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## HIGH DOSE HYDROXOCOBALAMIN ADMINISTERED AFTER H<sub>2</sub>S EXPOSURE COUNTERACTS SULFIDE POISONING INDUCED CARDIAC DEPRESSION IN SHEEP

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### Abstract

**Context**—Severe H<sub>2</sub>S poisoning leads to death by rapid respiratory and cardiac arrest, the latter can occur within seconds or minutes in severe forms of intoxication.

**Objectives**—Determine the time course and the nature of H<sub>2</sub>S induced cardiac arrest and the effects of high dose Hydroxocobalamin administered after the end of sulfide exposure.

**Materials and methods**—In 16 sedated mechanically ventilated sheep, NaHS was infused to reach concentrations of H<sub>2</sub>S in the blood we previously found to lead to cardiac arrest within minutes following the cessation of H<sub>2</sub>S exposure. High dose Hydroxocobalamin (5 g) or saline solution was administered intravenously, one minute after the cessation of NaHS infusion.

**Results**—All animals were still alive at the cessation of H<sub>2</sub>S exposure. Three animals (18%) presented a cardiac arrest within 90 seconds and were unable to receive any antidote or vehicle. In the animals that survived long enough to receive either Hydroxocobalamin or saline, 71% (5/7) died in the control group by cardiac arrest within 10 minutes. In all instances, cardiac arrest was the result of a pulseless electrical activity (PEA). In the group that received the antidote, intravenous injection of 5 g Hydroxocobalamin provoked an abrupt increase in blood pressure and blood flow; PEA was prevented in all instances. However, we could not find any evidence for a recovery in oxidative metabolism in the group receiving Hydroxocobalamin, as blood lactate remained elevated and even continued to rise after one hour, despite restored hemodynamics. This, along with an unaltered recovery of H<sub>2</sub>S kinetics, suggests that Hydroxocobalamin did not act through a mechanism of H<sub>2</sub>S trapping.

**Conclusion**—In this sheep model, there was a high risk for cardiac arrest, by PEA, persisting up to 10 minutes after H<sub>2</sub>S exposure. Very high dose of Hydroxocobalamin (5 g), injected very early after the cessation of H<sub>2</sub>S exposure, improved cardiac contractility and prevented PEA.

### INTRODUCTION

The treatment of Hydrogen sulfide (H<sub>2</sub>S) intoxication must resolve a difficult challenge: as already mentioned in the early 20<sup>th</sup> century by Haggard (1), H<sub>2</sub>S produces its deleterious effects, e.g. coma, apnea, cardiac arrest - within minutes or sometimes seconds (2, 3), while it vanishes, at least in its soluble/diffusible forms, very rapidly as soon as the exposure

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ceases (4, 5). More specifically, we found that H<sub>2</sub>S is present in essentially two pools during sulfide exposure: one pool of free/exchangeable sulfide and one larger compartment of sulfide likely combined with proteins or metallo-proteins. When H<sub>2</sub>S exposure ceases, the free/exchangeable form of sulfide disappears within one minute from the blood and thus from the tissues (1, 5–7). Combined H<sub>2</sub>S also decreases rapidly in the blood to a few microM, regardless of the initial H<sub>2</sub>S level, but remains present at very low concentrations for at least one hour (4, 5). The potential benefits of any antidote in patients severely intoxicated is thus limited by the very rapid disappearance of soluble H<sub>2</sub>S following hydrogen sulfide poisoning and the low and slow accessibility of the pools of combined intracellular H<sub>2</sub>S. Just like ferric compounds, Cobalt compounds such as Hydroxocobalamin, when used at a very high dose, do combine free H<sub>2</sub>S and catalyze its oxidation *during* H<sub>2</sub>S exposure (4, 8–10). Five grams of Hydroxocobalamin, corresponding to 10<sup>6</sup> times the recommended daily intake, can drop the concentration of soluble H<sub>2</sub>S to almost zero during infusion of moderate to severe levels of H<sub>2</sub>S infusion (4). This observation supports the view that Hydroxocobalamin or other Vitamin B12 analogues (11), could exert a protective effect against H<sub>2</sub>S toxicity since not only can they diffuse within cells (12) via non-transcobalamin mechanisms (13–15), but they also oppose the effects of Nitric oxide (16–19), recently shown to potentiate the effects of H<sub>2</sub>S (20, 21). Finally, intravenous infusion of large dose of Hydroxocobalamin is already FDA approved for the treatment of cyanide poisoning (22, 23). There is, however, no evidence that hydroxocobalamin can oppose any of the persisting toxic effects of sulfide related to its combination on cysteine residues or metallo-proteins *after* an exposure (24) or could enter cells rapidly enough to interact *in vivo* with the intra-cellular pool of combined sulfide. More specifically, since one of the immediate dangers of H<sub>2</sub>S toxicity relates to the production of a rapid shock, it remains to be shown that high dose Hydroxocobalamin can counteract the consequences of H<sub>2</sub>S on circulation. This study intends to determine 1- the natural history of cardiac arrest following H<sub>2</sub>S exposure in a large mammal, 2- whether hydroxocobalamin can affect the immediate outcome of sulfide poisoning, if administered just after H<sub>2</sub>S exposure, at a time wherein most soluble sulfide has already vanished. To answer these questions, we have developed a model of H<sub>2</sub>S exposure in the sheep, which has a high mortality *after* the cessation of sulfide exposure, using repetitions of short bouts of high levels of NaHS, but allowing enough time after complete cessation of H<sub>2</sub>S exposure to administer an antidote.

## METHODS

### Animal preparation

Sixteen sedated, adult Dorset sheep (*Ovis aries*, ewes, 1–2 years old) weighing  $54 \pm 13$  kg were used in these experiments. 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. The study was approved by the Pennsylvania State University College of Medicine Institutional Care and Use Committee.

The animals were pre-medicated with Ketamine (20 mg/kg) intramuscularly, then anesthesia was induced by an intravenous loading dose of Sodium Thiopental (Pentothal 10 mg/kg).

Anesthesia was subsequently maintained by an intravenous solution containing urethane (80 mg/kg) and alphachloralose (15 mg/kg), as previously described (4).

The animals were tracheostomized and an inflatable-cuff tracheal cannula (Shilley #7) was inserted through the tracheostomy. Systemic arterial blood pressure and heart rate were monitored by placing a catheter in one of the carotid arteries, which was also used for sampling arterial blood. Venous catheters (Plastimed 3F) were placed in one jugular and one femoral vein for injection of antidotes and NaHS infusion, respectively. One of the femoral arteries was isolated and a transonic flow probe (Transonic Systems Inc. Ithaca, NY) was placed around the artery for blood flow measurement. Animals were mechanically ventilated throughout the experiments to prevent H<sub>2</sub>S induced apnea (4, 5).

## Measurements

Respiratory flow, along with the pulmonary gas exchange rate, was recorded using a Hans-Rudolph pneumotachograph (Hans-Rudolph, Kansas City, MO) connected to the tracheostomy tube and to a differential pressure transducer. The animals were all ventilated in volume control mode (servo Siemens 900C) with a mandatory ventilation of 8 l/min. FIO<sub>2</sub> was maintained at 21% throughout the protocol.

The expiratory valve of the ventilator was connected to a mixing chamber where the mixed expired O<sub>2</sub> and CO<sub>2</sub> fractions were measured continuously using O<sub>2</sub> (Oxystar-100, CWE) and CO<sub>2</sub> (model 17630, VacuMed, Ventura, CA) analyzers. H<sub>2</sub>S expired fractions were also measured continuously from the same mixing chamber (Interscan RM series, Simi Valley, CA; 1–200 ppm).

The gas analyzers were calibrated immediately before use with different gas mixtures containing a known concentration of CO<sub>2</sub> and O<sub>2</sub> in air and H<sub>2</sub>S in N<sub>2</sub>. Arterial blood pressure and respiratory flow signals were digitized at 200 Hz (Power Lab system). The respiratory flow signal was integrated for breath-by-breath calculation of tidal volume and minute ventilation and oxygen consumption. At the end of the experiment, animals were euthanized by lethal injection of barbiturate (Pentothal 200 mg/kg) in the jugular catheter.

The arterial catheter was connected to a pressure transducer (TA-100, CWE Inc., Ardmore, PA), while the transonic flow probe was connected to a perivascular flowmeter (TS420, Transonic Systems Inc., Ithaca, NY). Electrocardiogram was recorded by using a bioelectric amplifier (Veterinary PC-ECG System, VacuMed, Ventura, CA).

## Determination of dissolved concentrations of H<sub>2</sub>S in the arterial blood

Mixed expired H<sub>2</sub>S (F<sub>E</sub>H<sub>2</sub>S) was determined as previously described (5). The partial pressure of expired H<sub>2</sub>S (P<sub>E</sub>H<sub>2</sub>S) was calculated as  $F_E H_2S \times (P_B - P_{H_2O})$ , allowing the determination of the alveolar partial pressure of H<sub>2</sub>S (P<sub>A</sub>H<sub>2</sub>S) as  $P_A H_2S = P_E H_2S \times (V_E / V_A)$ . V<sub>E</sub>/V<sub>A</sub> was determined from the P<sub>E</sub>CO<sub>2</sub>/P<sub>A</sub>CO<sub>2</sub> ratio assuming that P<sub>A</sub>CO<sub>2</sub> equates PETCO<sub>2</sub>. PaH<sub>2</sub>S was assimilated to P<sub>A</sub>H<sub>2</sub>S and the concentration of gaseous H<sub>2</sub>S in the blood was calculated as:  $C_g H_2S = (0.00012 \times P_a H_2S)$  (see (5) for details and references). Following the assumption that H<sub>2</sub>S is under the form of H<sub>2</sub>S gas (30%) and HS<sup>-</sup> (70%) at a pH of 7 (25, 26).

The time constant of the response of the circuit, included the sulfide analyzer was determined using a 2 liter balloon which was connected to the ventilator (minute ventilation of 10 l/min), with a constant flow of a mixture containing 200 ppm H<sub>2</sub>S at 1 l/min). F<sub>E</sub>H<sub>2</sub>S was monitored from the expiratory chamber connected to the expiratory circuit. When F<sub>E</sub>H<sub>2</sub>S reached a steady state, the flow of H<sub>2</sub>S was stopped and the time constant of the circuit, including gas analyzer and dilution of the gas in the balloon was computed as the time needed to drop H<sub>2</sub>S concentration by 64 % and was found to average 140 seconds.

## Protocol

**NaHS infusion and recovery**—We needed a model producing an oxygen deficit and increased lactic acidosis, without killing the animal before or immediately after the end of exposure, therefore allowing enough time to inject the antidote. To achieve this goal, we established, based on a series of pilot experiments, a protocol consisting in repetitive bouts of “supra-lethal” iv infusion.

Every animal received NaHS, until the level of expired H<sub>2</sub>S reached 110 ppm or greater, a level, which we previously found lead to a lethal outcome minutes after the end of infusion. This level of expired H<sub>2</sub>S was achieved by exposing the animals to bouts of NaHS of 40 seconds at a rate of 4 ml/min NaHS (20 mg/ml in saline) separated by 30 seconds with no infusion. As presented in the results section, 3–4 bouts were necessary to reach a level of expired H<sub>2</sub>S of 110 ppm. This protocol was compatible with immediate survival while it led to the development of an O<sub>2</sub> deficit and lactic acid accumulation with a very high mortality by cardiac arrest occurring within less than 10 minutes *after* the end of infusion (See result section). NaHS solutions were prepared a few minutes before the infusion and kept in airtight syringes. The solution was infused intravenously in the femoral vein using an infusion pump (Fusion 100; Chemyx Inc; Stafford, TX).

**Hydroxocobalamin infusion**—One minute after the end of NaHS infusion, a solution of Hydroxocobalamin (Cyanokit 5 g in 200 ml saline, Merck Santé, licensed by Meridian Medical Technologies, Inc.) or 200 ml saline were infused over 3 min in the catheter placed in the jugular vein. Arterial blood was sampled before the antidote infusion and then at 5, 10 and 15, 30 and 60 minutes into recovery for determination of Hydroxocobalamin concentrations, blood gases and blood lactate. For Hydroxocobalamin measurement, blood was centrifuged and 1 ml of supernatant was mixed with 500 µl of PBS. The concentration in Hydroxocobalamin present in the blood was estimated by measuring the plasma absorbance at 525 nm as previously described (27).

## Data analysis

Breathing frequency (f) and tidal volume (V<sub>T</sub>) were determined using peak detection and integration of the respiratory flow signal, respectively, and minute ventilation (V<sub>E</sub>) was computed as f×V<sub>T</sub>. Oxygen consumption (VO<sub>2</sub>) was computed using V<sub>E</sub>, the inspiratory and mixed expiratory fraction of O<sub>2</sub>.

All signals were digitized at 200 Hz using an analog-to-digital data acquisition system (Power Lab 16/35, AD Instruments, Colorado Springs, CO). Data were stored for later

analysis. Mean arterial blood pressure, femoral blood flow, minute ventilation, expired fraction of H<sub>2</sub>S, the electrocardiogram (ECG) and VO<sub>2</sub> were displayed online for monitoring.

Arterial blood gases partial pressure of O<sub>2</sub> (PaO<sub>2</sub>), CO<sub>2</sub> (PaCO<sub>2</sub>), and lactate concentration were measured using i-STAT1 blood gas analyzer (ABAXIS, Union city, CA).

All data are expressed as mean ± SD. The relationship between CgH<sub>2</sub>S and the time to death was established for every animal. The number of deaths was compared between the 2 groups using a Fisher's exact test, while the survival rate as a function of time (post infusion) was analysed using a log-rank (Mantel Cox) test and presented with Kaplan Meier curve. P<0.05 was considered as significant. Second, CgH<sub>2</sub>S, VO<sub>2</sub> and blood pressure were compared within each group, before NaHS infusion then at 1, 2, 3, 5, 10, 15 and 20 minutes after the end of NaHS infusion or up to the moment where the first animal died, using repeated ANOVA, followed by a post-hoc multiple comparison procedure (Turkey's multiple comparison). A two-way ANOVA was also used to compare animals between the saline and the Hydroxocobalamin groups, but only over the shortest period of survival (see result section for further details).

Blood gases and lactate were compared between and within groups at 5, 10, 15, 30 and 60 minutes using a two-way ANOVA followed by a Turkey's multiple comparison.

## RESULTS

### Effects of NaHS infusion in control group (n=8)

Repetition of 3–4 bouts of NaHS led to a level of expired H<sub>2</sub>S above 110 ppm in 7 out of 8 control animals. Indeed, in one animal the peak level of mixed expired H<sub>2</sub>S reached only 61 ppm despite a number of bouts similar to the other sheep. This animal survived the intoxication. In the 7 remaining animals, peak H<sub>2</sub>S ranged from 122 to 176 ppm (corresponding to a CgH<sub>2</sub>S between 13 and 22 microM). One of these 7 animals was unable to receive the saline solution in keeping with the protocol, since this sheep survived only 90 seconds after the end of NaHS infusion. This animal was therefore excluded from the comparisons. All of the 6 other animals, along with the animal that reached low levels of expired H<sub>2</sub>S, received a saline injection. All animals except two (including the animal with the low level of H<sub>2</sub>S) died by pulseless electrical activity (PEA), as illustrated in figure 1. All of the animals that did not survive displayed a complete disappearance of blood pressure or flow fluctuations between 140 and 613 seconds, while ECG signal was clearly sustained well beyond the period of cardiac arrest (figure 1). Repeated ANOVA was thus performed to analyze data up to 2 minutes following the end of NaHS exposure. Clearly a rapid decrease in blood pressure occurred by the end of NaHS infusion and continued to develop after the cessation of sulfide exposure (drop from 112 ± 8 to 70 ± 32 Torr, 60 seconds after the end of NaHS infusion, p<0.01) along with a severe reduction in femoral blood flow. This was associated to a rapid decrease in VO<sub>2</sub> from 203 ± 51 to 148 ± 136 ml/min after one minute (figures 1, 2 and 3) along with a significant rise in blood lactate levels (figure 4). Of note is that in the sheep in which mixed expired H<sub>2</sub>S reached 61 ppm, no reduction in VO<sub>2</sub>, blood pressure or increase in lactate levels was observed. In summary, out of the 8 control animals,

7 survived long enough to receive the saline solution. One of these 7 animals did not reach the levels of H<sub>2</sub>S intended (above 110 ppm) and survived, in the 6 remaining animals, 5 died.

### Hydroxocobalamin infusion (n=8)

Following the same protocol as in the control group, two out of the 8 animals presented a PEA 36 and 90 seconds into recovery and could not receive any injection of Hydroxocobalamin, they were therefore not used for comparison and were excluded from the data analysis. One animal reached only 52 ppm (C<sub>g</sub>H<sub>2</sub>S = 6 microM) and, just like in the saline group, did not display any drop in blood pressure and VO<sub>2</sub>, or increase in lactate. In all of the remaining animals, peak levels of H<sub>2</sub>S reached 110 to 180 ppm at the end of exposure, corresponding to C<sub>g</sub>H<sub>2</sub>S ranging from 13 to 22 microM. All of these animals presented a rapid reduction in blood pressure ( $120 \pm 8$  to  $94 \pm 31$  Torr) and VO<sub>2</sub> ( $222 \pm 69$  to  $167 \pm 66$  ml/min) one minute into recovery (figures 2 and 3). There was no difference between this group of animals and the control/saline group in terms of the severity of the circulatory failure, until the infusion of Hydroxocobalamin was performed (figure 2). The 6 animals that received Hydroxocobalamin presented a very rapid and significant increase in blood pressure to  $199 \pm 48$  Torr, 120 seconds after the onset of Hydroxocobalamin injection. Blood pressure and blood flow after this initial overshoot returned towards baseline values within 5 minutes (figure 3). In major contrast to the control group, all of the animals survived (figure 5). Survival was thus significantly different from the control group (Fisher tests and Log Rank test). Comparison with control animals could be performed using repeated ANOVA in all animals up to 120 seconds and is shown in figure 4. Note that since VO<sub>2</sub> and circulatory data were recorded up to 20 minutes in all animals, data have been averaged over this entire period in the saline group but data obtained after the first animal died (140 seconds) are shown without standard deviation for visual comparison only.

Although VO<sub>2</sub> and blood pressure returned to their baseline values (figure 3), lactic acid, which increased up to 5.38 mmol/l one minute into recovery, did not decrease over the entire hour following H<sub>2</sub>S poisoning as shown on figure 5. This was associated with a progressive increase in base deficit (Figure 4). No hypoxemia was observed over the hour following the cessation of infusion (Figure 4). The concentration of Hydroxocobalamin sampled 2 minutes after the onset of infusion was found to reach levels above 1000 microM before declining progressively. Blood Hydroxocobalamin concentrations remained above 400 microM for at least one hour (figure 6).

## DISCUSSION

We found that in a large animal model, wherein H<sub>2</sub>S is rapidly risen to levels able to produce a cardiac arrest, 3 out of 16 adult sheep died within 90 seconds following the end of sulfide exposure and were not able to receive any antidotes. Among the 13 animals that received the antidote or a saline infusion, 71% of the animals died in the saline group by PEA, while all animals survived following Hydroxocobalamin administration, when administered between one and four minutes after the end of H<sub>2</sub>S exposure.

## H<sub>2</sub>S intoxication

H<sub>2</sub>S exposure remains an occupational hazard in gas production, well drilling and gas refining industries (28, 29). H<sub>2</sub>S poisoning has also become a dreadful method of suicide over the last few years (2, 30–32). The incidence of this form of suicide has increased due to a phenomenon of imitation or “copy-cat” following reports in the media (33) and is committed by mixing in a closed environment (usually in a car or in a small room) a source of sulfur and various types of acidic solutions, readily available in most household chemicals. This method of suicide has also created new risks for neighbors and first responders (34, 35). Finally, since it is very easy to manufacture, H<sub>2</sub>S is also regarded as a possible threat by the Department of Homeland Security (36), and can create a large-scale panic due to its distinguishable smell of rotten egg.

At high levels of exposure - typically around 1000 ppm- hydrogen sulfide has been shown to be rapidly fatal by primary cardiorespiratory arrest. At lower levels, which, for obvious reasons, are not well established in humans, patients can present a reversible coma, often referred as “knock-down”. However, some patients can develop severe post-anoxic/toxic injuries (37). These neurological after-effects, which combine cognitive as well as motor or sensory deficits, result from the combination of the acute inhibition of mitochondrial cytochrome C oxidase in neurons (38, 39) and the depressive effects of sulfide on the cardio-vascular system (40). The latter appears to potentiate the mitochondrial insult produced by H<sub>2</sub>S on neuron, per se.

## H<sub>2</sub>S induced cardiac toxicity

In the present study, when levels of CgH<sub>2</sub>S reached 12 to 16 microM, death occurred between 30 seconds and 10 minutes *following* H<sub>2</sub>S exposure. A reduction in cardiac function produced by sulfide has been proposed to be one of the main factors dictating the outcome of animals and possibly patients surviving H<sub>2</sub>S induced coma (40). Cardiac arrest may be precipitated by a terminal apnea induced anoxia, although typically this form of cardiac arrest takes time to develop, or through an acute episode of ischemia-induced ventricular fibrillation. This study revealed that cardiac arrest results from a typical pulseless electrical activity (PEA) phenomenon, which can still occur *after* the period of exposure. This PEA seems to be the final stage of a very rapid deterioration in cardiac function. Sun et al. and Zhang et al. (41, 42) have established in-vitro that one of the main effects of H<sub>2</sub>S on cardiomyocytes at toxic concentrations is to inhibit L-Type Calcium channels. This inhibition of L-Type Calcium Currents could be accounted for by the interaction of H<sub>2</sub>S with free sulfhydryl group present on the channels. The redox modulation of L-Type Calcium channel activity (43) could also be affected by the presence of sulfide leading to a rapid inhibition of the mechanical contractions of the cardiomyocytes (41, 42). This inhibition has been studied in vitro and is clearly present at 100 microM of soluble H<sub>2</sub>S, a figure higher than what was required in-vivo to produce a toxic effect. Interestingly, this makes the effects of H<sub>2</sub>S intoxication on the heart very similar to those produced by calcium channels blockers poisoning (44). In the 2 animals, where CgH<sub>2</sub>S only peaked at 6–7 microM, no significant decrease in blood pressure or VO<sub>2</sub> was produced. Putting these results in the context of our previous studies (4, 5), concentrations of gaseous H<sub>2</sub>S around 1 microM are associated with a stimulation in breathing, concentrations between 6–8 microM

produces an apnea, and as soon as levels of H<sub>2</sub>S increase above 10 microM, PEA can occur. Such concentrations of gaseous H<sub>2</sub>S correspond to a total level of sulfide 10 to 20 times higher (5).

### Effects of Hydroxocobalamin

We have recently found that the ferric or cobalt compounds (methemoglobin solution or very high dose of hydroxocobalamin) currently proposed as antidotes against H<sub>2</sub>S poisoning are very effective in trapping gaseous/free H<sub>2</sub>S and thus decreasing the amount of H<sub>2</sub>S diffusing into tissue to exert its toxic effects (4). The impressive effects of these antidotes are, however, somehow misleading, as the pool of free H<sub>2</sub>S, i.e. gaseous H<sub>2</sub>S and its sulfhydryric anion HS<sup>-</sup>, is mostly -if not exclusively- present in the blood *during* H<sub>2</sub>S exposure (5). The very high ability of the mitochondria from all tissues to oxidize sulfide (45) explains why this pool vanishes within less than one minute after exposure (1, 4). In conditions associated with low cardiac output (or even cardiac arrest), one can imagine that H<sub>2</sub>S could still be found in the blood, but as soon as circulation is restored, whether by cardio-pulmonary resuscitation or spontaneously, free H<sub>2</sub>S should disappear very rapidly. As illustrated on figure 3, the time constant of the off-kinetics of arterial H<sub>2</sub>S was not different from the time constant of the circuit and the response time of the analyzers (140 seconds, see method section). Since the effects of any antidote on H<sub>2</sub>S poisoning is to be primarily dictated by kinetics of the pools of soluble H<sub>2</sub>S in the blood and tissues *after* H<sub>2</sub>S exposure, i.e. when patients are withdrawn from the source of intoxication and taken care of by paramedic units, one may question the benefit of any treatment based on trapping agents, which includes Hydroxocobalamin (8, 27) and methemoglobinemia (46). In other words, it remains to be explained how Hydroxocobalamin could be effective when no free H<sub>2</sub>S is present. Indeed, following H<sub>2</sub>S exposure, VO<sub>2</sub> rapidly increased after Hydroxocobalamin administration along with the restoration of blood pressure and flow, markers of the improvement of the cardiac function. These rapid changes in VO<sub>2</sub> very likely reflect the increase in cardiac output, rather than an improvement in cellular metabolism since lactate levels remained very high and were no different from control animals. Whether Hydroxocobalamin enters cardiac cells fast enough even via a non-transcobalamin mechanism (13) and can affect the concentrations of H<sub>2</sub>S already combined on proteins, remain unanswered questions.

Alternative mechanisms unrelated to the ability of Hydroxocobalamin to combine sulfide H<sub>2</sub>S can be proposed. For instance, the potent anti-NO effects of Hydroxocobalamin (16), as illustrated by the rapid and powerful increase in blood pressure, both in the present study and in the literature (19), could have counteracted some of the toxicity of H<sub>2</sub>S, which have been shown to be enhanced in the presence of NO (21). The latter can significantly contribute to the reduction in cardiac contractility in various pathological states from septic shock to chronic heart failure (47–50). NO and H<sub>2</sub>S may thus in concert affect cardiac contractility which could be antagonized by Hydroxocobalamin. Such a mechanism does not require the presence of high level of Hydroxocobalamin in the cells and does not necessarily require the persistence of free H<sub>2</sub>S. Whether Hydroxocobalamin could counteract the effects of sulfide on Calcium channels (41, 42) is an outstanding question. Finally, in this study, we



used a dose of Hydroxocobalamin of about 100 mg/kg, it remains to be determined if lower levels of Hydroxocobalamin can be as protective.

### Limitations of the model used

Creating a model of H<sub>2</sub>S poisoning faithful to a relevant clinical scenario is always challenging. Using a ventilated sedated animal may certainly affect the cardiovascular, metabolic and neuronal responses to the anoxic insult produced by sulfide differently from an un-anesthetized human. This point is, however, to be considered in the light of the use of urethane-chloralose in our model, as this “old” modality of sedation maintains intact a large part of the medullary reflexes (51–54). The initial injections of ketamine and barbiturate may still affect the response to sulfide, though equally in both groups; the doses we used were relatively small and only administered during the initial surgical period. In our view, the major limitation of this study relies on the “artificial” scenario created by our experimental setting in keeping with the specificity of sulfide poisoning. Indeed, a small difference in H<sub>2</sub>S concentrations can allow survival or can produce death within seconds or minutes. The steepness of the dose-toxicity relationship for H<sub>2</sub>S along with the fast disappearance of free/soluble forms of H<sub>2</sub>S from the blood and tissue (within a minute), make the conditions of success of any treatment of sulfide poisoning very difficult to evaluate. To substantiate any potential effect of a Hydroxocobalamin, we had to create specific conditions allowing most animals to survive long enough to be able to receive an antidote, while the model would still be lethal. Any antidote found to be effective one to 4 minutes after the end of exposure may therefore prove to be deceiving in real life conditions, due to this very short window of opportunity. Similarly, scenarios wherein an experimental model would still be exposed to toxic levels of H<sub>2</sub>S while receiving an antidote do not necessarily correspond to a realistic situation, since the basis for treating H<sub>2</sub>S poisoning – and reducing its concentration in the blood and tissue- is to allow the very effective sulfide oxidation to take place by preventing new molecules of sulfide to diffuse into blood. The rapid drop of H<sub>2</sub>S shown in figure 3 and previously reported (4, 5) supports the view that the most effective approach for treating sulfide poisoning is still to remove the subjects from any possible new contamination.

In conclusion we found that 1) H<sub>2</sub>S can kill by producing PEA even *after* the cessation of H<sub>2</sub>S exposure, 2) high dose Hydroxocobalamin (5 g) administered intravenously counteracts this effect, if injected between one to 4 minutes after the cessation of exposure, 3) the effect of large dose of Hydroxocobalamin does not seem to rely on the trapping of H<sub>2</sub>S.

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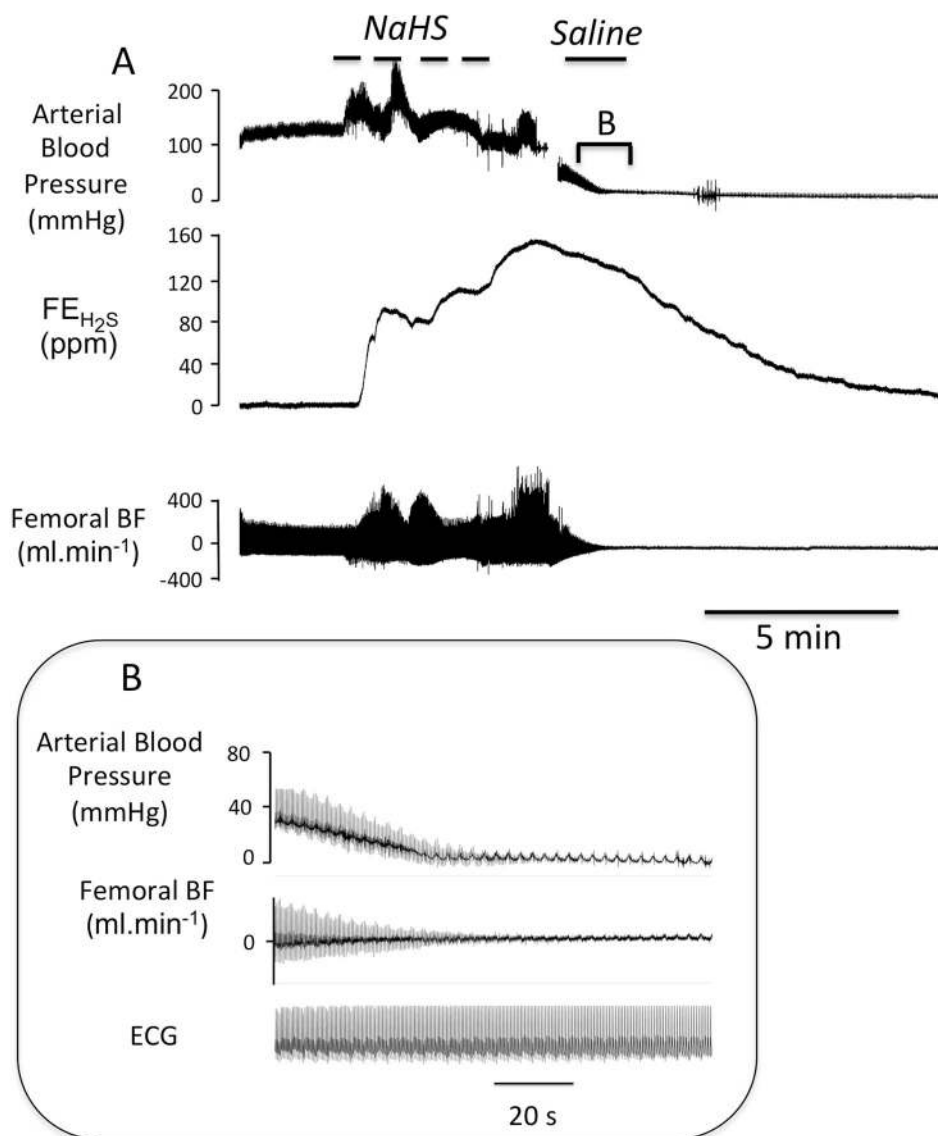
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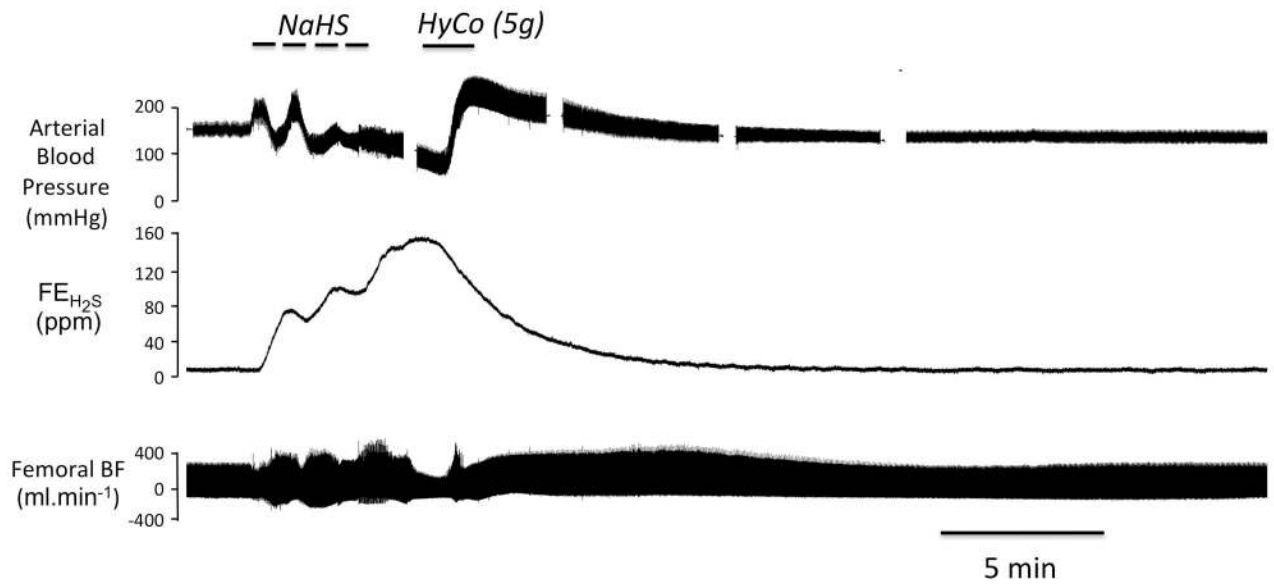
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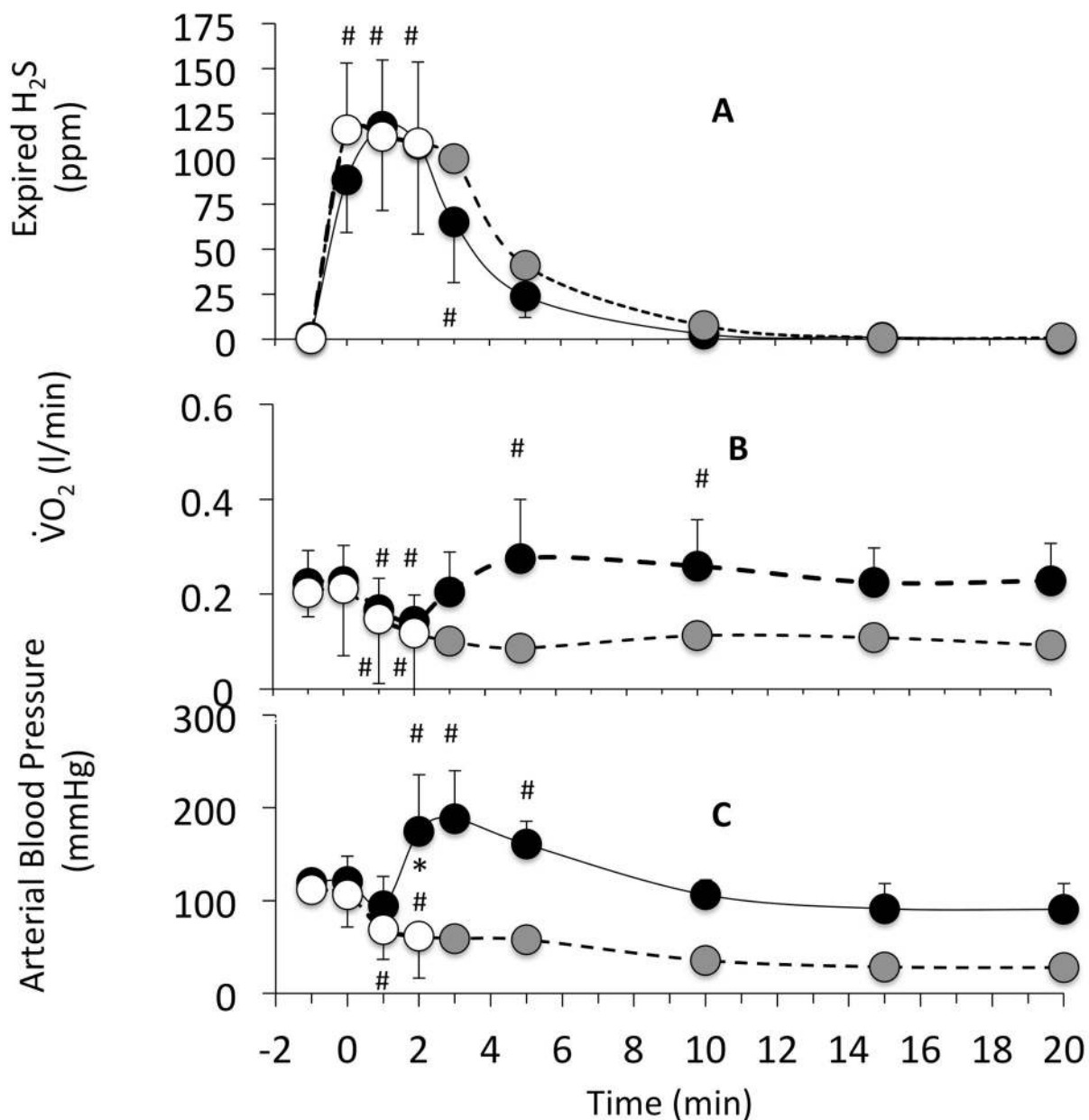
**Figure 1.**

**A** Example of a recording obtained in one sheep showing the effects of intravenous infusion of saline administered one minute after the cessation of sodium sulfide (NaHS) infusion. The changes in arterial blood, mixed expired fraction of H<sub>2</sub>S (FE<sub>H<sub>2</sub>S</sub>) and femoral blood flow are shown. In this example, the level of expired H<sub>2</sub>S reached more than 120 ppm at the end of exposure (see text for corresponding level in the blood). Note that arterial pressure and femoral blood flow decreased very rapidly during the recovery phase from NaHS infusion leading to death by pulseless electrical activity (PEA) within minutes. The inset **B** is the magnification of period of reduction in blood pressure and cardiac output leading to cardiac arrest, showing the persistence of the normal electrical activity with no arterial pulse pressure defining a PEA. All animals that presented a cardiac arrest kept a persistent electrical activity for several more minutes.



**Figure 2.**

Example of a recording obtained in one sheep showing the effects of intravenous infusion of 5 g Hydroxocobalamin (HyCo), administered one minute after the cessation of NaHS infusion. The changes in arterial blood, mixed expired fraction of H<sub>2</sub>S (FEH<sub>2</sub>S) and femoral blood flow are shown. In this example - like in figure 1- the level of expired H<sub>2</sub>S also reached more than 120 ppm by the end of period of exposure. In contrast to the animal receiving saline, blood pressure and femoral blood flow, which initially decreased very rapidly during the recovery phase from NaHS infusion, rose during and after Hydroxocobalamin infusion, restoring a normal circulation and preventing death by pulseless electrical activity for the total duration of the protocol.

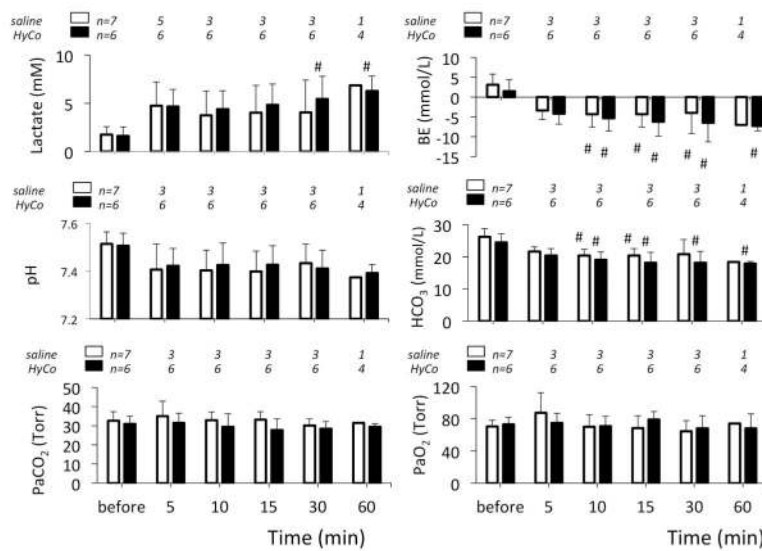


**Figure 3.**

Time course of expired H<sub>2</sub>S concentration (**panel A**),  $\dot{V}O_2$  (**panel B**) and arterial blood pressure (**panel C**), following the cessation of NaHS exposure. Data are shown as mean  $\pm$  SD. The infusion of sulfide was interrupted at time zero. Injections of saline (open circles) or hydroxocobalamin (closed circles) were performed 60 seconds following the end of NaHS infusion. In the hydroxocobalamin group, since all animal survived, averaged data are shown up to 20 minutes and have been compared to baseline data, using a one-way repeated ANOVA. For saline experiments, data points are shown up to 120 seconds as animals started to die thereafter. Averaged data in the saline group are shown using a dotted line after 120 seconds as data were obtained from animals already in cardiac arrest while some were still alive. Data from the saline group were therefore compared to the

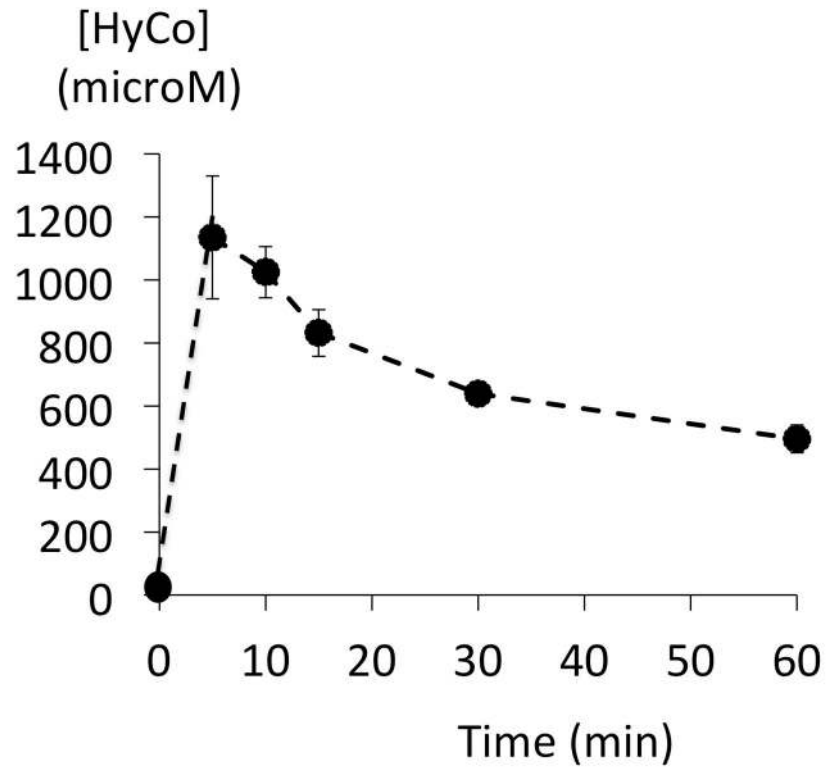
Hydroxocobalamin group (two-way ANOVA) up to 120 seconds. Expired H<sub>2</sub>S decreased very rapidly with no difference between the Hydroxocobalamin or saline groups. In contrast both arterial blood pressure and VO<sub>2</sub> increased significantly after Hydroxocobalamin, while in the saline group all animals, but 2 displayed a fatal drop in blood pressure. Values are shown as mean ± SD. #significantly from baseline, \*significantly different between the 2 groups, p<0.05.





**Figure 4.** Arterial lactate, base excess (BE), pH, bicarbonate (HCO<sub>3</sub><sup>-</sup>) and blood gases, before NaHS infusion then at 5, 10, 15, 30 and 60 minutes following the cessation of NaHS exposure in the animals receiving saline or Hydroxocobalamin. The number of animals studied is shown. # significantly different from baseline values at P<0.05.





**Figure 6.**

Time course of the concentrations of Hydroxocobalamin (HyCo) in the blood following HyCo infusion. Data are shown as mean  $\pm$  SD. Blood was collected in the 6 animals that received HyCo. Concentration in HyCo increased within minutes up to 1000 microM and remained above 400 microM up to an hour after the end of infusion.