Original Investigation

The Effect of Hydrogen Sulphide on Experimental Cerebral Vasospasm

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ABSTRACT

AIM: Cerebral vasospasm is the primary cause of morbidity and mortality after subarachnoid hemorrhage (SAH). Hydrogen Sulfide (H_2S), a gaseous neurotransmitter, is produced in many tissues including the central nervous system (CNS). The vasodilatatory effect of H_2S has been shown in the CNS; however, its role in cerebral vasospasm has not been investigated before.

MATERIAL and METHODS: The rats were divided into 8 groups: control, SAH, sodium hydrosulphide (NaHS), propargylglycine (PPG), aminooxy acetic acid (AOAA), SAH+NaHS, SAH+PPG, and SAH+AOAA. After establishing experimental SAH, the basilar artery and brainstem were harvested at 24th hours. The diameter and wall thickness of basilar artery were measured. Production of H_2S was assessed by showing the activity of cystathionine β-synthase (CBS) and cystathionine γ-lyase enzymes (CSE).

RESULTS: NaHS treatment significantly reduced vasospasm at 24 hours following SAH. This vasodilatatory effect was correlated with the CSE expression in basilar artery. CSE and CBS enzyme expressions were significantly lower in brain stem and basilar artery in PPG and AOAA-treated groups. PPG and AOAA treatments exerted a vasoconstrictive effect in the basilar artery. There were statistically significant differences between NaHS, PPG and AOAA groups, in terms of basilar artery luminal diameter.

CONCLUSION: H_2S may have a therapeutic potential in the treatment of vasospasm with its vasodilatator activity.

KEYWORDS: Cerebral vasospasm, Hydrogen sulfide, Subarachnoid hemorrhage

INTRODUCTION

Subarachnoid hemorrhage (SAH) is the fourth most frequent cerebrovascular disease and it constitutes 5% of patients with stroke (2). Of the patients who survive the initial bleed; up to 30% will experience vasospasm over the course of their recovery. Almost for half a century, researchers performed a wide range of cerebral vasospasm studies, but the pathophysiological mechanisms are still not known precisely. Vasospasm remains a leading cause of morbidity and mortality in relation to SAH. Current data suggest a role for calcium (Ca^{2+}) channels as a leading cause of vasospasm; however, Ca^{2+} channel blockers have had a limited impact on the overall morbidity and mortality rates (3).

Hydrogen sulphide (H_2S) is the third gaseous signalling molecule after nitric oxide and carbon monoxide in the central nervous system (CNS). It is produced by endogenous enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) from cysteine. CBS is mostly produced in astrocytes and microglia, particularly in the hippocampus and the cerebellum (1,17). Although CSE is mainly produced

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in cardiovascular tissue, its expression also in neural tissue has been shown previously (6,10). While aminooxyacetic acid (AOAA) is the major inhibitor of CBS, CSE is mainly blocked by propargylglycine (PPG) (16).

Although there are many studies showing the role of H$_2$S in cerebral injury (4), the effects of H$_2$S in experimental cerebral vasospasm has not been investigated before. The aim of the present study was to show the vasodilatory effect of H$_2$S and discuss its potential mechanisms after experimental SAH.

■ MATERIAL and METHODS

Animals

Forty-eight Wistar rats weighing between 200-300 gr were used in this study. The animals were kept under constant laboratory conditions of 18 to 21^{0}C room temperature, a 12-hour light dark cycle, and were allowed free access to standard rat feed and tap water. All experimental procedures were approved by Gazi University Board of Ethics and performed in accordance with the local guidelines in research to minimize animal discomfort.

Surgical Procedure

General anesthesia was induced by intraperitoneal injection of 35 mg/kg ketamine (Ketalar, Pfizer, Istanbul, Turkey) and 15 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey). Autologous arterial blood injection into cisterna magna is a widely used model for experimental cerebral vasospasm after SAH (15)**.** Under general anesthesia, 2 cm midline incision was performed between occiput and cervical spine. The paraspinal muscles were dissected and the craniospinal junction and occipitocervical membrane were exposed. An insulin injector was inserted into the cistern and after removing 0.1-0.3 cc cerebrospinal fluid, 0.3 cc autologous arterial blood was injected into the cistern. After SAH, the rats stayed in the Trendelenburg position for 45 minutes. All of the rats were sacrificed with 100 mg/kg intraperitoneal phenobarbital (Pentotal Sodyum, Abbott, Istanbul, Turkey) 24 hours after surgery or drug administration. After decapitation, the brain-brain stem was removed en bloc. The brain stem and basilar arteries were removed. The tissues were kept in 10% formaldehyde at 4^{0}C until further analyses.

Experiment groups

Group 1 (Control) (n=6): The brain stem and basilar arteries were removed after the sacrifice.

Group 2 (SAH) (n=6): SAH was performed as explained above.

Group 3 (NaHS) (n=6): Rats were treated with a single dose of NaHS (0.18 mmol/kg, i.p.) which is a H$_2$S donor (Sodium hydrosulfide monohydrate, Product No: 13590, Sigma-Aldrich). The brain stem and basilar arteries were removed to analyze the effect of H$_2$S on normal basilar artery and brain stem.

Group 4 (PPG) (n=6): Rats were treated with a single dose of PPG (100 mg/kg, i.p.), which is a cystathionine γ-lyase (CSE) inhibitor (DL-Propargylglycine 1gr, P7888, Sigma-Aldrich).

The brain stem and basilar arteries were removed to show the effects of inhibition of H$_2$S production in normal tissues.

Group 5 (AOAA) (n=6): Rats were treated with a single dose of AOAA (0.05 mmol/kg, i.p.) which is a cystathionine β (CBS) synthase inhibitor (O-(Carboxymethyl) hydroxylamine hemihydrochloride 98% 1gr, C13408, Sigma-Aldrich). The brain stem and basilar arteries were rapidly removed to show the effects of inhibition of H$_2$S production in normal tissues.

Group 6 (SAH+NaHS) (n=6): Rats were treated with a single dose of NaHS (0.18 mmol/kg, i.p.) immediately after SAH.

Group 7 (SAH+PPG) (n=6): Rats were treated with a single dose of PPG (100 mg/kg, i.p.) immediately after SAH.

Group 8 (SAH+AOAA) (n=6): Rats were treated with a single dose of AOAA (0.05 mmol/kg, i.p.) immediately after SAH.

The doses of NaHS, PPG and AOAA were determined according to the data collected from the previous studies (13,14).

Measurement of wall thickness and luminal diameter of the basilar artery

The brain stem tissues of each animal were fixed in 10% formalin for 24 hours and processed for routine paraffin embedding. The 4 μm sections were cut from the paraffin blocks and stained with hematoxylin-eosin (H&E). The diameter and the wall thickness of the basilar artery were measured by computerized digital image analysis for each animal. Digital images were obtained from H&E stained sections using a 3 CCD color video camera (Olympus DP70, Olympus Optical Co. Ltf, Tokyo), connected to the light microscope (Olympus BX51, Olympus Optical Co. Ltd) at an original magnification of x40. Images were processed with image-analysis software. The widest and the narrowest segments of the basilar artery were detected by eyeballing and the luminal diameters of these segments were measured for each case. Two high-power fields (X40) were selected for digital acquisition and the wall thickness of the basilar artery was measured on these captured images. Each measurement was recorded in the system automatically.

Immunohistochemical Analysis

Immunohistochemistry was applied on 4 micron-thick tissue sections, which were de-waxed in xylene and re-hydrated with a graded series of ethanol concentrations, and washed in tap water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 minutes. Heat-mediated antigen retrieval was performed by incubating the slides in a 0.01 M citrate buffer (pH 6.0) for 20 minutes in a microwave oven. Following the application of blocking serum (non-immune) for 20 minutes, the sections were incubated in primary antibodies (CTH monoclonal antibody (M03), clone S51-M01), CBS monoclonal antibody (CBS monoclonal antibody (M01), clone 3E1), for 2 hours. After washing with phosphate-buffered saline (PBS), the sections were incubated with a secondary antibody (Multi-species Ultra streptavidin detection system-HRP; Signet, Massachusetts, USA), streptavidin- biotin complex (Signet) and DAB (diaminobenzidine tetrachloride, Novocastra,

Newcastle-upon-Tyn, UK) respectively. Counterstaining was performed by Harris hematoxylin. Finally, the slides were dehydrated through graded alcohols to xylene and mounted in mounting medium.

Human hepatocellular carcinoma and human pancreatic tissues were used as positive controls for CBS and CTH antibodies, respectively. Cytoplasmic staining was recorded as positive for both. Staining intensities for both antibodies were compared with the positive control and graded as follows: weak staining= (+); moderate staining= (++); strong staining= (+++). For negative controls, the primary antibodies were omitted.

Statistical Analysis

Commercially available software package SPSS (IBM Corp. Released 2012.SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) was used for statistical analyzes. Descriptive statistics included mean, standard deviation, median and percentage values. Non-parametric data comparisons between groups were performed using the Kruskal-Wallis test. Pairwise comparisons were performed using Dunn's procedure with Bonferroni correction for multiple comparisons and probability level (p) less than 0.05 was accepted as statistically significant.

■ RESULTS

Basilar artery luminal diameter

The luminal diameter (LD) and wall thickness (WT) samples of basilar artery from Control, SAH and SAH+NaHS groups are shown in Figure 1A-C. The LD and WT values of basilar artery are shown in Figure 2A, B. In terms of LD, there were statistically significant differences between the groups (χ2 (7) = 32.083, p<0.001). LD values were significantly lower in group 2 (SAH) compared to group 1 (control) (p=0.027). SAH significantly reduced luminal diameter in rats. LD values were significantly high both in group 3 (NaHS) and group 6 (SAH+NaHS) comparing to the group 2 (SAH) (p=0.001 and p=0.003, respectively). NaHS treatment showed a vasodilatory effect on basilar artery in normal rat brain, and significantly reduced vasospasm at 24 hours following SAH. LD values

were significantly lower in group 5 (AOAA) compared to group 3 (NaHS) (p=0.013).

No significant luminal diameter change was observed after administration of PPG and AOAA in rats without SAH.

Basilar artery wall thickness (WT)

WT values of each group are shown Figure 2A,B. In terms of WT, there were statistically significant differences between the groups (χ2 (7) = 17.582, p=0.014). WT values were significantly high in group 2 (SAH) compared to group 1 (control) (p=0.023). SAH significantly increased WT in the basilar artery. There was also a significant difference between group 2 (SAH) and group 3 (NaHS) (p=0.009). NaHS treatment significantly decreased WT in basilar artery.

### CBS Expressions in Brain Stem and Basilar Artery

CBS expression in the brain stem and basilar artery was detected in all groups; however, the most significant expression was detected in Group 3 (NaHS). NaHS treatment significantly increased CBS expressions in both normal and SAH groups (Figure 3A-C).

CSE Expressions in Brain Stem and Basilar Artery

Although CSE expression was slightly higher in Group 3 (NaHS), no significant difference was detected between the groups in terms of brain stem CSE expression. Similarly, no significant difference was detected between groups in terms of CSE expression in the basilar artery (Figure 4A-C).

■ DISCUSSION

Hydrogen sulfide (H_2S) targets different ion channels and receptors, and fulfills its various roles in modulating the functions of different systems (19). It appears to be involved in numerous biological processes, including regulation of blood pressure and vascular tone. Although the vasodilatatory effect of H_2S on the cardiovascular system has been shown previously (8,12,21,23), the cellular mechanisms responsible for the vascular effect of H_2S, as well as its physiological significance still remain to be elucidated. The endogenous H_2S/CSE system has been reported to be one of the key factors in maintaining basal systolic blood pressure (21).
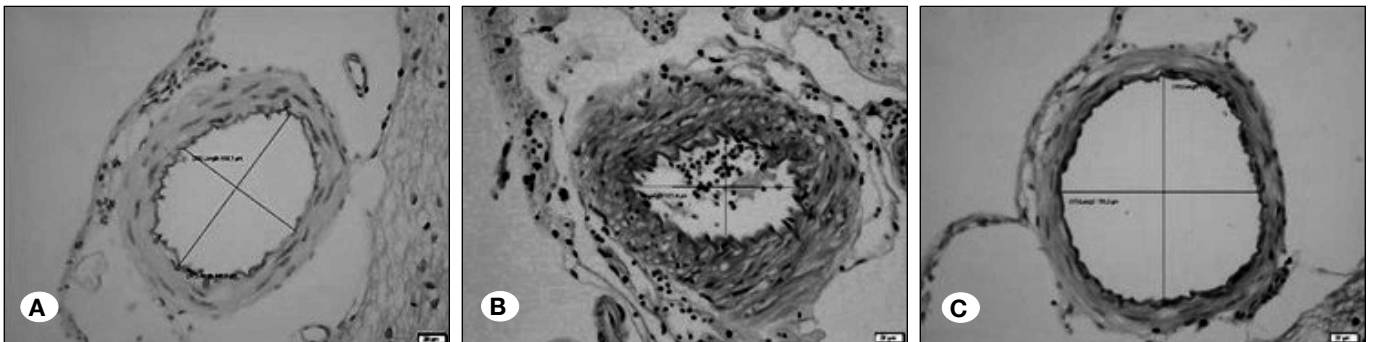

Figure 1: Basilar artery transections on the surface of the pons. **A)** Normal animal; **B)** SAH animal; **C)** SAH+NaHS treated animal. In the SAH animals, the vessel cavity was decreased, and the vessel wall thickness was increased when compared with samples from normal rat (H&E, 40X).

Exogenous administration of H$_2$S increases the plasma level of H$_2$S and enhances the CSE activity of aorta in spontaneous hypertension rats (8). H$_2$S also attenuates the elevation of pressure and lessens the aorta structural remodeling during the development of hypertension. H$_2$S causes vasodilatation through K (ATP) channels in the rat mesenteric artery vascular smooth muscle cells (18). NaHS induces CSE and CBS activity, and thus increases H$_2$S levels (13-16).

A variety of cells, including astrocytes, neurons and microglia can produce H$_2$S. It is an essential physiological product in the brain and acts as an endogenous anti-inflammatory and neuroprotective agent in the CNS (5). CBS and CSE are the key endogenous enzymes involved in H$_2$S synthesis. H$_2$S is synthesized in the brain primarily by the enzyme CBS (9). As the predominant source of H$_2$S in brain tissue, CBS may play a distinct role in cerebrovascular pathophysiology (7). H$_2$S is a dilator of the newborn cerebral circulation and that
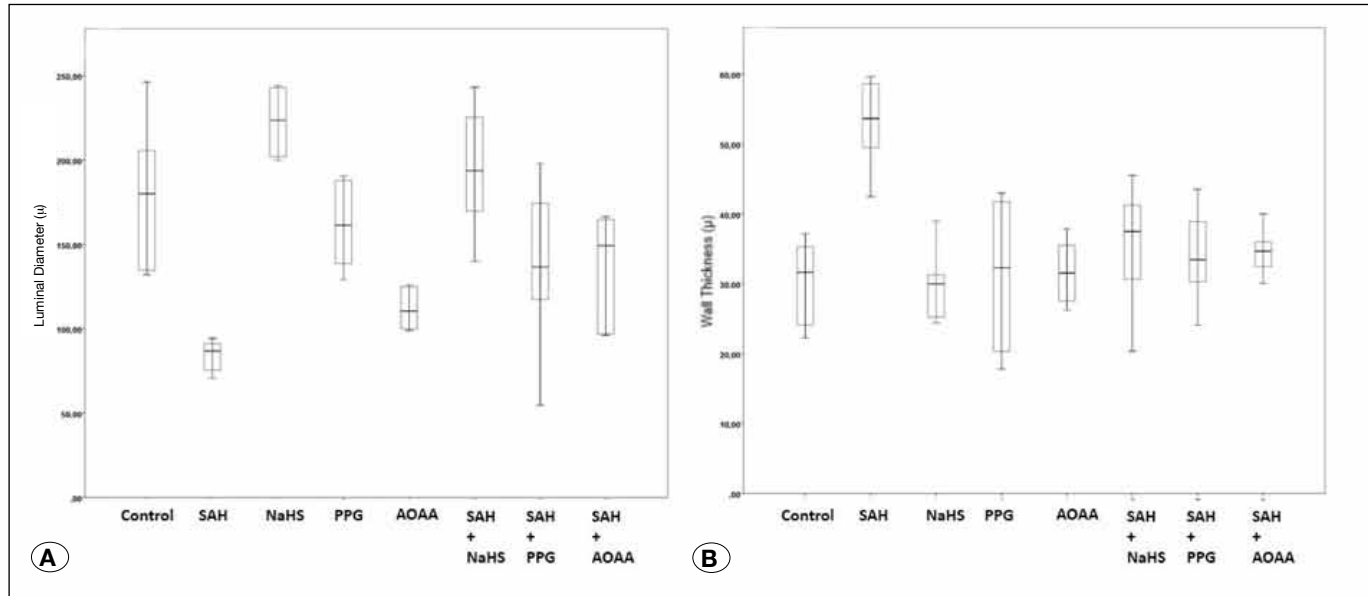
Figure 2: A) The luminal diameter and **B)** wall thickness values of basilar artery of each group. NaHS treatment showed a vasodilatory effect on basilar artery in normal rat brain, and significantly reduced vasospasm at 24 hrs following SAH. SAH significantly increased WT in the basilar artery. NaHS treatment significantly decreased WT in basilar artery.
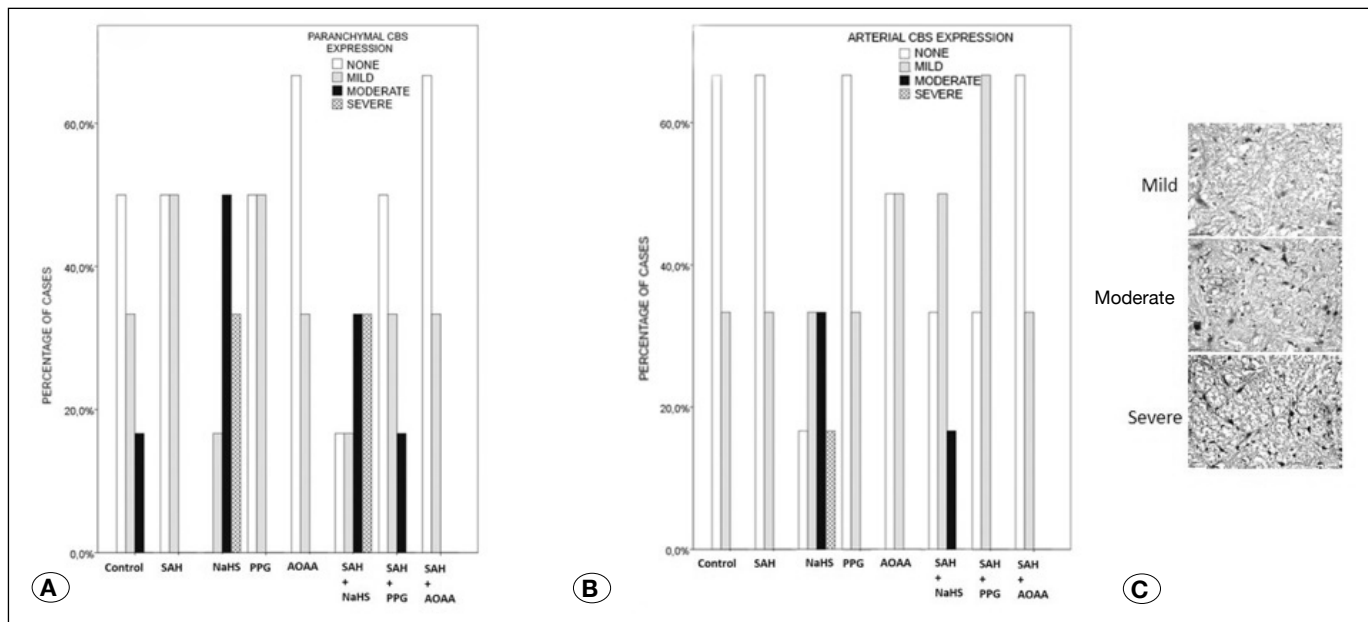
Figure 3: A) Brain stem and **B)** basilar artery CBS expressions in groups. **C)** Representative CBS expressions as mild, moderate and severe in brain stem parenchyma. CBS expression was highest in NaHS treated group comparing to SAH group.
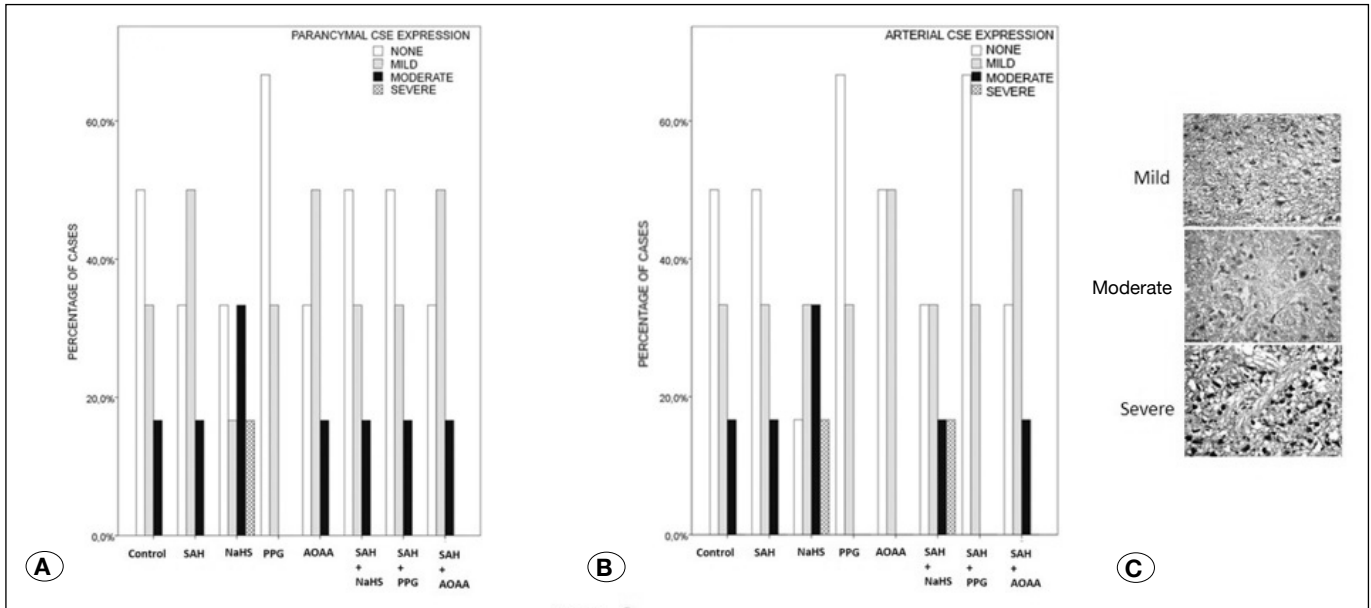
Figure 4: A) Brain stem and **B)** basilar artery CBE expressions in groups. **C)** Representative CBE expressions as mild, moderate and severe in brain stem parenchyma. CSE expression was highest in NaHS treated group comparing to SAH group.

endogenous CSE can produce sufficient H$_2$S to decrease vascular tone (11). H$_2$S-mediated dilatation of middle cerebral arteries is partly mediated by inhibition of L-type Ca^{2+} channels, with an additional contribution by K channels. Recently, Liu et al. have reported that H$_2$S decreased the myogenic response of cerebral arterioles in vivo, and this effect was endothelium-dependent and was partially mediated by K (ATP) channels (14). A recent study showed that NaHS, a H$_2$S donor, dilated rat cerebral arteries primarily through inhibiting Ca^{2+} influx via Ca^{2+} channels (20).

It has been suggested that H$_2$S plays a crucial role in the pathogenesis of cerebral injury following SAH s well. The relationships between angiographic vasospasm, delayed cerebral ischemia (DIND) and the CBS gene genotype was analyzed in 87 aneurysmal SAH patients (7). The patients with the gain of function genotype 844WT/ins were less likely to experience DIND compared to patients with the 844 WT/WT genotype. The decrease in function genotype 1080TT was also more likely to experience DIND compared to patients with the 1080 CC and CT genotypes (7).

As the H$_2$S/CBS complex induces vasodilatation, we hypothesized that administration of H$_2$S donor NaHS might reverse vasospasm following SAH. However, cerebral vasospasm and DIND after SAH are unsolved problems with a complex pathophysiology. It is well known that there is an association between the presence of vasospasm and development of a DIND in clinical practice. We have shown here that administration of NaHS exerts a vasodilatory effect on the basilar artery in the normal rat brain, and significantly reduces vasospasm at 24 hours when given immediately following SAH.

To further evaluate the roles of H$_2$S in the vasodilatation, we used PPG, a specific inhibitor of CSE in both the control and SAH groups. The role of CSE blockade was evaluated

by determining the effects of PPG on the basilar artery LD. Although NaHS produced significant vasodilation, PPG had no effect on arterial diameter in control rats. Similarly, AOAA, a CBS inhibitor, also had no effect on LD in control rats. When H$_2$S inhibitors and donor were administered to the SAH animals, only NaHS exerted significant vasodilatory effect on basilar artery LD, suggesting that H$_2$S plays a distinct role in cerebrovascular pathophysiology. The mechanism by which H$_2$S acts to inhibit vasospasm might relate to its ability to inhibit Ca^{2+} influx via Ca^{2+} channels in rat cerebral arteries (20). However, the underlying cellular mechanisms related to the vascular effect of H$_2$S remain to be examined in detail. H$_2$S might modulate not only microvascular cerebral blood flow but also direct neuronal cytoprotection following SAH (7).

Vessel wall thickness is another important parameter in evaluating the vasospasm. It is well documented that smooth muscle proliferation is related to endothelial cell death and detachment of the endothelium following SAH (3,22). We have shown here that SAH caused a significant increase in wall thickness. However, when given immediately after SAH, NaHS caused a decrease in basilar artery wall thickness. These actions might result from both direct and indirect activity of H$_2$S. In the NaHS group, expression was higher whereas in PPG and in AOAA groups it was lower. SAH did not affect the CBS expression in the brain stem. CSE expression was highest in NaHS-treated group. AOAA decreased CSE expression in basilar artery. In rats with SAH, only NaHS treated animals show slightly higher CSE expression in basilar artery.

The major limitation of the immunohistochemical analysis in this study is that the number of rats in the groups is not enough to statistically show the enzyme expression according to the grading scale. Because of this limitation, the degree of enzyme expression is presented by percentages. However the

immunohistochemical findings show consistent results with basilar artery luminal diameter. The experimental setting in this study is different than in clinical conditions like many of the SAH studies. Post-SAH cerebral vasospasm occurs between the third and seven days. However, in experimental SAH, the animals are generally sacrificed 24 hours after SAH. Because of the high mortality, the researchers prefer to remove the tissues after 24 hours. This is an important reason for failed clinical results for many agents, although they showed effectiveness in experimental conditions.

In further studies, a dose-response analysis can be performed and better results can also be achieved with different dose regimes and intervals.

Overall, the findings of this study suggest that the vasospasm that occurs following SAH can be attenuated by a H_2S donor, NaHS. Since vasospasm play an important role in mortality and morbidity, NaHS has emerged as a new therapeutic strategy for SAH-related vasospasm.

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