Oxygen deficit and H$_2$S in hemorrhagic shock in rats

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Abstract

**Introduction:** Hemorrhagic shock induced O$_2$ deficit triggers inflammation and multiple organ failure (MOF). Endogenous H$_2$S has been proposed to be involved in MOF since plasma H$_2$S concentration appears to increase in various types of shocks and to predict mortality. We tested the hypothesis that H$_2$S increases during hemorrhagic shock associated with O$_2$ deficit, and that enhancing H$_2$S oxidation by hydroxocobalamin could reduce inflammation, O$_2$ deficit or mortality.

**Methods:** We used a urethane anesthetized rat model, where 25 ml/kg of blood was withdrawn over 30 minutes. O$_2$ deficit, lactic acid, tumor necrosis factor (TNF)-alpha and H$_2$S plasma concentrations (Siegel method) were measured before and after the bleeding protocol in control animals and animals that received 140 mg/kg of hydroxocobalamin. The ability to oxidize exogenous H$_2$S of the plasma and supernatants of the kidney and heart homogenates was determined in vitro.

**Results:** We found that withdrawing 25 ml/kg of blood led to an average oxygen deficit of 122 ± 23 ml/kg. This O$_2$ deficit was correlated with an increase in the blood lactic acid concentration and mortality. However, the low level of absorbance of the plasma at 670 nm (A$_{670}$), after adding N, N-Dimethyl-p-phenylenediamine, that is, the method used for H$_2$S determination in previous studies, did not reflect the presence of H$_2$S, but was a marker of plasma turbidity. There was no difference in plasmatic A$_{670}$ before and after the bleeding protocol, despite the large oxygen deficit. The plasma sampled at the end of bleeding maintained a very large ability to oxidize exogenous H$_2$S (high μM), as did the homogenates of hearts and kidneys harvested just after death. Hydroxocobalamin concentrations increased in the blood in the μM range in the vitamin B12 group, and enhanced the ability of plasma and kidneys to oxidize H$_2$S. Yet, the survival rate, O$_2$ deficit, H$_2$S plasma concentration, blood lactic acid and TNF-alpha levels were not different from the control group.

**Conclusions:** In the presence of a large O$_2$ deficit, H$_2$S did not increase in the blood in a rat model of untreated hemorrhagic shock. Hydroxocobalamin, while effective against H$_2$S in vitro, did not affect the hemodynamic profile or outcome in our model.

**Introduction**

The severity of a shock secondary to an acute hemorrhage is not simply dictated by the volume of blood loss [1,2]. Rather, the prognosis of a hemorrhagic shock is linked to a cascade of events, occurring during both the phase of bleeding and resuscitation, related to the magnitude of the oxygen deficit [3-6] and the resulting ischemic and post-ischemic inflammatory response [7,8]. Indeed, hemorrhagic shock precipitates inflammatory cascades that comprise the activation of stress transcriptional factors and up-regulation of cytokines synthesis [9,10] leading to multiple organ failure [10]. Among the putative actors involved in the fatal course of an acute hemorrhage induced tissue ischemia/hypoxia, a novel candidate has been recently put forward: endogenous hydrogen sulfide [11,12]. Endogenous H$_2$S, a newly described gasotransmitter [13], has been shown to increase during and following an acute hemorrhage [11] and to act as a powerful pro-inflammatory agent in various animal models [14-17]. In humans, endogenous H$_2$S has been proposed 1) to increase in the blood up to 100 μM concentrations during various forms of shock [18] and 2) to be a predictor of
survival [18]. Although the mechanism of H2S production remains to be clarified in shock, this by-product of cysteine metabolism appears to increase under hypoxic conditions [19,20] and has been more recently suggested to contribute to the response to hypoxia [19,21–23], although this notion has been challenged [24–26]. One of the working hypotheses is that in hypoxic conditions, the level of H2S oxidation in the cells and mitochondria is diminished [23]; in turn, the accumulation of this gas was proposed to transduce the physiological response to hypoxia in the vessels or the arterial chemoreceptors [23], but also an unwanted inflammatory response in other tissues [27].

Many questions on the role of H2S in hemorrhage, however, remain to be clarified: there are, for instance, many reasons to believe that H2S cannot accumulate in the blood [28,29]. Indeed, the view that H2S increases in conditions associated with a hemorrhagic shock must be reconciled with the ability of the blood, the cytoplasm of most cells and the mitochondria to oxidize very large amounts of sulfide [29,30], which should prevent H2S from rising even at low PO2 [24]. One should also reconcile the view that H2S concentrations could rise in the body and have deleterious effects with 1) the levels of sulfide found during H2S intoxication (see [31] for discussion), which are much lower than those reported in shock [18], and 2) the observations that exogenous H2S appears to be beneficial [32–34]. The clinical significance of such a beneficial effect remains the subject of debate [35].

We have recently investigated the effects of cobalt in the form of hydroxocobalamin on H2S oxidation [30]. Injection of a large dose of vitamin B12 (at a level similar to that used in cyanide intoxication) dramatically increases the oxidative capacity of the blood and tissues (the kidney and to a lesser extent, the heart) for H2S in the rat, possibly via the presence of oxidized cobalt [30]. Acting on H2S oxidation in conditions associated with a reduction in oxidative mitochondrial metabolism may, however, represent a way to: 1) test the possible role of endogenous H2S in clinically relevant conditions, such as hemorrhage induced tissue ischemia, and 2) evaluate potential novel therapeutic approaches in hemorrhagic shock.

The aim of this study was to determine in a model of untreated hemorrhagic shock in spontaneously breathing urethane anesthetized rats, wherein a large O2 deficit can be produced; 1) the putative changes in H2S concentration in blood induced by this model of shock and 2) the potential benefit of large doses of vitamin B12 injected before the onset of the hemorrhage. The effects of the presence of μM levels of vitamin B12 in the blood and tissues on the survival at one hour, on the level of lactic acidosis, TNF-alpha and on O2 deficit accumulated during and following the period of hemorrhage were investigated, in keeping with the ability of blood and tissues (kidney and heart) to oxidize H2S. The possibility of bias accounting for the discrepancy between the low-expected and high-reported changes in H2S in humans was also investigated using the same methodological approaches as in published studies [11,16,18]. The hypothesis tested in this study is that H2S increases along with inflammatory markers when O2 deficit develops during a hemorrhagic shock, and that increasing the oxidative property of the blood and tissues for H2S by the presence of vitamin B12 could decrease these markers and improve survival.

**Materials and methods**

**Animal preparation**

After approval from the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee, a total of 17 adult Sprague-Dawley rats (470 ± 43 g) were prepared as follows: anesthesia was induced with 3.5% isoflurane in O2 followed by intra-peritoneal injection of 1.2 g/kg of urethane (Sigma-Aldrich, St Louis, MO, USA). A polyethylene PE-50 catheter was inserted into the left femoral artery for blood withdrawal and arterial blood pressure (ABP) monitoring (Cybersense, Nicholasville, KY, USA). The animals were tracheostomized and the tracheostomy was connected to a small dead space two-way valve [24]. The inspiratory port of the valve was connected to a calibrated pneumotachograph (Hans Rudolph Inc., KS, USA, 8420 series, Kansas city, MO, USA) to measure inspiratory flow. The rats were breathing spontaneously in room air during the entire protocol. Their body temperature was monitored using a rectal probe and was kept at 35 to 36°C throughout the surgery and the hypovolemia using a pad heated at a constant temperature.

**Protocol**

Immediately after surgery, the rats received an intraperitoneal (I.P.) injection of either 140 mg/kg hydroxocobalamin (vitamin B12a, Sigma-Aldrich, 60 mg/ml) in saline (vitamin B12 group, n = 9), or an equivalent volume of saline (2.3 ml/kg, control group, n = 8). Each rat receiving saline or vitamin B12 was randomly chosen among a homogenous group of rats of similar age and weight. Thirty minutes after I.P. injection, hemorrhage was initiated by withdrawing 2.5 ml/100 g of blood over about 30 minutes as follows: 0.5 ml/100 g were withdrawn over 3 minutes, every 6 minutes (5 sessions). Blood gas analysis and lactate measurements were performed just before and at the end of the hemorrhage period (i-STAT-1 blood gas analyser, Abaxis, Union City, CA, USA). The first and last samples of blood withdrawn were also used for H2S and vitamin B12 determinations.
(see below). Plasma was collected by centrifuging the blood 15 minutes at 13,000 rpm, and then frozen for the determination of TNF-alpha levels and of the ability of the plasma to oxidize H$_2$S (see below). No fluid was administered except for flushing the arterial catheter with a fixed volume of 0.2 ml of heparinized saline after each period of bleeding. After the hemorrhage period, data were continuously recorded until the death of the animal.

**Measurements and data analysis**

The inspiratory flow (\(\dot{V}_I\)) and arterial pressure signals were digitized by analog-to-digital converter at 200 Hz (LabView 8.5, National Instruments, Austin, TX, USA). Analysis of data was performed offline using Powerchart software (Chart 5, AD Instruments, Colorado Springs, CO, USA). Breathing frequency (\(f\)) and tidal volume (\(V_T\)) were respectively determined using peak detection and integration of the inspiratory flow signal. Minute ventilation (\(\dot{V}E\)) was computed in body temperature and pressure saturated (BTPS) conditions as \(f \times VT\).

In 10 animals (4 controls and 6 hydroxocobalamin-treated rats), a 7 ml mixing chamber was connected to the expiratory port of the valve, where mixed expiratory gas composition was continuously sampled and analyzed (GEMINI, CWE Inc., Ardmore, PA, USA). O$_2$ uptake (\(\dot{V}O_2\)) was computed in standard temperature and pressure, dry (STPD) condition using \(\dot{V}I\), the inspiratory and expiratory fractions of O$_2$ and CO$_2$. \(\dot{V}E\) was computed as \(\dot{V}I_{BTPS} \cdot (1-FIO_2-FICO_2/1-FEO_2-FECO_2)\) and \(\dot{V}O_2\) as \((\dot{V}I_{STPD}\cdot FEO_2) - (\dot{V}E_{STPD}\cdot FEO_2)\). The same approach was used to calculate \(\dot{V}CO_2\) as \(\dot{V}E_{STPD}\cdot FEO_2\). Oxygen deficit (ml/kg) was computed as the integral of difference between pre-hemorrhage \(\dot{V}O_2\) (averaged over five minutes) and \(\dot{V}O_2\) throughout the hemorrhagic period, then until death occurred. All signals were also displayed on line for monitoring.

Hydroxocobalamin concentrations in plasma and tissue homogenates were determined by spectrophotometric reading of the plasma at 525 nm (DU 530, Beckman Coulter, Danvers, MA, USA) as previously described [30].

The methylene blue method [36] was used for H$_2$S measurements in the plasma since this method was the one chosen in previous studies to establish the blood levels of H$_2$S increases in humans during shock [16,18]. We followed a similar protocol: after centrifuging 2.5 ml of blood at 13,000 rpm for five minutes, 1 ml of plasma was collected and 0.4 ml of zinc acetate (1%) was added to the plasma to trap H$_2$S. Then, 100 \(\mu\)l of a 20 mM solution of N,N-Dimethyl-p-phenylenediamine sulfate (Sigma, St Louis, MO, USA) in 7.2 N hydrochloric acid (Sigma), and 100 \(\mu\)l of a 30 mM iron chloride solution (Sigma) in 1.2 N hydrochloric acid were added to the plasma, producing a blue dye proportional to H$_2$S concentration. Throughout the procedure, every precaution was taken to prevent the samples from being in contact with air: the blood was collected in syringes that were immediately capped, and was then transferred to vials, which were completely filled, capped and centrifuged. After centrifugation, the plasma was collected and the reagents were immediately added. The same procedure (including centrifugation) was applied to phosphate-buffered saline (PBS) solution containing a known amount of H$_2$S (concentration 100 \(\mu\)M). The H$_2$S concentration was obtained after 20 minutes by adding 0.5 ml of trichloroacetic acid (TCA) 10% (to remove the proteins), centrifuging the solutions (10 minutes at 13,000 rpm) and reading the absorbance of the supernatants at 670 nanometers (spectrophotometer Beckman Coulter DU 530). A calibration curve for H$_2$S concentration was established, and for each experiment, a PBS solution was used as the blank.

In order to assess the ability of the plasma to oxidize exogenous H$_2$S, 0.6 ml of plasma was mixed with 0.6 ml of PBS. NaHS (sodium hydrosulfide hydrate, Sigma-Aldrich) solution was added to the diluted plasma, so that the final concentration of H$_2$S was 100 \(\mu\)M. The vials were entirely filled with diluted plasma and as soon as H$_2$S was added, the vials were capped to avoid contact with air. Two minutes later, residual H$_2$S concentration was measured at ambient barometric pressure after application of 0.5 ml of TCA 10% (Sigma-Aldrich) prior to the final centrifugation (13,000 rpm for 10 minutes), and the absorbance was read at 670 nm.

The same approach was used with the homogenates of hearts and kidneys harvested immediately after cardiac arrest. The organs were thoroughly rinsed in PBS, frozen in liquid nitrogen and stored at -80°C for later analysis. They were then thawed in ambient air, weighed and homogenized (Tissue-Tearor, Biospec, Bartlesville, OK, USA) in PBS (50% w/v for kidney, 25% w/v for heart). The homogenates were then centrifuged at 13,000 rpm for 15 minutes; 1.35 ml of supernatant was collected and mixed with the corresponding volume of NaHS solution to obtain a final concentration of 100 \(\mu\)M following the same procedure as for the plasma. Again, the vials were entirely filled with supernatant and as soon as H$_2$S was added, the vials were capped to avoid contact with air. Residual H$_2$S concentration was determined after two minutes.

As whole blood is known to readily oxidize H$_2$S, we also sought to determine the resolution of the methylene blue method applied to the blood by adding known concentrations of H$_2$S in fresh blood from three sham rats and then measuring, for two minutes, H$_2$S concentrations using the
were significantly and were also analyzed in each group, and lactate before, which occurred within 3. The O2 deficit which averaged 122 ± 23 ml/kg at the one hour. The survival rate was compared between the two groups using a logrank test [37]. For all comparisons, P < 0.05 was considered statistically significant.

Statistical analysis
All results are presented as mean ± SD. All parameters were compared between pre-bleeding and post-bleeding periods using a one-way ANOVA in each group. ABP, VO2 and Vl were also analyzed in each group before and after each of the five bleeding periods using ANOVA for repeated measurements; post-hoc comparisons were performed using a Bonferroni correction (SigmaStat 2.0, SPSS Inc, San Jose, CA, USA). Finally, the control and vitamin B12 groups were compared with ANOVA, while survival rates were compared between the two groups using a logrank test [37]. For all comparisons, P < 0.05 was considered statistically significant.

Results
The shock model
Control animals (n = 8)
Figure 1 displays recordings of the response to the hemorrhage protocol in two different rats, while Table 1 reports the averaged data. Hemodynamic, ventilatory and metabolic responses were qualitatively similar in all control rats. Typically, each of the five three-minute bleeding periods induced a drop in arterial pressure, minute ventilation, VO2 and VCO2. Between the bleeding periods, all the parameters tended to return progressively to their baseline values (Figures 1 and 2). This recovery was interrupted by the subsequent bleeding periods repeated after six minutes and was blunted over time. At the end of the bleeding periods (30 minutes), mean ABP, VO2 and VCO2 were significantly reduced by 68%, 44%, 56% and 51% respectively (see actual data in Table 1). Blood lactic acid increased significantly (P = 0.001) (Table 1). Two different profile patterns (Figure 1) were observed following the bleeding procedure: in five animals, arterial pressure increased slowly towards pre-bleeding levels before subsiding again until death occurred from primary respiratory or cardiac arrest, within two hours following the onset of bleeding. In the three remaining animals, arterial pressure, minute ventilation, VO2 and VCO2 continued to decrease until death (Figure 1), which occurred within one hour. The survival rate vs. time is shown in Figure 3. The O2 deficit which averaged 122 ± 23 ml/kg at the end of the bleeding period (Figure 4 and Table 1) reached 338 ± 88 ml/kg at the moment of death.

Vitamin B12 treated rats (n = 9)
The absorbance spectra of the plasma of the animals treated with vitamin B12 clearly showed a peak of absorbance at 525 nm (Figure 5A), corresponding to a concentration of 185 ± 216 μM/l. No peak of absorbance at 525 nm was observed in the plasma of any of the control rats (Figure 5A).

As shown in Figure 2 and Table 1, the changes in ABP, minute ventilation, VO2, VCO2 and lactate before, during and after the bleeding periods were similar in the group treated with vitamin B12 and in control animals. The time course of O2 deficit was also the same in the two groups of rats (Figure 4A). O2 deficit accumulated progressively during the bleeding period, reaching 118 ± 45 at 30 minutes (Table 1, NS vs controls, P = 0.98). When vitamin B12 and control rats were combined, O2 deficit and lactate level at the end of bleeding were significantly correlated (Figure 4B, r² = 0.79). The O2 deficit at the time of death was 265 ± 30 ml/kg (Table 1, NS vs controls, P = 0.10).

H2S measurements
Control animals
According to our standard curve, a concentration of 100 μM H2S resulted in an absorbance of 1.41 at 670 nm; the relationship between the concentration of H2S and the absorbance was linear up to 3 μM while it was possible to identify the presence of H2S at a minimal value of 1.5 μM (absorbance 0.005). We did not find any changes in the level of H2S added to PBS which were analyzed following the very same procedure as the blood (including centrifugation): the absorbance of a solution of H2S in PBS analyzed immediately after sampling from the “mother” solution dropped by 3.2% following the procedure applied to the blood (n = 12). Centrifugation for 10 minutes decreased the absorbance by 1.2%. Absorbance readings of the plasma before the shock averaged 0.014 ± 0.015 (Figure 5B). According to the standard curve, such an absorbance would correspond to a theoretical H2S concentration of 8.5 ± 2.9 μM. However, the profile of absorbance over the visible spectrum was markedly different from that of a PBS solution containing H2S at a concentration that would reach a similar absorbance at 670 nm; as shown in Figure 5B, the absorbance of the plasma was high at 400 nm and decreased continuously as the wavelength was increased with a lack of peak of absorbance at 670 nm, in major contrast to the PBS solution. In other words, in the absence of absorbance peak at 670 nm, the value of absorbance of 0.014 ± 0.015 in the plasma did not reflect the presence of methylene blue - and thus H2S at approximately 8 μM - but should be viewed as a marker of turbidity.
Figure 1 Examples of recordings in two rats during and following acute hemorrhage. The breath-by-breath inspiratory flow ($\dot{V}$), arterial blood pressure (ABP), minute ventilation ($\dot{V}I$), body temperature ($\Theta$), carbon dioxide production ($\dot{V}CO_2$) and oxygen uptake ($\dot{V}O_2$) are displayed. Interruptions in ABP recording are due to blood withdrawal during each of the bleeding periods. Note the drop in arterial pressure, minute ventilation, $\dot{V}O_2$ and $\dot{V}CO_2$, during each bleeding period (see text for more details). At the end of the five bleeding periods, all variables continued to either decrease slowly until death occurred (panel A, this response was observed in three out of eight control rats), or ABP, $\dot{V}I$, $\dot{V}CO_2$ and $\dot{V}O_2$ rose transiently before subsiding again, leading to a fatal outcome (panel B, this response was observed in five out of eight control rats). The vertical arrow corresponds to the final cardio-respiratory arrest.

Table 1 Hemodynamic and metabolic variables before and at the end of the bleeding period.

|                          | Control ($n=8$) | Vitamin B12 ($n=9$) |
|--------------------------|----------------|---------------------|
|                          | Pre-bleeding   | End of bleeding     | Pre-bleeding   | End of bleeding     |
| Mean ABP (mmHg)          | 80 ± 12        | 26 ± 3*             | 79 ± 7         | 29 ± 7*             |
| Minute ventilation (ml/min) | 212 ± 22   | 118 ± 50*           | 194 ± 31       | 123 ± 39*           |
| $\dot{V}O_2$ (ml/min)    | 7.18 ± 0.45    | 3.19 ± 0.60*        | 6.79 ± 0.67    | 2.63 ± 0.97*        |
| $\dot{V}CO_2$ (ml/min)   | 6.55 ± 0.60    | 3.23 ± 0.70*        | 6.42 ± 1.14    | 2.66 ± 1.03*        |
| Lactates (mM/l)          | 1.88 ± 0.50    | 6.35 ± 1.44*        | 1.53 ± 0.21**  | 6.63 ± 2.09*        |
| PO$_2$ (mmHg)            | 81 ± 5         | 90 ± 11              | 72 ± 9**       | 86 ± 11*            |
| PCO$_2$ (mmHg)           | 36 ± 4         | 31 ± 7               | 33 ± 8         | 29 ± 5              |
| TNF-alpha (pg/ml)        | -              | 1,301 ± 1,175        | -              | 732 ± 869           |
| O$_2$ deficit (ml/kg)    | -              | 122 ± 23             | -              | 118 ± 45            |

Values are mean ± SD. *$P < 0.05$ vs pre-bleeding values. **$P < 0.05$ vs control rats.
Similar results were found at the end of the bleeding period (when O$_2$ deficit reached 122 ± 23 ml/kg) with an absorbance of 0.016 ± 0.009 at 670 nm. No peak was observed and, just like before the bleeding period, a progressive decrease in absorbance from 400 to 700 nm was found (Figure 5B). This profile of absorbance and the lack of peak at 670 nm were observed in every animal with no exception.

**Vitamin B12 treated animals**

There was no significant difference between the absorbance at 670 nm in control animals and following vitamin B12, both prior (0.017 ± 0.009) and following (0.029 ± 0.018) the period of bleeding (Figure 5A). Just like in the control group and in major contrast to the PBS solution, no peak could be identified at 670 nm suggesting that H$_2$S concentration in the plasma, if any, could not be higher than a few μM.

**Oxidation of 100 μM H$_2$S by the plasma before and during shock**

As shown on Figure 6A, two minutes after adding 0.1 ml H$_2$S to pre-bleeding plasma to reach a final concentration of 100 μM, residual H$_2$S concentration was 9.2 ± 0.9 μM in the control plasma and 7.7 ± 0.8 μM in the plasma of the animals which received vitamin B12 ($P < 0.01$). No change in H$_2$S concentration was found in the PBS solution over two minutes. The ability of the plasma to oxidize H$_2$S remained unchanged at the end of the bleeding period, with residual plasma H$_2$S concentrations of 10.0 ± 1.0 and 7.2 ± 1.7 μM for the control and vitamin B12 groups respectively ($P < 0.01$). For the whole blood, the absorbance spectra of H$_2$S added in sham rat blood at three different concentrations (50, 100 and 150 μM) is displayed on Figure 6B along with the corresponding residual H$_2$S concentrations. Within five minutes, initial
H₂S concentrations in blood of 50, 100 and 150 μM dropped by 90, 92 and 75% respectively.

**Oxidation of 100 μM H₂S by tissue homogenates**

As shown on Figure 7B, two minutes after adding 0.15 ml H₂S to the supernatant of kidney homogenates to reach a final concentration of 100 μM, residual H₂S concentration was 50.2 ± 4.7 μM in control kidney homogenates and 34.8 ± 12.9 μM in kidney homogenates of the animals which received vitamin B12 (P < 0.01). In the same conditions, residual H₂S concentrations in heart homogenates were 31.3 ± 0.9 and 30.5 ± 0.9 μM for control and vitamin B12-treated rats respectively (Figure 7C, NS). In vitamin B12-treated rats, hydroxocobalamin concentrations were 47 ± 57 μM in kidney homogenates (Figure 7B), and below the threshold of detection (30 μM) in heart homogenates except for two animals (Figure 7C). Concentrations did not change in the PBS solutions.

**Mortality and vitamin B12**

There was no difference in time to death (59 ± 26 vs 65 ± 26 min) or mortality rates over time between vitamin B12 and control rats (Figure 3).

**Plasma TNF-alpha**

There was very large intra-group variability but no statistically significant difference in TNF-alpha plasma levels between vitamin B12 treated and control rats (P = 0.398, Table 1).
Discussion
In major contrast to previous reports in humans [18] and in animal models [11], we did not observe any increase in blood \( \text{H}_2\text{S} \) concentration in a model of lethal untreated hemorrhagic shock in urethane anesthetized rats, despite major \( \text{O}_2 \) deficit, hyperlactacidemia and systemic inflammation. In addition, following the bleeding period, the plasma of every animal, as well as the supernatants from the heart and kidney kept a very high ability of oxidizing/complex large (toxic) amounts of sulfide. Finally, following injection of a very large dose of vitamin B12, the ability to oxidize \( \text{H}_2\text{S} \) by the plasma and the kidney was enhanced in proportion to the local vitamin B12 concentration. However, the survival rate, \( \text{O}_2 \) deficit or the various markers of the severity of the shock were not affected by the presence of \( \mu \text{M} \) levels of hydroxocobalamin. These findings do not support the hypothesis that endogenous \( \text{H}_2\text{S} \) does accumulate in the blood or in most tissues and contributes to the severity of hemorrhagic shock induced oxygen deficit [11,18].

Rat model
To investigate the putative role of endogenous hydrogen sulfide during hemorrhagic shock induced cellular hypoxia, we used the urethane-anesthetized rat as an experimental model. Withdrawing 25 ml/kg of blood within 30 minutes produced a dramatic reduction in ABP, \( \dot{\text{V}}\text{O}_2 \) and \( \dot{\text{V}}\text{CO}_2 \) along with an increase in lactic acid and in the respiratory quotient ratio. This hemorrhage protocol led to a fatal outcome in 50% of the animals within one hour following the bleeding onset, which paralleled the magnitude of \( \text{O}_2 \) deficit and hyperlactacidemia. All animals died within two hours. This relatively low survival rate of hemorrhagic shock in rats compared to larger [38,39] or non-anesthetized animals [40] is not unexpected. Indeed, not only anesthesia alone is likely to affect the normal ability of the circulatory and respiratory systems to respond to an acute reduction in volemia [31,41], but urethane, by itself, significantly blunts the normal cardio-vascular regulation [42]. Nevertheless, the fatal outcome we observed in our study appears to be
Figure 5 Absorbance of the plasma and H$_2$S concentrations. Panel A, absorbance spectra of the plasma collected at the end of the overall bleeding period in control (open circles) and vitamin B12-treated (closed circles) rats. There was a clear peak of absorbance at 525 nm in vitamin B12-treated rats corresponding to a vitamin B12 plasma concentration of 185 ± 216 μM/l. No peak was observed in any of the control rats. At 670 nm, that is, the absorbance wavelength of the methylene blue, no peak was observed, neither in control nor in the vitamin B12-treated rats. Panel B, absorbance spectra, between 400 and 700 nm, of the plasma of the control rats, before (open triangles) and at the end of the bleeding period (closed squares). The observed absorbance values at 670 nm would theoretically correspond to a H$_2$S concentration of around 4 μM/l in the dilute plasma (or 8 μM/l in the plasma, see text for additional comments), as illustrated using a control solution (phosphate-buffered saline, PBS) containing H$_2$S (4 μM) (open diamonds). The lack of peak of absorbance in the plasma at 670 nm along with the pattern of absorbance over the visible spectrum (continuous decrease of absorbance from 400 nm) strongly suggest that it is the turbidity of the medium which could account for this apparent presence of H$_2$S in the plasma.

Figure 6 Plasma and H$_2$S. Panel A, residual H$_2$S concentration ([H$_2$S]$_r$), two minutes after addition of 100 μM H$_2$S in the plasma of control (open circles) and vitamin B12-treated (closed circles) rats. [H$_2$S]$_i$ is expressed in percentage of the concentration of 100 μM present in a control PBS solution analyzed at the same time as the plasma. Within two minutes, H$_2$S concentrations decreased by more than 90% in control and vitamin B12-treated rats respectively, with a significant difference between the two groups (*P < 0.05). Panel B, absorbance spectra of H$_2$S added to sham rat whole blood at 50, 100 and 150 μM and measured after two minutes. In the inset, the percentage of residual [H$_2$S] ([H$_2$S]$_r$), corresponding to the absorbance at 670 nm, is shown for each initial concentration. Depending of the initial [H$_2$S] ([H$_2$S]$_i$), exogenous H$_2$S concentrations decreased between 92 and 75%.
both quantitatively and qualitatively similar to that observed in larger mammals [1,3,5]. A number of characteristics of our model should, however, be discussed. First, O2 deficit per kilogram was much larger for a given volume of blood withdrawn than in larger animals, such as pigs [1] or dogs [4,5]. This larger reduction in VO2 during and following the hemorrhage was associated to a higher baseline specific (per kilogram) metabolic rate, typical of small mammals, akin to hypoxia induced metabolic depression [43-46]. More specifically, resting VO2 in our rat model averaged 15 ml/kg (about four times the expected VO2 level in humans), as previously reported [45,47], with a O2 deficit reaching 300 ml/kg over a one-hour period, up to three times the deficit reported in sheep or dogs during bleeding protocols leading to similar survival rates [2]. This large VO2 deficit can be accounted for by the magnitude of blood flow redistribution in small vs large animals [42,48], a reduction in uncoupling protein activity, specific to small-sized mammals [49] and, eventually, a genuine reduction in oxidative mitochondrial activity resulting in lactic acidemia. Although extremely variable between animals, an elevation in blood level of TNF-alpha was found in all rats at the end of the bleeding period.

It is interesting to note that just like in hypoxia-induced hypometabolism [43] or when unloading venous return [50], ventilation decreased with VO2 and VCO2 but with a relative hyperventilation (Figure 2); this discrepancy between the drop in gas exchange rate and VO2 resulted in all animals in a progressive reduction in PaCO2 and increase in PaO2 (Table 1). Finally, the relative higher values of VCO2 than VO2 at the end of the bleeding protocol are likely to be accounted for by the equimolar transformation of the bicarbonate into CO2 (buffering of the developing metabolic acidosis). This could have resulted in a relative increase in CO2 output, akin to the rise in the respiratory quotient ratio typical of heavy exercise with hyperlactacidemia [51].

**Hemorrhagic shock and H2S**

H2S has been shown to be present in the plasma at concentrations between 25 and 50 μM and to increase up to 100 μM in humans during various types of shock [18]. Plasma H2S concentration in these patients [18] correlates with the severity of the shock. The very presence of H2S in the plasma has already been challenged on methodological and physiological grounds [20,28]. Since the proteins present in the blood (hemoglobin)
complex and/or catalyze very large amounts of sulfide [20], trivial levels of H2S, if any, are expected to be found in the plasma in baseline conditions, as shown by Furne et al. [28] and Whitfield et al. [20]. Whitfield et al. reported no measurable level of H2S [20] after addition of 10 μM of H2S in rat blood before applying a method similar to that used in the present study. Besides, the levels reported both in baseline conditions and in shock [11,16,18] appear to be higher than those expected to be found during severe H2S intoxication [52], wherein mitochondrial activity is inhibited. The method used to determine H2S in previous studies [11,16,18] was developed by Siegel et al. [36] and relies on the transformation of one molecule of H2S and two molecules of N,N-dimethyl-p-phenylenediamine into one molecule of methylene blue (MB). H2S concentration can then be determined by measuring the absorbance of the solution at 670 nm (methylene blue). One of the limits of this method is directly related to the fact that the absorbance is proportional to the concentration of a given molecule - which color is the complementary of the light wave absorbed - if and only if none of the incident light is scattered by dispersed particles or molecules. The presence of a minimal level of turbidity can alter the absorbance of light at any wavelength irrespective of the actual “color” of the plasma, unless a genuine peak of absorbance can be found between 600 and 700 nm (Figure 5). We found that, even after application of TCA to remove the proteins and multiple centrifugations, a significant absorbance can be found at 670 nm. Using a broader spectrum of wavelengths, one can show that the Beer-Lambert law cannot be applied to identify small concentrations of H2S in plasma. This is also illustrated in Figure 5 where the spectrum of absorbance of the plasma after reaction with the reagents to form MB is very different from that of a solution containing H2S at the hypothetical concentration corresponding to a similar absorbance. These data suggest that if the method developed by Siegel is to be used in the plasma, determination of H2S concentrations based on the absorbance of light at only 670 nm can be misleading, yielding erroneous findings of H2S. This issue has already been highlighted by Hughes et al., who reported that the linear dependence of absorbance on the MB concentration was only valid for concentrations of H2S much lower than those reported in all these studies; these authors also recommended the use of the spectra of absorbance between 550 and 700 nm [53]. Using a different method, based on the monobromobimine derivatization [54], Tokuda et al. reported H2S plasma concentrations ranging at best between 2 to 4 μM, consistent with the present results, and more importantly that H2S levels decreased, if anything, in endotoxic shock in mice [55]. Volatilization of H2S observed by De Leon et al. [56] when samples are left in open air is unlikely to have occurred (see Method and Result sections); since we took the precaution to entirely fill all the vials and to cap them immediately, to prevent any significant volatilization. The procedure used for plasma H2S measurements (including centrifugation) was applied as well to PBS solutions containing a known amount on H2S (concentration 100 μM), and the level of H2S was not affected. In addition, we previously found that using this procedure, H2S concentration (in PBS or saline solution) remained stable with a few percent drop in concentration over one hour [30]. DeLeon et al. reported similar results when they took the precaution to close their chambers [56]. For the determination of the ability of the plasma and tissues to oxidize exogenous H2S, PBS solutions containing the same initial amount of H2S were analyzed at the very same time as the plasma or supernatant; H2S concentrations were unchanged in PBS within the two-minute period we chose for our determination and all results have been expressed in percentage of the concentration of 100 μM present in the PBS solution analyzed at the same time and following the same procedure.

Endogenous H2S in the tissues, vitamin B12 and hemorrhagic shock

There is a spontaneous oxidation/complexation of H2S in the plasma and the supernatant of tissues, which was enhanced by μM concentrations of vitamin B12. The latter was obtained following intraperitoneal injection of vitamin B12 at a dose used during cyanide intoxication (105 times the normal daily intake), as previously reported [46,57,58]. The kidney and heart were chosen as they are among the most important organs exposed to the consequences of hemorrhage-induced ischemia. In addition, our previous study [30] showed that the ability of tissue homogenates of these organs to oxidize H2S was clearly enhanced in vitamin B12-treated rats. In that previous study, we found that vitamin B12 could oxidize large amounts of H2S in direct relation to its concentration, likely due to the presence of oxidized cobalt [30]. We could establish that the presence of 10 μM vitamin B12 was able to oxidize 20% of a 100 μM solution of H2S; at 50 μM, about 80% of the H2S was oxidized within five minutes. This is consistent with our present results, where vitamin B12 concentrations in the plasma were found to be about 180 μM and could decrease the exogenous H2S levels to 7.7 ± 0.8 μM (significantly lower than control plasma; initial concentration 100 μM), after two minutes. Similarly, about 50 μM of hydroxocobalamin were found in our kidney homogenates, which in turn decreased H2S concentrations by 65% (vs only 50% in control; initial concentration 100 μM). For the heart and, very likely, for some other tissues, the spectrophotometric method of detection of
vitamin B12 was not sufficient to demonstrate the presence of vitamin B12 (the threshold is about 30 μM [30]). Incidentally, pM - and not μM - concentrations of hydroxocobalamin are expected to be present in the body [59,60]; therefore, with the methodology used in the present study we were unable to demonstrate whether low μM concentrations of vitamin B12 were able to oxidize H2S at the concentrations likely to be present in the heart [28]. This is a very important point to consider as our present results did not show that vitamin B12 was absent from the heart or could not oxidize sulfide, but within the very poor resolution of our method, no reliable conclusion could be drawn.

Hypoxic conditions have been proposed to decrease H2S oxidation resulting in the accumulation of H2S [23]. This increase in H2S concentrations may, however, occur only if PO2 decreases to extremely low levels [24], similar to those expected to be found in the vicinity of the mitochondria, suggesting to Olson [23,61] that the site of action for endogenous H2S can only be the mitochondria. Studies trying to establish the actual amount of H2S present and endogenously produced revealed that at best nM changes in H2S concentrations can be observed [46]. We speculate that low levels of vitamin B12 could still be able to decrease such concentration of H2S in our study [30].

The present study did not address the effect of reperfusion, wherein production of cytokines and oxidative stress are prominent. Patients or animal models who showed an increase in plasma H2S concentrations [16,18] were resuscitated, and the question of the putative role of endogenous H2S will need to be tested during the critical period of reperfusion.

Conclusions
There is no evidence that H2S can accumulate in the high micromolar range in the blood and tissues (extravascular compartment) during a lethal form of hemorrhagic shock. The presence of cobalt (hydroxocobalamin) did not affect any of the outcomes of the shock. These results imply that H2S in the blood cannot be used as a marker of hemorrhagic shock. The hypothesis that H2S could accumulate during hemorrhagic induced tissue hypoxia hypoxia must be reconciled with the ability of tissues to oxidize H2S.

Key messages
- Even during a severe form of hemorrhagic shock in the rat, where a major O2 deficit is present, there is no evidence for an increase in H2S concentration in the blood.
- Injection of high doses of hydroxocobalamin, although enhancing the ability of blood and kidneys to oxidize exogenous H2S in vitro, does not improve the survival, O2 deficit, lactacidemia or TNF-alpha levels in this model of shock.

Abbreviations
ABP: arterial blood pressure; BTPS: body temperature and pressure saturated; f: breathing frequency; IP: intraperitoneal; MB: methylene blue; MOF: multiple organ failure; NS: non-significant; PBS: phosphate buffer saline; STPD: standard temperature and pressure: dry; TCA: trichloroacetic acid; V: inspiratory flow; Vt: minute ventilation; VCO2: carbon dioxide production; VO2: oxygen uptake; VT: tidal volume.

Authors’ contributions
AV and PH conceived of the study, performed the animal experiments, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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