의학 박사 학위 논문

백서 뇌 허혈-재관류 손상 모델에서 황화수소의 보호효과에 대한 연구

Protective Effect of Hydrogen Sulfide in Cerebral Ischemia-Reperfusion Injury Model of Rat

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Abstract

Background: Hydrogen sulfide (H_2S) has been found to be a potent vasodilator and has a protective role in the ischemia-reperfusion injury of several organs, including heart, liver, and kidneys. In the present study, we investigated the protective effect of H_2S in a rodent cerebral ischemia-reperfusion injury model.

Methods: The ischemia-reperfusion injury model was induced by intraluminal middle cerebral artery occlusion for 120 minutes followed by reperfusion. Either sodium hydrosulfide (NaHS, 1 mg/kg, 5 mg/kg and 10 mg/kg), the donor of H_2S, or normal saline (1mL) was slowly injected via tail vein immediately after reperfusion.

Results: The injection of NaHS significantly reduced the infarct size relative to contralateral hemisphere (5 mg/kg, 43 ± 10% vs. Vehicle, 58 ± 11%; $p < 0.05$). However, high dose of NaHS (10 mg/kg) did not reduce infarct volume. NaHS increased the expression of Nrf2, an important molecule in antioxidative responses, thereby attenuating oxidative stress following cerebral ischemia-reperfusion. Immunofluorescence staining showed that the administration of H_2S reduced inflammatory cell infiltration in the peri-infarct area. ELISA of multiple cytokines showed that TNF-α levels at 3-hour and IL-6 levels at 48 hours following reperfusion were significantly higher in the NaHS group compared to the vehicle group. Further, NaHS treatment decreased TUNEL positive cells and the expression of cleaved caspase3, suggesting that H_2S reduces apoptotic cell death.

Conclusion: In the present study, we showed that administration of NaHS, the

donor of H_2S, protects the brain from ischemia-reperfusion injury. This effect may be related to the antioxidative and anti-apoptotic effects of H_2S. However, anti-inflammatory effect of H_2S in the cerebral ischemia-reperfusion injury model is uncertain in the present study.

Keywords

Hydrogen sulfide, Antioxidants, Apoptosis, Brain Ischemia, Inflammation, Focal Ischemia

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Introduction and background

Hydrogen sulfide (H_2S) is an endogenously produced gaseous signaling molecule. Production of H_2S in mammalian systems has been attributed to two key enzymes in the cysteine biosynthesis pathway: cystathionine β-synthase and cystathionine γ-lyase (1). A landmark study using cystathionine γ-lyase knockout mice clearly demonstrated that H_2S is a physiologic vasodilator and regulator of blood pressure (2). Afterwards, a number of studies have reported that, in the physiological concentrations, H_2S is cytoprotective in various models of cellular injury (3, 4). The cytoprotective effects of H_2S have been reportedly associated with its ability to mitigate oxidative stress, to inhibit leukocyte-endothelial cell interactions, to promote vascular smooth muscle relaxation, to reduce proapoptotic signaling, and to preserve mitochondrial function (5). In relation to cardiovascular disease, the administration of H_2S at the time of reperfusion decreased the infarct size and preserved left ventricular function in an *in vivo* model of myocardial ischemia-reperfusion injury (6-9). In those studies, attenuating oxidative stress has been suggested as a principal mechanism of protective effect of H_2S.

Reactive oxygen species (ROS) are a crucial cause of neuronal injury after cerebral ischemia-reperfusion injury, in which there is an overproduction of ROS, inactivation of antioxidant enzymes, and consumption of antioxidants, such that innate defense mechanisms fail to protect neurons from oxidative damage (10, 11).

Considering the anti-oxidative effect of H_2S, we hypothesized that H_2S has a protective role in the brain ischemia-reperfusion injury. With regard to cerebral ischemia, a few studies have examined the effect of H_2S (12-14). One such study showed that the inhibition of H_2S synthesis reduced the infarct volume induced by permanent middle cerebral artery occlusion (14). In contrast, two other studies demonstrated the protective effect of H_2S in a global cerebral ischemia model (12, 13). However, the effect of H_2S in the focal cerebral ischemia-reperfusion injury is unknown. The present study aimed to evaluate the protective effect of exogenous H_2S in the focal cerebral ischemia-reperfusion injury model and to investigate the protective mechanism of H_2S.

Material and methods

Animals and a transient focal cerebral ischemia-reperfusion injury model

This study was carried out according to the *National Institutes of Health Guide of the Care and Use of Laboratory Animals*, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital and adhered to the guidelines set by the Canadian Council for Animal Care and ARRIVE (Animal Research: Reporting In Vivo Experiments). Sprague-Dawley male rats (N = 137) weighing 250 to 300 grams (KOATECH, South Korea) were used.

The transient focal cerebral ischemia-reperfusion injury model was induced with a minor modification of the endovascular internal carotid artery (ICA) suture method developed by Longa et al, as previously described (15). After inhalation anesthesia using 3% isoflurane in 30% oxygen and 70% air, the left common carotid artery (CCA) was exposed at its bifurcation using a midline cervical incision. The external carotid artery (ECA), ICA, and CCA were ligated using a 5-0 silk suture. The CCA was then transected, and a 5-0 nylon monofilament suture (with its tip rounded by heating) was inserted into the CCA. To occlude the origins of the MCA and proximal anterior cerebral artery, the suture was advanced into the ICA for a distance of 20 mm. The suture was secured in place using a ligature, and the wound

was closed. The monofilament was removed 120 minutes after the occlusion. Cerebral blood flow was monitored throughout the procedure by laser Doppler flowmetry (PeriFlux system 5000, Perimed, Sweden). Physiological parameters, including mean arterial blood pressure, blood gases, and glucose concentration, were measured during the experiment. During the recovery period, the rats were assessed for forelimb flexion and contralateral circling to confirm the induction of focal cerebral ischemia. Seizure events were not observed during the experiments. Rectal temperature was maintained at 37 ± 0.5 ℃ with the use of a thermistor-controlled heating blanket. Free access to food and water was allowed following recovery from anesthesia.

Materials

H_2S was administered in the form of sodium hydrosulfide (NaHS, Sigma-Aldrich Co., USA). Just prior to administration, NaHS was diluted in normal saline to the desired concentration in a rapid fashion. Normal saline or NaHS was administered at the time of reperfusion via infusion pump. For all experiments, either normal saline or NaHS (1 mg/kg, 5 mg/kg and 10 mg/kg) in a final volume of 1mL was injected intravenously into the tail vein using a 32-gauge needle. As a bolus injection of NaHS resulted in profound hypotension (Figure 1), we slowly administered NaHS and normal saline for 5 minutes. Slow infusion of NaHS for 5 minutes did not decrease blood pressure (Green line at Figure 1). As shown in figure

2, slow of infusion of NaHS did not alter cerebral blood flow during procedure.

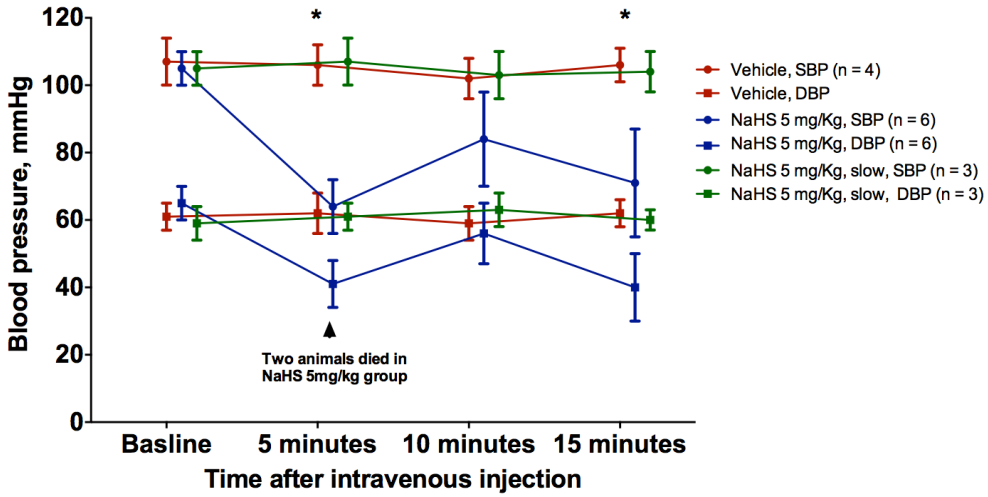


Figure 1. Change of blood pressure after the infusion of NaHS

Bolus injection of NaHS (5 mg/kg) decreased sytolic and diastoloc blood pressure (SBP and DBP, respecively) compared with the vehicle injection. At five and fifteen minutes following NaHS injection, systolic and diastolic blood pressure were significantly different between NaHS and vehicle groups. Slow infusion of NaHS (5 mg/kg, green line) did not decrease blood pressure. *$p < 0.05$

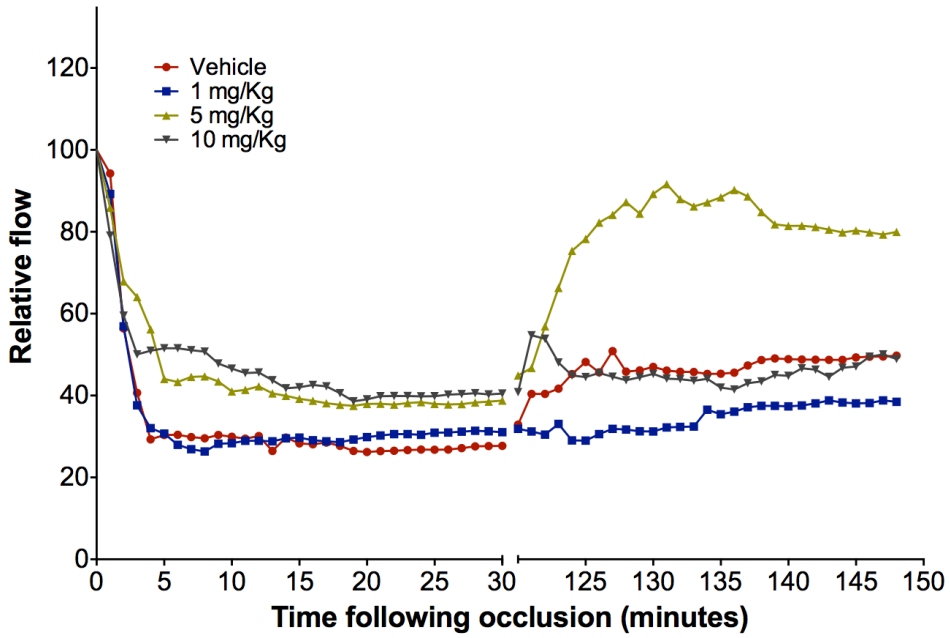


Figure 2. Change of cerebral blood flow following occlusion and reperfusion of middle cerebral artery

Within ten minutes after occlusion, cerebral blood flow dropped below 50% of basal cerebral blood flow. At 120 minutes following occlusion, MCA was reperfused and NaHS was slowly given via tail vein. There is no evidence suggesting that the administration of NaHS reduces cerebral blood flow. Rather, 5 mg/kg of NaHS tended increase cerebral blood flow compared to vehicle albeit statistically not significant. n = 4 for each group.

Infarction volume

Infarct volume was measured at two time points of 48 hours and 14 days following ischemia-reperfusion injury. After a cardiac perfusion-fixation with 4% paraformaldehyde, brains were cut into 30-μm-thick coronal sections on a freezing microtome (CM 3050S, Leica, Germany). A total of seven brain sections were processed for Nissl staining. Infarction volume was measured with an image analyzer, ImageJ (National Institutes of Health, USA). The percentage of infarction was calculated by comparing the infarction volume and the total volume of the contralateral hemisphere. At 14 days following ischemia-reperfusion injury, substantial tissue loss would hamper an accurate assessment of infarct volume. Thus, we used the contralateral hemisphere as a reference to ensure the outline of infarcted hemisphere.

Oxidative stress

To assess the extent of oxidative stress, quantifications of glutathione and lipid peroxidation were performed. At 24 hours following ischemia-reperfusion injury (n = 5 per group), brain glutathione was measured using commercially available kits (Cell Biolabs, USA). Glutathione (GSH) and GSH disulfide (GSSG) values were used to calculate the steady-state redox potential using the Nernst equation: $E_h = E_0 + (RT/nF) \times \ln([GSSG]/[GSH]^2)$ (where E_0 is the standard

potential for the redox couple at a defined pH, *R* is the gas constant, *T* is the absolute temperature, *F* is Faraday's constant, and *n* is the number of electrons transferred) (16, 17).

Malondialdehyde (MDA) was estimated as an indicator of lipid peroxidation (18). The MDA level in the brain tissue was measured using the thiobarbituric acid reduction method with a commercially available kit (Cell Biolabs, USA).

Western blot analysis

The rats were sacrificed by decapitation, and the brains were extracted at either 24 or 48 hours following ischemia-reperfusion injury (n = 4-5 per group). After the centrifugation of hemisphere homogenates, 50μg of the protein was separated on a 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. These membranes were incubated in a blocking buffer (5% skim milk in TBST [50 mM Tris, 0.15 mM NaCl, 0.05% Tween-20) and the blots were probed with antibodies recognizing signal transducer and activator of transcription 3 (STAT3; 1:1000, Cell Signaling Tech.,USA), pSTAT3 (1:1000, Cell Signaling Tech.,USA), extracellular signal-regulated kinase (ERK; 1:200, Santa Cruz Biotech., USA), pERK (1:1000, Cell Signaling Tech., USA), protein kinase B (Akt; 1:500, Santa Cruz Biotech., USA), pAkt (1:2000, Cell

Signaling Tech., USA), nuclear factor E2–related factor (Nrf2; 1:1000, Abcam, USA), cleaved caspase3 (1:1000, Cell Signaling Tech., USA), caspase3 (1:1000, Cell Signaling Tech., USA), bcl-2(1:1000, Cell Signaling Tech., USA), and gelsolin (1:1000, Abcam, USA). The protein level was expressed as the relative optical density, representing the optical density divided by the optical density of β-actin within the same lane.

Cytokine analysis

MILLIPLEX MAP kits (Millipore Corporation, USA) were used for microsphere-based multiplex immunoassays to measure the concentration of different proteins (n = 4 per group). The following cytokines were measured in tissue homogenates at 1, 3, 6, 24 and 48 hours after reperfusion using a ten-plex Rat Cytokine Kit: Interleukin-1 beta (IL-1β), IL-6, IL-10, and tumor necrosis factor alpha (TNF-α). PBS containing 0.1% Triton-X 100 and 5mg/ml bovine serum albumin was used as a matrix. The tissue homogenates were standardized to the smallest protein concentration detected in the homogenates. At least 100 beads were counted per analyte. Analysis of raw data was done with the BIO-PLEX manager software (Version 4.1, BioRad Laboratories, Hercules, USA).

TUNEL assay

Paraffin-embedded sections were dewaxed, rehydrated, and then incubated in 20 mg/ml of proteinase K for 15 minutes. TUNEL was accomplished using a DNA fragmentation detection kit (QIA33; Oncogene, USA). After the sections were immersed in 100 μl of 3 % H_2O_2 for 5 minutes, they were incubated in a TdT labeling reaction mixture (supplied with kit) in a humidified chamber for 90 minutes at 37 ℃. Then, they were incubated in the stop buffer at 37 ℃ for 5 minutes. Sections were washed with PBS before being incubated in a blocking buffer (supplied with kit) for 30 minutes at room temperature, colored with diaminobenzidine– H_2O_2 solution, and counterstained with methyl green. According to morphologic criteria, TUNEL positive nuclei with chromatin condensation and fragmented nuclei were considered as probable apoptotic cells, and TUNEL-positive cells with diffuse light brown labeling of the nucleus and cytoplasm were considered probable necrotic cells.

Immunofluorescence staining and cell quantification

Immunofluorescence staining of the brain tissue was performed using cryopreserved 40 μm coronal sections. Each section was incubated with 0.5 % bovine serum albumin/0.3 % Triton-X followed by 10 % normal serum in PBS for one hour for blocking. Sections with a primary antibody were placed at 4 ℃ for 16 hours. After they were washed, each section was subsequently incubated for two hours at room temperature with the fluorophore-conjugated secondary antibody. The

following primary antibodies were used: monoclonal antibodies against MHC class II (Ox6; 1:200, BD bioscience, USA) to label activated microglia/macrophages and myeloperoxidase (MPO; 1:50, Santa Cruz Biotech., USA) to stain neutrophils. Cell nuclei were visualized with 4, 6-diaminodino-2-phenylnodole (DAPI) staining. Stained cells were then examined under a confocal laser scanning biological microscope (LSM 410 META; Carl Zeiss, Germany).

A quantitative analysis of the positively stained cells was performed in the peri-infarct regions. To count the activated microglia/ macrophages and neutrophils, high-power fields were taken from the six predefined sections through the peri-infarct area at 24 and 48 hours following ischemia-reperfusion injury (Figure 3).

Behavior test

At 1, 3, 7, and 14 days following ischemia-reperfusion, we assessed neurological severity of rats using modified neurological severity score. The modified neurological severity score rates neurological functioning on a scale of 18. This includes a composite of motor (muscle status and abnormal movement), sensory (visual, tactile and proprioceptive), reflex and balance tests.

Statistical analysis

Values are expressed as means ± standard error. The nonparametric Mann-Whitney U test or Wilcoxon signed-rank test was used for unpaired or paired samples, respectively. A 2-tailed *p*-value of less than 0.05 was considered significant. All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

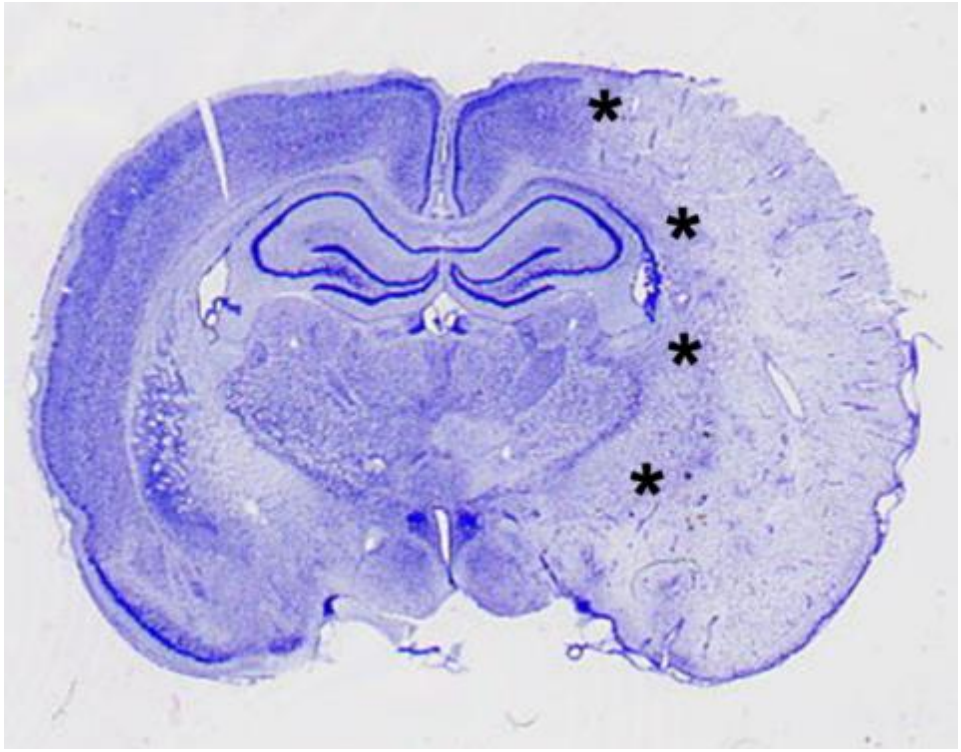


Figure 3. Definition of peri-infarct area

High-power field images that fully covered peri-infarct areas were taken from

sections stained through the infarct core for counting activated microglia/macrophages, neutrophils and TUNEL-positive cells. The asterisks indicate peri-infarct area.

Results

H_2S ameliorates the extent of brain injury following ischemia-reperfusion

The physiological parameters, including mean arterial blood pressure, blood gases, serum glucose, and body temperature, were comparable between vehicle and NaHS groups (Table 1).We investigated whether the administration of NaHS reduces the extent of brain injury following ischemia-reperfusion. Compared with the vehicle group, 5 mg/Kg of NaHS significantly reduced infarction volume (55 ± 11% versus 42 ± 8%, $p < 0.01$; Table 2, Figure 4). However, 10 mg/kg of NaHS tended to increase infarct volume compared to vehicle group albeit not significant. In addition, we also found that the administration of 5 mg/kg of NaHS reduced chronic infarct volume at 14 days following ischemia-reperfusion injury (34 ± 2% versus 8 ± 3%, $p < 0.01$; Figure 5). For the following experiments, we used 5 mg/Kg of NaHS which was designated as the H_2S group.

Table 1. Physiological parameters

	Control		NaHS					
			1mg/Kg		5mg/Kg		10mg/Kg	
	Baseline	After I/R	Baseline	After I/R	Baseline	After I/R	Baseline	After I/R
pH	7.34 ± 0.01	7.33 ± 0.01	7.33 ± 0.02	7.34 ± 0.01	7.34 ± 0.01	7.34 ± 0.01	7.33 ± 0.02	7.34 ± 0.01
PaO_2, mmHg	108 ± 11	108 ± 6	106 ± 9	106 ± 7	107 ± 10	109 ± 7	108 ± 7	106 ± 9
$PaCO_2$, mmHg	38.2 ± 0.6	37.4 ± 1.0	38.1 ± 0.5	37.6 ± 0.9	38.1 ± 0.5	37.8 ± 0.8	38.2 ± 0.4	38.1 ± 0.7
Glucose, mg/dL	123 ± 63	118 ± 78	124 ± 62	119 ± 74	121 ± 68	119 ± 79	116 ± 64	120 ± 73
Rectal temperature	36.6 ± 0.2	36.8 ± 0.2	36.8 ± 0.3	36.4 ± 0.3	36.7 ± 0.3	36.4 ± 0.3	36.5 ± 0.4	36.7 ± 0.4
MABP, mmHg	80 ± 5	79 ± 6	80 ± 7	78 ± 6	81 ± 6	79 ± 5	78 ± 7	77 ± 4

After I/R indicates the time point at 30 minutes after reperfusion of middle cerebral artery; MABP, mean arterial blood pressure and I/R, ischemia-reperfusion injury. Values are mean ± standard deviation.

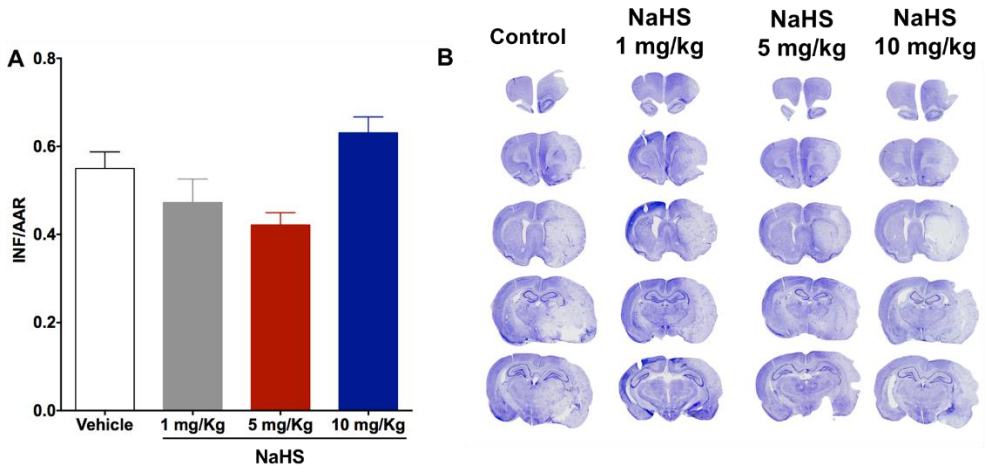


Figure 4. Infarct Volume at 48 hours following ischemia-reperfusion and representative Nissl stain

NaHS reduces the extent of injury at 48 hours following cerebral ischemia-reperfusion (n = 4-11 for each group). (A) Percentage of infarction area relative to the contralateral hemisphere. Compared to vehicle group, 5 mg/kg of NaHS significantly reduced infarct volume ($p < 0.01$). (B)Representative Nissl stains of each group

Table 2. Comparison of infarct volume between vehicle and NaHS (1 mg/kg, 5 mg/kg, and 10 mg/kg) groups

	Vehicle	NaHS		
		1 mg/kg	5 mg/kg	10 mg/kg
Infarction/Area at risk				

Mean	0.55	0.47	0.42	0.57
Std. Error	0.04	0.05	0.03	0.10

Data represent relative infarct volume compared with contralateral hemisphere.

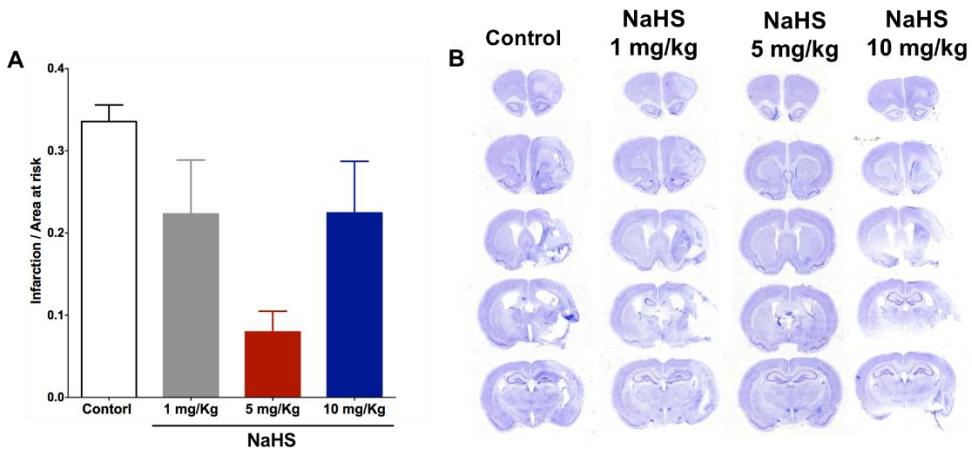


Figure 5. Infarct Volume at 14 days following ischemia-reperfusion and representative Nissl stain

NaHS reduces the chronic infarct volume at 14 days following cerebral ischemia-reperfusion (n = 4 for each group). (A) Percentage of infarction area relative to the contralateral hemisphere. Compared to vehicle group, 5 mg/kg of NaHS significantly reduced infarct volume ($p < 0.01$).

(B)Representative Nissl stains of each group

H_2S increased phosphorylation of Akt and STAT3

As the NaHS treatment attenuated infarction volume, we performed western blot to elucidate protective mechanisms of H_2S at 24 and 48 hours following ischemia-reperfusion injury (Figure 6). At 24 and 48 hours following ischemia-reperfusion injury, the phosphorylation of STAT3 increased in the NaHS group (Figure 7A) compared to the vehicle group. We also found an increased phosphorylation of Akt in the NaHS group following ischemia-reperfusion injury (Figure 7B). However, the phosphorylation of ERK1 and ERK2 did not differ between the two groups (Figure 7C).

Previous studies have shown that phosphorylation/activation of Akt reduce oxidative stress in cerebral ischemia (19, 20). Additionally, in animal cerebral ischemia model, STAT3 phosphorylation promotes survival signaling and reduces ROS production (21, 22).

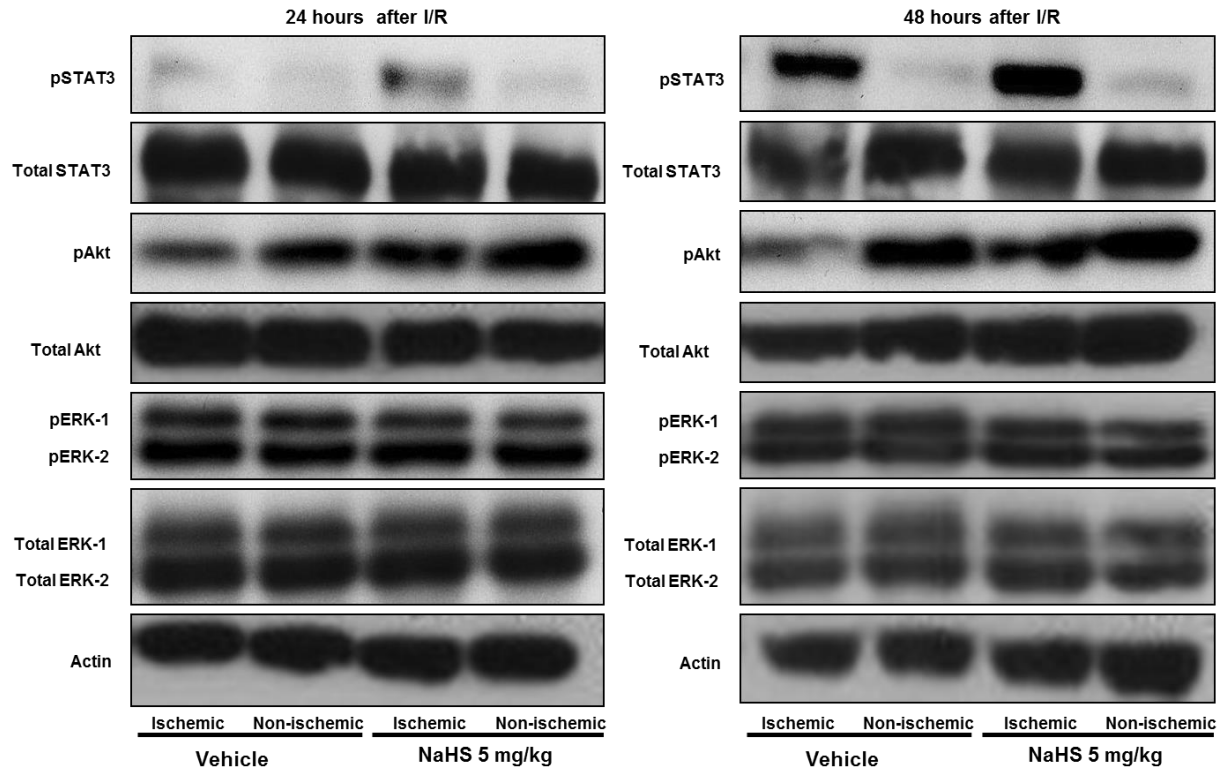


Figure 6. Western blots at 24 and 48 hours after ischemia-reperfusion injury

I/R indicates ischemia-reperfusion.

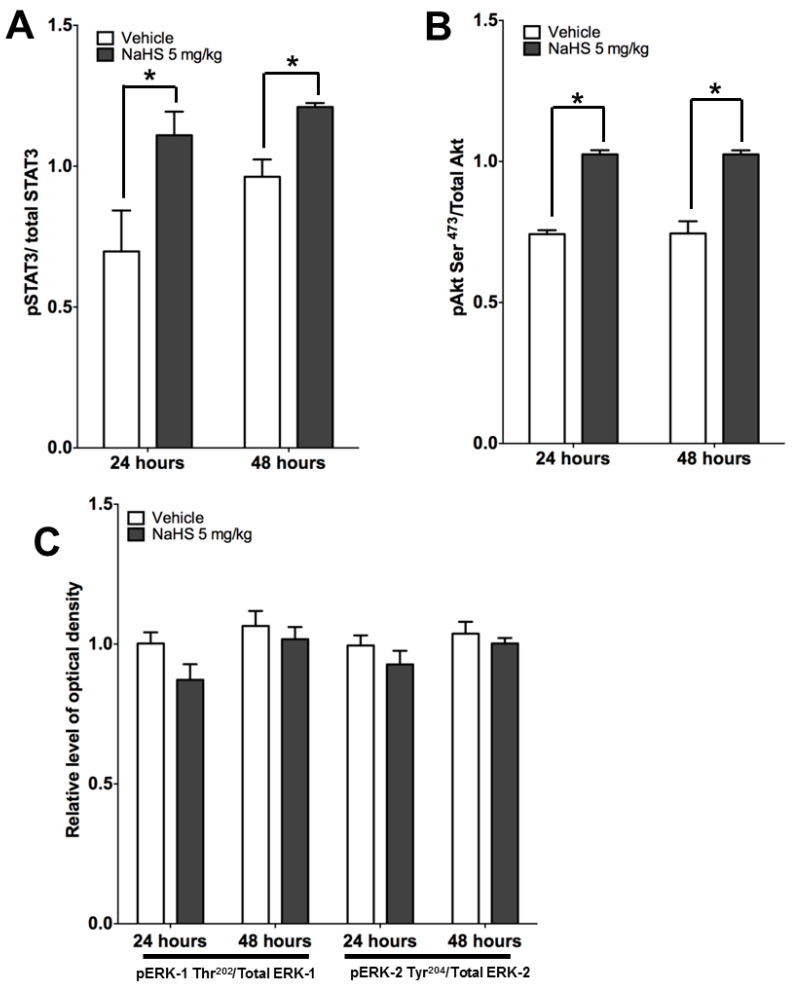


Figure 7. Relative optical density of STAT3, Akt, and ERK 1/2 at 24 and 48 hours following ischemia-reperfusion injury

NaHS increases the phosphorylation of Akt and STAT3. (A) Quantification of expression level of pSTAT3/total STAT3 (B) Quantification of expression level of pAkt/total Akt (C) Quantification of expression levels of pERK-1/total ERK-1 and pERK-2/total ERK-2. n = 4 for each group. *$p < 0.05$ vs. vehicle.

H_2S induced the Nrf2 expression and thereby reduced oxidative stress after cerebral ischemia-reperfusion

Nrf2 is a key transcription factor that regulates antioxidant genes as an adaptive response to oxidative stress and pharmacological stimuli. In previous reports showing the cytoprotective role of H_2S, Nrf2-mediated anti-oxidative pathway has been crucial. Thus we investigated Nrf2 expression and oxidative stress following ischemia-reperfusion injury. At 24 hours following ischemia-reperfusion injury, Nrf2 expression significantly increased in the NaHS group compared to the vehicle group and remained at an elevated level 48 hours after ischemia-reperfusion injury (Figure 8A and B).

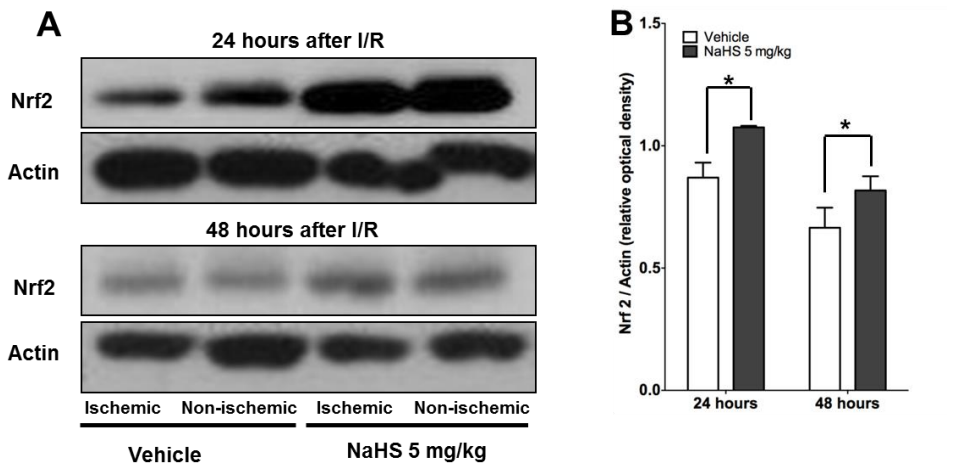


Figure 8. Expression of Nrf2 at 24 and 48 hours following ischemia-reperfusion injury

NaHS increases Nrf2 expression. (A) Representative immunoblot of Nrf2 (B) quantification of the expression level of Nrf2 (n = 4 for each group). *p < 0.05**.** I/R indicates ischemia-reperfusion injury.

To evaluate the oxidative stress in the tissue, we harvested the brain at 24 hours following ischemia-reperfusion injury. GSH, GSSG, and lipid hydroperoxide levels were determined and the steady-state redox potential was calculated using the Nernst equation. The brain GSH levels increased to a greater extent in the NaHS group than the vehicle group (Figure 9A), whereas the GSSG levels decreased more in the brains of the NaHS group than the vehicle group (Figure 9B). Accordingly, the redox potential (E_h) significantly decreased in the brains of the NaHS group compared to the vehicle group (-195.1 ± 2.5 mV versus -181.9 ± 4.3 mV, $p < 0.05$; Figure 9C). Lipid hydroperoxide levels in the brain were also lower in the NaHS group than the vehicle group (5.85 ± 1.31 versus 3.20 ± 0.95, $p < 0.05$; Figure 9D).

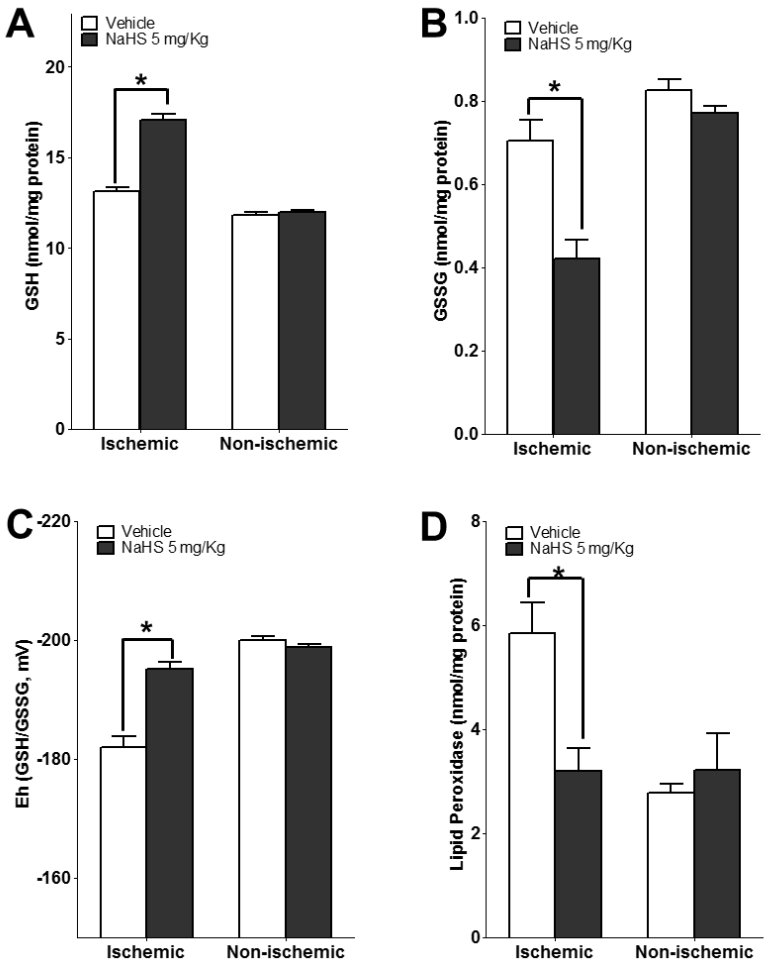


Figure 9. Oxidative stress at 24 hours following ischemia-reperfusion injury

(A) Brain GSH levels (nmol/mg protein) (B) Brain GSSG levels (nmol/mg protein) (C) Brain redox state (E_h) for GSH and GSSG (D) Brain lipid hydroperoxide level (nmol/mg protein). n = 4 or 5 for each group. *$p < 0.05$

Effect of H_2S on inflammatory responses following ischemia-reperfusion

To investigate the inflammatory response following ischemia-reperfusion injury, we measured brain cytokine levels at 1, 3, 6, 24 and 48 hours following ischemia-reperfusion injury. At 1-hour after reperfusion, there is no significant difference of cytokine levels between the NaHS and vehicle groups. At 3-hour, brain TNF-α levels were significantly higher in the NaHS-treated infracted hemispheres than the vehicle-treated infracted hemispheres (Figure 10A), whereas IL-6, IL-1β, and IL-10 levels were similar between two groups. Throughout 6 and 24 hours following reperfusion, no significant difference was found between two groups. At 48 hours after reperfusion, IL-6 levels were significantly higher in the NaHS-treated infracted hemispheres (Figures 10B). At 24 hours after reperfusion, MPO-positive neutrophil infiltration was lower in the NaHS group (Figure 11A and B), whereas at 48 hours, both MPO-positive and OX-6 positive cells (Figure 11C and D) infiltrations were attenuated in the NaHS group. Cell counts were significantly different between NaHS and vehicle groups (Figure 12A and B).

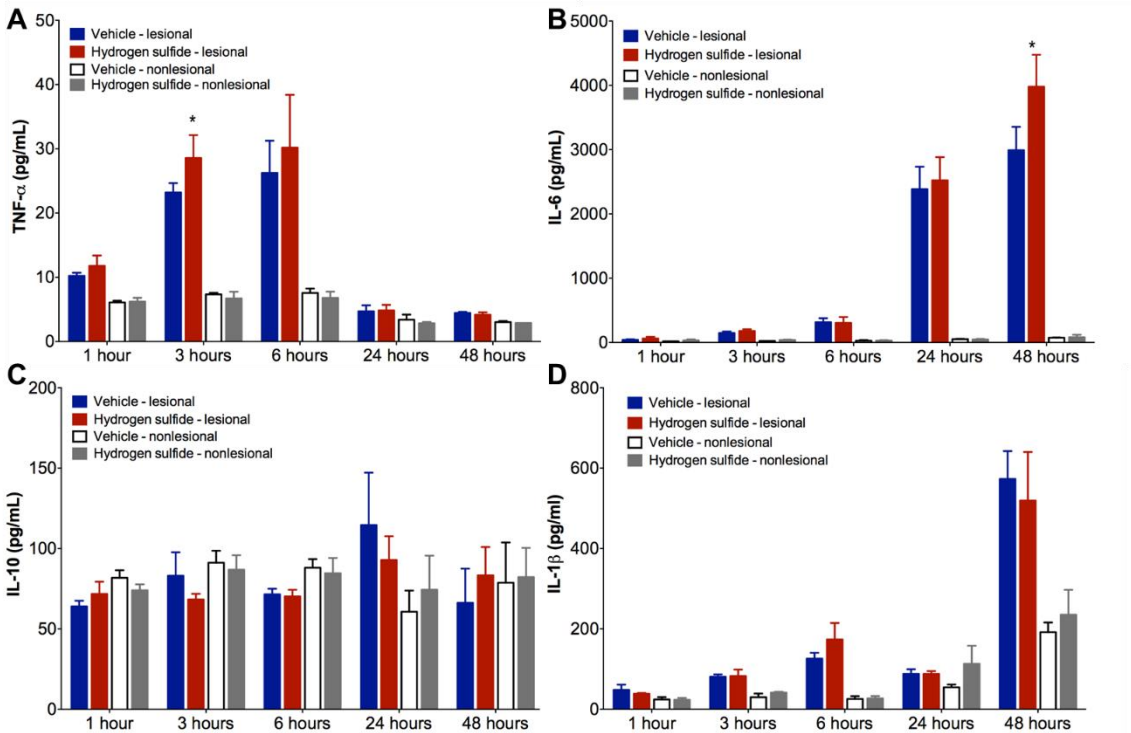
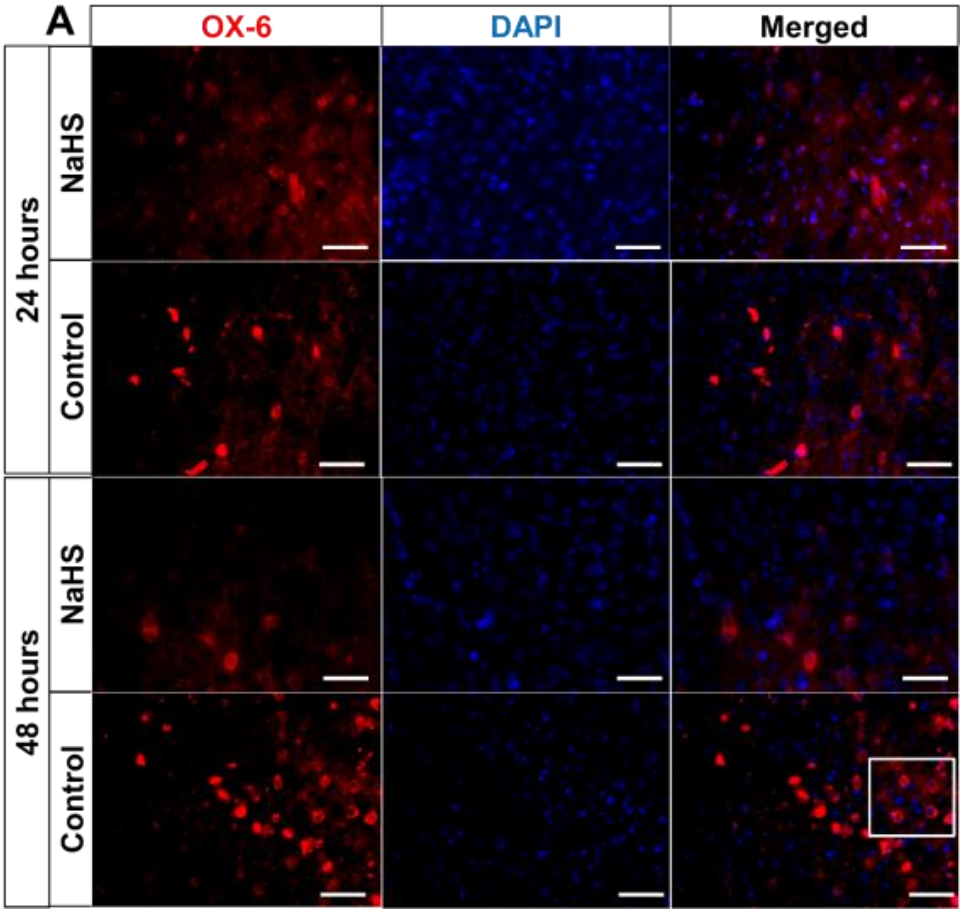
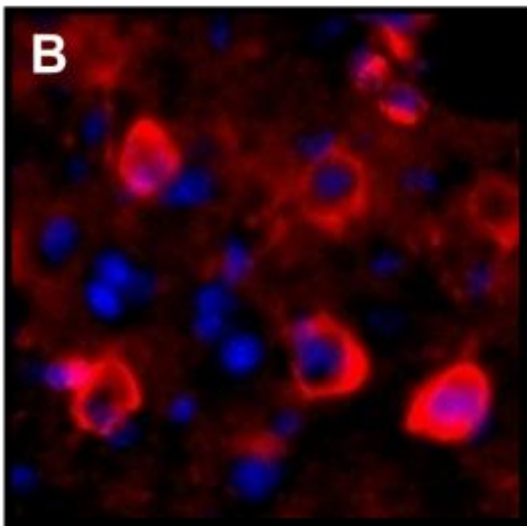


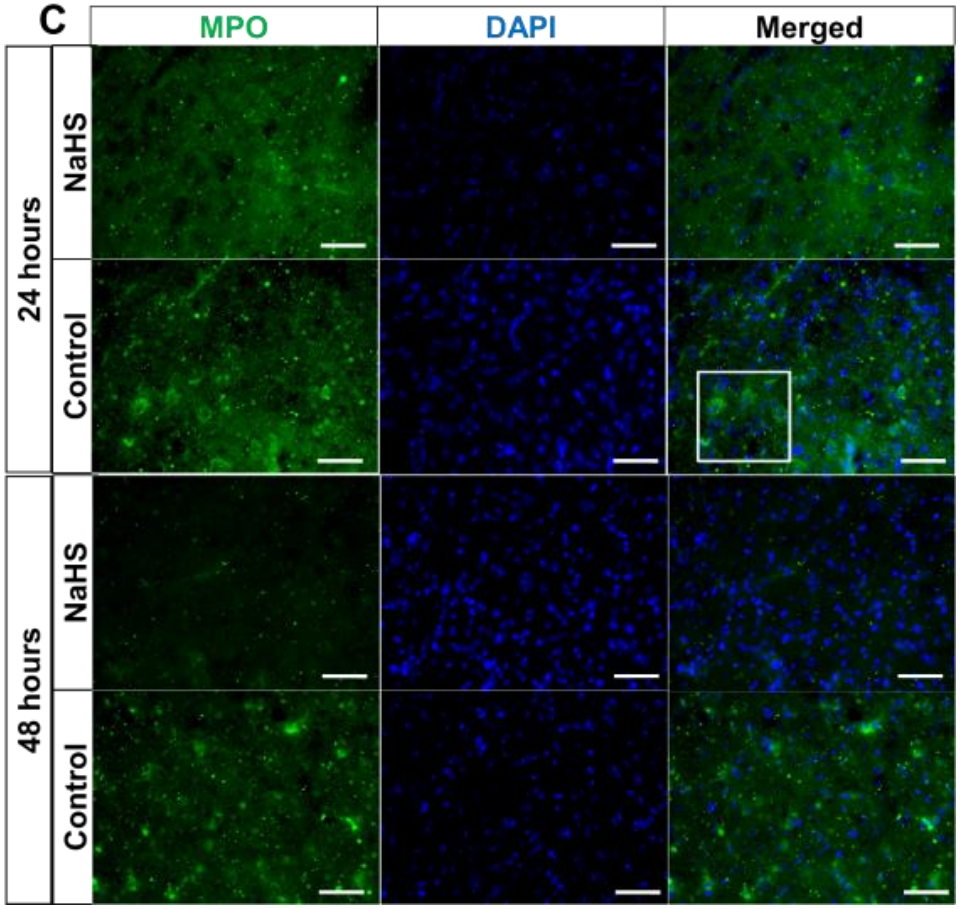
Figure 10. Levels of inflammatory cytokines at 1, 3, 6, 24 and 48 hours after ischemia-reperfusion injury

(A) IL-1β levels (pg/ml) (B) IL-6 levels (pg/ml), (C)IL-10 levels (pg/ml), (D) TNG-α levels (pg/ml) n = 4 for each group.

*$p < 0.05$


A
OX-6
DAPI
Merged
24 hours
NaHS
Control
48 hours
NaHS
Control


B


C
MPO
DAPI
Merged
24 hours
NaHS
Control
48 hours
NaHS
Control

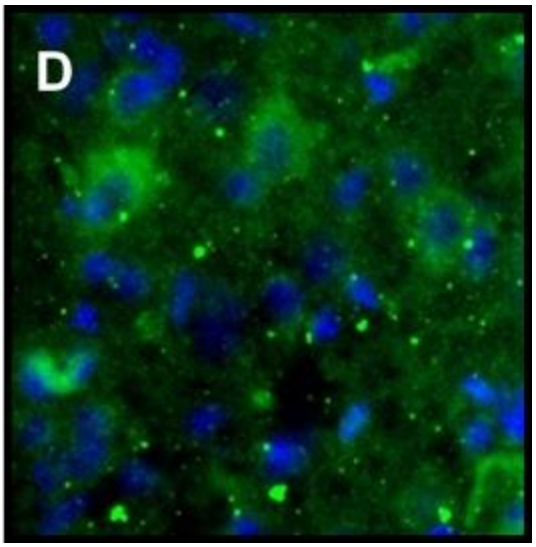

D

Figure 11. Immunofluorescence staining for inflammatory cells at 24 and 48 hours after ischemia-reperfusion injury

Immunofluorescence staining for OX-6 (A and B) and MPO (C and D) Bar indicates 50 μm. B and D were enlarged picture of selected area in A and C, respectively.

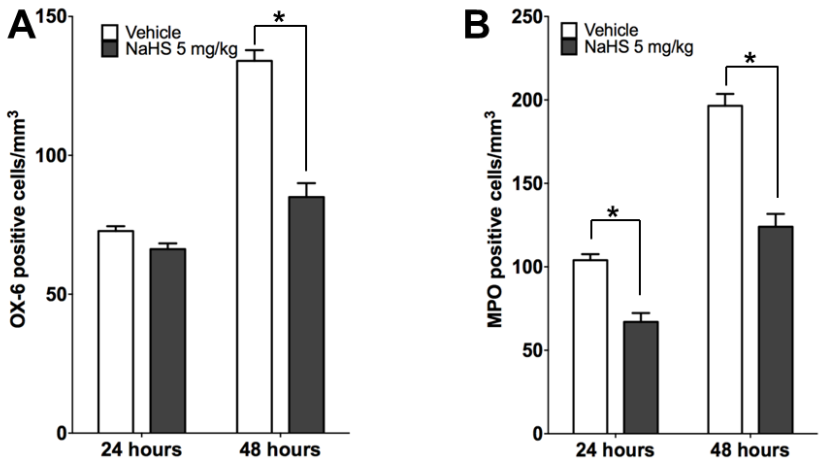


Figure 12. Infiltration of inflammatory cells infiltration at 24 and 48 hours after ischemia-reperfusion injury

(A) Number of double positive cells for OX-6 and DAPI. (B) Number of double positive cells for MPO and DAPI. n = 4 for each group. *$p < 0.05$ vs. vehicle

H_2S attenuated apoptotic cell death

We examined apoptotic pathways following ischemia-reperfusion injury because oxidative stress is well known to lead apoptosis and STAT3 phosphorylation increases expression of pro-survival signaling in cerebral ischemia-reperfusion. Bcl-2 and gelsolin, anti-apoptotic signaling molecules, were significantly higher in the NaHS-treated group (Figures 13A-C). We also found that NaHS treatment decreased an expression of cleaved caspase3 (Figure 13D). We performed TUNEL stains at 48 hours following ischemia-reperfusion injury to investigate cell death. Figures 14A and 14B showed the representative TUNEL stains of the H_2S and vehicle groups, respectively. As shown in Figure 14C, the TUNEL positive cell count was significantly lower in the H_2S group (817 ± 93 /mm^2 versus 457 ± 74 /mm^2, $p < 0.01$).

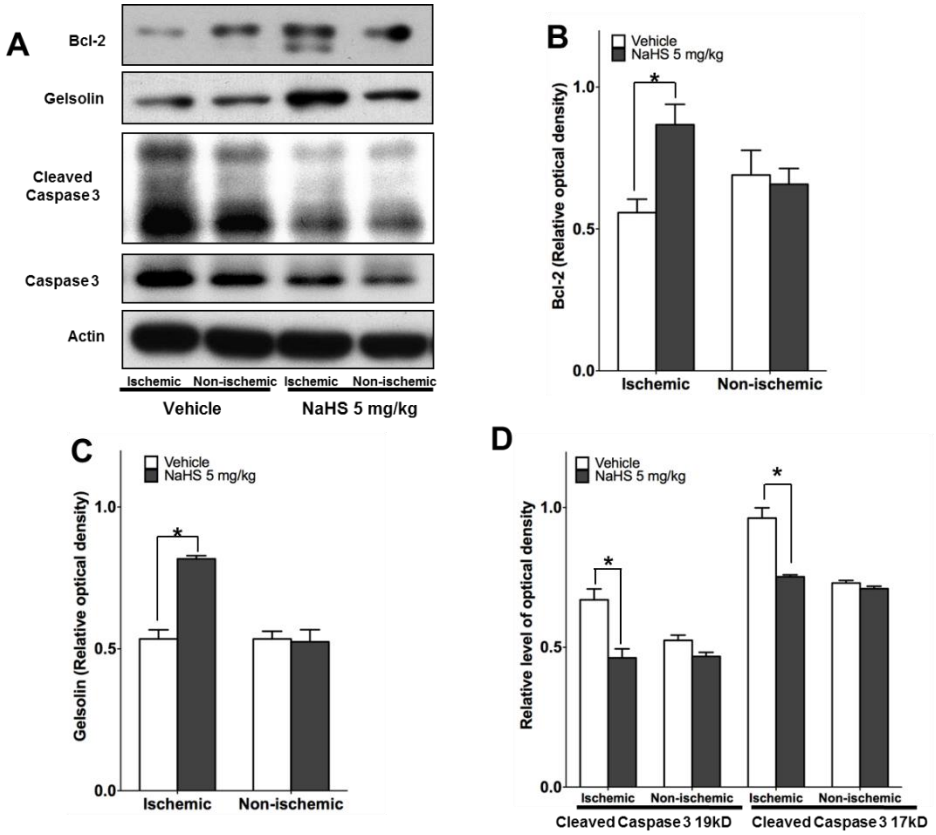


Figure 13. Proteins related to apoptosis at 24 hours following ischemia-reperfusion injury

NaHS attenuates the apoptotic signaling. (A) Immunoblots of Bcl-2, gelsolin, caspase3 and cleaved caspase3 at 24 hours following ischemia-reperfusion injury. (B) Densitrometric analysis of Bcl-2. (C) Densitrometric analysis of gelsolin. (D) Densitrometric analysis of cleaved caspase3. n = 4 for each group. *$p < 0.05$ vs. vehicle.

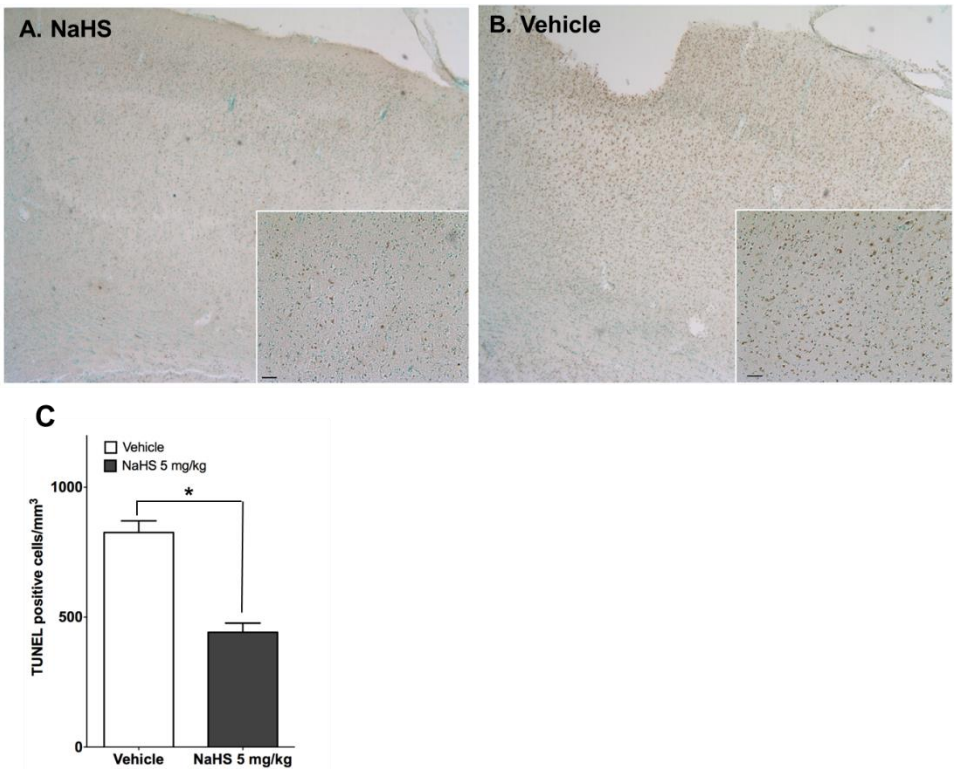


Figure 14. Comparison of TUNEL stain between vehicle and NaHS groups

(A) TUNEL stain of peri-infarct area from the NaHS group (B) TUNEL stain of peri-infarct area from the vehicle group (C) Pooled data showing significantly fewer TUNEL-positive cells after ischemia-reperfusion injury in the NaHS treated rat than in the vehicle group.

NaHS improved functional outcome

Figure 15 showed the reulsts of behavior test at 1, 3, 7, and 14 days following ischemia-reperfusion injury. Administration of 5 mg/kg of NaHS significantly improved functional outcome at 7 and 14 days following ischemic-reperfusion injury compared with the vehicle gorup. However, 1 mg/kg and 10 mg/kg of NaHS did not improved funational outcome.

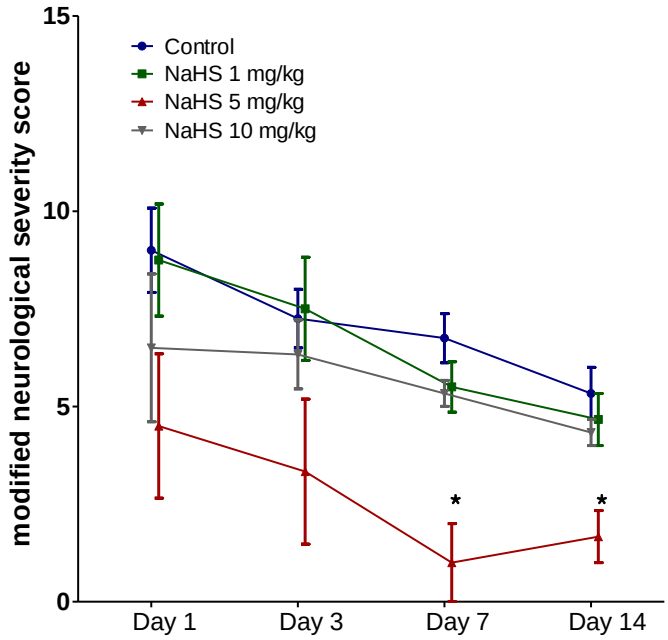


Figure 15. Comparison of neurological score between NaHS 1 mg/kg, 5 mg/kg, 10 mg/kg and vehicle

* $p < 0.01$ compared with vehicle

Discussion

In the present study, we demonstrated that the slow infusion of NaHS (5 mg/kg), a donor of H_2S, reduced infarct volume following cerebral ischemia-reperfusion at 48 hours and 14 days after reperfusion. However, a high dose of NaHS (10 mg/kg) did not reduce infarct volume. NaHS increased expression of Nrf2, a key regulator of anti-oxidant system, thereby reducing oxidative stress. In addition, NaHS exerted phosphorylation of Akt and STAT3, which in turn may increase anti-apoptotic protein and decrease pro-apoptotic protein. Further, NaHS decreased infiltrations of inflammatory cells around peri-infarct area, although NaHS increased inflammatory cytokines, IL-6 and TNF-α.

Hydrogen sulfide reduces oxidative stress via activation of Nrf2

The production of ROS and subsequent related to cellular damage is an initial cause of injury to the brain following ischemia-reperfusion injury (23, 24). During the ischemia-reperfusion injury process, the activity of the endogenous antioxidant enzyme systems is diminished. Therefore, the upregulation of the antioxidative cascade is critical to protect the brain against reperfusion damage. Likewise, during the cerebral ischemia, a blood clot blocked blood flow to the oxygen-starved tissue for a period. After blood clot being broken down, the blocked vessel is reperfused. Although reperfusion is mandatory for salvage of

brain tissue, the reintroduction of molecular oxygen triggers the formation of cytotoxic reactive oxygen species by the mitochondria. These reactive oxygen species inevitably drive downstream signaling cascades that lead to inflammation, necrosis and apoptosis (25). During recent several decades, numerous efforts have therefore been made for attenuating oxidative stress after the reperfusion therapy. Previous studies have shown that H_2S protects neurons from cell death by increasing GSH levels through an enhancement of γ-glutamylcysteine synthetase activity and an upregulation of cystine transport (26). Calvert et al. demonstrated that the cardioprotective effects of H_2S are mediated in large part by expression of Nrf2 in *in vivo* model of myocardial infarction (6). Peake et al. indicated that exogenous administration of H_2S attenuates myocardial ischemia-reperfusion injury in db/db mice, suggesting the potential therapeutic effect of H_2S in treating a heart attack in the setting of type 2 diabetes (27). Moreover, an in vitro study showed that H_2S protects against cellular aging via S-sulfhydration of Keap1 and Nrf2 activation in association with oxidative stress (28). Nrf2, a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, regulates the gene expression of various enzymes that serve to detoxify prooxidative stressors (29). This regulation is mediated by Nrf2 binding to the antioxidant responsive element, a *cis*-acting regulatory element or enhancer sequence found in the promoter region of certain genes. In our study, H_2S induced a signaling pathway to withstand oxidative stress, as evidenced by the ability of H_2S to upregulate cellular antioxidants in the brain.

Hydrogen sulfide increases STAT3 phosphorylation

The intracellular signal transduction induced by IL-6 involves the activation of JAK tyrosine kinase family members, leading to the activation of STAT3. The JAK–STAT3 pathway is the main intracellular signaling pathway of the IL-6 cytokine family (30). Jung et al. recently demonstrated that IL-6 has a neuroprotective effect against cerebral ischemic injuries through IL-6R-mediated STAT3 activation and manganese–superoxide dismutase expression (22). They also showed that manganese superoxide dismutase, a key regulator of oxidative stress, is a direct target of STAT3 in reperfusion-induced neuronal cell death, indicating that STAT3 is a novel transcription factor of manganese superoxide dismutase gene and plays a crucial role as a neuroprotectant in regulating levels of ROS in the rodent brain (31). In addition, a recent study by Kinouchi, et al, suggested that the activation in the peri-infarct region of STAT3 phosphorylation and peroxisome proliferator-activated receptor gamma by pioglitazone is essential for neuroprotection after ischemia (32). In the present study, the administration of NaHS increased phosphorylation of STAT3 and IL-6 levels. These results confer a notion that protective effect of H_2S in cerebral ischemia-reperfusion injury may be in part mediated by IL6/STAT3 signaling.

Hydrogen sulfide increases Akt3 phosphorylation

Another finding of our study relates to H_2S-medaited activation of Akt

signaling, a key pathway to confer neuroprotection against the cerebral ischemia (33-36). Yin et al. showed that suppression of inflammatory responses mediated in part via upregulating PI3-K/Akt activity may contribute to preconditioning-related neuroprotection against neonatal ischemic brain injury (34). A recent report demonstrated that the protective mechanisms of H_2S therapy are related to increased phosphorylation of Akt in the ischemia-induced heart failure model (37). Furthermore, Shao et al showed that H_2S induces cAMP-mediated Akt expression and increases cell survival in the rat hippocampal oxygen deprivation/reoxygenation, an ex vivo model of ischemia-reperfusion injury (38). In line with these previous reports, our results suggest that H_2S induces Akt expression and may consequently mitigate apoptotic cell death via activation of anti-oxidative pathway.

Hydrogen sulfide and inflammation after ischemic stroke

Contrary to our expectation, IL-6 and TNF-α were higher in the NaHS-treated group compared with the vehicle group. Several possible reasons could account for this unexpected finding. First, differences of cytokine levels between vehicle and NaHS groups were minimal albeit statistically significant. Prior studies focusing on an anti-inflammatory effect of a therapeutic agent have shown a 2-4 fold increase or decrease of cytokine levels in the brain (39, 40). Therefore, small differences of cytokine levels in the present study may have negligible impact on ischemia-reperfusion injury. Second, ambivalent effect of

cytokines on cerebral ischemia-reperfusion injury should be considered. Clinical studies based on patients with ischemic stroke have shown several implications of inflammatory cytokines. Higher levels of IL-6 were positively associated with an early neurological deterioration, infarct volume, unfavorable outcome and mortality in patients with ischemic stroke (41-45). However, these clinical studies are unable to draw a direct causal relationship between IL-6 and stroke and their results should be interpreted with a caution. Experimental studies have shown inconsistent and conflicting results regarding the role of IL-6 in cerebral ischemia (46-48). Hence, it has been suggested that, in the brain, IL-6 acts as a double-edged sword (46). In the acute phase of cerebral ischemia, IL-6 elicits an inflammatory pathway and consequently destructs brain tissue. However, in prolonged phase, IL-6 controls inflammatory response by decreasing level of the proinflammatory cytokines and increasing anti-inflammatory molecules (46). Moreover, a number of studies have indicated that IL-6 exerted cell survival and neurotropic factors in the chronic phase of cerebral ischemia (49-51).

To date, accumulating evidence was unable to conclude the effect of inflammatory cytokine on neuron after cerebral ischemia. Thus, our results may be in favor of a neuroprotective role of inflammatory cytokines. Alternatively, the anti-oxidative and anti-apoptotic effects of H_2S may counterbalance and overwhelm the toxic effect of H_2S although exogenous H_2S may aggravate inflammation after cerebral ischemia.

Explanations for conflicting results between ours and Qu et al.

Major findings of our study directly contradict those of the prior study by Qu et al. showing that H_2S mediates cerebral ischemic damage (14). Different dose of NaHS would be the most probable explanation. In the prior study, 0.18 mmol/kg (≈ 6.3 mg/kg) of NaHS was used to investigate the effect of H_2S on cerebral ischemia. This is relatively large compared with other studies of H_2S. Zhao et al. showed that 0.14 mmol/kg of H_2S decreased mean blood pressure of 30 mmHg (52). As shown in the present study, a rapid injection of large volume of H_2S dramatically lowered blood pressure, which in turn may lead to low perfusion pressure and large infarct volume. In addition, we also showed that higher dose (10 mg/kg) of NaHS did not reduced infarct volume. Thus, we speculate that, in the study by Qu et al.(14), blood pressure lowering effect of H_2S may overpower the protective of H_2S. Alternatively, the difference in the specific disease models used would be another explanation. In the prior study, Qu et al used the permanent cerebral ischemia model. Given the fact that their model exerts less oxidative stress than ischemia-reperfusion injury model, and that the Nrf2-mediated antioxidative process is an important and consistent mechanism in the cellular protective of H_2S, ischemia-reperfusion injury model in our study is more desirable to evaluate the cytoprotective effect of H_2S.

Limitation

A few limitation of the present study deserve comment. First, monitoring of cerebral blood flow yielded inconsistent results. A transducer should be located in the same location of rat's skull between procedures because the experimental device measures a relative blood flow. The time gap between occlusion and reperfusion hindered us to locate transduce at the same location. At least, however, we found that 5 mg/kg of NaHS did not alter cerebral blood flow compared to normal saline. Second, our experiment was unable to lend a direct causal relationship between mechanism suggested in the experiment and neuroprotection in cerebral ischemia-reperfusion injury because we did not use blocking agents of Nrf-2, Akt, and STAT3 and knock-out mice. However, this hypothesis generating study may open the door to future studies to investigate the possibility of H_2S as a novel therapeutic agent. Finally, we did not evaluate vasodilatory effect of H_2S on cerebral ischemia. Considering a potent vasodilatory effect of H_2S, the administration of H_2S might increase micro-circulation and collateral blood flow in the penumbra, thereby enhancing neuronal survival.

Conclusion

Strokes remain the third leading cause of death and the most important cause of serious, long-term disabilities (53). To date, there is no proven effective therapy for strokes, with the exception of an intravenous recombinant tissue plasminogen activator. The findings of our study provide evidence that H_2S administration significantly attenuates the extent of infarctions in cerebral ischemia in rats by reducing oxidative stress and attenuating inflammatory responses. Therefore, these findings further support the emerging concept that H_2S therapy may be of clinical importance in the treatment of vascular disease and may have the practical clinical use of alleviating morbidity and mortality after cerebral ischemia.

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국문초록

배경. 황화수로는 최근 발견된 새로운 가스 전단물질로 강력한 혈관 확장효과가 있는 것으로 알려져 있다. 또한, 최근 간, 심장, 콩팥 등을 포함한 다양한 장기의 허혈성 모델에서 황화수소의 세포 보호효과가 입증되었다. 그러나, 뇌경색 동물 모델에서 황화수소의 효과에 대해서는 잘 알려져 있지 않았다. 본 연구에서는 폐색-재관류 동물 모델에서 황화수소의 세포 보호 효과와 그 기전에 대해 알아보고자 한다.

실험재료 및 방법. 백서를 사용하여 동맥 내로 실을 집어 넣어 뇌혈관을 막아 혈관을 폐색시켰다. 120 분간의 혈관 폐색 뒤 다시 혈관을 개통시키는 폐색-재관류 (Ischemia-reperfusion) 모델로 실험을 진행하였다. 황화수소의 전구물질인 수황화나트륨(sodium hydrosulfide)를 1 mg/kg, 5 mg/kg, 혹은 10 mg/kg을 생리식염수 1ml에 녹여 정맥 주사한 군과 생리식염수 1ml를 주입한 군을 비교하였다.

결과. 황화수소 5 mg/kg를 주사한 군은 대조군에 비해 뇌경색의 부피가 뚜렷하게 감소하였다. 그러나, 10 mg/kg에서는 보호효과가 없었다. 황화수소를 주사한 군에서 항산화반응이 증가되어 있었고 이의 신호를 전달하는 단백질인 Nrf2의 발현이 증가되어 있었다. 허혈을 유발한지 24시간과 48시간 째, pAKT 와 STAT3의 발현이 황화수소를 주입한 실험군에서 대조군에 비해 현저하게 감소되어 있었다. 그러나, ERK와

pERK의 발현은 양 군사이에 차이를 보이지 않았다. ELISA 검사와 면역현광염색에서 염증관련 사이토카인과 MPO와 OX-6을 발현하는 미세아교세포의 숫자가 실험군과 대조군 사이에 뚜렷한 차이를 보였다. 또한, 아포토시스 관련 단백질의 발현이 실험군에서 감소되어 있음을 확인하였다. TUNEL 염색으로 실제 파괴된 세포의 숫자도 대조군에 비해 실험군에서 감소되어 있음을 확인하였다.

결론. 황화수소가 뇌 폐색-재관류 동물 모델에서도 세포 보호효과가 있음을 확인하였다. 그 기전으로는 항산화 및 항세포사 반응이 관연하는 것으로 보인다.

주요단어: 황화수소, 뇌경색, 뇌졸중, 염증, 항산화, 세포사

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