Hydrogen sulfide and its cardioprotective effects in myocardial ischemia in experimental rats

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Running title: Hydrogen sulfide is cardioprotective

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Abstract:

The role of hydrogen sulfide (H$_2$S) in myocardial infarction (MI) has not been previously studied. We therefore investigated the effect of H$_2$S in a rat model of MI in vivo. Animals were randomly divided into 3 groups (n=80) and received either vehicle, 14 μmol/kg of NaHS or 50mg/kg propargylglycine (PAG) everyday for 1 week before surgery and the treatment continued for a further 2 days after MI when the animals were sacrificed. The mortality was 35% in vehicle-, 40% in PAG- and 27.5% in NaHS-treated (p<0.05 vs vehicle) groups, respectively. Infarct size was 52.9±3.5% in vehicle-, 62.9±7.6% in PAG- and 43.4±2.8% in NaHS-treated (p<0.05 vs vehicle) groups. Plasma H$_2$S concentration was significantly increased after MI (59.2±7.16 μM) compared to the baseline concentration (i.e. 38.2±2.07 μM before MI, p<0.05). Elevated plasma H$_2$S after MI was abolished by treatment of animals with PAG (39.2±5.02 μM). We further showed for the first time cystathionine-gamma-lyase (CSE, an enzyme responsible for endogenous H$_2$S formation) was detected in the myocardium of the infarct area using immunohistochemical staining. In the hypoxic vascular smooth muscle cells, we found that cell death was increased under the stimuli of hypoxia but the increased cell death was attenuated by the pre-treatment of NaHS (71 ± 1.2% cell viability in hypoxic vehicle vs 95 ± 2.3% in non-hypoxic control, p<0.05).

In conclusion, endogenous H$_2$S was cardioprotective in the rat model of MI. PAG reduced endogenous H$_2$S production after MI by inhibiting CSE. The results suggest that H$_2$S might provide a novel approach to the treatment of myocardial infarction.

Keywords:
Hydrogen sulfide, cardioprotection, myocardial ischemia, rat
Introduction

For many years, hydrogen sulfide (H$_2$S) has been considered solely as a broad-spectrum environmental toxicant with effects on many major organ systems including the lung, brain and kidney (2, 11). However, the possible physiological role(s) of H$_2$S in the cardiovascular system have only recently come to light. It has been suggested that H$_2$S interferes with cardiovascular function as a result of anoxia rather than a direct action on cardiac myocytes or vascular smooth muscle cells (13). However, this possibility now appears less certain in light of more recent research. The localisation of H$_2$S generating enzymes and the detection of biologically significant levels of H$_2$S in plasma and tissue homogenate from animals have recently been reported (20). Endogenous H$_2$S is formed locally by the activity of two pyridoxal-5’-phosphate-dependent enzymes namely cystathionine $\beta$-synthase (CBS) and cystathionine $\gamma$-lyase (CSE) each of which utilize L-cysteine as substrate (17). While CBS does not appear to play a major role in generating H$_2$S in cardiovascular tissue under physiological conditions (21), the expression of CSE and the associated production of endogenous H$_2$S has been demonstrated in rat mesenteric artery and other vascular tissue several years ago. Hosoki and colleagues (7) reported that NaHS, an H$_2$S donor, relaxed vascular smooth muscle (including rat portal vein and aorta) in vitro. By analogy with other endogenous gaseous molecules such as nitric oxide (NO) and carbon monoxide (CO), H$_2$S has been implicated as a gaseous transmitter fulfilling a physiological role in regulating cardiovascular function. The mechanisms underlying the vascular relaxant effect of H$_2$S are incompletely understood, although opening of K$_{ATP}$ channels in vascular smooth muscle cells may play an important role (21). Similarly, the effect of H$_2$S on cardiac function was reported by
Geng et al. (6) and their subsequent study on isoproterenol induced myocardial injury (5). Additional studies suggesting a role for H<sub>2</sub>S in the pathogenesis of spontaneous hypertension (19) as well as hypoxic pulmonary hypotension (12) prompted us to study the potential role of H<sub>2</sub>S in myocardial infarction (MI). We hypothesized that H<sub>2</sub>S may protect the ischemic myocardium after MI possibly through the unique mechanisms on reducing cell damage.

**Materials and Methods**

All animal experiments were approved by the Animal Research Ethics Committee, National University of Singapore. The animals were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996).

**In vivo ischemic model (myocardial infarction)**

Male Wistar rats (250-300 g) were anesthetized with 7% w/v chloral hydrate (350 mg/kg, *i.p.*) followed by endotracheal intubation. After thoracotomy, the heart was exteriorized and the left anterior descending coronary artery was permanently ligated using 6-0 silk suture 2-3 mm from its origin between the pulmonary artery conus and the left atrium as previously reported (15, 21). Successful ligation of the coronary artery was verified by the color change immediately in the ischemic area (anterior ventricular wall and the apex) of the heart and MI was confirmed by electrocardiography (ECG) (4, 21).

**Protocol 1**

Wistar rats (250-300 g) were randomly assigned into three groups (n=80), namely MI with vehicle, MI with NaHS pretreatment and MI with PAG (DL–propargylglycine). PAG blocks the effect of CSE and leads to lower the endogenous production of H<sub>2</sub>S by
CSE. In brief, rats were injected i.p. either with saline (1 ml/kg/day), NaHS (14 µmol/kg/day) or PAG (50 mg/kg/day) for 7 days prior to the occlusion of coronary artery to induce MI as we previously described (15, 22, 25). Blood pressure and ECG were measured on the day of experiment initiation, on the day of MI induction and on the day of sacrifice. Surviving rats were killed 48 h after MI induction and the ventricular myocardium was harvested and separated into LV and RV for total RNA extraction. In a separate experiment for the preparation of paraffin tissue sections, the hearts were perfused with PBS for 20 mins and then fixed in paraformaldehyde followed by tissue processing and paraffin embedding, then sectioned at a thickness of 4 µm.

**Homodynamic parameter measurement**

Rat blood pressure (BP) was measured in anaesthetized rats using a tail-cuff Non-invasive Blood Pressure (NIBP) System (ML125/R, AD Instruments PowerLab System) and BioAmp Amplifier (ML 136, AD Instruments PowerLab) respectively as described previously (22, 24, 25). ECG and BP measurements were performed on day 1 (baseline), day 7 (the day of surgery) and day 9 (shortly before killing the animals, 48h after surgery).

**Infarct size measurement**

The entire ventricular tissue was dissected, cut into four horizontal slices and sections. After incubation in 1% w/v 2,3,5 triphenyl tetrazolium chloride (TTC) solution for 15 min (37°C), the sections were immersed in formalin (4% w/v) for another 30 minutes. After fixing, slices were photographed and captured separately as digital images. Total area and infarct area were determined by computerized planimetry (Scion Image, CA, USA). Percentage infarct size was calculated as the infarct area/total left ventricular area.
(22). The infarct area was corrected for tissue weight, summed for each ventricle and calculated as the ratio of the area of the necrotic zone to the left ventricle free wall.

**Cell viability and cell damage measurement**

Cell viability of vascular smooth muscle cells (VSMC, from ATCC) was determined by measuring exclusion of trypan blue. Cells were detached with 1 ml of trypsin, pelleted by 2,000 g (5 min) and resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma, USA). After staining with trypan blue, viable cells in five random fields of view were counted and quantified using a hemocytometer. The percentage of trypan blue exclusive viable cells was determined as a percentage of the total number of cells.

The lactate dehydrogenase (LDH) *in vitro* toxicology assay kit (TOX-7, Sigma-Aldrich, USA) was further used to measure membrane viability as a function of the amount of cytoplasmic LDH released into the medium. LDH assay mixture (30µl) was added to sample aliquots (60µl) taken from the receiver chamber at each sampling point time, followed by mixtures incubated at room temperature for 30 min, 1 M HCl (9 µl) added, and absorbance was measured at 490 nm using a spectrophotometer (TECAN Systems Inc., USA). LDH activity is shown as percentage of LDH release compared with normoxic control.

*Protocol 2*

Vascular smooth muscle cells (VSMC) (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in a humidified incubator with 95% air and 5% CO₂ in DMEM supplemented with 10% (wv⁻¹) heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 0.4% (wv⁻¹) Genticin (Invitrogen). The experiments were performed when the cells reached 80-90% confluence. The cells were placed in serum-free DMEM
for 48 hours. They were then exposed to DMEM supplemented with either no addition (group 1 and 2), 300µM NaHS or 1000µM PAG. The concentrations of NaHS and PAG were chosen based on their effective dosages in the previous studies (17). After one hour treatment, hypoxia was induced by placing treated or non-treated culture into a BBL GasPak Pouch System (Becton Dickinson, USA), which was then tightly sealed and placed back into the incubator for a further 8 hours. Pouches contain an iron-based oxygen consuming chemical, an O2 indicator strip and a CO2 generator. The GasPak Pouch System generates a CO2-enriched hypoxic microenvironment with an oxygen concentration of <2% and a CO2 concentration of > 4% within 2 hours incubation. The cells were used thereafter for viability measurement, LDH assay or RNA isolation. The concentration of H2S release was detected from culture medium. Hypoxia induced cell death and this can be seen from the cell viability test. We tested time-dependent conditions for inducing hypoxia in cultured cells and 8 hours hypoxia was most suitable condition (cell viability at about 70% for vehicle treated hypoxic group).

Measurement of H2S concentration in plasma and culture medium

H2S concentrations were measured in plasma as described previously (9, 10). Briefly, plasma was collected 48 hours after surgery from rat blood before sacrifice followed by centrifugation. We modified the methods for measuring in cell culture medium recently. Culture medium was collected from VSMC flask. 75 µl plasma or medium were mixed with 250 µl of 1% (w/v) zinc acetate and 425 µl distilled water, depending on the volume of plasma used, in a glass test tube. Then 20 mM N-dimethyl-p-phenylenediamine sulphate in 7.2 mM HCl (133 µl) and 30 mM FeCl3 in 1.2 mM HCl (133 µl) were also added to the test tube for 10 minutes incubation at room temperature. The protein in the
plasma was removed by adding 250 µl of 10% tricholoacetic acid, to the reaction mixture and pelleted by centrifugation at 14,000 g (5 min). The absorbance of the resulting solution at 670 nm was measured with a spectrophotometer (TECAN Systems Inc., USA) in a 96-well plate. All samples were assayed in duplicate and concentration in the solution was calculated against a calibration curve of sodium hydrosulfide (NaHS, 3.125 – 250 µM). Results show plasma or medium H₂S concentration in µM.

**RNA extraction and Reverse Transcription – Polymerase Chain Reaction (RT-PCR) analysis of CSE mRNA in ventricular myocardium**

Total RNA from each LV, right ventricle (RV) myocardium or harvested cells were extracted using the TRIzol reagent as in the manufacturer’s instructions (Invitrogen, USA). The concentration of isolated nuclei acid was determined spectrophotometrically by measuring the absorbance at 260 nm.

RT-PCR was performed with QIAGEN® OneStep RT-PCR kit (QIAGEN Inc., U.S.A.). Each reaction contained 0.5 µg total RNA as template, 4 µl of 5× RT-PCR buffer, 0.8 µl of dNTP mix (400 µM), 1.2 µl of each primer (0.6 µM), 0.8 µl of enzyme mix (contains Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase and HotStarTaq® DNA Polymerase) and RNase-free water in a final volume of 20 µl. Rat β-actin served as an internal control gene. The RT-PCR profile was one cycle of cDNA synthesis at 50°C for 30 min and one cycle of initial PCR activation at 95°C for 15 min, followed by 20 cycles (β-actin) or 33 cycles (CSE) of penetration at 94°C for 30 s, annealing at 60°C (β-acting) or 61°C (CSE) and extension at 72°C for 35 s (β-acting) or 30 s (CSE) followed by one cycle of final extension at 72°C for 10 min. After RT-PCR, aliquots of the RT-PCR products were electrophoresed through 1.2% agarose gels (Bio-
Rad, Hercules, CA, U.S.A.) containing 0.5 g/ml of ethidium bromide (Bio-Rad, Hercules, CA, U.S.A.) and visualized under UV light and photographed. Semi-quantitative analysis was obtained using Gel analysis software (Syngene, UK). The primer sequences of β-actin and CSE which were designed to be intron spanning such that genomic DNA contamination was excluded and were as followed: β-actin (forward) 5’-GGG CTG TAT TCC CCT CCA TC-3’, β-actin (reverse) 5’-GTC ACG CAC GAT TTC CCT CTC-3’; CSE (forward) 5’-GAC CTC AAT AGT CGG CTT CGT TTC-3’; CSE (reverse) 5’-CAG TTC TGC GTA TGC TCC GTA ATG-3’. The RT-PCR product size for β-actin and CSE were 552 bp and 618 bp.

**Morphological examination and CSE localization identification**

After dewaxing with serial concentrations of xyline and rehydration with serial concentrations of ethanol (from high to low concentrations gradually), the heart sections for vehicle or PAG pre-treatment groups were stained in Gill’s haematoxylin for 1 min followed by staining in alcoholic eosin for 30 sec., dehydrated with serial concentrations of ethanol (from low to high concentrations gradually) and mounted in Permount (BDH Laboratory Supplies, Poole, England).

In another experiment, a two-step immunostaining Vectastain® ABC kit was employed for detecting the expression of CSE antigens in heart sections. Briefly, after dewaxing and rehydration as described above, sections were treated with 3% (wv⁻¹) H₂O₂ for 10 min to quench endogenous peroxidase activity, and incubated with normal serum for a further 30 min at room temperature. Sections were then sequentially incubated with 1:1,000 diluted CSE antibody (immunized from an antagonistic piece of rat CSE sequence designed by ourselves and serum collected and purified by Biogenes GmbH
(Berlin, Germany) for 1 h. Biotinylated secondary antibody (Vectastain) was then added for 30 min at room temperature. Slides were subjected to microwave antigen retrieval prior to each round of immunohistochemistry as specified individually. Colorimetric detection was performed with 0.02% (w/v) 3′, 3′-diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl buffer. After washing, sections were mildly counterstained with methyl green, and then dehydrated, cleared and mounted. The specificity of labeling was confirmed by the absence of staining upon substitution of sodium phosphorlyate buffer (PBS) or an equal concentration of an irrelevant nonimmune species matched serum for the primary antibody (1:200), or upon omission of secondary antibody (1:200).

Statistics
Data show mean ± s.e. mean. Statistical analysis was by one-way ANOVA followed by post hoc Tukey test. A p value of <0.05 was taken to indicate statistical significance. Chi-square test was employed for calculating the significance of mortality.

Result
The mortality (within 48h after MI) of animals in the vehicle group was 35% after coronary artery ligation. In contrast, a significant reduction in mortality was apparent in animals treated with NaHS (27.5%) (p<0.05 vs vehicle). In contrast, PAG pre-treated animals showed an increase in mortality after MI (40%, p<0.05 vs NaHS treated animals) (Fig. 1) (n=80).

Size of myocardial infarction
To evaluate the effect of H2S on myocardial infarction, we measured infarct size by TTC staining (Fig. 1). The infarct size/total area of myocardium was significantly less in rats subjected to NaHS treatment than in vehicle-injected rats (43.4 ± 2.8% vs. 52.9 ± 3.5%,
However, infarct size was greater (62.9 ± 7.6%, \( p < 0.05 \)) in PAG treated animals. This result was further confirmed by the ratio of weight of ischemic area verse weight of whole left ventricle (0.29±0.05 in NaHS treated animals vs 0.42±0.06 in vehicle treated animals, \( p < 0.05 \), Fig. 1B).

**Hemodynamic parameters**

Hemodynamic measurements of systolic blood pressure in three groups (n=20-21) are shown in Fig. 2. Only significant change was observed in the NaHS pretreated group before MI induction (114.8 ± 7.9 mmHg vs vehicle, \( p < 0.05 \)). Rat HR measured from ECG showed an average HR between 440 ± 24.9 (baseline) and 474 ± 20.0 (beat/min) (day 9) in all rats. However, there was no significant difference on HR among groups.

**Cell viability and cell cytotoxicity assay**

Cell viability measurement (n=12) demonstrated that cell death was increased under the stimuli of hypoxia (71 ± 1.2% cell viability in hypoxic vehicle vs 95 ± 2.3% in non-hypoxic control, \( p < 0.05 \)). The increased cell death was attenuated by the pre-treatment of NaHS (85 ± 1.8%), but this effect was abolished significantly by PAG pre-treatment (62.2 ± 2.1%) (Fig. 3A).

LDH activity was measured (n=12) as NADH oxidation in order to correctly quantitate the observed cytotoxicity. Fig. 3B shows the LDH activity was increased (1.65-fold compared to control, \( p < 0.05 \)) in vehicle treated hypoxic cells. In contrast, when cells was pre-treated with NaHS, the LDH activity decreased sharply (NaHS 0.93 ± 0.12% vs vehicle 1.65 ± 0.18%, \( p < 0.05 \)) but remained at the same level for the PAG pre-treatment group when compared to vehicle group (Fig. 3B).

**Plasma and culture medium \( \text{H}_2\text{S} \) level measurement**
Plasma H$_2$S concentration in the vehicle rats 48 h after MI induction was elevated to 59.2 ± 7.16 µM as compared to the baseline value at 38.2 ± 2.07 µM (n=12, p<0.05). Plasma H$_2$S concentration was significantly decreased in animals treated with PAG  (39.2 ± 5.02 µM, n=12, p<0.05) but was significantly increased in animals administered with NaHS group (92.2 ± 12.40 µM, n=12, p<0.05). The plasma H$_2$S concentration in NaHS-treated rats was approx. 2.4 times higher than PAG pre-treated rats (Fig. 4A). The measurement of H$_2$S content from culture medium was shown in Fig. 4B. H$_2$S content was 81.2 ± 3.2 µM in the culture medium from vehicle and 69.3 ± 1.2 µM in the PAG treated cells. NaHS treated cells has highest H$_2$S content in their culture medium (107.6 ± 5.3 µM).

**CSE mRNA expression**

To further verify the role of endogenous H$_2$S in myocardium and identify the alternations after ischemic injury, we accessed the expression of CSE mRNA by RT-PCR (Fig. 5) at 618 bp. An isoform (486 bp) of CSE gene was detected as we reported by GenBank (access no. AY641456). The relative amount of CSE mRNA in myocardium of control rats subjected to MI group decreased 2.70-fold compared with that in the sham control group (61.75 ± 3.45 vs. 166.84 ± 1.18 [arbitrary unit, gene expression density] in LV and 66.84 ± 2.36 vs. 147.56 ± 2.36 in RV, respectively, n = 4, p<0.05), which indicated that CSE gene expression was down-regulated in the development of myocardial ischemia. Consistently, CSE gene expression was remarkable suppressed after PAG pretreatment (2.3-fold in LV and 3.1-fold in RV decrease respectively when compared with MI vehicle, p<0.05).

**Morphological change and the localization of CSE in myocardium**
Histologically, at 48 hours after MI, the infarcted cardiac muscle fibres exhibited patchy loss or blurring of striation, which were expressed intensely eosinophilic and most had lost their nuclei (Fig 6A, left). Nonetheless, there was marked infiltration by neutrophils into the oedematous interstitium with the necrotic myocardium undergoing autolysis and fragmentation in the PAG-pretreated myocardium after MI induction (Fig. 6A, right).

CSE immunoactivity was detected in the vehicle treated myocardium of the infarct area (Fig 7A) and in the endothelium of small vessels of area-at-risk (Fig 7B) after MI induction. In contrast, CSE was rarely expressed in the sham group (Fig 7C), suggesting the overexpressed CSE was mainly effected in the injured myocardium.

Discussion

The pathophysiology of mechanisms underlying in MI has yet been elucidated. The recent discovery of H2S’s effect in different diseases, that has functions in vasodilation (21), as a neuromodulator (1, 18) as well as a novel mediator of lipopolysaccharide-induced inflammation (9), have put forward possibilities of this gas with roles to play in cardiovascular and neurological diseases. H2S was found to be endogenously produced in heart and vascular tissue, and could act as a regulator of physiological cardiac function an effect suggested to be mediated by KATP channels (6, 21). A potential protective effect by H2S on cardiac myocytes during isoproterenol-induced MI was also demonstrated (5). All these suggest the potential of H2S as an important modulator in cardiovascular physiology and pathophysiology. Since the possible role of H2S in the pathogenesis of ligature-induced MI has not been investigated, our current study demonstrates for the first time, a role of endogenous H2S in ligature-induced MI in rats.
Role and protective effects of H$_2$S in MI

Beneficial effects were produced by H$_2$S on MI in rats, as seen from a decreased mortality rate of MI rats that underwent NaHS treatment, with only 27.5% of the rats dying after induction of MI. This was approximately three-quarters that of the vehicle group, which had a mortality rate of 35% (Fig. 1). The size of the infarct area is an important gauge of the extent of ischemia as well as remodeling in MI, and thus is critical for prognosis. Therefore, beneficial effects of H$_2$S were seen again as the infarct size was also diminished in MI rats treated with NaHS, whereas decreased levels of H$_2$S produced by inhibiting CSE with PAG resulted in a larger infarct area (Fig. 1). Administration of PAG caused significantly higher mortality rate in animals.

Cardiac function was evaluated by looking at the hemodynamic parameters such as BP, ECG and HR. HR was not affected, both before and after MI. Systolic BP was observed to be reduced from the NaHS 7 days after treatment (Fig. 2) which indicated a hypotensive effect of H$_2$S after chronic administration.

H$_2$S has been found to be a potent vasodilator (21) and its inhibition or downregulation should produce the opposite effects. This was indeed the case, as shown by systolic BP measurements in PAG treatment rats (Fig. 2). PAG successfully decreased the levels of H$_2$S in the plasma (Fig. 4A) and in the medium of cultured cells (Fig. 4B), most likely via an irreversible inhibition of its producing enzyme – CSE, and this effectively increased the systolic BP after seven days of PAG administration in rats.

The possible role of H$_2$S in the pathogenesis of MI has not been reported. This study demonstrates for the first time, a role of H$_2$S in a rat model of MI. NaHS significantly decreased the infarct size of the left ventricle and mortality after acute MI in
rats. It could be assumed that vessel dilating/relaxing effects of NaHS could dilate coronary arteries and increase coronary blood flow in ischemic diseases, thus reducing cellular damage from ischemia. This result is also consistent with our finding that NaHS treated group had lower mortality rate.

Similar results have been noted in the hypoxic in vitro cell culture model. The cell viability of vehicle treated hypoxic cells was the lowest, which was correlated with high levels of LDH level being detected at the same time. This may offer a probable explanation in that hypoxic cells in vitro, similarly, produce a lower amount of H$_2$S and thus more cellular damage was inflicted. Expectedly, the addition of NaHS significantly increased the cell viability as compared to the cells treated with vehicle in hypoxia. This result was consistent with the LDH assay, where the LDH level was significantly decreased in NaHS treated group. NaHS treatment elevated the plasma concentrations of H$_2$S, while PAG caused a considerable drop in plasma H$_2$S levels (Fig. 4A). Similar pattern of changes were observed in culture medium of H$_2$S content (Fig. 4B).

Results for the assays for plasma H$_2$S concentration and H$_2$S biosynthesis activity in myocardial tissue, as well as in CSE gene expressions opened an avenue for understanding the possible role of H$_2$S in ischemic conditions. MI and PAG treatment resulted in a decreased CSE gene expression (Fig. 5). However, Geng et al. reported upregulated CSE gene expression in isoproterenol (ISO) injected rat hearts induced myocardial ischemic injury model (5). A possible explanation could be that the animal models were different: surgically-induced cardiac ischemia was used in our current study while Geng and colleagues used the model of chemically-caused cardiac damage.
Another possible reason could be the different timing of tissue collection: our animal hearts were collected 48 h after MI but Geng and colleagues collected their samples on the last day of ISO treatment. It is well-known that most genes (including CSE) are transiently expressed.

In another study, Geng et al. showed that mRNA of CSE was expressed in myocardial tissues and H2S could endogenously be produced in myocardial tissues (6). Beside detecting CSE gene expression in heart, we have also localized CSE protein expression using our own raised CSE antibodies. In the infarct area, obvious increased CSE expression was observed (Fig. 7) which could be a ‘compensatory effect’ to produce H2S to cope with the damage of LV. CSE expression was also found at the area-at-risk beside the necrotic tissue while few staining was seen in cardiomyocytes. The results demonstrated further that production of H2S should be cardioprotective against ischemic insult. Our morphological study confirmed again this statement: more significant autolysis and fragmentation were seen under the microscope in the PAG pre-treated myocardium when compared with NaHS treated myocardium (Fig. 6).

In conclusion, we demonstrated for the first time decreases in infarct size and mortality are linked to elevated plasma H2S concentrations after MI, and the opposite, where decreased H2S levels in the plasma was associated with an increased infarct size and mortality. Similar observation were found in our in vitro study whereby higher level H2S content in the culture medium increased cell viability after exposing in hypoxic conditions. Our results suggested a significance of H2S in the reduction of cellular damage inflicted on cells exposed to hypoxia. Exogenously administered H2S via NaHS showed improved conditions in MI rats compared to the vehicle, while PAG
administration showed worsened conditions. Same was seen in the in vitro study where inhibition of endogenous H$_2$S had a negative consequence on the cell viability as well as a subsequent increase in LDH production. Lastly, this study strengthens the possibility of H$_2$S to be a potent cardioprotective agent, however, exact mechanisms remain to be further investigated.

**Acknowledgement:**

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References


Figure legends:

**Fig 1. (A)** Mortality and myocardial infarct size changes in vehicle, PAG and NaHS pre-treatment groups. *p*<0.05 infarct size in NaHS group vs vehicle and PAG treated groups; #p<0.05 infarct size in PAG group vs vehicle and NaHS treated groups. §p<0.05 mortality in NaHS group vs vehicle and PAG treated groups. (B) Comparison of the ratio of ischemic area against free wall in vehicle treated rat, in PAG pretreated rats and in NaHS pretreated rats. PAG= DL-propargylglycine. *p*<0.05 vs PAG treated group. (C) Representative image of infarct area and free wall.

**Fig 2.** Blood pressure change in vehicle, PAG and NaHS pre-treatment groups. PAG= DL-propargylglycine. *p*<0.05 vs vehicle and PAG treated rats.

**Fig.3 (A)** Cell viability measurement (n=12) in control (non-hypoxia) and hypoxic vehicle, NaHS and PAG pre-treatment groups. (B) Relative LDH liberation (n=12) in control (non-hypoxia) and hypoxic vehicle, NaHS and PAG pre-treatment groups. (*Hypoxic vehicle vs control, p*<0.05; #NaHS treated vs vehicle, p<0.05; §PAG vs NaHS treated, p<0.05). PAG= DL-propargylglycine.

**Fig. 4. (A)** Alteration of plasma H2S level in vehicle, PAG and NaHS pre-treatment groups after 48 h of MI induction (*PAG vs vehicle, p*<0.05; #NaHS vs vehicle, p<0.05; †NaHS vs PAG, p<0.01; n = 12 each). (B) The measurement of H2S content from culture medium 8 h after hypoxia (*PAG vs vehicle, p*<0.05; NaHS vs PAG, p<0.05; n=12 each). PAG= DL-propargylglycine.

**Fig 5.** Image of RT-PCR showing the CSE mRNA expression in PAG-pretreatment, MI rat ventricles and MI with PAG pretreatment rat ventricles (n = 4 each) (A) and its Semi-
quantitative measurement (B); M=marker, LV=left ventricle, RV=right ventricle, MI=myocardial infarction, PAG= DL-propargylglycine.

**Fig. 6** Morphological expression change under the effect of PAG. (A) MI induction without pretreatment of PAG. (B) The edema myocardium in intense sarcoplasmic eosin staining expressed with inflammatory cell infiltration in NaHS pretreated rat heart. (C) PAG pretreatment followed by MI induction in LV myocardium (the box in the bottom right corner [magnification x 400] demonstrated the amplified infiltrated neutrophils/inflammatory cells). There was marked infiltration by neutrophils into the edematous interstitium with the necrotic myocardium undergoing autolysis and fragmentation in the PAG pretreated myocardium. PAG= DL-propargylglycine.

**Fig. 7.** Distribution of CSE in the ischemic myocardium. The CSE immunoactivity was detected in the infarct area (A) and in the endothelium of small vessels of area-at-risk (B) (both A and B are vehicle treated group). In contrast, CSE is slightly detected in the cardiomyocytes (C). The negative staining was presented in (D).
Fig. 1

(A)

![Graph showing mortality and infarct size](image)

n=80 in each group for mortality, n=10-12 for infarct size

(B)

![Graph showing area (IA)/free wall (LV)](image)

n=10-12 in each group
Fig. 2

![Bar chart showing systolic blood pressure (mmHg) before treatment, before MI, and 48h after MI for Vehicle, PAG, and NaHS groups.]

Systolic BP (mmHg)

- Vehicle
- PAG
- NaHS

n= 20-21 in each group
Fig. 3

(A) Cell viability (%)

![Bar graph showing cell viability with data points marked with asterisks and hashtags.]

(B) LDH activity (%)

![Bar graph showing LDH activity with data points marked with asterisks and hashtags.]

n=6
Fig. 4

(A) 

Plasma H$_2$S Concentration (uM)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
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<td>NaHS</td>
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n=12

(B) 

Medium H$_2$S content (uM)

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<td>PAG</td>
<td>60.0</td>
</tr>
<tr>
<td>NaHS</td>
<td>120.0</td>
</tr>
</tbody>
</table>

n=12
Fig. 5

(A)

CSE
618 bp

M  sham-LV  sham-RV  MI-LV  MI-RV

CSE
618 bp

M  sham-LV  sham-RV  PAG-LV  PAG-RV

β-actin
552 bp

M  sham-LV  sham-RV  MI-LV  MI-RV  PAG-LV  PAG-RV

n = 4

(B)

Relative CSE mRNA content (arbitrary unit)

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV</td>
<td>160</td>
<td>180</td>
</tr>
<tr>
<td>RV</td>
<td>120</td>
<td>140</td>
</tr>
</tbody>
</table>

Relative CSE mRNA content (arbitrary unit)

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>PAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV</td>
<td>120</td>
<td>140</td>
</tr>
<tr>
<td>RV</td>
<td>80</td>
<td>100</td>
</tr>
</tbody>
</table>

* *
Fig. 6

A: MI

B: MI-NaHS

C: MI-PAI

100 μm

25 μm
Fig. 7