died due to the failure of ROSC, and 10 rats died before the test.

Therefore, 190 rats were involved in the statistics. The whole experiment consists of two parts. In part one, arterial blood for blood gas analysis was obtained at baseline at 10 and 30 minutes after ROSC in each group, the neurological function was evaluated at 1 day, 3 day and 7 day after ROSC and their survival rate was monitored up to 7 days after ROSC (n=5 for the sham group; n=15 for each group of the other 4 groups). In part two, the brain edema, blood-brain barrier integrity, the protein expression and the BBB ultrastucture alteration were measured at 24 hours after ROSC (n=25 for each group).

Assessment of Survival Rate and Neurologic Outcome

The survival rate was monitored up to 7 days after ROSC or sham operation, and the neurological function was evaluated at 1, 3, and 7 day after ROSC or sham operation. A modified tape removal test described previously was conducted to evaluate neurologic outcome [23]. In brief, 10 mm by 12 mm adhesive tapes were affixed to each of the animal's front paws. The time to remove both adhesive tapes was recorded. The test was truncated at 180 seconds and all times > 179 seconds were recorded as '180 seconds'. Before the experiment, all animals were familiarized with the neurologic test for 3 consecutive days. All evaluations were performed by the same investigator who was masked to treatment.

Determination of Brain Water Content

Cortical and hippocampal water content was determined by wet-dry method at 24 hours after ROSC or sham operation. The left hemisphere of the brain was used for here, right cerebral hemisphere was for western blot analysis. Brain tissue was immediately divided into cortex and hippocampus after decapitation and weighed to obtain the wet weight. The tissues were slow evaporation in a laboratory oven (80°C) for 72 hours and reweighed to determine the dry weight. Brain water content (%) was calculated as (wet weight – dry weight) / (wet weight) × 100%.

Evaluation of Blood–Brain Barrier Permeability

Evans blue (EB) and fluorescein isothiocyanate–dextran (FITC-dextran) were used to assessed macromolecular proteins and small solute permeability at 24 hours after ROSC or sham operation respectively. Evans blue dye (Sigma Chemical Co., St. Louis, USA; 2% solution in saline, 2 ml/kg), which can bind to albumin (molecular weight, about 68 kDa) and FITC–dextran (Sigma Chemical Co., St. Louis, USA; 5% solution in saline, 2 ml/kg) (average molecular weight, 4 kDa) were administered intravenously and allowed to circulate for 30 or 2 minutes, respectively. Rats were then administered transcardial saline to remove intravascular EB or FITC-dextran. The brains were removed and rinsed with phosphate - buffered saline, and two 4-mm wide coronal slices (1.8 mm anterior to the bregma to 6.2 mm posterior to the bregma) were made. The cerebral cortex above the rhinal fissure from the first slice and the hippocampus from the second slice were dissected as shown according to previous studies (Hoffmann *et al.*, 2011). After weighing, the cortex and hippocampus were homogenized in 50% trichloroacetic acid. For EB measurement, samples were centrifuged at 21,000 g for 20 minutes. The supernatant was collected, and evans blue per weight of sample was measured at 620 nm with a spectrophotometer, and quantified by a series of standard EB solution (100-1000 ng/mL). For FITC-

dextran measurement, samples were centrifuged at 10,000 g for 20 minutes, the supernatant was collected, and FITC–dextran fluorescence (ng/mL) was measured at 538 nm using 485 nm excitation (PerSeptive Biosystems, USA). Total fluorescence of each sample was calculated from concentrations of external standards (100-8000 ng/mL) and presented as percentage of change from the sham group.

Ultrastructure alteration of BBB

BBB ultrastructure alterations were observed by a transmission electron microscopy. The rats were decapitated at 24 hour after reperfusion, the hippocampus were cut into 1 mm^3, fixed in 1% freshly made paraformaldehyde and 2% glutaraldehyde for 24 hours at 4 °C, washed with 0.1 mol/L phosphate buffer for 3 times. Then the samples were post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 2 hours at 4 °C. After fixation, the samples were dehydrated in grade acetone and embedded in Epon 812. The ultra-thin sections of hippocampus were stained with uranium acetate and lead citrate and then examined with a transmission electron microscope (JEM 1230, JEOL, Japan).

Western blot analysis

Western blot analysis was used to assess expression of tight junction occludin in the cortex and hippocampus, the samples were harvested at 24 hour after ROSC, frozen in liquid nitrogen and stored at −80°C for western blot analysis. Protein homogenates of samples were prepared by rapid homogenization in Tissue Extraction Reagent II (Invitrogen Corporation, Carlsbad, USA), according to the manufacturer's instructions. After homogenization, tissue samples were centrifuged at 15 000 g for 20 minutes at 4°C. Protein concentration was determined using a BCA protein assay kit (Bio-Rad, Hercules, USA). Proteins (30 μg) were electrophoresed on 12% Tris-glycine gels, and then transferred onto polyvinylidene difluoride membranes. Membranes were incubated with primary anti-occludin (1:1000, Abcam, Cambridge, USA), anti-β-actin (1:2000, Abcam, Cambridge, USA) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000, Cell Signaling Technology, USA). Protein bands were visualized with an enhanced luminescence reagent (Millipore) and photographed with ChemiDoc XRS+ (Bio-Rad, Hercules, USA). Final results were normalized to β-actin and expressed as the ratios of target proteins/β-actin.

Immunohistochemical procedures

Immunohistsochemical analyses were performed as previously described [24]. Rats were perfused through the left ventricle of the heart with phosphate - buffered saline and then with 4% paraformaldehyde in 0.01 mol/L phosphate - buffered saline. Hippocampus were fixed in 4% paraformaldehyde and embedded in paraffin wax prior to sectioning. The fixed brains were immersed in 20% sucrose in phosphate - buffered saline overnight, then tissues were sectioned at 4-μm thickness. After antigen retrieval treatment and 5% BSA blocking, sections were incubated overnight at 4°C with the primary antibody anti-MMP-9 (1:200, Abcam, Cambridge, USA), followed by rabbit anti-rat IgG-HRP antibody (1:100, Cell Signaling Technology, USA) at room temperature for 2 hours. Thereafter, the sections were incubated with streptavidin-peroxidase (Fuzhou Maixin Biotech Co. Ltd., China), and

visualized with diaminobenzidine stain. For each tissue, four fields (×400) were selected for each section, and the number of positive cells was counted. The average positive cells of each slice were obtained by dividing the sum of the positive cells counted from each field by four. The average of each sample was used for statistical analysis.

Statistical Analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL) were used for statistical analyses. Survival was expressed as a percentage and the Kaplan-Meier survival curves were compared using log-rank testing, and we used a Bonferroni correction for multiple comparisons. Date from the tape removal test were presented as the median (quartiles); and analyzed using the Kruskal-Wallis test, a Nemenyi test was performed when the overall P value was significant. The data of therapies during CPR and the ROSC rate and data of physiological variables were represented as mean ± SD, Other data were presented as the mean ± SEM. Normal distribution data were confirmed using the Kolmogorov-Smirnov test, and analyzed by one-way analysis of variance (ANOVA) or ANOVA for repeated measures followed by Bonferroni test for intergroup comparisons. The Bonferroni-adjusted P value was defined such that the raw P value multiplies the number of comparisons. $P < 0.05$ was considered statistically significant.

Results

Physiological Parameters and Therapies During Cardiopulmonary Resuscitation

At baseline, physiological parameters including arterial blood gas samples, heart rate, blood pressure and core body temperature were in the normal range, there were no differences between the groups (Supplement Figure 1, Supplement Table 2). During induction of CA, no significant differences were observed with regard to the duration of CPR time, the number of defibrillations, the dose of adrenaline or the rate of successful resuscitation between the groups (Supplement Table 1).

Compared to the baseline in each group, blood gas variables analyzed at 10 and 30 minutes after resuscitation revealed severe changes in all groups: a severe degree of acidemia occurred, the pH and negative BE were significantly lower after successful resuscitation. The heart rate, blood pressure both decreased slightly after resuscitation, furthermore, the blood glucose increased after resuscitation. There were no significant differences in arterial pH, PaO_2, $PaCO_2$, base excess, hematocrit, glucose, blood pressure among the groups. Heart rate and lactate in the hypothermia groups were lower than in the normothermia groups after resuscitation (Supplement Table 2).

Time Course of Rectal Temperature

During CA and CPR, rectal temperature in rats was maintained at 38°C. In the TH and H_2S+TH groups, rectal temperature was rapidly decreased to 34°C within 15 minutes with surface cooling. Subsequently, temperature was well maintained for 6 hours. In the CAR and H_2S groups, rectal temperature was

maintained at 38°C during the experiment. During the period of rewarming, temperature was increased at approximately 1°C per hour (Supplement Figure 1).

Hypothermia and Hydrogen Sulfide Improved the Survival Rate

The survival rate 7 days after ROSC was 100% (5/5) in the sham group, 40% (6/15) in the CAR group, 60% (9/15) in the H_2S group, 67% (10/15) in the TH group, and 80% (12/15) in the H_2S+TH group (Fig. 2). Compared with group CAR, the survival rate was significantly increased in the H_2S group, TH group and H_2S+TH group ($P < 0.05$). Compared with H_2S group and TH group, the survival rate was increased in the H_2S+TH group ($P < 0.05$). The results indicated that the effect of combined of hypothermia and hydrogen sulfide treatment on the survival rate after CPR was more beneficial than the individual treatment.

Hypothermia and Hydrogen Sulfide Improved the Neurological Function

On all testing days up to 7 days after ROSC, resuscitated animals needed significantly more time to remove the tapes when compared to sham-treated animals (Figure 2). All resuscitated animals remained severely impaired at day 1 after ROSC, the time for all animals removed the adhesive tapes were near 180 s. Compared with group CAR, the time needed on day 3 was decreased in the H_2S group, TH group and H_2S+TH group ($P < 0.05$). Test on day 7 also revealed a significantly better performance of the group treated with hypothermia and (or) NaHS. Moreover, the time needed in the group H_2S+TH was shorter than the group H_2S ($P < 0.05$) and group TH ($P < 0.05$). The results indicated that neurological function was improved by treatment with hydrogen sulfide or hypothermia during CPR in post-CA rats. Combine treatment hydrogen sulfide with hypothermia had additive effect on neurological function after ROSC.

Hypothermia and Hydrogen Sulfide Diminished Brain Edema

The water content of cortex and hippocampus was measured at 24 hours after ROSC or sham operation. Compared with sham group, the water content was significantly increased in the CAR group, H_2S group, TH group and H_2S+TH group ($P < 0.05$). Compared with group CAR, the water content of cortex and hippocampus were markedly reduced in the H_2S group, TH group and H_2S+TH group ($P < 0.05$), and water content in the H_2S+TH group was less than the groupH_2S ($P < 0.05$) and group TH ($P < 0.05$). These results indicated that NaHS treatment plus hypothermia had additive effect on ameliorate brain edema after CA and CPR (Figure 3).

Hypothermia and Hydrogen Sulfide Decreased the Blood–Brain Barrier Permeability

The blood-brain barrier permeability was evaluated by evans blue and fluorescein isothiocyanate–dextran extravasation at 24 hours after ROSC or sham operation. Compared with the sham group, evans blue extravasation in the whole brain was significantly increased the CAR group, H_2S group, TH group and H_2S+TH group ($P < 0.05$). In contrast, compared with group CAR, evans blue extravasation in the whole brain was markedly reduced in the H_2S group, TH group and H_2S+TH group ($P < 0.05$), and evans blue

extravasation in the H_2S+TH group was less than the group H_2S ($P < 0.05$) and group TH ($P < 0.05$). (Figure 3).

Similar to evans blue extravasation, fluorescein isothiocyanate–dextran extravasation in the cortical and hippocampal in the normothermia plus saline treatment was significantly greater than in the sham group ($P < 0.05$), and was diminished by treatment of hypothermia and (or) hydrogen sulfide ($P < 0.05$) (Figure 3).

Hypothermia and Hydrogen Sulfide Reduced the Degradation of Occludin

To further evaluate BBB disruption following cardiac arrest, we assessed the loss of tight junction proteins in the cortex and hippocampus. Compared to sham group, the levels of occludin in the cortex and hippocampus at 24 h after ROSC were significantly decreased in the CAR group, H_2S group, TH group and H_2S+TH group ($P < 0.05$). Moreover, the loss of occludin in the groupH_2S+TH was much less than the groupH_2S ($P < 0.05$) or group TH ($P < 0.05$) (Figure 4).

Hypothermia and Hydrogen Sulfide Suppressed the Matrix Metalloproteinase-9 Expression

To elucidate the possible molecular mechanisms underlying the BBB disruption that occured after resuscitation, we researched the MMP-9 expression in the hippocampus obtained at 24 hours after resuscitation. Compared to sham group, the expression of MMP-9 was significantly up-regulated in CAR, H_2S, TH and H_2S+TH groups. Notably, compared with H_2S and TH groups, H_2S+TH group had fewer positive cells (Figure 5).

Effect of Hypothermia and Hydrogen Sulfide on Ultrastructure Alteration of BBB

The ultrastructure of BBB in sham operated group was normal, as shown in Figure 6. Of notice, the base membrane of BBB was disrupted and the tight junctions were unclear in CAR group. Whereas, in H_2S and TH groups, continuous base membrane and tight junctions were observed although the electron density of basement membrane was low, indicating a restoration of the BBB integrity. Remarkably, the combination of hypothermia and hydrogen sulfide resulted in a better structure.

Discussion

The aim of the present study was to test the hypothesis whether sodium hydrosulfide treatment amplifies the effects of therapeutic hypothermia in regard to the BBB permeability and brain edema induced by cardiac arrest and resuscitation. The key findings were that: global brain ischemia induced by CA and CPR resulted in brain edema and BBB disruption; therapeutic hypothermia or sodium hydrosulfide treatment diminished brain edema, decreased the permeability and preserved the structure of BBB during the early period of CA and resuscitation, and more importantly, improved the neurologic function, increased the 7-day survival rate after resuscitation; the combination of therapeutic hypothermia and

sodium hydrosulfide treatment was more beneficial for reducing BBB permeability and brain edema than that of hypothermia or hydrogen sulfide treatment alone.

Sudden cardiac arrest is an important cause of death worldwide, many individuals who survive CA experience long-term disabilities [1, 3]. Ventricular fibrillation induced cardiac arrest is a well characterized model to research the physiopathologic mechanism after CA and CPR, and the model is closer to clinical cardiac arrest types, and the recovery rate is high. In our experiment, the physiological parameters demonstrated that we successfully produced a rat model of CA, and the overall successful recovery rate was about 85%. Hypothermia has been used to limit brain injury in certain clinical settings and animal models of brain insult, and recommended by international guidelines for CA survivors [1]. Up to now, the clinical efficacy of hypothermia in improving neurological outcome is incomplete, in part, by the delay in instituting cooling and access to equipment. The therapeutic time window is an important factor for the effects of cooling. In models of focal cerebral ischemia, protection is only observed if it is initiated within 1–2 hours of insult onset [25]. In view of the reasons, it is likely to be feasible to combine hypothermia with other neuroprotectants to extend the therapeutic window and amplify the effects of hypothermia. In a rat model of cerebral ischemia using Pulsinelli 4-vessel occlusion, our group demonstrated that combination of mild hypothermia and sodium hydrosulfide treatment for resuscitation following ischemia-reperfusion injury was more beneficial for improving neuron survival than that of hypothermia or hydrogen sulfide treatment alone [26, 27]. Now, in this experiment, we found that injection of sodium hydrosulfide or implemented with therapeutic hypothermia both improved the survival rate and neurological function after CA and CPR.

The restoration of blood flow after ischemia can lead to secondary injuries including brain edema and disruption of BBB. Disruption of the BBB after resuscitation has been shown one of the major factors leading to brain damage [5, 9]. A study of ischemic stroke found that patients with BBB disruption had a significantly reduced chance of major neurologic improvements and significantly higher risks of mortality and complications after endovascular therapy [28]. An inevitable consequence of BBB disruption is the increase in the permeability of the BBB and the subsequently formation of brain edema [4]. Disruption of the BBB is not an “all-or- nothing” phenomenon, different sizes of exogenous tracers have been used to estimate the magnitude of the BBB opening. In our study, BBB permeability was evaluated by measuring the Evans blue and Fluorescein isothiocyanate (FITC)–dextran content extravasated in the brain. Evans blue dye, which binds to albumin (molecular weight, ≈ 68 kDa), is an imaging marker for protein permeability, and the extravasation of FITC-dextran (4 kDa) was used to test both solute and ion permeability. In our model, we found global brain ischemia increased the permeability of BBB at 24 hours after resuscitation, which was similar to other studies [5, 29]. Both therapeutic hypothermia and sodium hydrosulfide treatment decreased the EB and FITC-dextran penetration significantly. The formation of brain edema induced by cerebral ischemia has been fully demonstrated. With the permeability of BBB increased, the proteins and solutes and ions permeated into extracellular spaces, and leading to vasogenic edema, thereby increased intracranial pressure, reduced the perfusion pressure, ultimately, exacerbated the ischemic state. Similarly, severe brain edema was observed in the cortex and hippocampus at 24 hours after resuscitation, and both therapeutic hypothermia and sodium hydrosulfide

treatment lightened the water content. Here, we also demonstrated that ischemia impaired the ultrastructure of BBB by electron microscopy, and both therapeutic hypothermia and sodium hydrosulfide treatment restored the integrity of BBB. These results indicate the efficiency of therapeutic hypothermia and sodium hydrosulfide as a therapeutic strategy for brain edema and BBB disruption in a global cerebral ischemia and reperfusion rat model.

The neuroprotective effects of cooling following cerebral ischemia including affect pathways leading to excitotoxicity, free radical production, inflammation and apoptosis, as well as blood flow, metabolism, furthermore, hypothermia could protect BBB integrity by reducing the extracellular protease expression and activity, stabilizing the biological membrane [12, 29]. Some of the pathways have also been shown to exert the neuroprotective actions mediated by hydrogen sulfide [16, 17]. As the third novel gasotransmitter, H_2S can permeate cell membranes freely without specific transporters. It modulates neurotransmission and synaptic activity, and show neuroprotective effects in models of brain injury.[17] H_2S treatment may be a feasible approach for extending the therapeutic window for hypothermia treatment. The BBB breakdown after ischemia is caused by structural and functional impairment of components of the neurovascular unit, including tight-junction proteins, basement membrane, endothelial cells, astrocytes, pericytes and neurons [4]. Tight-junction protein degradation is a crucial step in ischemic BBB breakdown, and occludin was the first integral transmembrane protein identified that localized to tight junction composition, the level of occludin protein decreased will lead to increased permeability of BBB [4, 30]. The expression of MMP-9 is a well-established destructive mediator of BBB disruption in cerebral ischemia, and MMP-9 has been shown to degrade several tight junction proteins that make up the BBB, leading to edema formation [7]. Models of brain ischemia have shown mild to moderate hypothermia protects the BBB and prevents edema formation, specifically, inhibits the expression of proteases and prevents the activation of MMPs. Lee et al reported that, using a middle cerebral artery occlusion rat model, mild hypothermia (33˚C) immediately after the onset of ischemia, attenuates BBB disruption, decreases MMP expression, and suppresses MMP activity [18]. Increasingly, more studies indicate that H_2S plays an important role in the regulation of MMP-9. Wang et al found that, H_2S protected BBB integrity following experimental stroke possibly by suppress the expression of MMP-9[31]. Geng et al showed that inhalation of 80-ppm H_2S immediately after CPR attenuated BBB permeability and brain edema, and the benefits could be associated with suppression of MMP-9 expression [5]. Similar to these studies, our study showed that the expression of MMP-9 was upregulated after brain ischemia, the degradation of occludin was serious, importantly, both hypothermia and hydrogen sulfide treatment decreased the MMP-9 expression, normalized the expression of occludin, more importantly, the combination of hypothermia and hydrogen sulfide showed the expression of MMP-9 was least, and occludin increased the most. These results indicated that the protective effect on BBB integrity of hypothermia and hydrogen sulfide may rely on the inhibition of MMP-9 and preservation of the occludin of BBB. It is likely that the same and different mechanisms involved in hypothermia and H_2S neuroprotection may be effective at different times after global cerebral ischemia and, therefore, add to the profound effect that has been observed.

The optimal target temperature, timing and duration are important factors for the effects of hypothermia. In our current study, we selected the rectal temperature (34˚C) as target temperature, and chose a 6 hours hypothermic period induced within 15 minutes of initiating reperfusion. Slow rewarming is also considered important in avoiding harmful systemic responses, the optimal rate of warming is not clear. Here, we chose the rate as approximately 1˚C/h over 4 hours. H_2S salts, sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S) are often used as donors in researches. Study is generally considered that therapeutic range of H_2S is relatively narrow, a lower concentration of H_2S exerts protective effect while higher levels of H_2S exposure lead to damage effect. In our earlier study, we injected NaHS intraperitoneal, and using a methylene blue colorimetric assay, we found the concentration of H_2S in the hippocampus tissue was 1.7-fold higher than that of ischemia-reperfusion control group [26]. Here, we selected a bolus injection of NaHS (0.5 mg/kg) at the beginning of CPR, followed by a continuous infusion of NaHS (1.5 $mg \cdot kg^{-1} \cdot h^{-1}$) for 6 hours based on a previous study with minor modification. Our results were consistent with which Kida et al reported, mice received Na_2S (0.55 mg/kg) before CPR improved the neurological function and 10-day survival rate [32]. However, Knapp et al sought to evaluate the impact of Na_2S on core body temperature and neurological outcome after CA in rats, they found that, after 6 min of global cerebral ischemia, a bolus of Na_2S (0.5 mg/kg) 1 min before the beginning of CPR, followed by a continuous infusion of Na_2S (1 $mg \cdot kg^{-1} \cdot h^{-1}$) for 6 h, sulfide therapy was associated with only a short term beneficial effect on neurological outcome, furthermore, sulfide had been shown to have no additive effect of the spontaneous hypothermic reaction after CA [2]. In their experiment, CA seems to trigger a spontaneous hypothermic response in rats, and we should exclude the confounding effect of spontaneous hypothermia in the experiment. In a mouse model of cardiac arrest, Shin Nakayama et al. demonstrated that post-CPR treatment with NaHS exerted neuroprotection in mice while maintaining a normal cranial temperature, indicating that NaHS-related neuroprotection is independent of the known protective effect of spontaneous hypothermia [33]. In contrast, we investigated whether sodium hydrosulfide treatment amplifies the effects of deliberate hypothermia in regard to the neurological function and survival, and blood-brain barrier disruption and brain edema induced by cardiac arrest and resuscitation.

There were also some limitations in this study. Firstly, the results were obtained in healthy rats, however, many of the patients suffering CA usually have underlying diseases. Secondly, due to the cooling technical reasons, the experimental procedure could not be blinded, but samples analysis and neurological function evaluation were performed by blinded. Thirdly, in this experiment, we used pure oxygen during resuscitation, but high concentration of oxygen inhalation may lead to a large number of reactive oxygen species, and may further damage the neurons. Reducing the concentration of oxygen inhalation or adjusting the oxygen concentration according to the oxygen saturation may be more feasible.

Conclusion

This study showed that combination of therapeutic hypothermia and H_2S after resuscitation was more beneficial for attenuated BBB disruption and brain edema, and improved neurologic function and 7-day survival rate than that of hypothermia or hydrogen sulfide treatment alone. The protective effects were associated with decreased the expression of MMP-9, and preserved of the tight junction protein occludin. The finding suggested that combined use of hypothermia and H_2S treatment during resuscitation of cardiac arrest patients could be a potential strategy to improve clinical outcomes and survival rate.

Declarations

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Author Contributions CSQ and DML designed all the experiments and revised the paper; CSQ, LQ, ZH and CLB performed the experiments; and CSQ and FJJ wrote the manuscript. All authors read and approved the final manuscript.

Data Availability The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics Statement The present study was approved by the Research Ethics Committee of Jinling Hospital, School of Medicine, Nanjing University (Nanjing, China).

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Figures

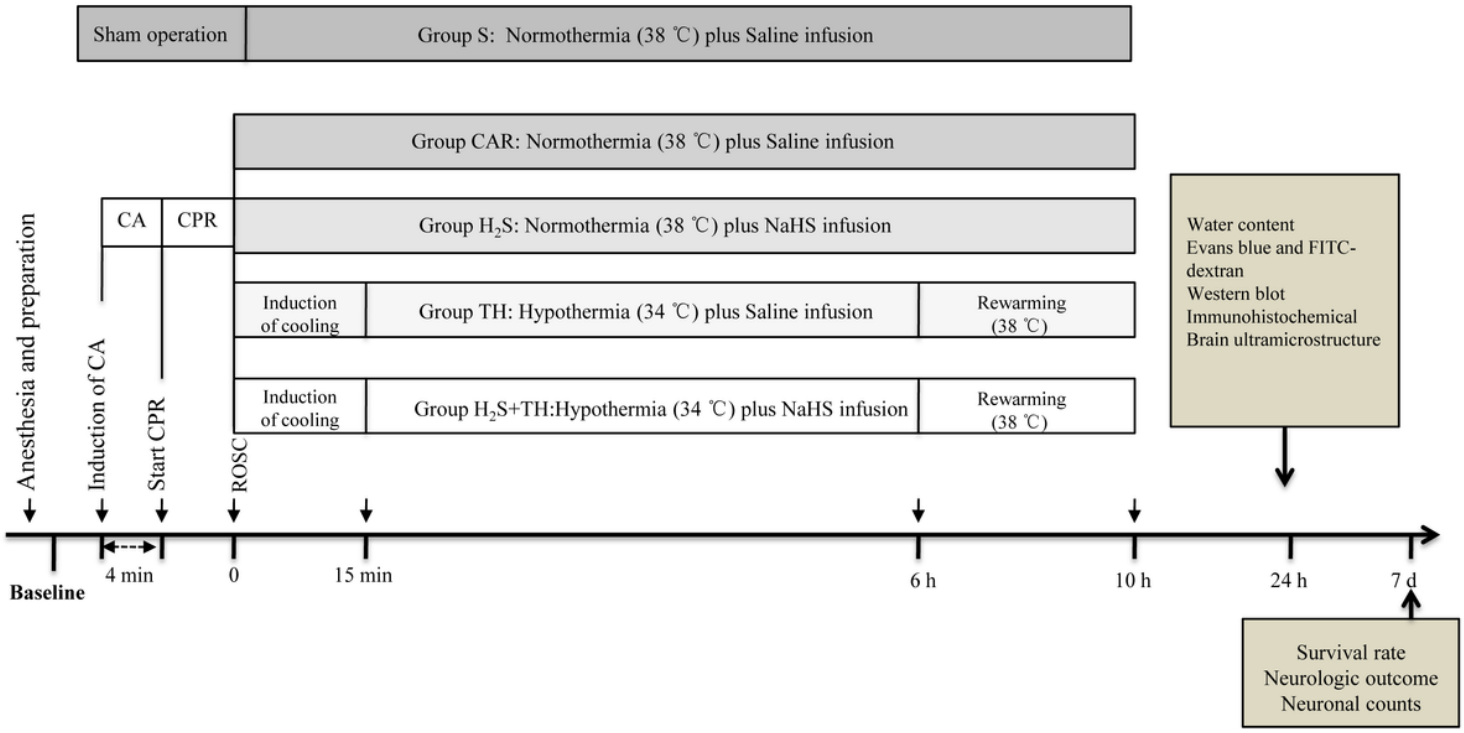

Figure 1

Experimental time line. Rats were subjected to cardiac arrest induced by electrical stimulation or sham operation. After four minutes of CA, rats were resuscitated by chest compression and mechanical ventilation. After successful return of spontaneous circulation (ROSC), rats were randomized to one of four groups: Group S, Group CAR, Group H2S, Group TH and Group H2S+TH.

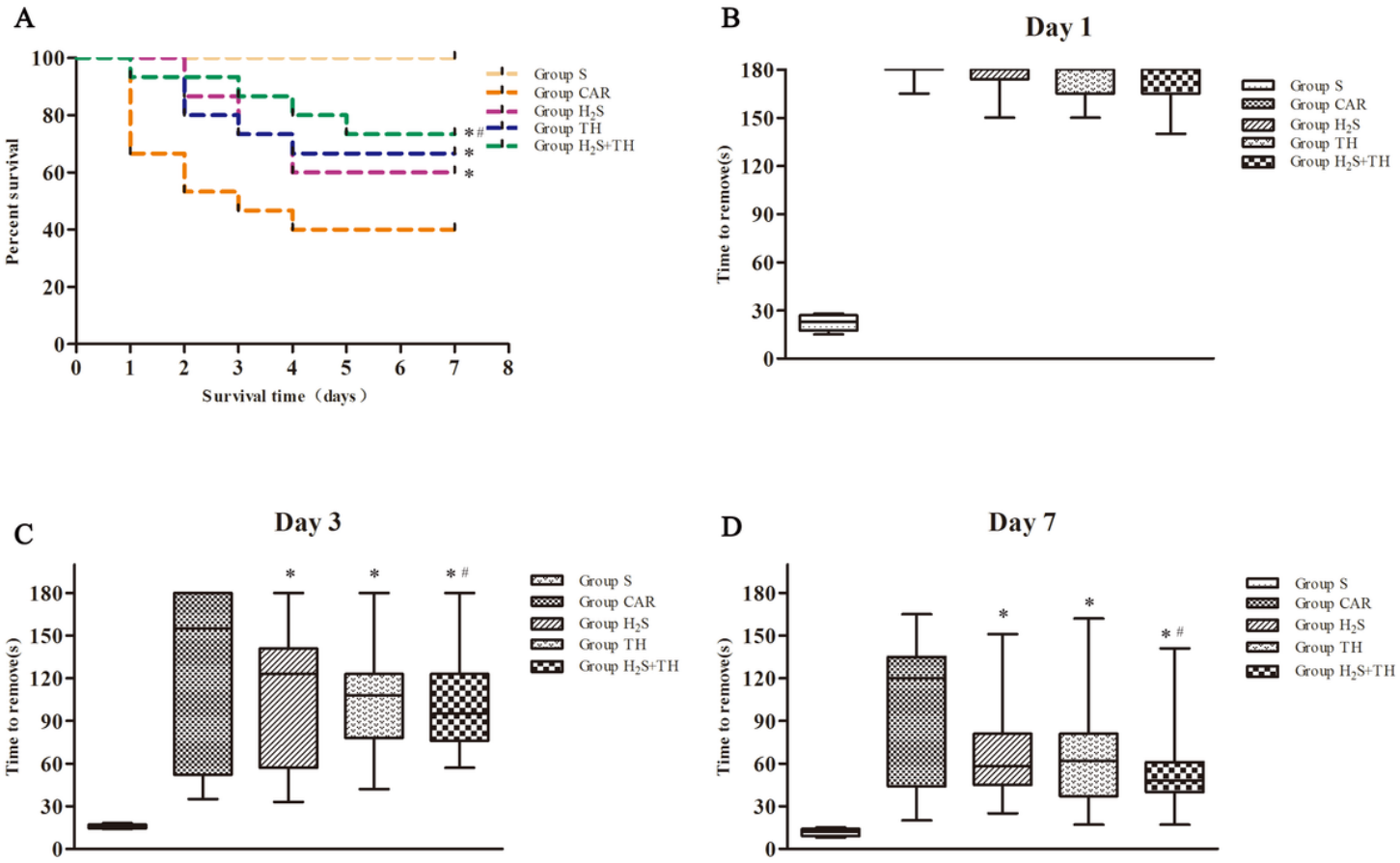

Figure 2

Effects of hypothermia and hydrogen sulfide treatment on survival rates and neurological function of rats with cardiac arrest and resuscitation. (A) Kaplan–Meier plot of cumulative survival 7 days after cardiac arrest and resuscitation in Group S (n=5), Group CAR(n=15), Group H2S (n =15), Group TH (n=15), and Group H2S+TH (n=15). Data was presented as a percentage. (B-D) Time needed in the tape removal test at day 1, day 3, and day 7 after resuscitation. Date was presented as median (quartiles). *$P < 0.05$ versus Group CAR, #$P < 0.05$ versus Group H2S or Group TH.

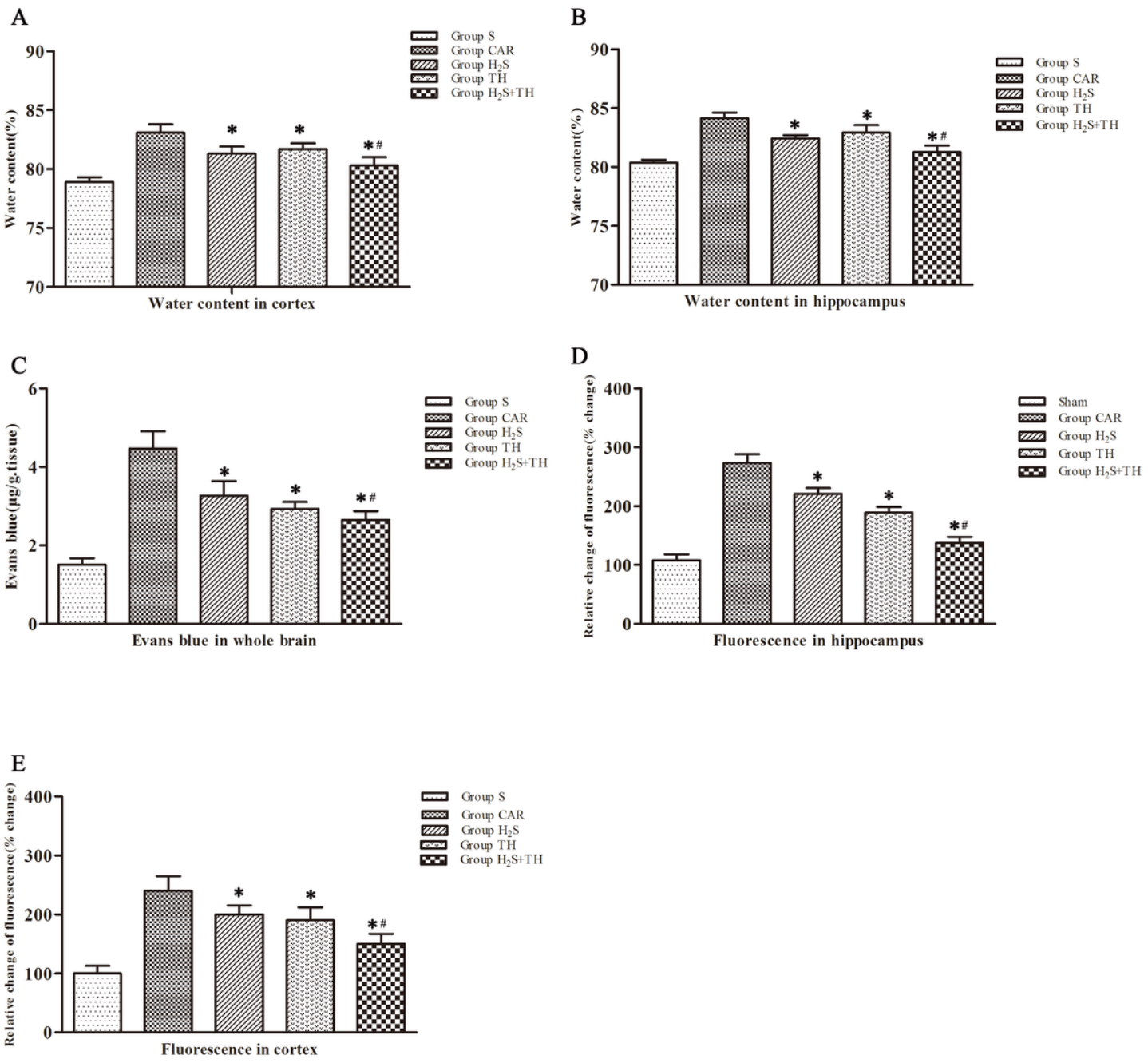

Figure 3

Effects of hypothermia and hydrogen sulfide treatment on brain edema and blood-brain barrier integrity of rats with cardiac arrest and resuscitation. (A, B) Brain water content in the cortex and hippocampus. Brain water content, an indicator of brain edema, was measured with wet-dry method at 24 hours after resuscitation or sham operation (n = 5 rats per group). (C-E)Evans blue and FITC–dextran permeability in the brain. Blood–brain barrier permeability was evaluated using Evans blue in the whole brain, and FITC–dextran permeability in the cortex and hippocampus at 24 hours after resuscitation or sham operation (n = 5 rats per group). Data are presented as mean ± SEM.

*P < 0.05 versus Group CAR, #P < 0.05 versus Group H2S or Group TH.

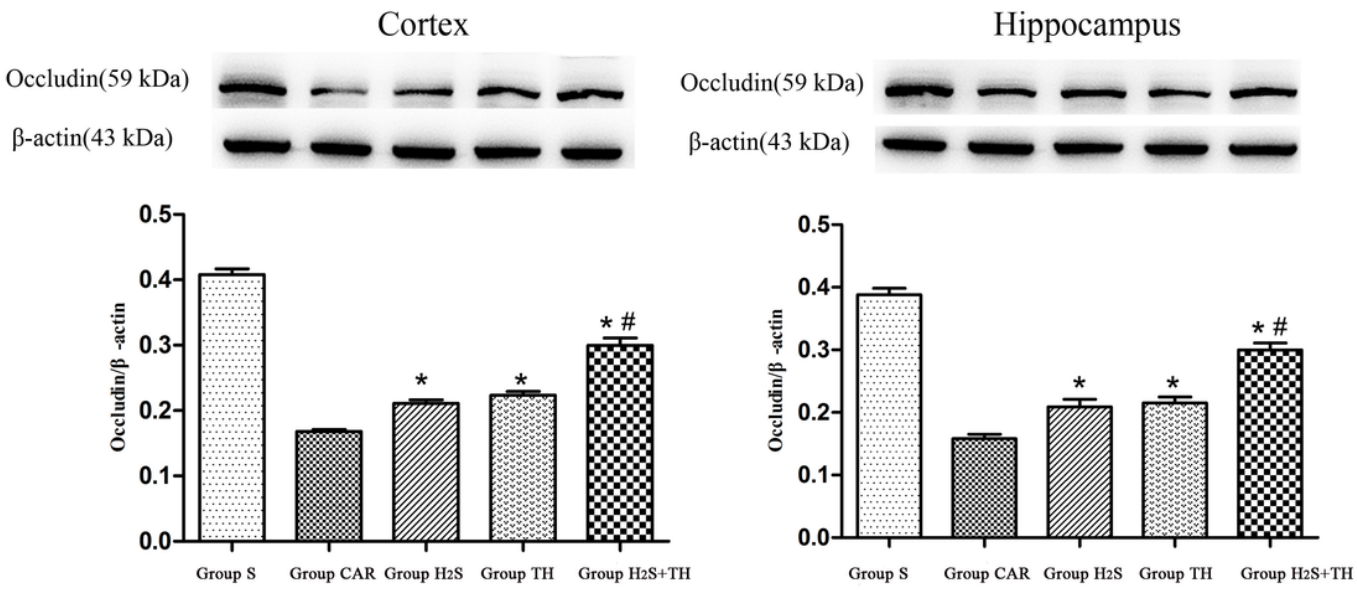

Figure 4

The expression of occludin protein in the cortex and hippocampus at 24 hours after resuscitation among groups (n = 5 rats per group). (A, B) Representative western blot images of occludin. (C-D) The bars of semi-quantitative. Results are expressed as the ratio of occludin and β-actin among groups. Data are presented as mean ± SEM.

*P < 0.05 versus Group CAR, #P < 0.05 versus Group H2S or Group TH.

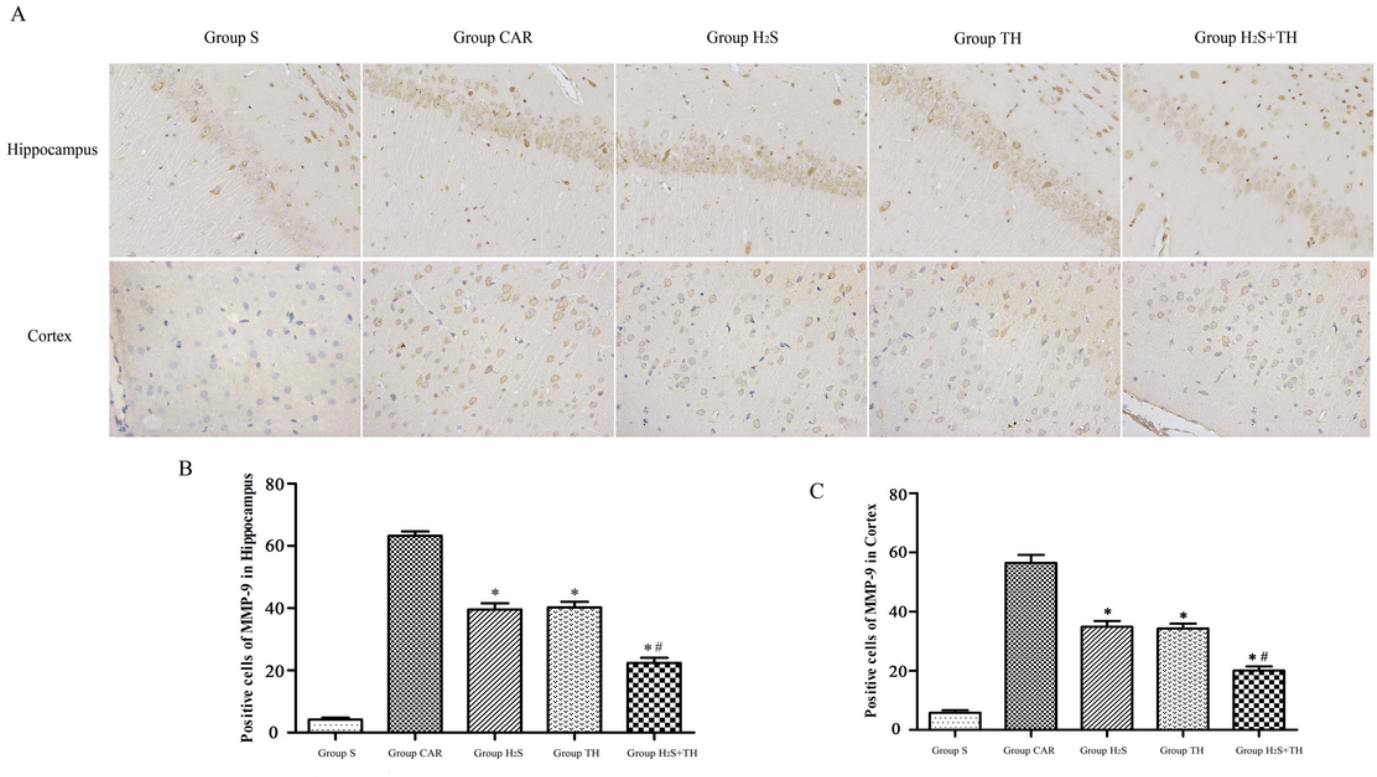

Figure 5

MMP-9 immunohistochemical staining and positive cell counts in the cortex and hippocampus in rats at 24 hours after resuscitation (n = 5 rats per group). (A, B) MMP-9 immunohistochemical staining in the cortex and hippocampus, respectively. (C, D) MMP-9 positive cell counts in the prefrontal cortex and the cornu ammonis (CA-1) area of the hippocampus. Data are presented as mean ± SEM. *P < 0.05 versus Group CAR, #P< 0.05 versus Group H2S or Group TH.

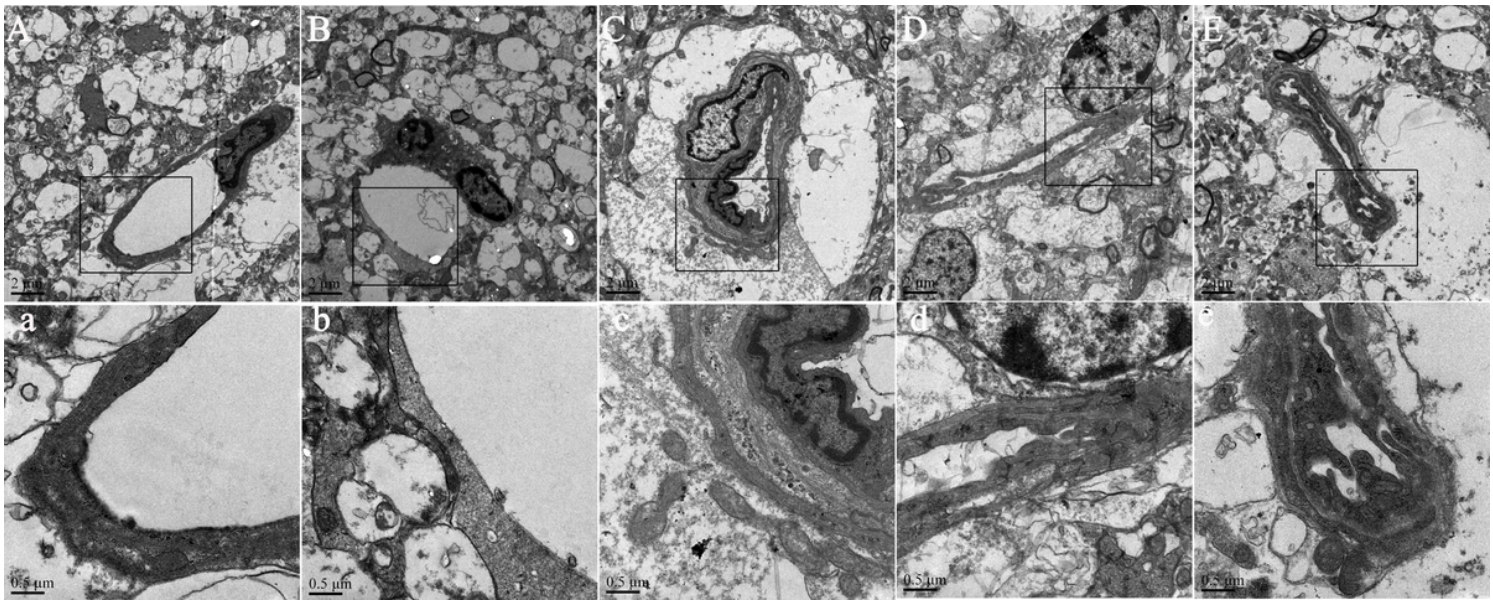

Figure 6

Ultrastructure alteration of blood-brain barrier in the hippocampus of rats at 24 hours after resuscitation or sham operation. The representative transmission electron micrographs of BBB are displayed, the micrographs in a-e are the high magnification of the area inside the boxes in A-E, respectively. (A)Group S, (B) Group CAR, (C) Group H2S, (D) Group TH, (E) Group H2S+TH.

Scale bar = 2 μm (A-E) or 0.5μm (a-e).

Supplementary Files

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