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Inhaled Hydrogen Sulfide Improves Graft Function in an Experimental Model of Lung Transplantation

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Abstract

Objectives: Ischemia-reperfusion(IRI) is a common complication of lung transplantation(LTx). Hydrogen sulfide(H_2S) is a novel agent previously shown to slow metabolism and scavenge reactive oxygen species, potentially mitigating IRI. We hypothesized that pre-treatment with inhaled H_2S would improve graft function in an ex vivo model of LTx.

Methods: Rabbits(n=10) were ventilated for 2 hours prior to heart-lung bloc procurement. The treatment group(n=5) inhaled room air(21% O_2) supplemented with 150 ppm H_2S while the control group(n=5) inhaled room air alone. Both groups were gradually cooled to 34 C. All heart-lung blocs were then recovered and cold-stored in low potassium dextran solution for 18 hours. Following storage, the blocs were reperfused with donor rabbit blood in an ex vivo apparatus. Serial clinical parameters were assessed and serial tissue biochemistry was examined.

Results: Prior to heart-lung bloc procurement, rabbits pre-treated with H_2S exhibited similar oxygenation(p=0.1), ventilation(p=0.7), and heart rate(p=0.5); however, treated rabbits exhibited consistently higher mean arterial blood pressures(p=0.01). During reperfusion, lungs pre-treated with H_2S had better oxygenation($p<0.01$) and ventilation(p=0.02) as well as lower pulmonary artery pressures($p<0.01$). Reactive oxygen species levels were lower in treated lungs during reperfusion(p=0.01). Additionally, prior to reperfusion, treated lungs demonstrated more preserved mitochondrial cytochrome c oxidase activity(p=0.01).

Conclusions: To our knowledge, this study represents the first reported therapeutic use of inhaled H_2S in an experimental model of LTx. After prolonged ischemia, lungs pre-treated with inhaled H_2S exhibited improved graft function during reperfusion. Donor pre-treatment with inhaled H_2S represents a potentially novel adjunct to conventional preservation techniques and merits further exploration.

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Keywords

Transplantation, Lung; Ischemia/reperfusion injury, Lung; Animal model; Hydrogen sulfide

INTRODUCTION

Although lung transplantation(LTx) is an effective and life-saving treatment for end-stage lung disease, 15-25% of LTx recipients experience severe post-transplant respiratory failure known as primary graft dysfunction(PGD).(1-5) PGD is associated with impaired short-term graft function, an 8-fold increase in 30-day mortality, and an increased risk of bronchiolitis obliterans syndrome.(3) A proposed cause of PGD is ischemia-reperfusion injury(IRI).(1,3,4) Despite many experimental and clinical therapies aimed at mitigating IRI, PGD is a persistent problem.

Exogenously administered hydrogen sulfide(H_2S) gas has recently been shown to reduce metabolism and induce a state of suspended animation in several animal models.(6-11) This state is characterized by significantly reduced oxygen requirements, allowing animals to survive severe hypoxia without apparent detriment.(8-10,12) Therefore, H_2S-induced suspended animation may represent a novel means of augmenting conventional organ preservation techniques. Theoretically, inducing a state of reduced metabolism prior to allograft procurement could decrease oxygen requirements during the ischemic phase of transplantation, thus mitigating IRI. Therefore, we undertook this study to investigate whether pre-treatment of donor lungs with H_2S would improve graft function in an experimental model of LTx.

METHODS

Model

These experiments were conducted using an *ex vivo* model of lung reperfusion whereby rabbit lungs were pre-treated with inhaled H_2S, subjected to cold ischemia, and then externally ventilated and perfused with donor rabbit blood. Our protocol has been described in detail previously.(1) The experiment utilized 4 kg, male New Zealand White rabbits(Myrtle's Rabbitry Inc., Thompson Station, TN). This study was approved by the Animal Care and Use Committee at the Johns Hopkins University.

A total of 10 rabbits were randomly divided into 2 treatment groups and underwent en bloc heart-lung harvest followed by 18 hours of cold storage. These blocs were subsequently reperfused with donor rabbit blood for 120 minutes while physiologic data were recorded and tissue biopsies were taken.

Pre-Treatment and Heart-Lung Bloc Harvest

All rabbits were anesthetized with an intramuscular injection of ketamine(35mg/kg) and xylazine(6.5mg/kg). Additional sedation was given with acepromazine(5mg/kg) as needed. A rectal probe was placed for continuous temperature monitoring. A tracheotomy was performed to facilitate endotracheal intubation and mechanical ventilation was

initiated(Harvard ventilator apparatus, model 665; Harvard Apparatus Co., Holliston, MA). The initial experimental ventilator settings were: Volume Control mode; rate, 20 breaths/minutes; tidal volume, 10mL/kg; Fractional inspired concentration of oxygen(FiO_2), 100%. The left carotid artery was dissected, ligated distally, and cannulated with a 22Fr intravenous catheter for blood gas and hemodynamic monitoring. The chest was then entered through a median sternotomy.

After obtaining baseline arterial blood gas and lung tissue samples, pre-treatment commenced. In the experimental group(n=5), the rabbits were pre-treated with 150ppm H_2S mixed with room air(21% oxygen, 78% nitrogen; Praxair Inc., Danbury, CT). In the control group(n=5), the rabbits were pre-treated with room air alone(21% oxygen, 78% nitrogen). In both groups, pre-treatment continued for 2 hours. During pre-treatment, both groups were gradually externally cooled to 34°C. Throughout pre-treatment, serial arterial blood gases were taken and physiologic measures were recorded.

Intravenous heparin(1000U/kg) was given and 30ug of prostaglandin E1 was injected directly into the PA. The PA was cannulated through a right ventriculotomy and the left atrium was cannulated directly. The lungs were flushed with 250mL of cold(4°C) low-potassium dextran(Perfadex; Vitrolife, Englewood, CO) via gravity drainage through the PA cannula. The superior and inferior vena cavae and the aorta were ligated. Topical cold ice slush was placed around the heart-lung bloc which was then excised from the chest. A lung sample was taken and the lungs were inflated and preserved in low-potassium dextran at 4°C for 18 hours.

Perfusion Pump System

Two donor rabbits were heparinized(1000U/kg) and exsanguinated through a right ventriculotomy to obtain 300mL of whole blood for each reperfusion. Following cold storage, the heart-lung bloc was suspended by the trachea and ventilated at 10mL/kg, 20 breaths/minute, with an FiO_2 of 100%. All animals were reperfused for 120 minutes with the donor rabbit blood using a Sarns 5000 roller head pump(Sarns Inc., Ann Arbor, MI). Blood removed via the left atrial cannula was collected in a reservoir and deoxygenated to achieve a PO_2 and PCO_2 of 60 mmHg(to simulate venous blood) before being returned to the heart-lung bloc through the PA cannula.

Hemodynamic Measurements

Physiologic measurements were recorded at baseline and then every 15 minutes during pre-treatment and during reperfusion. Every 15 minutes, arterial blood gas samples were analyzed during pre-treatment, while PA and left atrial blood samples were analyzed with the same frequency during reperfusion.

Lung Samples

Lung samples were taken prior to pre-treatment, after 2 hours of pre-treatment, after cold storage but prior to reperfusion, after 1 hour of reperfusion, and after 2 hours of reperfusion. Samples were removed from similar regions of the lungs regardless of treatment for consistency. All lung samples were removed sharply after the application of hemoclips for

hemostasis. Samples were flash frozen in liquid nitrogen and stored at −80°C for biochemical analysis.

Wet to Dry Ratios

Wet to dry ratios were obtained to determine levels of pulmonary edema. Left lower lobe samples were removed and weighed after 120 minutes of reperfusion. These samples were dried at 80°C for 72 hours and subsequently reweighed for ratio determination.

Reactive Oxygen Species (ROS) Levels

ROS levels were examined in lung samples using a green fluorescence assay(OxiSelect *In Vitro* ROS Assay Kit, Cell Biolabs, San Diego, CA). Lung samples were homogenized on ice in a phosphate-buffered solution, centrifuged, and re-suspended in assay buffer. Cell permeable 2',7'-dichlorodihydrofluorescin diacetate fluorogenic probe was used to assay ROS levels. Fluorescence was read on a Spectramax M5 plate reader.

Cytochrome C Oxidase (COX) Activity

Mitochondria were isolated with a mitochondrial isolation kit(Mitoiso1; Sigma Aldrich, St. Louis, MO). Fresh lung tissue was washed with an extraction buffer, homogenized, centrifuged, and re-suspended in storage buffer. The isolated mitochondria were assayed for COX activity using a commercially available kit(Cytocox1; Sigma Aldrich, St. Louis, MO). The mitochondrial isolate was placed in a 10mM Tris-HCl buffer. 50uL of Ferrocytochrome c substrate solution was added and activity was read immediately with a spectrophotometer.

Cyclic Guanosine Monophosphate (cGMP) Levels

cGMP concentrations were determined by a commercially available enzyme immunoassay(Amersham cGMP Enzymeimmunoassay, GE Healthcare Life Sciences, Piscataway, NJ). Tissue samples were weighed and homogenized in 500uL of 6% trichloroacetic acid. Samples were centrifuged and supernatants recovered and washed five times in 2mL of water saturated ether. The aqueous layer was recovered and dried to recover a pellet which was re-suspended in assay buffer. The acetylation assay was performed according to the vendor's specifications.

Statistical Analysis

Physiologic reperfusion data was evaluated by two independent statistical tests to assess the longitudinal differences between the treatment groups. First, repeated-measures analysis of variance(RM-ANOVA) was performed to evaluate the effects of H_2S over time. Post hoc comparisons at specific time points were evaluated with the Tukey-Honest significant difference tests. Multilevel random effects modeling was also performed to account for interactions both between and within each animal. The model used(generalized estimating equation, GEE) estimated the variance in the physiological parameters between animals and across time.

Results of biochemical activity assays were compared using the Student's t-test(cGMP), the RM-ANOVA test(ROS, COX), and the GEE(ROS, COX). Data are presented as means ±

standard deviations. P-values<0.05 were considered statistically significant. All graphs are presented as mean values with error bars defining standard error. Statistical analysis was performed using Stata 12.0(Stata Corporation LP, College Station, TX).

RESULTS

Pre-Treatment Physiology

During pre-treatment, both experimental and control lungs exhibited similar levels of oxygenation(GEE, p=0.1) and ventilation(GEE, p=0.7). More specifically, lungs pretreated with H_2S had similar baseline oxygenation with an FiO_2 of 100%(460±48 vs. 416±70mmHg, p=0.2) and similar oxygenation after pre-treatment with an FiO_2 of 21% just prior to procurement(88±50 vs. 87±3 mmHg, p=0.9; Table 1). The pattern of cooling was also similar between the two groups(GEE: p=0.9). Although the groups had similar heart rates during pre-treatment(GEE: p=0.5), rabbits pre-treated with H_2S exhibited higher mean arterial blood pressures during cooling(GEE: p=0.01).

Reperfusion Physiology

Prolonged cold storage produced a significant deleterious effect on lung performance in the control group; however, lungs pre-treated with inhaled H_2S were relatively spared (Table 2). Lungs pre-treated with inhaled H_2S exhibited better oxygenation(RM-ANOVA: p< 0.001; GEE: p=0.003; Figure 1A) and ventilation(RM-ANOVA: p=0.005; GEE: p=0.02; Figure 1B) than controls. The superior oxygenation of lungs pre-treated with H_2S was apparent both at the beginning of reperfusion(659±64 vs. 456±226mmHg, p=0.06) and at the conclusion of reperfusion(544±37 vs. 158±146mmHg, p=0.005). Additionally, lungs pre-treated with H_2S exhibited lower pulmonary artery pressures(RM-ANOVA: p=0.002; GEE: p=0.002; Figure 1C) but similar airway pressures(RM-ANOVA: p=0.3; GEE: p=0.3; Figure 1D). Although treated lungs had lower wet:dry ratios, this difference did not reach statistical significance(7.1±1.9 vs. 8.7±2.0, p=0.3).

Biochemistry

Although ROS levels were similar at all points prior to reperfusion, lungs pre-treated with room air alone exhibited progressively higher levels of ROS during reperfusion(RM-ANOVA: p=0.07; GEE: p=0.01; Figure 2). By the end of reperfusion, ROS levels were significantly lower in lungs pre-treated with H_2S(3449±1832 vs. 885±574 relative fluorescence units, p<0.05).

The pattern of mitochondrial COX activity was different between the groups(RM-ANOVA: p=0.01; GEE: p=0.01; Figure 3). Specifically, the relative activity of COX was higher in the group pre-treated with H_2S after cold storage(1.38±0.28 vs. 0.98±0.18 arbitrary units, p<0.05). The activity was not different at other time points.

Although there were increased cGMP levels in lungs pre-treated with H_2S, this difference did not reach statistical significance(53.44±53.26 vs. 30.60±22.92 fmol/mg tissue, p=0.4; Figure 4).

DISCUSSION

In our experimental model of LTx, pre-treating donor rabbits with inhaled H_2S resulted in improved oxygenation and ventilation as well as decreased PA pressures during reperfusion. Despite having similar levels of ROS during pre-treatment and after ischemic storage, lungs pre-treated with H_2S demonstrated lower levels of ROS during reperfusion. Finally, pre-treated lungs exhibited higher levels of mitochondrial COX activity after ischemic storage.

In LTx, lung allografts are necessarily subjected to a period of ischemia in order to facilitate explantation, transport, and implantation, thus exposing lung allografts to IRI.(1,3,4) The ischemic period is characterized by oxidative stress which precipitates an inflammatory response, leading to the production of ROS by endothelial cells, macrophages, and other immune cells.(2-5) At the cellular level, the combination of ROS and inflammation results in mitochondrial injury, cell wall damage, and the induction of apoptosis.(2) This injury produces diffuse alveolar and vascular damage, causing increased microvascular permeability and pulmonary edema, leading to increased pulmonary vascular pressures and limited gas exchange.(2-4) The ischemic injury is then exacerbated by reperfusion. Cells primed for the low oxygen environment of cold storage are inundated with oxygen resulting in the generation of more ROS that overwhelm the lung's anti-oxidant defenses and trigger an inflammatory response which further damages the pulmonary alveoli and vasculature.(2,4) Clinically, this combined IRI can result in severe pulmonary hypertension and progressive hypoxia.(2-4)

Hypothermic flush and storage of organs has been shown to lower the metabolic rate of allografts, decreasing their oxygen requirement, and thus ameliorating the oxygen supply and demand imbalance of ischemia.(13) Although hypothermia is highly effective at slowing metabolism, the prevalence and severity of IRI after LTx demonstrates a remaining need to augment the protective effects of cold storage.(3)

Several studies have demonstrated that exogenous administration of H_2S can dramatically lower metabolism, inducing a state the authors refer to as suspended animation.(8-11) This state is characterized by a decreased metabolic rate, lower oxygen consumption, and reduced carbon dioxide production.(9) Moreover, when placed in lower metabolic state, animals are able to survive severely hypoxic conditions without obvious detriment.(9) Since IRI is initiated by an ischemic phase in which oxygen demand exceeds supply, we pre-treated our rabbit lung donors with H_2S in an effort to induce a state of suspended animation and thus lower oxygen demand and avoid ischemic injury.

Whether our experiment achieved animation state of reduced metabolism is unclear. During pre-treatment, oxygen consumption and carbon dioxide production were not measured. Moreover, core body temperature and heart rate were similar between the two groups. Thus, there is no empiric evidence that a state of suspended animation, as described by previous researchers, was achieved.(7)

However, pre-treatment with H_2S prevented ischemic injury and improved graft function during reperfusion. Although the numerous cytoprotective and physiologic mechanisms of H_2S are still being explored, an examination of our biochemical results suggests some

possible protective mechanisms.(6) First, prior to reperfusion, ROS levels were similar between the two groups; however, during reperfusion, ROS levels increased significantly and progressively in the control group while they remained stable in the treatment group. These findings suggest that ischemic injury may have been mitigated by H_2S, decreasing any subsequent inflammatory response and consequent damage during reperfusion. That ischemic injury may have been partially averted is also supported by the improved oxygenation and lower pulmonary artery pressures in the pre-treated group at the beginning of reperfusion. Whether this reflects the directly cytoprotective and anti-apoptotic properties of H_2S or resulted from a reduction in the oxygen requirement of the lungs during cold storage is unclear. Future studies will focus on identifying which cell types are injured in this model and how H_2S ameliorates this injury.

Second, a number of studies have demonstrated H_2S to be an effective scavenger of free radicals.(6,14-16) While this may have been a contributing factor to our improved outcomes, this mechanism alone does not fully explain our observations. There is no difference in ROS levels during H_2S treatment; rather, ROS levels do not diverge until reperfusion, when there was no further H_2S therapy. Although it is possible that during reperfusion, some small amount of H_2S was present in the lungs from the pre-treatment phase, we feel it is unlikely that this small quantity could fully account for the observed differences.

Third, several studies have demonstrated that in addition to functioning as a free radical scavenger, H_2S also results in the up-regulation and potentiation of other antioxidant compounds including glutathione, N-acetylcysteine, catalase, and superoxide dismutase.(6,17-19) Although we did not measure tissue levels of these compounds, it is possible that the 2 hours of H_2S pre-treatment increased lung anti-oxidant levels. Then when reperfusion triggered further release of ROS, the pre-treated lungs had an ample supply of anti-oxidants to scavenge these free radicals and thus prevent damage. This would also explain the rising level of ROS observed in the control lungs as the anti-oxidants were consumed, and the stable levels in the treated lungs which had significant anti-oxidant reserves.

Fourth, previous research suggests that H_2S slows metabolism by directly inhibiting mitochondrial COX activity, preventing the transfer of electrons from complex II to COX(complex III) in the electron transport chain, and thus inhibiting oxidative phosphorylation.(14,20) Moreover, H_2S has also been shown to decrease COX levels by down-regulating the production of its constituent subunits.(21) Decreased COX activity resulting in reduced metabolism and oxygen consumption could help explain the differences in ROS levels in our experiment; however, we observed increased COX activity in pre-treated lungs just prior to reperfusion. This could be partly explained by the complex relationship between H_2S and COX. Although at high concentrations, H_2S inhibits COX, at lower concentrations it acts as an electron donor for the electron transport chain, thus increasing oxidative phosphorylation.(20,22) Perhaps in the low oxygen environment of ischemia, H_2S acted as an electron donor, maintaining COX activity levels, increasing ATP production, and thus preventing cellular injury. Further complicating this relationship is the fact that COX can be released from mitochondria during apoptosis.(23,24) Thus the badly damaged control lungs in our experiment may have been experiencing significant apoptosis prior to reperfusion and thus have extruded significant quantities of COX from

mitochondria. Finally, given the relatively small size of our experiment and the isolated time point of significance, we cannot exclude the possibility that this finding is spurious.

Although the impact of H_2S on cGMP levels is unknown, previous research suggests that higher cGMP levels are associated with improved lung function.(1) Thus, we measured cGMP levels as a surrogate for a favorable biochemical profile during reperfusion. In our experiment, there were higher levels of cGMP in treated lungs after reperfusion but this difference did not reach statistical significant. Further investigation is warranted.

Previous Literature

Although studies have shown H_2S to exert a cytoprotective effect in models of IRI, most of these models have focused on post-injury treatment.(6) Organ transplantation presents a unique opportunity to anticipate the ischemic insult and administer therapies to mitigate or prevent ischemic injury. Along these lines, Fu et al. pre-treated rat lungs with intravenous H_2S prior to inducing IRI.(19) They found H_2S to be associated with improved histology, decreased edema, and preserved compliance. Similar beneficial effects of pre-treatment with H_2S have been demonstrated in models of myocardial infarction, stroke, and heart, liver, and kidney transplantation.(16,25-28) Post-injury treatment with H_2S has also been associated with beneficial results in lung models of ventilator-induced and smoke-induced lung injury.(29,30) Despite the potential advantages of H_2S in LTx, caution is warranted. Very high levels of H_2S can be directly toxic to the lungs.(7,31)

Limitations

In this study, we utilized an *ex vivo* model of LTx. We did not induce brain death in the donors nor did we utilize immunosuppression. Therefore, we did not completely replicate the *in vivo* LTx procedure. Moreover, since we only conducted 2 hours of reperfusion, we cannot evaluate long-term effects. Furthermore, although pre-treatment with H_2S resulted in improved graft function and decreased ROS, the underlying mechanism of this improved function remains unclear. Future experiments will focus on identifying which cell types suffered the most severe injury, which cells are responsible for the increased ROS production, and the way in which H_2S ameliorates this injury. Finally, although we planned to induce suspended animation, whether we achieved this state is uncertain.

CONCLUSION

To our knowledge, this study represents the first reported therapeutic use of inhaled H_2S in an experimental model of LTx. Pre-treatment of donor rabbits with inhaled H_2S is associated with significantly improved graft function during reperfusion. Donor pre-treatment with hydrogen sulfide appears to mitigate IRI and may represent a novel adjunct to conventional preservative techniques.

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ABBREVIATIONS

cGMP	Cyclic guanosine monophosphate
COX	Cytochrome c oxidase
FiO_2	Fractional inspired concentration of oxygen
GEE	Generalized estimating equation
H_2S	Hydrogen sulfide
IRI	Ischemia-reperfusion injury
LTx	Lung transplantation
PA	Pulmonary artery
PGD	Primary graft dysfunction
RM-ANOVA	Repeated-measures analysis of variance
ROS	Reactive oxygen species

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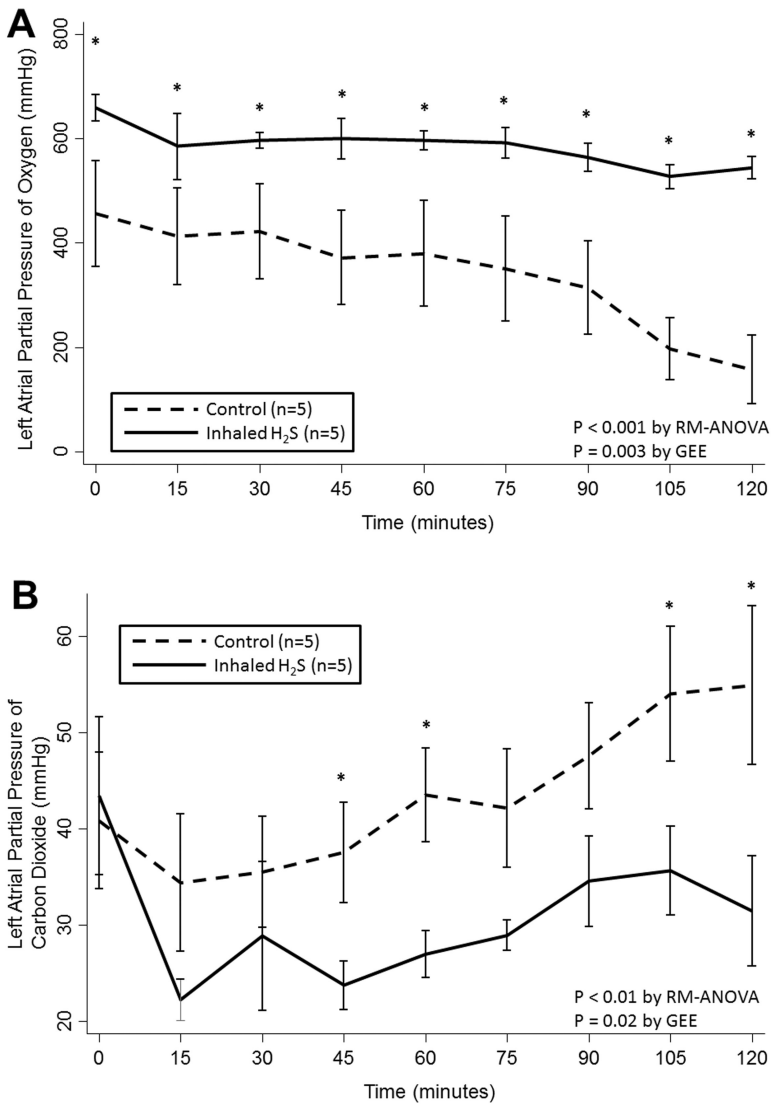

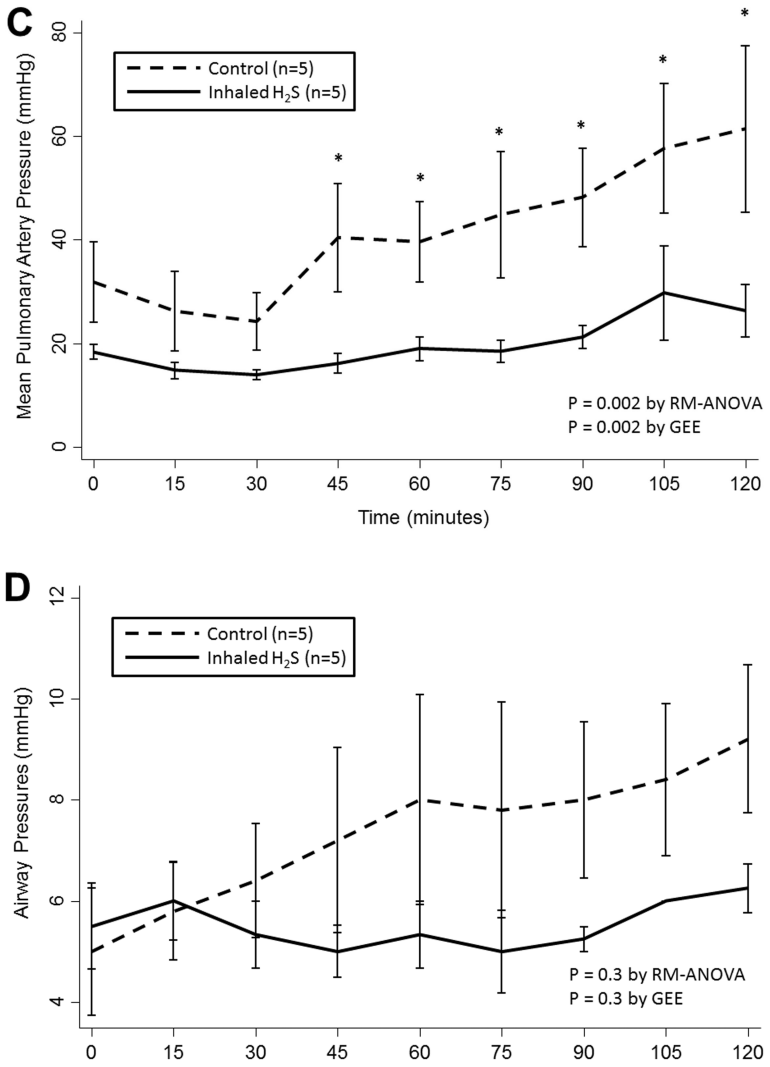

Figure 1.

(A) Partial pressure of oxygen and (B) carbon dioxide and (C) mean pulmonary artery and (D) airway pressures over time during reperfusion. Lungs pre-treated with room air are displayed with a dashed line. Lungs pre-treated with hydrogen sulfide are displayed with a solid line. Asterisks indicate a post hoc statistical difference at given time points as determined by the Tukey-Honest significance difference test. Abbreviations: n, number; RM-ANOVA, repeated measures analysis of variance; GEE, generalized estimating equation; H2S, hydrogen sulfide.

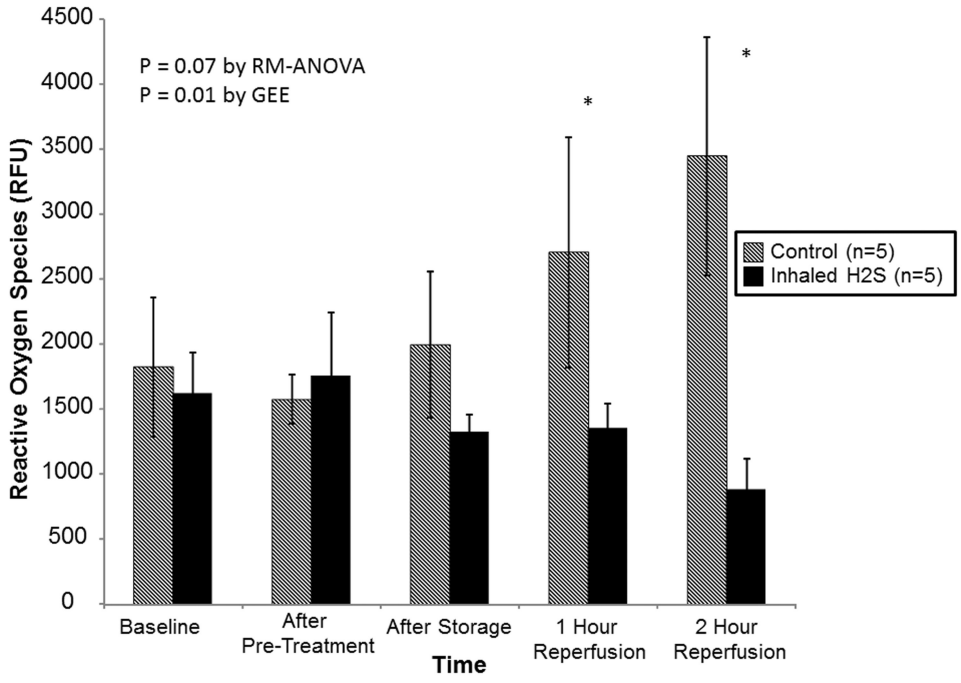

Figure 2.

Reactive oxygen species levels stratified by treatment. Lungs pre-treated with room air are displayed with shaded bars. Lungs pre-treated with hydrogen sulfide are displayed with solid bars. Asterisks indicate a post hoc statistical difference at given time points as determined by the Tukey-Honest significance difference test. Abbreviations: n, number; RM-ANOVA, repeated measures analysis of variance; GEE, generalized estimating equation; RFU, relative fluorescence units; H2S, hydrogen sulfide.

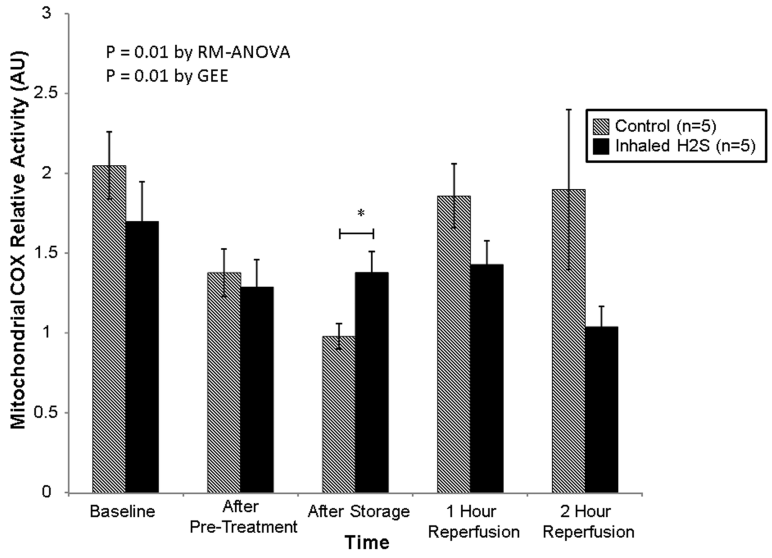

Figure 3.

Relative mitochondrial cytochrome c oxidase activity stratified by treatment. Lungs pre-treated with room air are displayed with shaded bars. Lungs pre-treated with hydrogen sulfide are displayed with solid bars. Asterisks indicate a post hoc statistical difference at given time points as determined by the Tukey-Honest significance difference test. Abbreviations: n, number; COX, cytochrome c oxidase; RM-ANOVA, repeated measures analysis of variance; AU, arbitrary units; H2S, hydrogen sulfide.

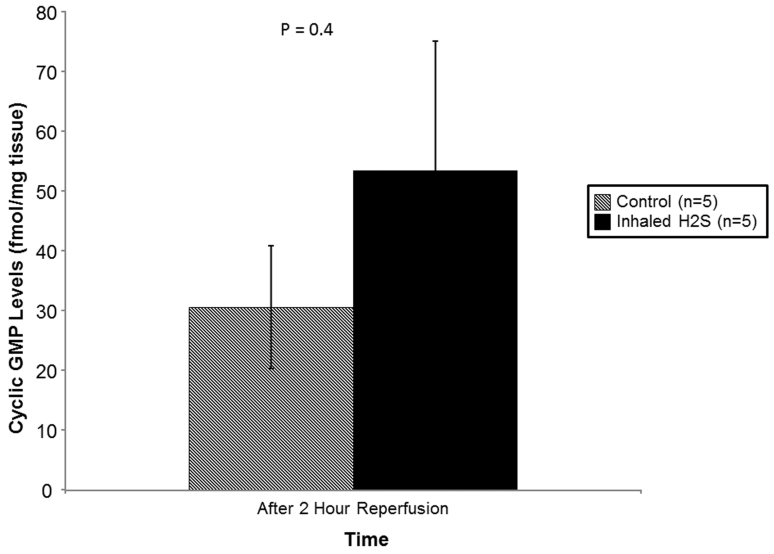

Figure 4.

Cyclic GMP levels at the conclusion of reperfusion stratified by treatment. Lungs pre-treated with room air are displayed with shaded bars. Lungs pre-treated with hydrogen sulfide are displayed with solid bars. P-value determined by Student's t-test. Abbreviations: n, number; GMP, guanosine monophosphate; H2S, hydrogen sulfide.

Table 1

Physiologic Parameters during Pre-Treatment Stratified by Pre-Treatment Strategy

Variable	Control (n=5)	Hydrogen Sulfide (n=5)	P-value
At Baseline			
Temperature (°C)	38.9 ± 0.5	39.0 ± 0.3	0.7
Initial PaO_2 (FiO_2 100%, mmHg)	460 ± 48	416 ± 70	0.2
$PaCO_2$ (mmHg)	46 ± 18	41 ± 7	0.5
Heart rate (beats/min)	184 ± 52	179 ± 52	0.9
Mean arterial pressure (mmHg)	52 ± 8	61 ± 12	0.3
At the Conclusion of Pre-Treatment			
Temperature (°C)	33.2 ± 0.9	33.9 ± 1.5	0.5
Heart rate (beats/min)	148 ± 21	168 ± 20	0.3
Mean arterial pressure (mmHg)	45 ± 4	40 ± 7	0.3
PaO_2 (FiO_2 21%, mmHg)	88 ± 50	87 ± 3	0.9
$PaCO_2$ (mmHg)	32 ± 14	40 ± 12	0.4

Table 2

Comparison of Physiologic Parameters during Reperfusion Stratified by Pre-Treatment

Variable	0 Minutes	30 Minutes	60 Minutes	90 Minutes	120 Minutes
PaO_2 (mmHg)					
Control (n=5)	456 ± 226	422 ± 205	379 ± 226	315 ± 200	158 ± 146
Treatment (n=5)	659 ± 64‡	596 ± 38‡	596 ± 46‡	564 ± 54‡	544 ± 37‡
$PaCO_2$ (mmHg)					
Control (n=5)	41 ± 16	36 ± 13	44 ± 11	48 ± 12	55 ± 19
Treatment (n=5)	43 ± 21	29 ± 19	27 ± 6 ‡	35 ± 9 ‡	31 ± 11 ‡
Mean PA Pressure (mmHg)					
Control (n=5)	32 ± 18	24.2 ± 12	40 ± 17	48 ± 21	61 ± 36
Treatment (n=5)	18 ± 1	14 ± 2	19 ± 6‡	21 ± 5‡	26 ± 10‡

*P<0.001 by RM-ANOVA;

*P<0.005 by RM-ANOVA;

*P<0.002 by RM-ANOVA;

Abbreviations: RM-ANOVA, repeated-measures analysis of variance.

‡P<0.05 for comparison between control and experimental groups