

Gaseous Hydrogen Sulfide Protects against Myocardial Ischemia-Reperfusion Injury in Mice Partially Independent from Hypometabolism

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Abstract

Background: Ischemia-reperfusion injury (IRI) is a major cause of cardiac damage following various pathological processes. Gaseous hydrogen sulfide (H₂S) is protective during IRI by inducing a hypometabolic state in mice which is associated with anti-apoptotic, anti-inflammatory and antioxidant properties. We investigated whether gaseous H₂S administration is protective in cardiac IRI and whether non-hypometabolic concentrations of H₂S have similar protective properties.

Methods: Male C57BL/6 mice received a 0, 10, or 100 ppm H₂S-N₂ mixture starting 30 minutes prior to ischemia until 5 minutes pre-reperfusion. IRI was inflicted by temporary ligation of the left coronary artery for 30 minutes. High-resolution respirometry equipment was used to assess CO₂-production and blood pressure was measured using internal transmitters. The effects of H₂S were assessed by histological and molecular analysis.

Results: Treatment with 100 ppm H₂S decreased CO₂-production by 72%, blood pressure by 14% and heart rate by 25%, while treatment with 10 ppm H₂S had no effects. At day 1 of reperfusion 10 ppm H₂S showed no effect on necrosis, while treatment with 100 ppm H₂S reduced necrosis by 62% (p<0.05). Seven days post-reperfusion, both 10 ppm (p<0.01) and 100 ppm (p<0.05) H₂S showed a reduction in fibrosis compared to IRI animals. Both 10 ppm and 100 ppm H₂S reduced granulocyte-influx by 43% (p<0.05) and 60% (p<0.001), respectively. At 7 days post-reperfusion both 10 and 100 ppm H₂S reduced expression of fibronectin by 63% (p<0.05) and 67% (p<0.01) and ANP by 84% and 63% (p<0.05), respectively.

Conclusions: Gaseous administration of H₂S is protective when administered during a cardiac ischemic insult. Although hypometabolism is restricted to small animals, we now showed that low non-hypometabolic concentrations of H₂S also have protective properties in IRI. Since IRI is a frequent cause of myocardial damage during percutaneous coronary intervention and cardiac transplantation, H₂S treatment might lead to novel therapeutical modalities.

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Introduction

Ischemia-reperfusion injury (IRI) is the most important cause of myocardial damage and subsequent heart failure. Although IRI is most frequently caused by acute myocardial infarction with (early or late) reperfusion, it can also be observed following surgical procedures such as cardiopulmonary bypass or cardiac transplantation. [1,2] Myocardial IRI causes acute tissue responses characterized by inflammation and upregulation of inflammatory mediators. This process ultimately leads to irreversible fibrotic damage. [3,4] Despite major therapeutical developments, cardiovascular disease remains the leading cause of death in the western world [5].

Hydrogen sulfide (H₂S) has drawn considerable attention for its role in various (patho)physiological processes. It is, in addition to nitric oxide and carbon monoxide, acknowledged as the third gasotransmitter, sharing many functions with these gases. [6] H₂S is endogenously produced and exerts fine, modulatory control over cellular functions by influencing an array of intracellular signaling processes. H₂S-producing enzymes and H₂S-plasma levels are reduced in various diseases.[7–9] Exogenously administered H₂S can reversibly induce a hypometabolic state in mice, during which it rapidly reduces O₂-consumption, CO₂-production, core body temperature, heart rate and breathing frequency. [10,11] The most probable mechanism for these properties is the reversible

inhibition of mitochondrial O₂-consumption and ATP-production through non-permanent binding of sulfide to the terminal enzyme in the electron transport chain, cytochrome c oxidase (complex IV). [12] It was thought that the reduced demand for oxygen during hypometabolism might be one of the protective mechanisms during ischemia. However, H₂S is also considered protective during other processes critically involved in myocardial IRI such as oxidation, inflammation and apoptosis. These cytoprotective features of H₂S make it an attractive candidate for therapeutic reduction of the damaging effects of hypoxia [13,14].

The influence of gaseous administration of H₂S and the effects of hypometabolic and non-hypometabolic concentrations on the outcome of myocardial IRI remains to be elucidated. Some studies have explored the beneficial effects of soluble H₂S donors such as NaHS and Na₂S in myocardial IRI and other models of cardiac damage.[15–18] The preference for gaseous administration above injection with H₂S donors lies within accurate management of the concentration. As opposed to injection with soluble H₂S donors, gaseous H₂S is less difficult to dose and has a short wash-out period, leaving its positive effects behind. [11] Moreover, gaseous administration has proven to induce a hypometabolic state, while this has not been shown for intra-peritoneal or intra-venous administration of soluble H₂S. [10,11] Although H₂S does not appear to have hypometabolic effects in ambiently cooled large mammals, thereby questioning its therapeutic applications in humans, the beneficial effects of non-hypometabolic concentrations of H₂S have not been studied. [19,20] Since minimizing myocardial IRI has broad clinical implications and may have beneficial effects on cardiac surgical outcomes [1], we investigated whether gaseous H₂S-treatment attenuates myocardial IRI in mice and whether non-hypometabolic concentrations exhibit similar protective properties.

Materials and Methods

Ethics Statement

Procedures were in agreement with institutional and legislator regulations and approved by the Committee on the Ethics of Animal Experiments of the University Medical Center Groningen (DEC 5276). Utmost effort was utilized to prevent suffering and minimize the numbers of mice required for each experiment.

Animals

Male C57BL/6 mice (6–8 weeks, Harlan, Zeist, the Netherlands) were housed at our animal research facility under standard conditions with a 12 h light:dark cycle with free access to water and chow.

Telemetry

Blood pressure was measured telemetrically (n = 4) using transmitters (TA11PA-C10; Data Sciences International, St Paul, MN, USA). Devices were placed through a midline abdominal incision under anesthesia (2% Isoflurane) and mice were placed on a heating pad to maintain body temperature at 37°C. The catheter was placed in the aorta and the transmitter body in the abdominal cavity. Animals recovered 7 days before commencing measurements. Data were recorded as 10-second averages every minute using Dataquest ART data acquisition system (Data Sciences International). Animals were treated with room air or a H₂S/air mixture in our respirometry system during measurements. For comparison of blood pressure and heart rate, the average of 20 minutes baseline measurement and 20 minutes of 10 ppm and 100 ppm H₂S treatment was determined. A crossover design was

used in which all animals received all treatments in randomized order.

Respirometry

Measurement of CO₂-production was performed as described. [21] Compressed air and 500 ppm H₂S/N₂ (Air Products, Amsterdam, the Netherlands) were mixed in a 4:1 ratio and in a 49:1 ratio resulting in a 100 ppm H₂S/17% O₂ mixture and a 10 ppm H₂S/17% O₂ mixture, respectively. CO₂-production was corrected for body weight and normalized to mean control values. Animals (n = 11) were treated in a crossover model in randomized order and all received room air, 10 ppm H₂S and 100 ppm H₂S on different days. Baseline CO₂ measurements with room air were performed for 30 minutes followed by treatment with either a 10 or 100 ppm H₂S/17% O₂ mixture for 30 minutes. Recovery with room air was measured for 30 minutes.

Myocardial Ischemia-reperfusion and H₂S Treatment

Mice were intubated and mechanically ventilated (n = 77) with an O₂/N₂ mixture in a 4:1 ratio, an O₂/100 ppm H₂S/N₂ mixture in a 4:1 ratio or a O₂/10 ppm H₂S/N₂ mixture in a 49:1 ratio at a frequency of 180/min with a tidal volume of 250 µl using a rodent ventilator (Harvard Midivent model 849). Treatment regimens (Sham n = 15; IRI n = 20; 10 ppm n = 21; 100 ppm n = 21) were randomly assigned and started 30 minutes prior to ischemia until 5 minutes pre-reperfusion. Myocardial IRI was inflicted by temporary ligation of the left anterior descending coronary artery (6.0 prolene suture) for 30 minutes through an incision in the fourth intercostal space under anesthesia (75 mg/kg ketamine, 1 mg/kg domitor). After removing the ligature the heart was inspected for restoration of blood flow and muscle and skin layers were sutured with 5.0 vicryl. Body temperature was monitored with a rectal probe and maintained at 37°C using heat pads. Sham operated animals underwent the same procedure, except the placement of the ligature. Post-operatively, all mice received a subcutaneous injection of 50 µg/kg buprenorphin (Schering-Plough) for analgesic purposes and were allowed to recover from surgery at 37°C in a ventilated incubator. After 1 and 7 days mice were anaesthetized with 2% isoflurane in O₂ for collection of blood and organs. Blood was collected in heparin containing tubes, centrifuged for 10 minutes at 1000 rcf and plasma was collected and stored at –80°C. The hearts were rapidly excised and mid-papillary slices were fixed in 4% paraformaldehyde, paraffin-embedded and sections were cut for immunohistochemical analysis. Apical parts of the heart were snap frozen in liquid nitrogen and stored at –80°C for molecular analysis.

Serum Analysis

Cardiac damage was assessed by measuring high sensitive (hs) Troponin-T in serum samples using a standard electrochemiluminescence immunoassay (Roche) in the clinical chemical laboratory.

Histopathological Scoring

At 1 day of reperfusion the extent of necrosis was determined in haematoxylin-eosin stained sections. At 7 days of reperfusion the extent of fibrosis was determined in Masson stained sections. Both were examined in a blinded fashion. Sections were scanned using an Aperio ScanScope GS (Aperio Technologies, Vista, CA, USA). Total cardiac area, necrotic cardiac area and fibrotic cardiac area were determined using Aperio Imagescope software, and the ratio of necrotic cardiac surface area and fibrotic surface area to total

cardiac surface area were determined. Representative photomicrographs were artificially colored indicating the extent of damage [11,21].

Immunohistochemistry for Ly-6G

For granulocytes, paraffin-embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G/C-FITC IgG2b antibody (AbCam, Cambridge, MA, USA), followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Slides were scanned using an Aperio ScanScope GL (Aperio Technologies, Vista, CA, USA) and analyzed for positive pixel area (Ly-6G) using the Aperio Positive Pixel Analysis v9.1 algorithm.

Qualitative Real-Time Polymerase Chain Reaction

RNA extraction, DNAase treatment [21] and cDNA synthesis [22] were performed as described. A relative quantification PCR was performed to determine gene expression (Applied Biosystems, Foster City, CA). β -actin and GAPDH were used as housekeeping genes. The primers used were: fibronectin (NM_010233.1) – Forward: AGGAAATGTAAGTGAATGCTAGTACCCA, Reverse: TCAGATGGCAAAGAAAGCAGA; ANP (NM_008725.2) – Forward: ACCCTCCTGGAGCTGCG, Reverse: ACCCCACTAGACCCTCATCTACAT; NOX2 (NM_007807.4) – Forward: GATGCAATAAGACTAGGCCAAACC, Reverse: CCATCTCATAACCAGAATAAATCTCAGGATA; NOX4 (NM_015760.4) – Forward: TGCACCAAACA-CAGAAGCACA, Reverse: AGCAGGGTATCACTCCATGAATTC. PCR was performed in a volume of 20 μ l containing 10 ng cDNA and 15 μ l PCR mastermix (SYBR GREEN Applied Biosystems; 5 ml P/N 4309155). The Thermal Profile was performed as described. [22] The average Ct-values for fibronectin, ANP, NOX2 and NOX4 were subtracted from the average β -actin Ct-values and the average of β -actin and GAPDH Ct-values to yield the delta Ct. Results were expressed as $2^{-\Delta Ct}$.

Cell Culture

The H9c2 cell line (ATCC) is an immortalized line with characteristics of rat heart myoblasts. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Germany) containing 4.5 g/l glucose, 10% fetal calf serum (FCS; Bodinco, Alkmaar, the Netherlands), L-glutamine and penicillin (100 U/ml) streptomycin (100 μ g/ml) (Lonza, Germany). Cells were cultured using 75 cm² collagen coated flasks (Corning, Schiphol-Rijk, Netherlands) in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37°C.

In vitro Model of Oxidative Stress

H9c2 cells grown to 80–90% confluency were harvested using 3 ml trypsin EDTA (200 mg/l) after washing twice with Hank's Buffered Salt Solution (HBSS) (Lonza; Germany). Cells were cultured in a 24-well plate at a density of ~10,000 cells/well in 0.5 ml medium. After 24 hours cells were loaded with 15 μ M Dihydroethidine (DHE). Culture plates were placed in a humidified chamber with 5% CO₂ on an automated inverted fluorescent microscope system (TissueFAXS system, TissueGnostics GMBH, Vienna, Austria) which makes sequential photomicrographs of 9 area's in each well every 5 minutes. After baseline measurements, cells were exposed to Antimycin (50 μ g/mL) and NaHS (donor of H₂S in solution) in a concentration of 1 mM. Fluorescence intensity of every cell was analyzed using the TissueQuest software (TissueGnostics).

Statistical Analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, CA, USA) using two-way ANOVA, Mann-Whitney U, Friedman or Kruskal Wallis tests where appropriate. Bonferroni or Dunns post-hoc analysis were applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirnov test. $p < 0.05$ was considered statistically significant. All data are expressed as mean \pm SEM (Standard Error of the Mean) unless otherwise indicated.

Results

Effect of H₂S on CO₂ Production, Blood Pressure and Heart Rate

Within 15 minutes of treatment with 100 ppm H₂S induced a state of hypometabolism, concomitant with a reduction in CO₂-production by an average of 72% compared to basal levels ($p < 0.001$). Cessation of H₂S resulted in a rapid recovery of CO₂-production, where CO₂ concentrations raised to basal levels within 30 minutes ($p < 0.001$). 100 ppm H₂S lowered blood pressure with 14% (103 *vs.* 120 mmHg, $p < 0.05$) and heart rate with 25% compared to baseline (502 *vs.* 670 beats per minute, $p < 0.05$) 10 ppm H₂S had no effect on CO₂ production, blood pressure and heart rate (Figure 1).

H₂S Reduces Myocardial Damage

At 1 day of reperfusion cardiac IRI induced significant necrosis (Figure 2A) in animals exposed to 0 ppm H₂S when compared to sham animals ($p < 0.001$) as indicated by infarct size. 10 ppm H₂S did not reduce the size of the necrotic area, while 100 ppm H₂S reduced infarct size by 62% ($p < 0.05$) (Figure 2B). In mice treated with 10 ppm H₂S hs Troponin-T levels were not reduced 1 day post-reperfusion, while 100 ppm H₂S reduced hs Troponin-T levels by 47% ($p < 0.05$) compared to IRI animals (Figure 2C). Fibrosis, as measured by collagen deposition in Masson stained sections at 7 days of reperfusion, was markedly increased in animals treated with 0 ppm H₂S when compared to sham-operated animals ($p < 0.001$). Treatment with either 10 or 100 ppm of H₂S reduced collagen deposition to comparable levels (10 ppm: 59%, $p < 0.01$; 100 ppm: 57%, $p < 0.05$) (Figure 3A,B). Cardiac mRNA levels of fibronectin, a marker of myocardial fibrosis, were massively increased in 0 ppm H₂S treated animals ($p < 0.01$), while no increase was detected in animals of both H₂S treated groups (10 ppm H₂S $p < 0.05$; 100 ppm H₂S $p < 0.01$) (Figure 3C). Seven days post-reperfusion hs Troponin-T levels were reduced by 59% ($p < 0.05$) and 75% ($p < 0.01$) in 10 ppm and 100 ppm H₂S treated mice, respectively (Figure 3D).

ANP-gene Expression

mRNA expression of atrial natriuretic peptide (ANP), a marker for induction of the fetal gene program, was significantly increased in hearts of mice treated with 0 ppm H₂S compared to sham-operated animals at 7 days of reperfusion. In hearts of 10 and 100 ppm H₂S treated mice the relative ANP expression was significantly reduced compared to mice treated with 0 ppm H₂S ($p < 0.05$) (Figure 3E).

Inflammation

One day post-reperfusion, Ly-6G-positive granulocytes were increased 12-fold in animals treated with 0 ppm H₂S compared to sham-operated animals ($p < 0.001$). Exposure to 10 ppm and

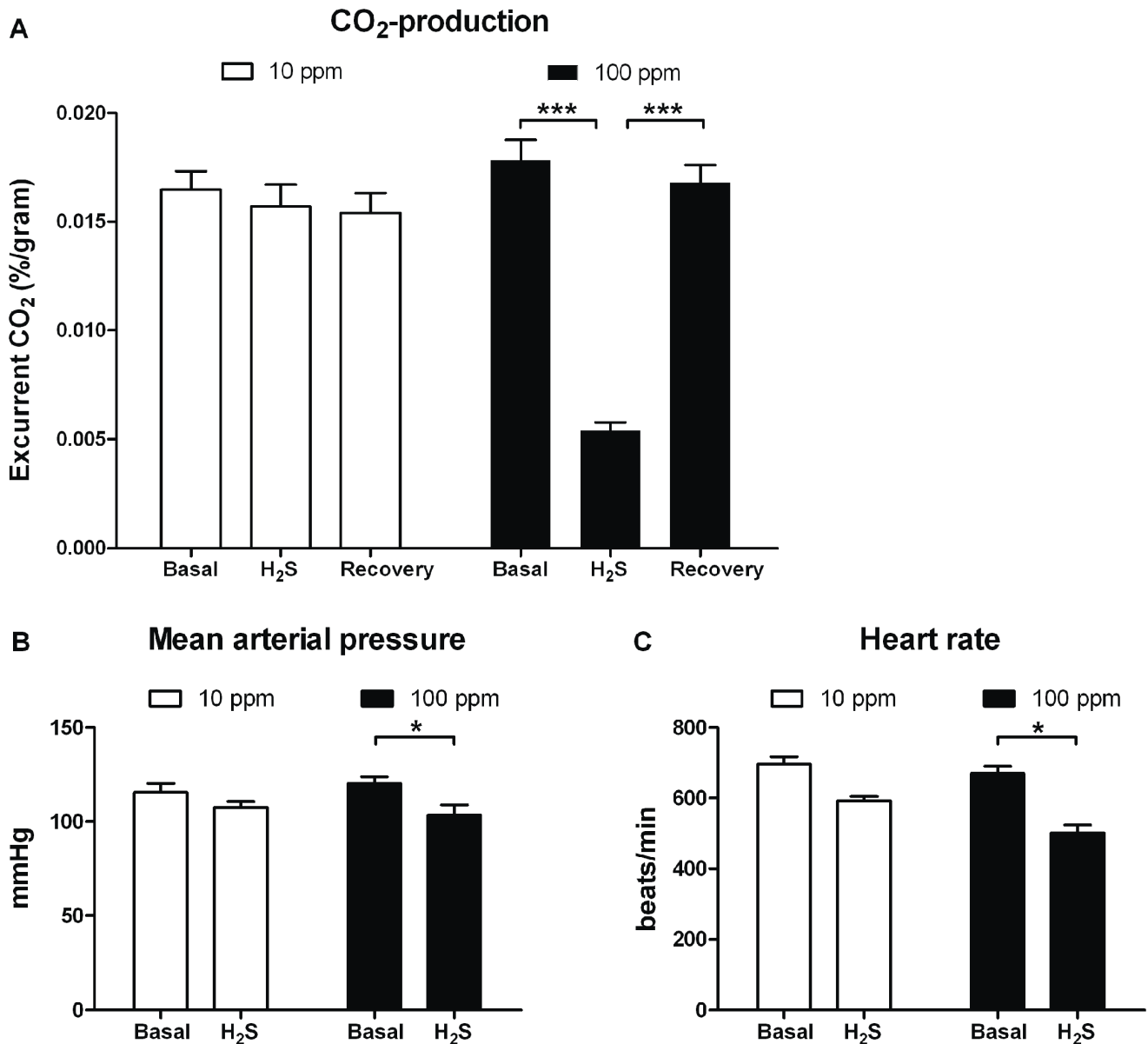


Figure 1. High concentrations of H₂S induce a state of hypometabolism. Within 15 minutes (A) CO₂-production decreased by 72% (**p<0.001) in mice (n=11) subjected to 100 ppm H₂S. Exposure to 10 ppm H₂S (n=11) did not induce a reduction in CO₂-production in these animals. Cessation of gaseous H₂S resulted in rapid recovery, within 30 minutes CO₂-levels returned back to baseline concentrations (**p<0.001). Administration of 100 ppm H₂S (n=4) resulted in a 14% decrease in (B) mean arterial pressure and a 25% decrease in (C) heart rate (*p<0.05). However exposure to 10 ppm H₂S (n=4) had no effect on mean arterial pressure or heart rate. doi:10.1371/journal.pone.0063291.g001

100 ppm H₂S reduced granulocytes by 43% (p<0.05) and 60% (p<0.001), respectively (Figure 4).

NOX2 and NOX4- Gene Expression

To investigate ROS-related genes *in vivo*, we measured mRNA expression of nicotinamide adenine dinucleotide phosphate oxidase 2 and 4 (NOX2 and NOX4). At 1 day of reperfusion no significant differences were found in NOX2 and NOX4 mRNA expression. Seven days post-reperfusion, NOX2 and NOX4 expression were significantly increased in hearts of mice treated with 0 ppm H₂S compared to sham operated animals (NOX2: p<0.05, NOX4: p<0.01). NOX2 and NOX4 expression were not amplified in hearts of mice treated with 10 and 100 ppm

H₂S compared to mice treated with 0 ppm H₂S (p<0.05) (Figure 5A and B).

Effect of H₂S on ROS Production *in vitro*

Antimycin A induced ROS production in cultured H9c2 rat cardiomyoblasts was significantly reduced by treatment with NaHS. Live cell imaging of DHE fluorescence showed a massive increase in cytoplasmic ROS production during treatment with Antimycin, whereas addition of NaHS to the medium markedly reduced this fluorescence signal (p<0.001) (Figure 6).

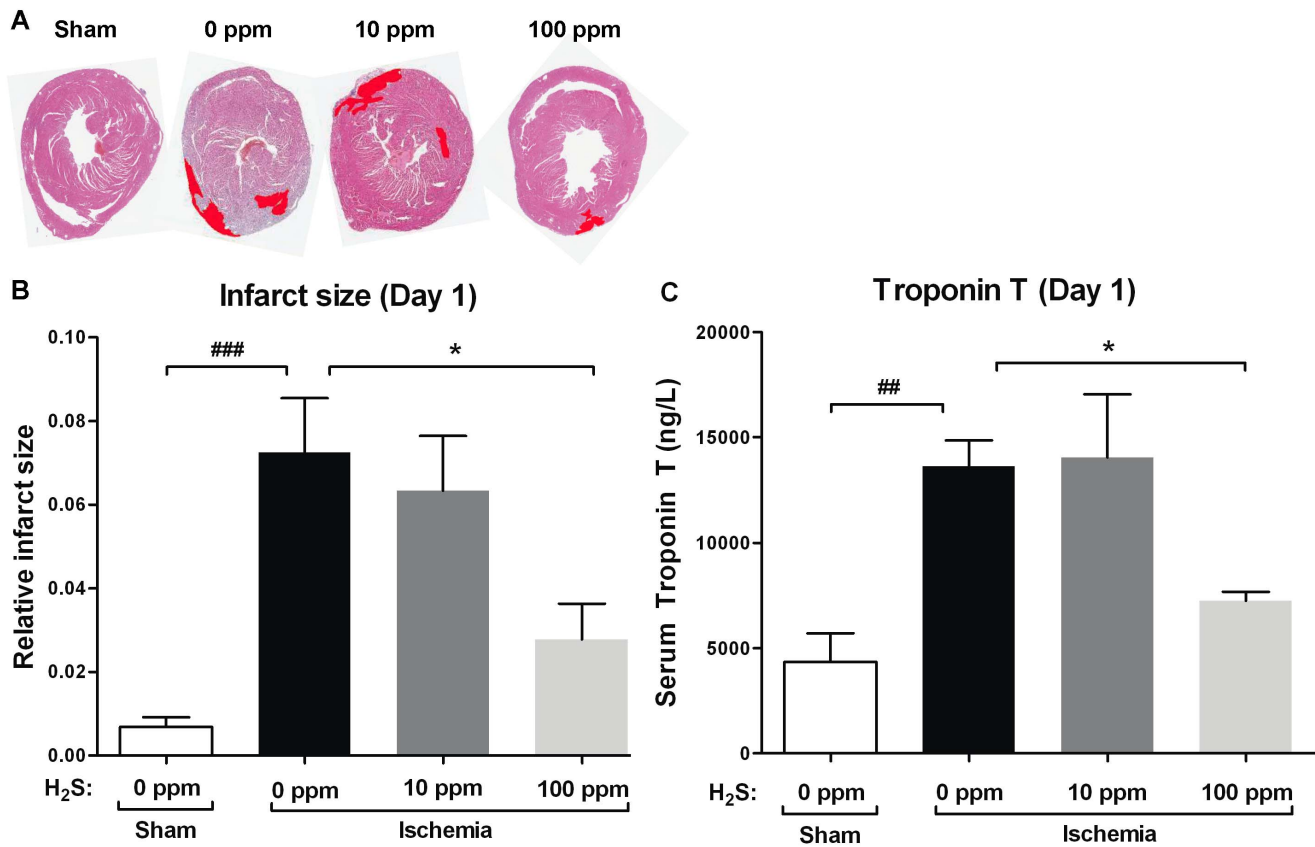


Figure 2. Cardiac damage is reduced by 100 ppm H₂S at 1 day of reperfusion. (A) Representative photomicrographs of haematoxylin-eosin stained cardiac sections with necrotic area artificially colored red, indicating the extent of necrotic damage found in each group at 1 day of reperfusion. (B) Cardiac IR induced a significant amount of necrosis in IRI animals exposed to 0 ppm H₂S (###*p*<0.001 vs. sham). In animals treated with 100 ppm H₂S necrosis was reduced by 62% (**p*<0.05 vs. IRI) where as 10 ppm H₂S had no effect on necrosis. (C) At 1 day of reperfusion hs Troponin-T levels were elevated in IRI animals exposed to 0 ppm H₂S (###*p*<0.01 vs. sham). In the 100 ppm H₂S treated group hs Troponin-T levels were reduced compared to 0 ppm treated animals (**p*<0.05), 10 ppm H₂S had no effect. doi:10.1371/journal.pone.0063291.g002

Discussion

The major finding of this study is that administration of hypometabolic concentrations of gaseous H₂S during myocardial IR limits the extent of myocardial damage. Furthermore, non-hypometabolic concentrations of H₂S do not seem protective in the early phase after myocardial infarction, but attenuate ischemia associated processes such as fibrosis and ROS formation. Gaseous administration of H₂S appears to be an effective way to attenuate the outcome of myocardial IRI, with multiple mechanisms seemingly underlying the protective properties.

H₂S is cytoprotective during hypoxia in multiple organs. Beneficial effects of H₂S treatment have been reported in models of shock [23] and intestinal- [24], pulmonary [25], hepatic- [21,26] and renal ischemia. [11] The cardio-protective effects of H₂S have been demonstrated in models of myocardial injury. However, most of these studies use injection with soluble H₂S donor compounds such as sodium hydrosulfide (NaHS) or sodium sulfide (Na₂S), while no results have been published on gaseous H₂S. [15–18,27] Gaseous administration might be applicable in patients who are being ventilated because of cardiogenic shock, a state of brain dead or during transplantation procedures. Inhaled H₂S has beneficial effects in endotoxin induced systemic inflammation [28] and in experimental Parkinson's disease. [29] We previously showed protective effects of gaseous H₂S during renal and hepatic IRI. [11,21] The benefits of gaseous administration

compared to injections with soluble H₂S donors lay within the management of the concentration. As opposed to injection with H₂S donors it is possible to administer the gas continuously with a stable dose over longer periods of time. Furthermore, when treatment is stopped the effects vanish rapidly while leaving its positive therapeutic effects behind. Also, gaseous administration at higher concentrations has proven to induce a hypometabolic, hibernation-like state in small animals like rodents [10,11].

As previously shown, 100 ppm H₂S induces a hypometabolic state and lowers blood pressure, heart rate and CO₂-production, whereas 10 ppm H₂S does not. [10,11,30] A suspended animation-like state induced by H₂S protects mice from lethal hypoxia for periods up to 6 hours [31], suggesting that the induction of regulated, reversible and well-controlled hypometabolism in organs holds clinical promise in ischemia-reperfusion related damage where oxygen demand exceeds oxygen availability. Although there was no difference in cardiac necrosis between the 10 ppm treated group and 0 ppm treated group, treatment with 100 ppm significantly reduced necrosis at 1 day of reperfusion. hs Troponin-T levels in serum were in line with this finding. This suggests that H₂S in non-hypometabolic concentrations is not effective in preventing short-term necrosis caused by ischemia, and that the additional value of H₂S-induced hypometabolism lies in the early phase of IRI. Although, the protective effects of 100 ppm H₂S on necrosis might also be caused by a larger amount of H₂S

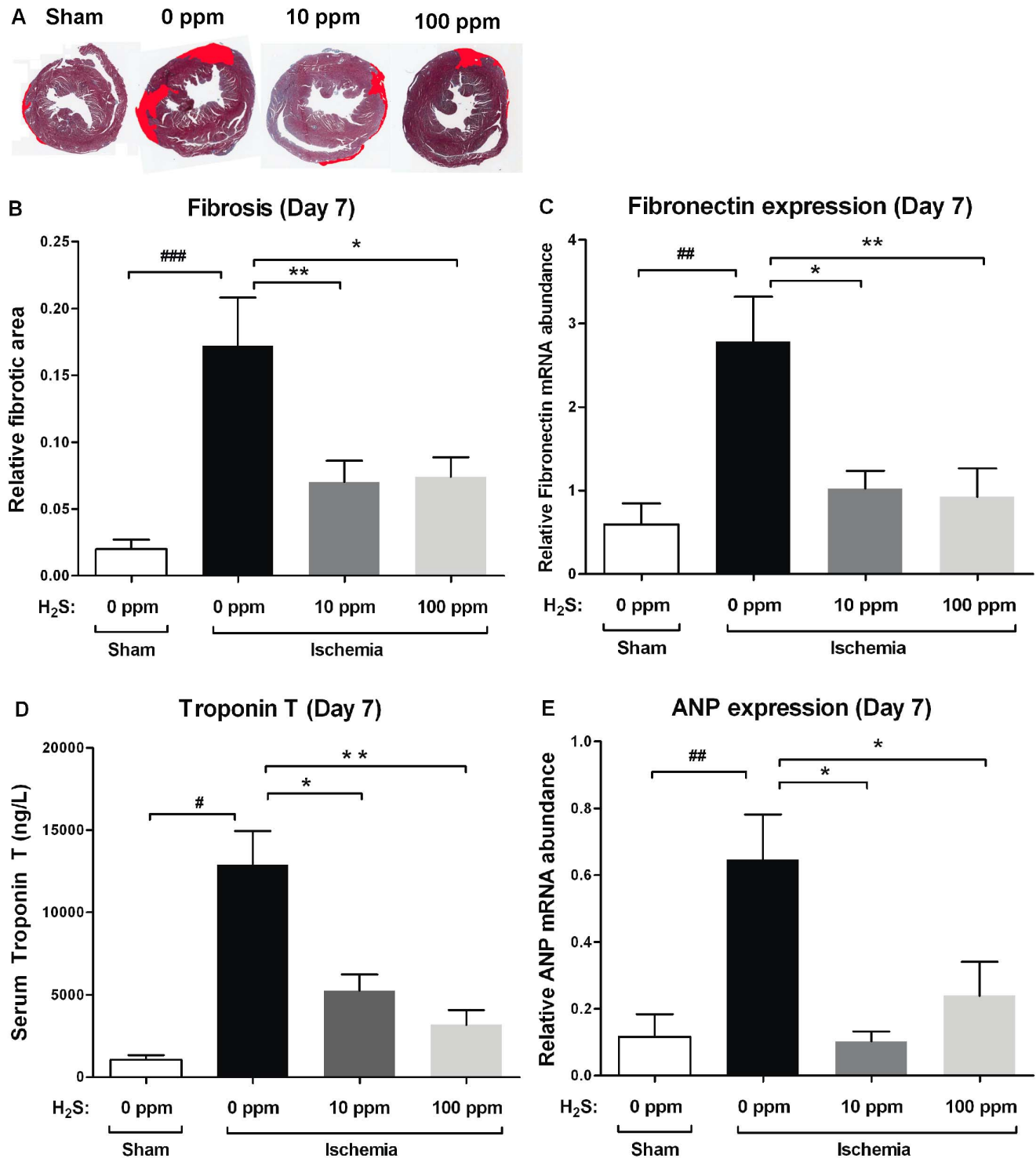


Figure 3. Cardiac damage is reduced by 10 and 100 ppm H₂S at 7 days of reperfusion. (A) Representative photomicrographs of Masson stained cardiac sections with fibrotic area artificially colored red, indicating the extent of fibrotic damage found in each group at 7 days of reperfusion. (B) Cardiac IR induced a significant amount of fibrosis in IRI animals exposed to 0 ppm H₂S (###p<0.001 vs. sham). In animals treated with 10 ppm and 100 ppm H₂S fibrosis was significantly reduced (**p<0.01, *p<0.05 vs. IRI). (C) Expression of fibronectin at 7 days of reperfusion was increased in IRI animals (##p<0.01 vs. sham). Treatment with 10 and 100 ppm H₂S reduced the expression of fibronectin (*p<0.05, **p<0.01 vs. IRI). (D) Seven days post-reperfusion hs Troponin-T levels were elevated in IRI animals exposed to 0 ppm H₂S (#p<0.01 vs. sham). Both 10 and 100 ppm H₂S reduced hs Troponin-T levels by 59% and 75%, respectively, as compared to animals treated with 0 ppm (*p<0.05, **p<0.01 vs. IRI). (E) Expression of ANP mRNA at 7 days of reperfusion was increased in IRI animals (##p<0.01 vs. sham). Treatment with 10 and 100 ppm H₂S reduced the expression of ANP (*p<0.05 vs. IRI).

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Granulocytes (Day 1)

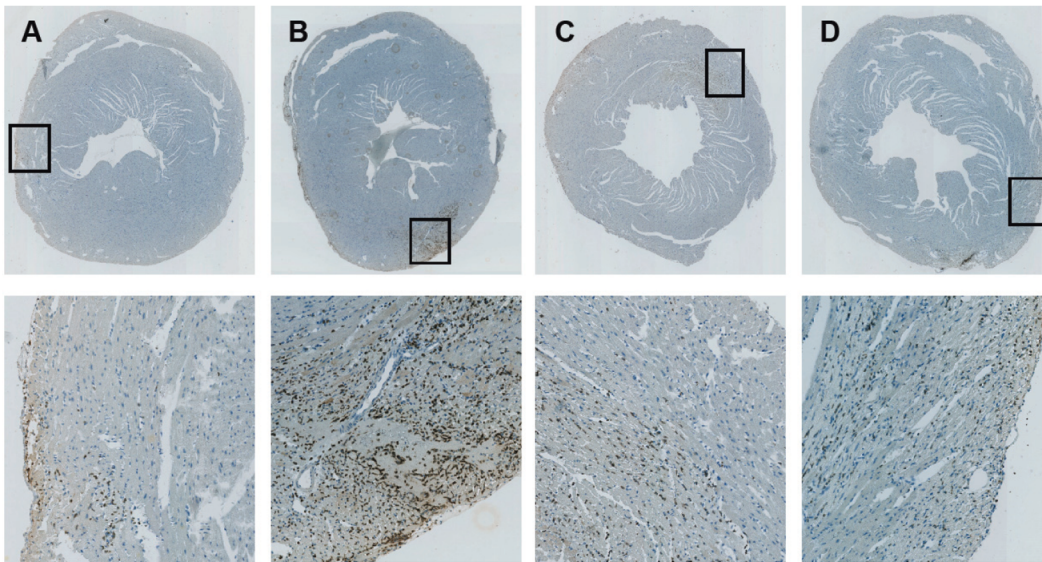
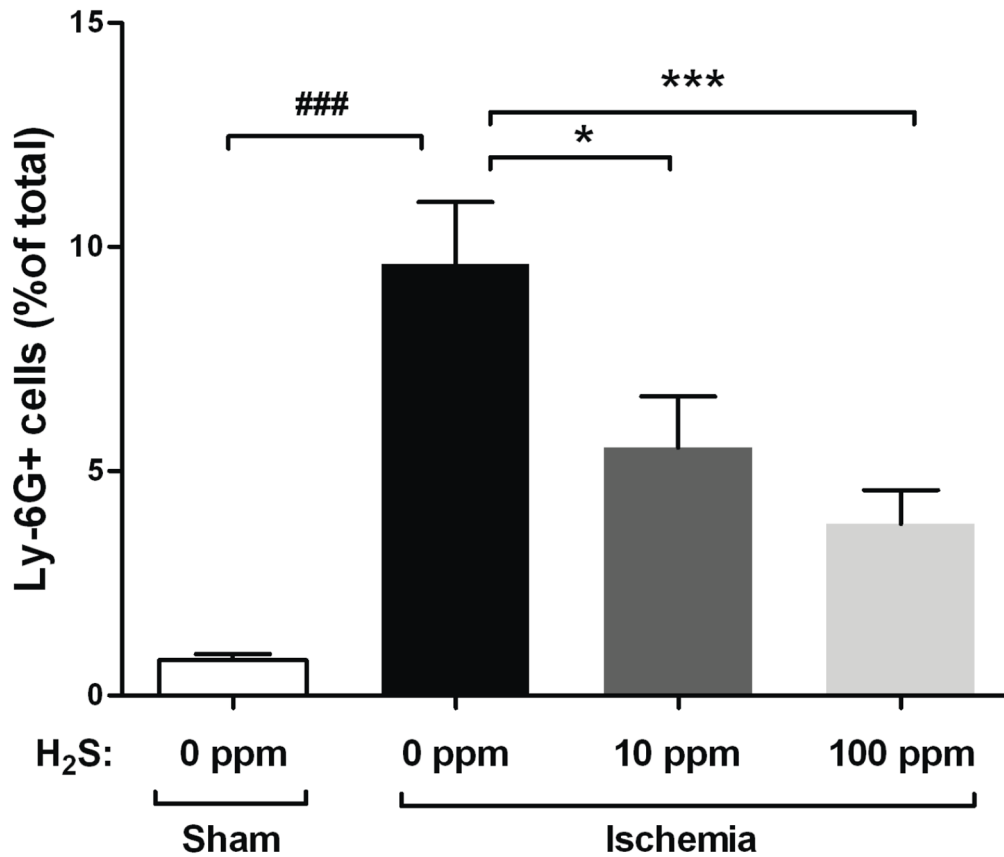


Figure 4. H₂S reduces cardiac IRI induced inflammation. There was a marked increase in granulocyte influx after cardiac ischemia ($p < 0.001$) compared to sham animals. Exposure to 10 ppm and 100 ppm H₂S significantly reduced the influx of granulocytes in the infarct area (10 ppm $p < 0.05$; 100 ppm $p < 0.001$). Below are representative images of these stainings: (A) Sham (B) IRI, 0 ppm H₂S (C) IRI, 10 ppm H₂S (D) IRI, 100 ppm H₂S. doi:10.1371/journal.pone.0063291.g004

leading to increased anti-oxidant effects, it is difficult to distinguish between the effects of hypometabolism and other effects attributed to a higher dose. However, inducing a suspended animation-like

state might be restricted to small animals like rodents. The applicability of hypometabolism in larger animals is still under debate and we are far from developing therapeutic applications in

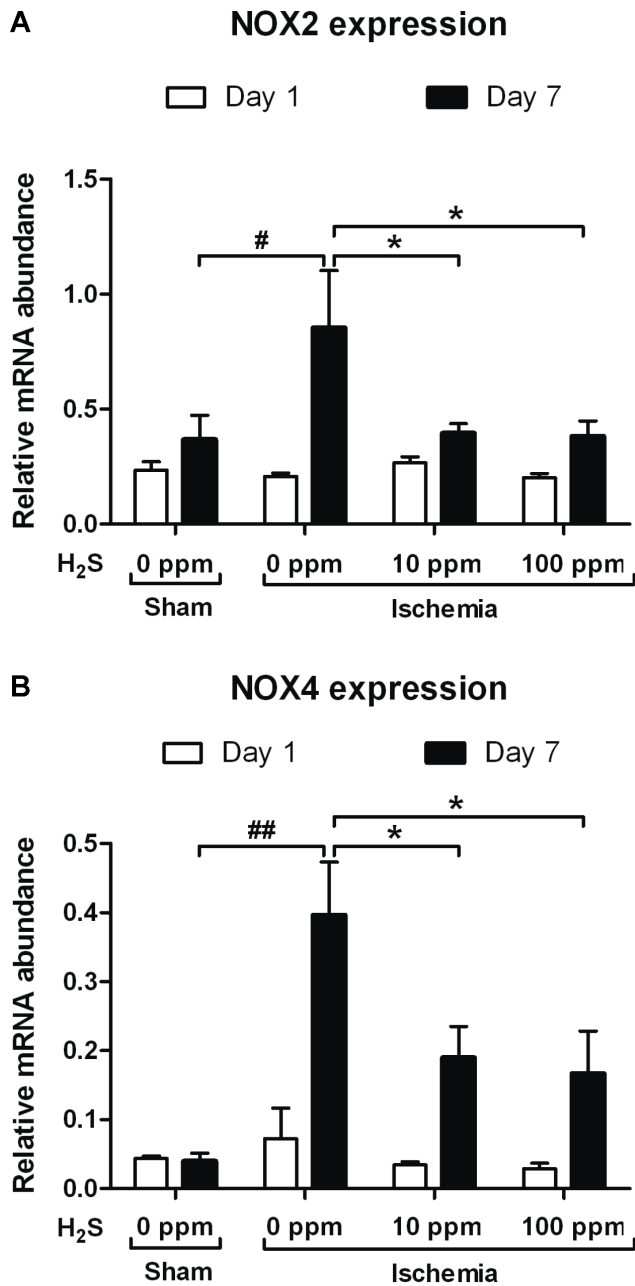


Figure 5. H₂S attenuates NOX2 and NOX4 upregulation. Expression of (A) NOX2 and (B) NOX4 mRNA was increased in IRI animals at 7 days post-reperfusion (NOX2: #*p*<0.05, NOX4: ##*p*<0.01 vs. sham). Treatment with 10 and 100 ppm H₂S reduced the expression of both genes (**p*<0.05 vs. IRI). After 1 day of reperfusion no differences were observed between all groups. doi:10.1371/journal.pone.0063291.g005

reducing metabolic rate in the clinical setting with the use of H₂S [19,20,32,33].

There are a number of potential mechanisms through which H₂S may exert its cardioprotective effects. Both 10 and 100 ppm of H₂S were proven anti-inflammatory as evidenced by reduced granulocyte influx into necrotic areas. Treatment with H₂S also lowered the influx of granulocytes after renal IRI. [11] Furthermore, H₂S inhibits neutrophil adhesion and activation in response to inflammatory stimuli and suppresses the release of the pro-inflammatory mediator tumor necrosis factor- α . [34,35] Other

studies report that H₂S mediates pro-inflammatory effects by potentiating sulfide production in neutrophils [36] and mediating leukocyte activation. [37] Although granulocyte influx seems to be reduced by treatment with H₂S, literature is inconclusive on the contribution of neutrophil invasion to final myocardial infarct size and appears not to be a dominant factor [38].

We show that treatment with H₂S protects against fibrosis at day 7 of reperfusion, as evidenced by reduced collagen deposition and fibronectin expression. Interestingly, the amount of necrosis differs between 10 and 100 ppm H₂S at day 1 of reperfusion, but this does not translate into differences in fibrotic area size after 7 days. This indicates that treatment with both concentrations of H₂S attenuate the onset of fibrosis. The prevention of fibrosis is in accordance with previous literature showing decreased cardiac remodelling and fibrosis in models of myocardial infarction and heart failure after H₂S treatment. [15,39,40] Although we find reduced fibrosis with both concentrations of H₂S, a balanced development of fibrosis remains essential. Suppressed fibrosis with no reduction in the extent of necrosis predisposes to infarct expansion and tissue rupture. [41] Since 10 ppm H₂S does not affect necrosis 1 day post-reperfusion, the anti-fibrotic effects at day 7 of reperfusion are not beneficial *per se*.

Another functional property of H₂S relates to the inhibition of ROS production, since the imbalance in redox status and oxidative stress contributes to fibrosis. [42] ROS-generating NOX2 and NOX4 are both increased after ischemic events in experimental models and their deficiency is protective in these models. [43,44] Seven days post-reperfusion, we found attenuated expression of NOX2 and NOX4 in both H₂S treated groups indicating less ROS production *in vivo*. We did not find an alteration of these genes at 1 day of reperfusion, which is in concordance with previous literature concerning NOX2. [45] Although it is not possible to distinguish whether these components originate from the myocardium or from phagocytes migrated into the myocardium, these results point towards increased oxidative stress in the infarcted heart, and a possible beneficial involvement for the effects of H₂S at the later time point. Furthermore, ROS production was markedly reduced in H₂S treated cardiomyoblasts in an *in vitro* model of Antimycin induced oxidative stress, indicating direct scavenging or reduction in production of ROS by mitochondria. H₂S has direct scavenging effects on ROS, but also has indirect effects via activation of antioxidant mechanisms, such as increasing glutathione levels. [46,47] Another mechanism that could be involved is the capacity of H₂S to modulate cellular respiration, as the inhibition of mitochondrial respiration has been shown to protect against myocardial IRI by limiting ROS production in mitochondria. [48] Antioxidant effects of H₂S may be of critical importance for the treatment of myocardial IRI because oxidative stress plays a prominent role in the development of cardiac damage and remodeling [42].

The effect of exogenous H₂S on blood pressure is still under debate. *In vivo* and *ex vivo* studies revealed conflicting responses to H₂S treatment. [49–53] The effects of H₂S on heart rate are also ambiguous; ranging from no change [54] to decreased heart rate in others. [50] Ufnal et al. noticed an increased heart rate upon NaHS infusion, however dependent on H₂S concentration in cerebrospinal fluid. [55] In additional support of this last view, suppression of H₂S production either pharmacologically [56] or genetically [57] leads to an increase in blood pressure. These opposing results might be attributable to differences in dose and route of administration.

In this study we show that 100 ppm of gaseous H₂S significantly lowers blood pressure and heart rate which might have affected

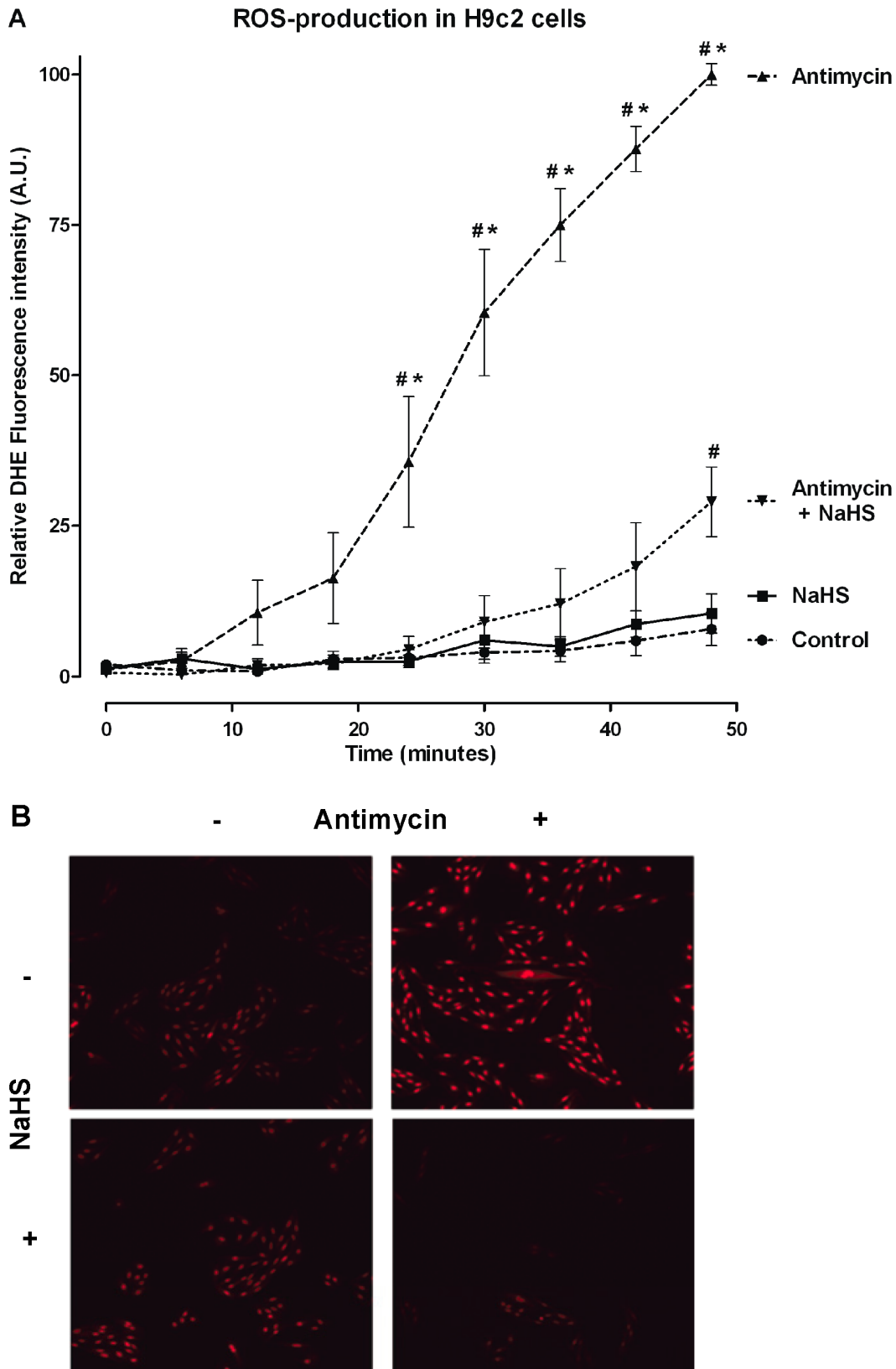


Figure 6. H₂S reduces ROS production in cultured cardiomyoblasts. (A) Antimycin (50 μ g/ml) significantly induced ROS production in H9c2 cells from 24 minutes onwards compared to untreated control cells. Addition of 1 mM NaHS prevented the increase in ROS production. ($\#$ $p < 0.001$ vs. control; $*p < 0.001$ vs. NaHS) (B) Representative photomicrographs of DHE stained H9c2 cells treated with antimycin and NaHS showing less DHE staining (red) in the NaHS treated cells as compared to cells treated with only antimycin. doi:10.1371/journal.pone.0063291.g006

cardiac workload and oxygen demand. Since we did not add a group with similar decrease in heart rate and blood pressure or a hypometabolic group with normal heart rate and blood pressure, we can not exclude this phenomenon to be responsible for the improved outcome. Aside from other protective effects of H₂S, it is thought that the reduced demand for oxygen during hypometabolism might be one of the protective mechanisms during ischemia based on the fact that oxygen availability and oxygen expenditure are more balanced. On the other hand the protective effects can not solely be explained by these effects since 10 ppm H₂S does not alter heart rate and blood pressure and has positive effects on several damage parameters. Another approach might be local delivery of H₂S by H₂S-donors thereby circumventing its systemic effects, which has previously been shown to be protective. [15] However, the highly volatile nature of H₂S and the associated difficulties in measuring this compound make it difficult to determine the exact dose and how long its effects endure, when given locally.

In conclusion, gaseous administration of H₂S protects the heart from IRI, likely through reduction of myocardial ROS production

and the inhibition of inflammation, necrosis and fibrogenesis. Hypometabolism-inducing concentrations of H₂S seem to have additional protective effects on necrotic cell death shortly after ischemia. H₂S treatment might be of clinical use in myocardial ischemia or cardiac transplantation, where it could lead to reduced myocardial damage related to hypoxia.

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Author Contributions

Conceived and designed the experiments: PMS RADB EMB MCRFVD JLH HGDL HVG. Performed the experiments: PMS JCVDB IVB. Analyzed the data: PMS RADB EMB JCVDB WTR MCRFVD. Contributed reagents/materials/analysis tools: RADB IVB MCRFVD JLH HGDL HVG. Wrote the paper: PMS RADB EMB JCVDB WTR IVB MCRFVD JLH HGDL HVG.

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