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EFFECTS OF INFUSION OF HUMAN METHEMOGLOBIN SOLUTION FOLLOWING HYDROGEN SULFIDE POISONING

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Abstract

Rationale—We have recently reported that infusion of a solution containing methemoglobin (MetHb) *during* exposure to hydrogen sulfide results in a rapid and large decrease in the concentration of the pool of soluble/diffusible H_2S in the blood. However, since the pool of dissolved H_2S disappears very quickly *after* H_2S exposure, it is unclear if the ability of MetHb to "trap" sulfide in the blood has any clinical interest and relevance in the treatment of sulfide poisoning.

Methods—In anesthetized rats, repetition of short bouts of high level of H_2S infusions were applied to allow the rapid development of an oxygen deficit. A solution containing methemoglobin (600mg/kg) or its vehicle was administered one minute and a half after the end of H_2S intoxication.

Results—The injection of methemoglobin solution increased methemoglobinemia to about 6%, almost instantly, but was unable to decrease the blood concentration of soluble H_2S, which had already vanished at the time of infusion, or to increase combined H_2S. In addition H_2S-induced O_2 deficit and lactate production as well as the recovery of carotid blood flow and blood pressure were similar in treated or control animals.

Conclusion—Our results do not support the view that administration of MetHb or drugs induced methemoglobinemia during the recovery phase following severe H_2S intoxication in sedated rats can restore cellular oxidative metabolism, as the pool of diffusible sulfide, accessible to MetHb, disappears rapidly from the blood after H_2S exposure.

Keywords

H_2S toxicity; metallo-compound; Methemoglobin; rat model

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Introduction

During hydrogen sulfide poisoning, H_2S produces an apnea, a coma and a refractory circulatory shock leading to cardiac arrest. These effects result from a direct inhibition the mitochondrial cytochrome c oxidase, preventing in turn mitochondrial ATP production [1-3].

Sodium nitrite-induced methemoglobinemia is certainly the antidote that has been the most extensively studied [5,6]. The oxidation of the molecule of ferrous iron contained in hemoglobin (Hb) dramatically increases the ability of Hb to combine H_2S [7]. Nitrite induced methemoglobinemia is effective to *prevent* H_2S toxicity, when administered *prior and during* H_2S exposure [6,8-11]. Anecdotal case reports suggest beneficial effects following exposure in humans as well [12].

To overcome some of the side effects of nitrite-induced methemoglobinemia (drop in blood pressure, unpredictable methemoglobin (MetHb) levels, potentiation of the effects of H_2S by Nitric Oxide, we used an infusion of a solution of methemoglobin [13] and have showed that, *during* H_2S infusion, the presence of methemoglobin dramatically decreases the amount of free/diffusible H_2S in the blood. MetHb is effective at concentrations starting at 4-5% [11] and is capable of combining with H_2S as soon as it enters in the blood, as shown in both sheep [14] and rat models [11]. Consequently, the pool of combined H_2S, which represents the largest pool of H_2S in the blood during sulfide exposure, increases in the presence of MetHb [13]. The “antidotal” properties of methemoglobin against H_2S poisoning must however be relevant when used in a scenario faithful to H_2S intoxication in human, that is *following* H_2S exposure. In other words, since the efficacy of a solution of methemoglobin is primarily dictated by the fate of H_2S in the blood *after* sulfide exposure, i.e. when patients are withdrawn from the source of intoxication, what benefit is to be expected from the presence of MetHb, when all soluble H_2S has already vanished from the blood [15,16].

To tackle this outstanding question, an important experimental challenge must be overcome: a balance must be found between levels of H_2S that would be high enough to alter the respiratory and cardiovascular system and create an oxygen deficit, without reaching lethal levels. To produce a severe form of H_2S poisoning, but without immediate lethal consequence, we used a repetition of short bouts of intravenous infusion of levels of H_2S able to kill animals within 5 min, if it were infused continuously, separated by very short periods of recovery. As developed in the method section, this approach allows the progressive buildup of a large O_2 deficit and lactate, akin to the creation of oxygen deficit by repetitive hemorrhage [17,18].

We developed a rat model of H_2S intoxication using NaHS infusion, which allowed us to study the systemic sulfide toxicity. Rats were chosen since they behave just like larger mammals in terms of their respiratory, circulatory and metabolic responses to H_2S poisoning, in marked contrast to mice [19,20]. We are presenting 1) the characteristics of this model during and following severe H_2S intoxication and 2) the effects of a direct infusion of a methemoglobin solution versus saline, administered after the cessation of H_2S exposure, on the kinetics and magnitude of the recovery of O_2 deficit/debt, lactate production and circulatory responses.

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Material and Methods

Animal preparation

Adult Sprague-Dawley rats were studied (see weight and number of animals used in the protocol section). All experiments were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was approved by the Pennsylvania State University College of Medicine Institutional Care and Use Committee. Rats were anaesthetized with 3.5% isoflurane in O_2, followed by urethane (1.2 g/kg, IP). Animals were then tracheostomized (14G surflo catheter, TERUMO, NJ) as previously described [15]. A catheter (PE-50 tubing) was inserted into the right femoral artery for continuous monitoring of systemic arterial blood pressure (ABP), while another catheter was inserted into the left carotid artery for arterial blood sampling. An additional catheter was inserted into the right femoral vein for NaHS infusion, while a double lumen catheter was inserted into right jugular vein for injection of methemoglobin solution and continuous infusion of muscular relaxant (10 mg/kg/h, Rocuronium Bromide, Hospira Inc. Lake Forest, IL). The left femoral artery and right carotid artery were exposed and isolated from the vein, nerve, and surrounding tissues and a transonic flow probe (MA1PRB, Transonic Systems Inc., Ithaca, NY) was placed around both vessels. Body temperature was monitored with a rectal probe (Thermalert TH-5, Physitemp, Clifton NJ) and maintained around 37°C using a heating pad and a lamp. At the end of the experiment, rats were euthanized by a lethal injection of barbiturate IV (200mg/kg) into the right heart through the jugular catheter, followed by an aortic dissection.

Measurements and data analysis

The tracheal catheter was connected to a small animal ventilator (SAR-1000, CWE) and rats were mechanically ventilated (frequency of breathing: 80-90 breath.min^{-1}, minute ventilation~350-400 ml/min). The expiratory flow was measured using a pneumotachograph (1100 Series, Hans Rudolph, Shawnee, KS). The expiratory circuit was connected to a series of two 5 ml mixing chambers. Mixed expired O_2, CO_2 and H_2S fractions were measured continuously from the second mixing chamber using O_2 (Oxystar-100, CWE), CO_2 (model 17630, VacuMed, Ventura, CA) and H_2S (Interscan RM series, Simi Valley, CA; range: 0-200.0 ppm) analyzers[15].

Breathing frequency (f) and tidal volume (V_T) were determined from the expiratory flow signal and minute ventilation (VE) was computed as f.V_T. Oxygen consumption (VO_2) was computed in Standard Temperature and Pressure, Dry (STPD) conditions as previously described [18].

The arterial catheter was connected to a pressure transducer (TA-100, CWE Inc., Ardmore, PA), while the transonic flow probes were connected to a perivascular flowmeter (TS420, Transonic Systems Inc., Ithaca, NY). All signals were digitized at 200 Hz using an analog-to-digital data acquisition system (Power Lab 16/35, AD Instruments, Colorado Springs, CO) and stored for later analysis. Mean ABP, VO_2, minute ventilation, carotid blood flow (CBF) and femoral blood flow (FBF) were displayed online for monitoring.

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Arterial partial pressures in O_2 (PaO_2) and CO_2 ($PaCO_2$), as well as lactate concentration were measured using i-STAT1 blood gas analyzer (ABAXIS, Union city, CA).

H_2S determination in the blood

We followed the procedure proposed and validated by Wintner et al. [21] which we have previously used and described [15]. In brief, arterial blood (200 μl) was added with a syringe to a solution of monobromobiname (MBB; 20 mM in 200 μl of acetonitrile) and 200 μl HEPES (50 mM, pH 8.0) in a sealed vial. After 10 minutes, a volume of 100 μl 0.1 N HCl was added to prevent any further reaction between MBB and H_2S. The mixture was then extracted and the residue was dissolved and purified by Supercritical Fluid Extraction. Sulfide-dibimane was measured by High Performance Liquid Chromatography (HPLC) analysis using a Shimadzu HPLC system consisting of two 10ADVP pumps, a SCL-10AVP controller, and a Rheodyne injector, interfaced with a Hitachi L 7485 fluorescence detector. Data were recorded using a Hitachi D2500 integrator. The fluorescence excitation wavelength was 390 nm and the emission wavelength was 470 nm. Under these chromatographic conditions sulfide-bimane eluted at 19.4 min. The levels of sulfide-bimane in rat blood were determined based on standard. Several examples of chromatograms, obtained with this technic, can be found in 2 previous publications from our laboratory[15,16].

Concentration of gaseous H_2S in the blood

Expired H_2S was determined as previously described [13,15]. The fraction of H_2S was continuously measured from the second mixing chamber, allowing the calculation of the partial pressure of expired H_2S (P_EH_2S) and of the alveolar pressure (P_AH_2S) assuming to be equivalent to H_2S arterial partial pressures (PaH_2S) (see [15] for further details). The concentration of gaseous H_2S in the blood (CgH_2S) was calculated as: $CgH_2S = 0.00012*PaH_2S$, with 0.00012 being the coefficient of solubility of H_2S (0.09 mol.l.760^{-1} mmHg at 37°C in saline) as previously described [15]. Assuming that H_2S is under the form of H_2S gas and its sulfhydryl anion HS^- at a ratio of 1:3 in the arterial blood [22,23], the concentration of total dissolved H_2S was estimated as three times CgH_2S.

Methemoglobin concentration in the blood

Arterial gas partial pressure in O_2 and CO_2 and MetHb concentrations were determined using a GEM Premier 4000 gas analyzer (Instrumentation Laboratory, Bedford, MA, USA). The accuracy of MetHb measurement was validated using a spectrophotometric method in duplicate [24]. This technique was also used to determine the concentration of MetHb in the Hb solution, which was found to be about 98% [11].

H_2S intravenous infusion

H_2S was administered intravenously as solution of sodium hydrosulfide hydrate (0.8 mg/ml NaHS, Sigma Aldrich, St Louis, MO) in sterile saline, prepared immediately prior each experiment and kept in airtight syringes. H_2S was infused using a syringe pump (Fusion 100, Chemyx Inc., Stafford, TX) in the femoral vein.

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Methemoglobin solution

Methemoglobin solution was prepared from human hemoglobin powder (Sigma Aldrich, St Louis, MO), mostly constituted (92-100%) of methemoglobin. Hemoglobin powder was diluted in sterile saline at the concentration of 100 mg/ml.

Experimental protocols

1. Effects of methemoglobin infusion on O_2 deficit, lactic acidosis and circulation following H_2S exposure (protocol 1)—This model was designed to produce a significant oxygen deficit and lactic acidosis along with a low blood pressure without killing the animal within one hour. To achieve this goal, we established a protocol consisting in repetitive bouts of potentially lethal IV dose. The lethal dose of NaHS was established in a series of pilot experiments, showing that 25% of animals died within minutes at a rate of infusion of 5-6 μmole/min while no animals survived more than 5 min at a rate of 10 μmole/min. Below this rate of infusion, no clear oxygen deficit would develop within at least 10 minutes of exposure. As presented below a intermittent infusion of NaHS at a rate of 20 micromole/min produces a progressive reduction in blood pressure and VO_2, allowing enough time to administer the antidote after sulfide exposure.

Twelve rats weighing 433 ± 50 g were studied in this protocol: after an adequate recovery period from surgical procedure for about one hour and following at least 10 minutes of hemodynamic and respiratory stability, a first arterial blood was sampled. Two minutes later, NaHS infusion was started at a rate of 2 $ml.min^{-1}$ (20-22 $\mu mol.min^{-1}$) and was sustained until mean arterial blood pressure reached 50 mmHg, 40 s was allowed before resuming H_2S infusion as illustrated in figure 1. The same sequence was repeated until an O_2 deficit of about 15 ml/kg was reached, then H_2S infusion was stopped. Arterial blood was sampled again at 1, 5, 10 and 15 min following H_2S exposure. One minute and 30 seconds into recovery, a methemoglobin (100 mg/ml, 3 ml in total) or a saline (3 ml, Control group) solution was infused intravenously.

2. Effects of methemoglobin infusion on H_2S kinetics following H_2S exposure (protocol 2)—This protocol was designed to characterize the changes in H_2S concentrations after moderate single steady infusion of H_2S, following a saline or MetHb injection. Twelve rats 569 ± 77 g were studied in this protocol. Each animal received an infusion of NaHS at a rate of 5.5-6.5 micromol/min, which allowed mean arterial blood pressure to drop by about 10%, which typically required 4-5 minutes. Then NaHS infusion was stopped; one minute and a half into recovery a 2ml saline solution was infused IV for 90 s. Thirty minute later, a second IV NaHS infusion was performed followed this time by the infusion of a MetHb solution (2 ml during 90 s) following the same protocol as with saline. H_2S concentrations were compared between the two conditions (Saline/control versus MetHb). Blood was sampled before NaHS infusion (baseline), 3 minutes into infusion and then 5, 10 and 15 min into recovery.

Statistical Analysis

All results are presented as mean± SD. All variables of interest were compared between the control animals (saline) and those receiving methemoglobin. They were also analyzed over

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time, i.e., prior to and at the end of each of the periods of H_2S infusion as well as before and after injection of saline or methemoglobin, using ANOVA for repeated measurements. All statistical analyses were conducted using GraphPad Prism 6 (Graphpad Software, La Jolla, CA, USA). Post-hoc comparisons were performed using a Bonferroni correction; $p < 0.05$ was regarded as significant.

Results

1) Effects of Methemoglobin infusion following repetition of short bouts of high levels of H_2S (protocol 1)

Figure 1 shows an example of the response to the repetition of several bouts of high level H_2S infusion (20 micromol/min). Among the twelve rats that were studied, 3 rats died during or just after the cessation of H_2S infusion and were excluded from the analysis. In order to minimize the number of rats used in the study, a second test was also performed each time H_2S exposure was followed by a saline injection. Following the second series of H_2S exposure, the injection of either saline or MetHb solution was randomly performed. In contrast, when MetHb was performed in first, no second H_2S exposure was completed. Accordingly, six rats received a saline injection following the first exposure to H_2S bouts. Among these six rats, a second bout of H_2S exposure was performed followed by a saline injection in two rats and a MetHb injection in the remaining four rats. The three other rats only received a MetHb injection following the first exposure to H_2S bouts. Overall, fifteen tests were analyzed in 9 rats, 8 using saline injection, and 7 with Methemoglobin injection, following H_2S exposure. As shown in table 1 (see also below), there was no difference in any of the variables of interest before injection in the rats that received MetHb or saline. In addition, no statistical difference was found between the first and second trial in the magnitude of oxygen deficit or peak/nadir values of hemodynamics (blood pressure, blood flow). However, a significant lower cumulative dose of H_2S was needed during the second bout of NaHS (42.1 ± 10.9 vs 27.1 ± 7.7 μmol (p=0.01)) to obtain the same level of O_2 deficit (−15.6 ± 4 vs −17.3 ± 4.2 ml.kg^{-1} ; NS) and blood lactic acidosis (6.0 ± 0.9 vs 5.3 ± 1.7 mM; NS) as compared to the first trial.

Typically, the series of repetitions of H_2S infusions resulted in a dramatic decrease in ABP, carotid and femoral blood flows and VO_2 (figure 1 and 2) along with an increase in lactic acid (table 2). After each bout of NaHS infusion, mean arterial blood pressure, carotid and femoral blood flows have the tendency to return, although not fully, towards baseline values during the 40 seconds in between two repetitions of NaHS infusion (Figure 1). Table 1 summarizes the baseline data during and at the end of NaHS infusion.

1.1) Recovery with Saline/control injection (n=8)—In the tests wherein saline was used, the repetitions of intravenous H_2S infusions provided an oxygen deficit of −16 ± 4.9 ml.kg^{-1} at the end of H_2S infusion along with an increase in blood lactates (5.7 ± 1.2 mM). On average ABP decreased by 45 ± 17% while mean CBF and mean FBF were reduced by 73.6 ±15.5% and 90.6 ±9.3%, respectively.

As illustrated in Figures 2A, following the cessation of NaHS infusion, mean ABP increased from 40.3 ± 10.5 to 125.8 ± 33.6 mmHg even before the injection of 3 ml saline, and

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remained higher than baseline until the 15th minutes of recovery, with an average of 103±22 mmHg (p=0.001). Femoral blood flow also rose above the baseline value gradually following the IV injection of saline (Figure 2B). Carotid blood flow increased progressively eventually reaching a higher level than baseline (p=0.0006; Figure 2C).

Within the first 15 min of recovery, oxygen consumption increased and remained higher than baseline by 25.1±9.3% (p<0.0001), 19.3±13.1% (p=0.002), 13±13.2% (p=0.02) at 5, 10 and 15 min of recovery respectively, as illustrated in Figure 2D. On average, VO_2 rose from 12.2 ±2.1 at baseline to 13.7 ± 2.4 $ml.kg^{-1}.min^{-1}$ at 15 min of recovery (p=0.002). It takes about 10 minutes to recover from the oxygen deficit in the saline group (Figure 3).

1.2) Recovery with Methemoglobin injection (n=7)—As mentioned above, no statistical difference was found between the groups that received saline vs MetHb prior to the injection of the antidote or its vehicle: we found the same level of oxygen deficit and lactic acidosis as in saline tests, 16.8 ±3.1 $ml.kg^{-1}$ just before the injection of the antidote (NS from saline) and 5.8 ± 1.5 mM (NS from saline) respectively. Mean ABP decreased by 52 ± 16%, while mean CBF and mean FBF were reduced by 76.3 ± 11.6% and 90 ±3.5%, respectively (NS from saline group). Oxygen consumption was dramatically reduced and reached its nadir within the 10 sec following the end of the last bout of NaHS infusion, at 4.8 ± 1.4 $ml.kg^{-1}.min^{-1}$ (p<0.0001; from baseline; NS from saline condition).

Following MetHb infusion, no differences were observed in the change in MABP (Figure 2A), carotid blood flow (CBF, Figure 2B) VO_2 (Figure 2D), or oxygen deficit (figure 3) when compared to saline/control group. However, femoral blood flow (FBF) returned to baseline more slowly following MetHb than following saline infusion (see Figure 2C). As in saline tests, oxygen consumption remained elevated following MetHb injection by 21.3 ± 20.2% (p=0.02), 15.2 ±10.5% (p=0.004), 12 ±7.9% (p=0.005) at 5, 10 and 15 min of recovery respectively.

As shown in Table 2, the use of MetHb did not affect the amplitude or the kinetics of recovery nor did it speed up the reduction in lactic acidosis. Of note is that the time to recover the oxygen deficit following H_2S intoxication appeared to be if anything longer following MetHb than after saline (533±242 s vs 395±130 s), but without reaching statistical significance (figure 3).

2) Effects of intravenous injection of methemoglobin on sulfide concentrations following constant infusion of H_2S (protocol 2)

Among the twelve rats studied according to this protocol, 4 rats died during or within one minute following H_2S infusion in control conditions, while one rat died just at the onset of MetHb injection. As a result, 8 control recovery periods and 7 recovery periods with MetHb were analyzed.

2.1) Recovery and saline infusion (n=8)—Intravenous NaHS infusion at a rate 5.9 ±2.4 $\mu mol.min^{-1}$ caused a rapid rise in both CgH_2S and $CMBBH_2S$. CgH_2S increased up to 2.3 ± 2.5 µM and $CMBBH_2S$ to 19.4 ±14.9 µM in keeping with the rate of H_2S infusion. This was associated with a moderate decrease in mean arterial blood pressure and mean

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femoral blood flow without reaching significance as illustrated on Figure 4. CgH_2S decreased by 90% in the very first minute following the cessation of NaHS infusion, while $CMBBH_2S$ dropped to 1.6 ±0.3 μM ($p<0.0001$) at 5 min (Figure 4A). Saline was infused one minute and half after the cessation of NaHS and $CMBBH_2S$ remained unchanged up to 15 min of recovery (Figure 4B).

2.2) Recovery and methemoglobin infusion (n=7)—In the rats that received methemoglobin solution during recovery, the rate of NaHS infusion was identical to the saline group, averaging 6.2 ±2.5 μmol/min. This infusion resulted in an increase in CgH_2S to 3.0 ±2.9 μM and $CMBBH_2S$ to 19.6 ±16.9 μM. CgH_2S decreased as soon as NaHS infusion was stopped. The injection of methemoglobin, which was performed 1.5 min into recovery, did not affect the normal decline in CgH_2S and $CMBBH_2S$ at 5, 10 and 15 min (Figures 4A and 4B). Blood MetHb concentration reached 5.9 ± 0.6 % 5 min after MetHb IV injection ($p<0.0001$). The only difference between saline and MetHb groups was a persistent increase in blood pressure following MetHb infusion. The difference remained significant up to 15 minutes.

Discussion

We found that in a rat model of hydrogen sulfide poisoning, the injection of a methemoglobin solution in the very first minutes of recovery, was unable to affect the concentration of H_2S in the blood, H_2S-induced O_2 deficit, and lactate accumulation.

Methodological considerations

The effects of a constant IV NaHS infusion in protocol 2, showed that almost 25% of rats died within minutes when using a rate of 5.5-6.5 μmol/min, but with little or no effects on circulation and the rate of oxygen uptake. Exploratory tests in a sample of rats have shown that no animals would survive more than 5 min at a constant IV NaHS infusion rate of 10 μM/min (100% mortality). H_2S administered intermittently at a must higher rate allowed a severe reduction in VO_2 and in arterial blood pressure along with lactate accumulation, but with the same mortality (25 %) as a during moderate rate of infusion, due to the very rapid off-kinetics of H_2S in the blood and the tissues.

Among the 12 rats that were studied in order to complete protocol 1, 3 rats died during or just after the cessation of H_2S infusion. Among the surviving rats, those “treated” by saline injections received a second injection of NaHS followed by a second treatment, in attempt to reduce the number of animals. Clearly lower cumulative doses of H_2S were needed during the second series of injections to produce a decrease in arterial blood pressure and VO_2 of same magnitude as in the first series. In that sense, this current protocol differs from the “traditional” way of looking at dose-effect response as we were primarily interested in the recovery of the circulatory and “metabolic” depression induced by H_2S and thus needed to produce similar effects on these variables in all exposed animals. The dose-effects relationship for H_2S is so steep that responses can be quite variable. Even during the first series of injection, the cumulative quantity of H_2S was rather variable, averaging 42.1 ± 10.9 micromol, with a coefficient of variability of 25 % (SD/Mean). The short bouts of infusions were thus repeated until our target reduction in arterial blood pressure was obtained. With

this approach there was no difference in any of the variables of interest before injection in the rats that received MetHb versus saline, as presented in table 1. In addition, no statistical difference was found between the first and second trial in the magnitude of oxygen deficit or peak/nadir values of blood pressure or blood flow.

Methemoglobin as an antidote following H_2S intoxication

Methemoglobinemia has long been proposed as a specific treatment of hydrogen sulfide poisoning not only in animals models [6,25], but also in humans [10,12,25]. Its counteracting effects are based on the combination of H_2S with the ferric iron (Fe^{3+}) present on MetHb [26] and also on the apparent ability of MetHb to catalyze the oxidation of H_2S [8]. This effect was found with either sodium nitrite-induced methemoglobinemia [27] or a methemoglobin solution [11]. We have shown that levels as low as 3% methemoglobinemia are sufficient to abolish the stimulation of breathing triggered by IV bolus of H_2S [11]. To our knowledge, all previous studies on the efficacy of MetHb have demonstrated beneficial effects against H_2S-induced lethality, particularly in rodents, when nitrite-induced methemoglobinemia was injected prior to [6,25] or during H_2S exposure[15]. We have recently found [13] that infusion of a similar levels of methemoglobin in rat *during* H_2S exposure produces an immediate reduction in CgH_2S by more than half, while $CMBBH_2S$ increased by several folds. The latter reflects the creation of a new sink (ferric iron) capable of combining free H_2S.

The absence of effects of methemoglobin infusion on H_2S concentrations *following* H_2S intoxication can be understood in light of the kinetics of H_2S in the blood[13]. Indeed, our group has already shown that at least two compartments of H_2S can be described in the body: a soluble form related to the H_2S partial pressure which disappears with virtually no delay to undetectable levels a soon as the exposure ceases, and complex combined forms, which remain present at low concentrations in the blood and tissue for quite sometimes after the cessation of exposure [15]. Since we injected MetHb solution 1-2 minutes following H_2S infusion, we can assume that little dissolved H_2S if any was present to be trapped in the blood.

Lack of improvement in circulation and O_2 deficit with MetHb infusion following H_2S infusion

We hypothetized that MetHb could improve the recovery of O_2 utilization by the mitochondria, following the cessation of hydrogen sulfide intoxication via its specific ability to combine soluble H_2S, but also possibly though its anti-NO effects [28-30]. All the animals, during severe forms of H_2S intoxication, presented signs of impediment of mitochondrial oxidative metabolism, as reflected by a decrease in VO_2 leading to a large O_2 deficit of about 16 ml/kg and a hyperlactacidemia of more than 7 mM in some animals. We were unable to observe any changes in the recovery of the markers of oxidative metabolism following MetHb infusion. This supports the view that, since the soluble forms of H_2S have already disappeared at the moment of methemoglobin infusion [13,15], MetHb is unable to alter the recovery from sulfide exposure. As MetHb does not diffuse inside the cells and can not interact with combined H_2S, no faster restoration of a normal activity of the mitochondrial electron chain is to be expected.

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Clinical relevance of the present study in the treatment of H_2S poisoning

The fact that no improvement in hemodynamics, O_2 deficit or lactate production could be demonstrated following a severe H_2S intoxication after MetHb infusion can not exclude, however that a clinical benefit would be produced by this antidote. Only long-term follow-up, i.e. in a chronic animal model, would help us to clarify such an effect. Indeed, human studies have shown that subjects surviving from acute H_2S poisoning present with severe post-anoxic injury responsible for long-term after-effects of sulfide intoxication, including motor and cognitive impairment [31], which may be explained as well by the associated shock and reduction in oxygen supply. The potential beneficial effects of an antidote injected following H_2S exposure on the long-term memory loss and cognitive impairment remains to be evaluated. The lack of intracellular effects of methemoglobin appears to be a fundamental limit for its use; other antidotes such as high dose hydroxocobalamin (Vit B12) for example [32], which can diffuse into the cells [33] via non-transcobalamin mechanisms [34] may prove to be better candidate than MetHb to treat sulfide poisoning after sulfide exposure [13].

In conclusion, our results have shown no acute benefits of MetHb infusion during the recovery phase following severe H_2S intoxication in sedated rats. No differences in hemodynamics or O_2 deficit recovery were observed when compared to saline injection. We postulate that this lack of effect is in large part accounted for by the natural fate of soluble H_2S in the body and the action of methemoglobin, which is limited to the intra-vascular compartment. However, the potential effects on the long-term neurological consequences in the animal surviving remain to be evaluated.

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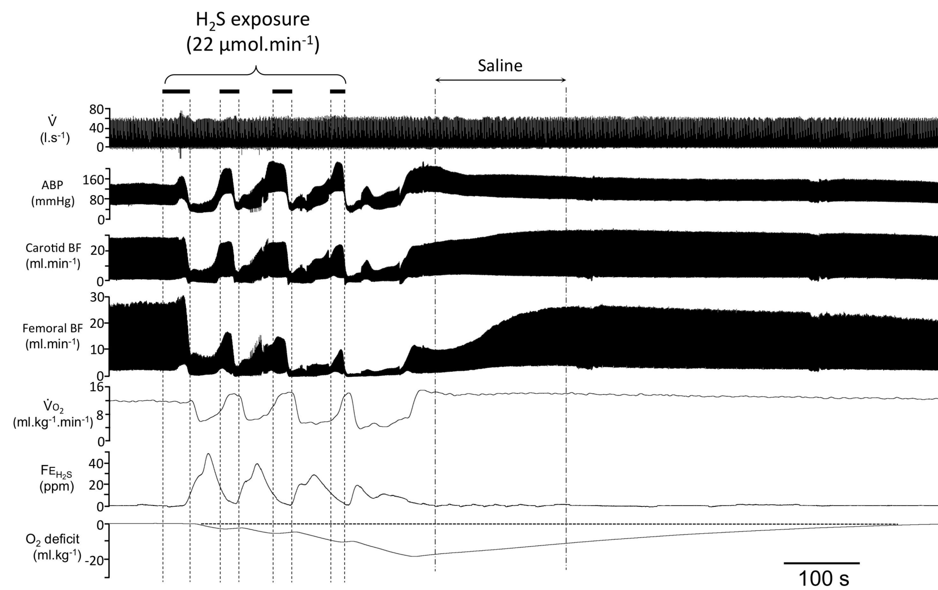

Figure 1.

Recording obtained in one rat during and following intoxication with H_2S produced by four repetitions of NaHS infusion at a rate of 22 µmol min^{-1}. This intoxication was in this example followed by a saline injection during the recovery. From top to bottom: respiratory flow ($\dot{V}$), Arterial Blood Pressure (ABP), Carotid Blood Flow (CBF), Femoral Blood Flow (FBF), Oxygen uptake (VO_2), expired fraction of H_2S (F_EH_2S), and O_2 deficit are shown. The solid horizontal bars indicate the periods of NaHS infusions. Arterial blood pressure and carotid blood flow dramatically decreased during NaHS infusion. The oxygen deficit accumulated during the NaHS infusion reached −18 ml.kg^{-1} along with an important lactic acidosis – in this example 7.4 mM, one minute following the cessation of NaHS infusion–. Following the cessation of sulfide infusion, ABP and blood flows increased progressively with an exponential like pattern towards baseline values. Note that VO_2 remained elevated during the whole recovery “repaying” the O_2 deficit/debt within about 10 minutes.

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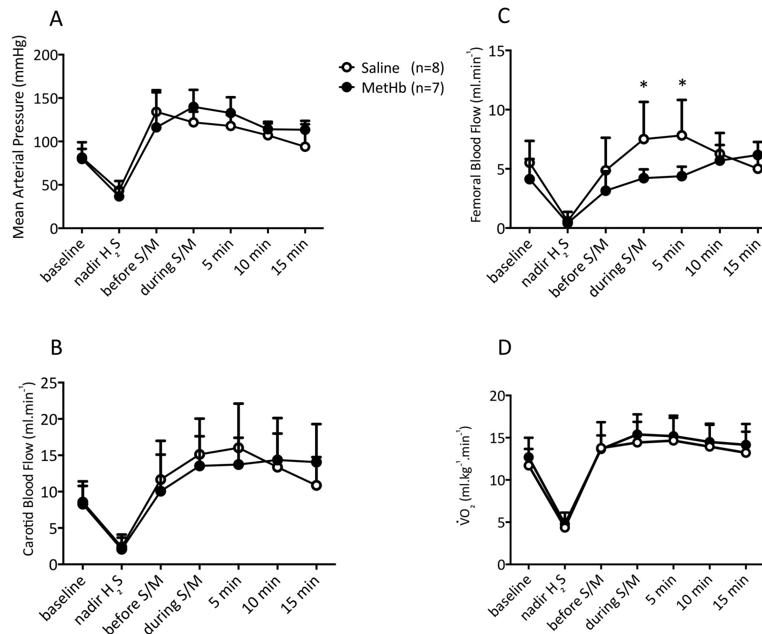

Figure 2.

Temporal evolution of mean ABP (panel A), carotid blood flow (CBF, panel B), femoral blood flow (panel C) and VO_2 (panel D) during and following H_2S intoxication with saline (open circles) or MetHb (closed circles). Mean ABP, CBF and VO_2 decreased during sulfide infusion but recovered during the recovery with a similar kinetics and magnitude following the injection of MetHb or saline. Femoral blood flow (FBF) increased less with MetHb than saline, so FBF was significantly lower during the MetHb ($p=0.01$) and at 5 min ($p=0.007$) compared to saline injection.

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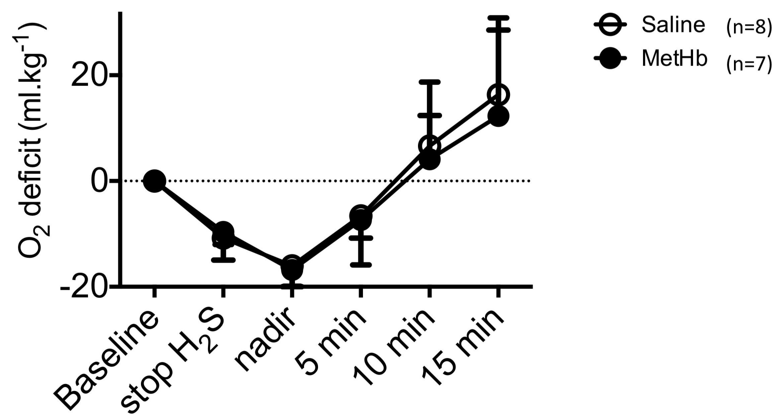

Figure 3.

O_2 deficit with saline injection (open circles) or MetHb injection (closed circles). The large O_2 deficit produced by H2S infusion recovered in both conditions, and was "repaid" with 15 min. MetHb had no effect on the magnitude or kinetics of the oxygen deficit (before injection) or its repayment (after injection).

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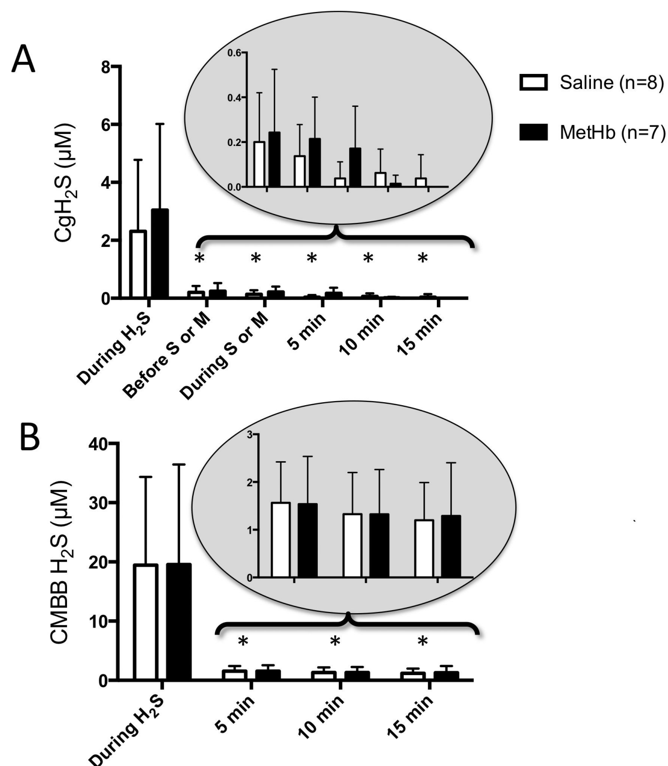

Figure 4.

Concentration of gaseous H_2S (CgH_2S, Panel A) and total H_2S ($CMBBH_2S$, Panel B), before and after saline or MetHb infusion. Both gaseous and total H_2S concentrations in the blood decreased within the first one minute following the cessation of H_2S infusion, i.e. before injection of MetHb or saline. No effect of MetHb on CgH_2S or $CMBBH_2S$ was found. *Significantly different from the data during H_2S infusion, $p<0.0001$.

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Table 1

Oxygen uptake, carotid and femoral blood flow, arterial blood pressure, arterial blood gases and lactates before (baseline) and at the end of repetition of intravenous H_2S infusions. Data are expressed as Mean±SD.

	Control (n=8)		Methemoglobin (n=7)		
Baseline	**mean**	**SD**	**mean**	**SD**	**p***
Weight (kg)	0.41	0.026	0.43	0.051	NS
VO_2 (ml/kg/min)	11.7	1.9	12.7	2.3	NS
Carotid BF (ml/min)	8.6	2.8	8.3	2.5	NS
Femoral BF (ml/min)	5.5	1.8	4.1	1.7	NS
Arterial BP (mmHg)	82	17	80	12	NS
PaO_2 (mmHg)	69	9	67	14	NS
$PaCO_2$ (mmHg)	37	6	36	14	NS
Blood lactates (mM)	2.38	0.6	1.9	0.7	NS
Repetition of H_2S infusions					
N° repetitions	4.8	1.4	4.3	1.5	NS
CgH_2S (µM)	15.6	2.9	15.8	2.6	NS
Cumulative dose (µM)	36.6	12.3	33.4	12.6	NS
Total duration of infusion (s)	101.6	34.1	92.8	34.9	NS
End H_2S infusion					
VO_2 nadir (ml/kg/min)	4.4	1.0	4.8	1.4	NS
O_2 deficit nadir (ml)	-16	4.9	-16.8	3.1	NS
Carotid BF nadir (ml/min)	2.4	1.7	2.1	1.6	NS
Femoral BF nadir (ml/min)	0.6	0.8	0.4	0.2	NS
Arterial BP nadir (mmHg)	44	11	37	9	NS
PaO_2 (mmHg)	63	9	76	21	NS
$PaCO_2$ (mmHg)	38	14	35	7	NS
Blood lactates (mM)	5.7	1.2	5.8	1.5	NS

VO2 = oxygen uptake; Carotid BF= Carotid Blood Flow; Femoral BF= Femoral Blood Flow; Arterial BP= Arterial Blood Pressure N° repetitions= number of repetitions of H_2S infusion, CgH2S=Concentration of gaseous H_2S in the blood; SD = Standard deviation;

*ANOVA

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Table 2

Mean ±SD of blood lactates and kinetics of recovery.

	Control (n=8)		Methemoglobin (n=7)		
Blood lactates (mM) in recovery	mean	SD	mean	SD	p*
1 min	5.7	1.2	5.8	1.5	NS
5 min	3.5	0.6	3.2	0.6	NS
10 min	2.1	0.6	2.3	0.6	NS
15 min	1.5	0.4	1.6	0.5	NS
Kinetics of recovery (s)					
Time from stop H_2S to nadir O_2 deficit	68	18	89	40	NS
Time from nadir to payment O_2 deficit	395	130	533	242	NS