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Publication Date

2022-11-01

DOI

10.1016/j.etap.2022.103998

Environmental Toxicology and Pharmacology 96 (2022) 103998

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Treatment of life-threatening H2S intoxication: Lessons from the trapping agent tetranitrocobinamide

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ARTICLE INFO

Keywords:
Hydrogen sulfide intoxication
Antidote

ABSTRACT

We sought to evaluate the efficacy of trapping free hydrogen sulfide (H_2S) following severe H_2S intoxication. Sodium hydrosulfide solution (NaHS, 20 mg/kg) was administered intraperitoneally in 69 freely moving rats. In a first group (protocol 1), 40 rats were randomly assigned to receive saline (n = 20) or the cobalt compound tetranitrocobinamide (TNCbi) (n = 20, 75 mg/kg iv), one minute into coma, when free H_2S was still present in the blood. A second group of 27 rats received TNCbi or saline, following epinephrine, 5 min into coma, when the concentration of free H_2S has drastically decreased in the blood. In protocol 1, TNCbi significantly increased immediate survival (65 vs 20 %, $p < 0.01$) while in protocol 2, administration of TNCbi led to the same outcome as untreated animals. We hypothesize that the decreased efficacy of TNCbi with time likely reflects the rapid spontaneous disappearance of the pool of free H_2S in the blood following H_2S exposure.

1. Introduction

Hydrogen sulfide (H_2S) is a chemical threat in oil and gas refining industries (Arnold et al., 1985; Toxicological, 2003) as well as in farming activities involving manure pits (Chenard et al., 2003). H_2S inhalation has also been used as a "method" of suicide (Reedy et al., 2011; Truscott, 2008; Kamijo et al., 2013; Sams et al., 2013). No current standard of care (Haouzi et al., 2016) exists to treat H_2S-exposed victims with life-threatening symptoms, i.e. presenting with circulatory shock (Sonobe and Haouzi, 2016a; Judenherc-Haouzi et al., 2016), coma, and respiratory depression.

Treating victims of acute H_2S intoxication is challenging (Haouzi et al., 2016). Indeed, H_2S produces symptoms that are rapidly and spontaneously reversible (transient syncopal episodes and decreases in blood pressure) (Beauchamp et al., 1984; Guidotti, 2010) or that can lead to a lethal outcome (Sonobe et al., 2015). Rapid death results from H_2S induced apnea and circulatory failure as well as from primary pulseless electrical activity (Judenherc-Haouzi et al., 2016, 2018; Cheung et al., 2018a; Haouzi et al., 2015; Haouzi and Sonobe, 2015; Sonobe and Haouzi, 2016b). Intervention in life-threatening intoxications must therefore be very rapid, requiring immediate access to techniques of life support (mechanical ventilation and cardio-pulmonary resuscitation) and/or antidotes that can reverse the effects of sulfide within minutes or seconds.

The traditional view is that the main mechanism of H_2S toxicity is from its binding to mitochondrial cytochrome C oxidase (CCO or complex IV) (Cooper and Brown, 2008; Nicholls et al., 2013; Jensen et al., 1984; Ball and Cooper, 1952). This effect prevents in turn CCO re-oxidation by O_2, impeding the activity of the mitochondrial electron transport chain. As it diffuses into the blood during exposure, H_2S is present in different forms. A free/diffusible pool of H_2S, which could be accessible to trapping agents, represents a small proportion total H_2S of

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https://doi.org/10.1016/j.etap.2022.103998
Received 25 November 2021; Received in revised form 5 October 2022; Accepted 7 October 2022
Available online 11 October 2022

in the blood and tissue (Sonobe and Haouzi, 2016a; Haouzi et al., 2014; Klingerman et al., 2013). The majority of H_2S is combined with proteins, including metallo-proteins in forms that are not "accessible" to trapping agents (Klingerman et al., 2013; Haouzi and Klingerman, 2013).

Molecules that decrease the pool of free H_2S/HS^- have been shown to reduce H_2S toxicity (Haouzi et al., 2015; Haouzi and Klingerman, 2013; Chenuel et al., 2015; Van de Louw and Haouzi, 2013; Smith, 1969; Smith and Gosselin, 1976; Haouzi and Van de Louw, 2013; Truong et al., 2007), however, because free H_2S spontaneously disappears from the blood and tissues rapidly after the end of exposure (Haouzi et al., 2014; Klingerman et al., 2013; Haouzi and Klingerman, 2013; Toombs et al., 2010; Haggard, 1921), it has been hypothesized that "trapping" agents must be administered as soon as possible after removing a victim from the source of sulfide. Different types of pharmacological agents belonging to this group of antidotes have been considered: nitrite compounds (Smith and Gosselin, 1976, 1966; Smith, 1981) induce methemoglobinemia to allow the binding of free H_2S in the red cells and prevent its diffusion into tissues. Of note, nitrites have been proposed to have a direct beneficial effect on mitochondrial function independent of the production of methemoglobin (Anantharam et al., 2017). However, the decrease in blood pressure due to nitric oxide generation can represent a limitation in severe forms of H_2S intoxication, which are typically associated with a cardiogenic shock (Sonobe and Haouzi, 2016a; Judenherc-Haouzi et al., 2016). The second family of trapping agents are cobalt compounds, such as hydroxocobalamin, which are also very effective in trapping free H_2S (Haouzi et al., 2015; Van de Louw and Haouzi, 2013; Smith, 1969; Smith and Gosselin, 1976; Truong et al., 2007) without decreasing blood pressure (Haouzi et al., 2015). Tetranitrocobinamide (TNCbi) is a cobalt compound recently proposed as a cyanide (Broderick et al., 2006, 2007; Chan et al., 2010; Bebarta et al., 2014) and H_2S antidote (see (Ng et al., 2019a) for review) that can be administered at a much lower injection volume than hydroxocobalamin (Sharma et al., 2003). It is also water soluble and can be administered intramuscularly (Brenner et al., 2010). This family of cobalt-containing molecules also possess protective properties against ROS production (Truong et al., 2006; Jiang et al., 2016), at least in vitro (Eghbal et al., 2004), which clinical impact remains poorly understood (Cheung et al., 2018a). Finally, new families of trapping agents have been more recently considered (Miyazaki et al., 2021).

In most experiments designed to test the efficacy of hydroxocobalamin or its "derivative", the antidote is typically administered 1- immediately after the end of exposure (Haouzi et al., 2015), 2- when animals are still exposed to some levels of exogenous sulfide (Brenner et al., 2014; Bebarta et al., 2017; Ng et al., 2019b; Hendry-Hofer et al., 2020) or 3- between periods of repetitive sulfide exposures (Anantharam et al., 2017). In the present study, we investigated in awake rats the short and long-term outcomes of the potent sulfide trapping agent TNCbi, when free/diffusible H_2S in the blood is either still present or has largely disappeared at the time of the antidote administration, following an acute and brief exposure to H_2S (via intraperitoneal injection, IP). We sought to determine whether, and with what timing, TNCbi could improve survival in a life-threatening scenario of H_2S intoxication. TNCbi was therefore administered either at 1 or 5 min after the onset of coma, which typically occurred ~2 min after IP injection. The 5-minute delayed injection was performed at a time when no or trivial concentrations of free H_2S were present in the blood. This model produces a very severe form of H_2S intoxication with rapid coma that leads to cardiac arrest within less than 10 min (Judenherc-Haouzi et al., 2016) in about 70 % of the animals (Judenherc-Haouzi et al., 2016; Sonobe et al., 2015). It also allows the study of the long-term neurological effects in the surviving rats (Sonobe et al., 2015). More importantly, this model produces rapid cardiogenic shock (Judenherc-Haouzi et al., 2016; Sonobe et al., 2015), which offers only a very short therapeutic window to administer an antidote, akin to some of the most severe clinical presentations of H_2S intoxication in humans.

2. Material and methods

2.1. Animals

A total of 69 male Sprague-Dawley rats (Charles River) weighing 407 ± 48 g were studied. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition (National Research Council (US) Institute for Laboratory Animal Research). The study was approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Rats were housed in open top cages in a temperature-controlled room (24 ± 1 °C) with 12-hour light/dark cycle and were provided with standard laboratory diet and water ad libitum. Three hours before experiments, rats were anesthetized using 1–2 % isoflurane in 100 % O_2 and a 22 g IV catheter (Jelco, Smiths Medical) was placed into a dorsal tail vein. The catheter was protected by a plastic tube placed around the tail. Three to four hours were given for the animals to recover after placement of the tail catheter.

2.2. H_2S-induced coma

H_2S solutions were prepared from sodium hydrosulfide hydrate (NaHS, Sigma Aldrich, St Louis, MO) in sterile physiological saline (5 mg/ml), which limitations have been previously described (Judenherc-Haouzi et al., 2016; Sonobe et al., 2015). The solution was prepared immediately prior to each experiment and kept in sterile airtight syringes. 20 mg/kg of the NaHS solution was administered IP. This dose typically produces a rapid coma within 2–3 min post injection in about 30 % of the rats (Sonobe et al., 2015). Rats that do not develop coma within 5 min receive a second injection, and some animals require three injections to induce coma. Repeated injections do not affect the outcome (Sonobe et al., 2015). In pilot studies, we examined the kinetics of H_2S in the blood following an IP injection in two rats as presented in Fig. 1. Rats were placed in a custom-designed, leak-proof, acrylic cylinder (internal volume 1.4 liters). Air was delivered through the inlet port of the plethysmographic chamber using a precision rotameter (flow of ~2.5 L/min) (Haouzi et al., 2009). The outlet port was connected through noncompliant tubing and to a Fleisch 01 pneumotachograph connected to a pressure transducer (Sensym, DCLX O1DN; Honeywell, Morristown, NJ) to determine the flow of air through the chamber (Haouzi et al., 2009; Bell and Haouzi, 2009). The fractions of expired H_2S (FEH_2S) Interscan RM series; range: 0–1 ppm, Interscan Corporation, Simi Valley, CA) was determined in the gas leaving the chamber (Haouzi et al., 2008). The time delay along with the on and off-kinetics of the circuit (from the chamber and the analyzer) were determined in a response to an acute step change in FEH_2S in the chamber. A gas with a known fraction of H_2S was delivered into the chamber and then abruptly interrupted as shown in Fig. 1A and B. We found that our equipment displayed a time delay between 10 and 20 s with an off time constant of 60 s. Animals receiving 20 mg/kg H_2S IP showed a rapid increase in expired H_2S with a return to baseline within less than 5 min (Fig. 1C) which after correction for the time constant of the equipment was not different from zero.

Coma was defined by the loss of righting reflex (Judenherc-Haouzi et al., 2016; Sonobe et al., 2015). Breathing pattern was monitored and cardiac pulse was examined by palpation of the chest, while the animal was in coma.

One minute into coma (protocol 1), the intoxicated animals received a solution of TNCbi (0.2 g/ml, 75 mg/kg) or the same volume of saline through the tail vein catheter (Fig. 2). Animals were clinically monitored until they recovered their righting reflex or became pulselessness. In a second group of animals (protocol 2, Fig. 3) we intended to administer TNCbi 5 min into the coma, at a time when all the H_2S in the blood and expired gas had disappeared. Since many animals were already pulseless at 5 min or were moribund, 2 injections of epinephrine (0.04 mg/kg) were performed one minute into coma. A total of 27 animals were

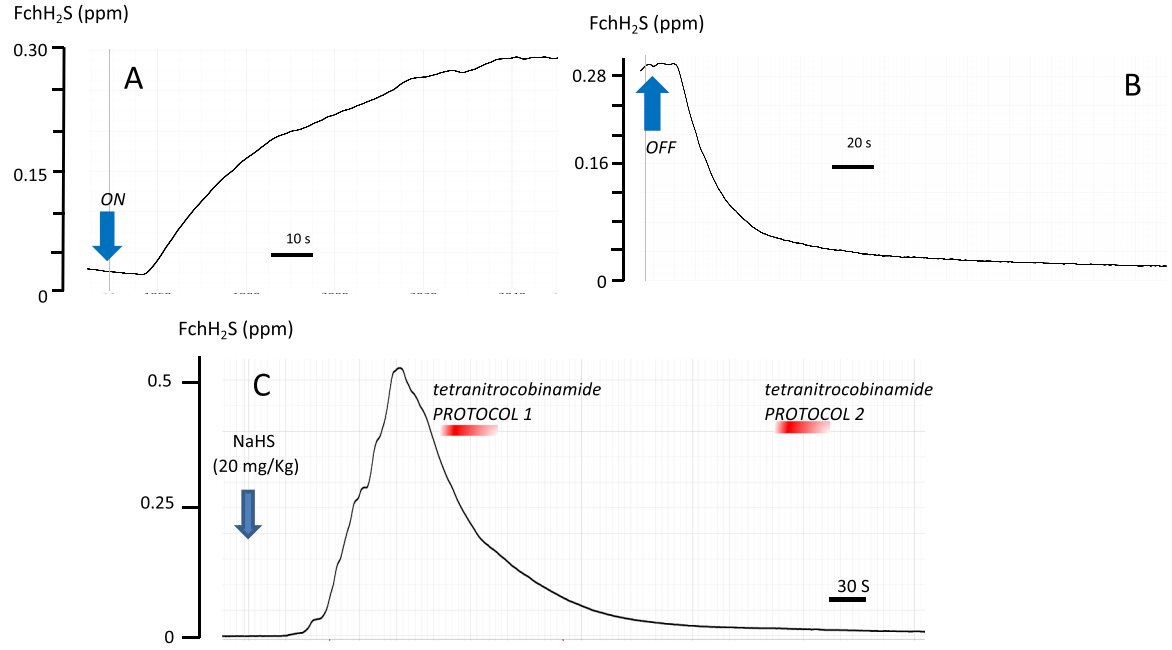

Fig. 1. Panel A: Example of the changes in the fraction of H_2S in the plethysmographic chamber ($FchH_2S$) in response to a step change in a gas mixture containing a known concentration of H_2S, using flow conditions like those used during our rat experiments. A time delay ranging from 10 to 20 s with an off-time constant of ~ 60 s (panel B) was observed. Panel C: Kinetics of change in expiratory fraction of H_2S in rats receiving an IP injection of NaHS (20 mg/kg, vertical blue arrow). A rapid increase in H_2S concentration occurred, which subsided very rapidly. No measurable level of H_2S could be identified after 5–7 min in this animal that remained alive. The 2 horizontal bars represent the timing at which tetranitrocobinamide was administered in protocols 1 and 2.

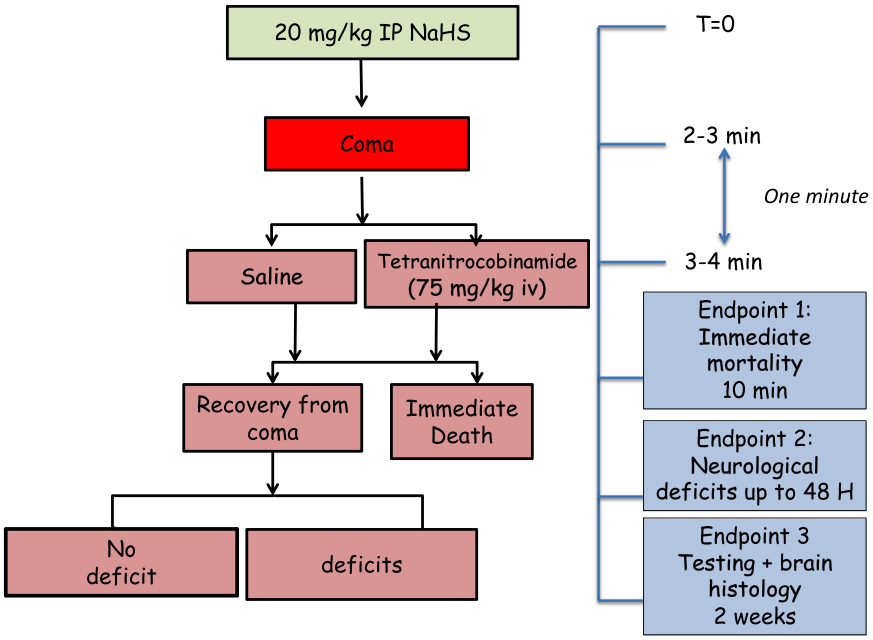

Fig. 2. Experimental design used in protocol 1: Forty rats received NaHS by IP injection and were followed for 2 weeks; one group (n = 20) received intravenous saline one minute into the coma while the other group received intravenous tetranitrocobinamide (n = 20).

studied in protocol 2, 13 received TNCbi at 5 min, and 14 received the same volume of saline.

2.3. Morris water maze task

For protocol 1, 2 weeks following the episode of sulfide-induced coma, surviving rats performed a Morris water maze (MWM) task (Sonobe et al., 2015). Briefly, the rats were trained, swimming in a circular pool (1.80 m diameter, 60 cm height), to locate a hidden transparent platform (11 cm diameter, 1.5 cm below surface) using extra-maze visual cues. Position of the platform remained constant in one quadrant throughout the study. Rats were released from one of the other three quadrants and allowed to search for the platform for up to 120 s. After a trial, rats were allowed to stay on the platform for 30 s. Each rat was given 4 trials per day for 4 consecutive days with an inter-trial interval of 15 min. The starting quadrant was changed in a random manner every day. On the last day, the 4th trial was replaced by a 120 s probe trial, i.e. with the platform removed (Sonobe et al., 2015).

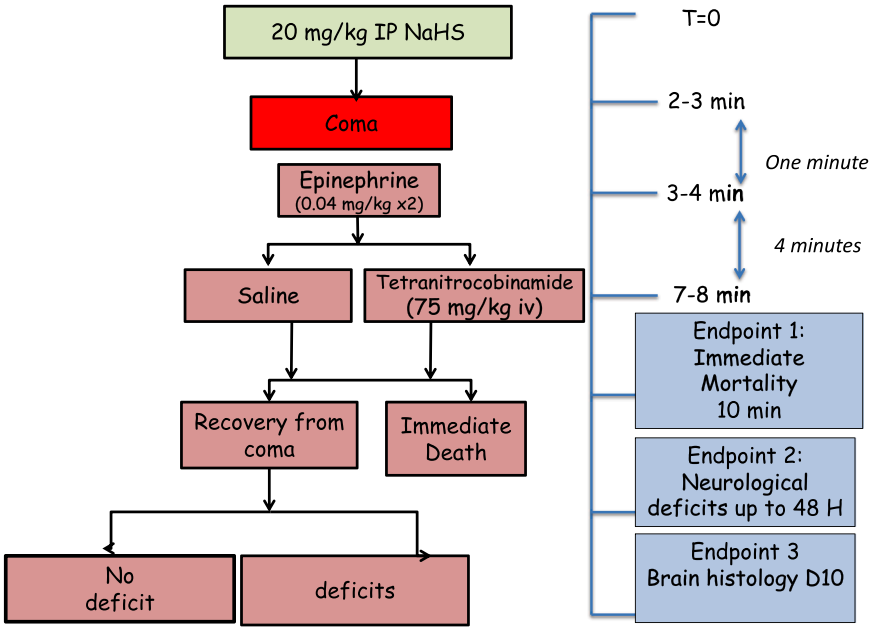

Fig. 3. Experimental design used for protocol 2: 27 rats received NaHS by IP injection ; all animals received epinephrine at one minute, and then the animals were divided into 2 groups. One group (n = 14) received saline 5 min into the onset of coma while the other group received tetranitrocobinamide (n = 13).

Swim paths of each animal were recorded using a ceiling mounted digital camera and ANY-maze video tracking software (ANY-maze, Stoelting Co., Wood Dale, IL).

2.4. Euthanasia and brain studies

For protocol 1, 3 days after the last day of MWM test, surviving rats were anesthetized with 3–5 % isoflurane in O_2 followed by urethane injection (1.2 g/kg, IP). The animals were perfused with transcardial phosphate buffered saline (60 ml) followed by 60 ml of 10 % formaldehyde solution. The brains and heart were fixed and processed as previously reported (Sonobe et al., 2015). For protocol 2, animals were euthanized 10 days after the exposure and autopsy was performed with histological studies of the brain and heart.

2.5. Data analysis

The primary outcomes of the sulfide-induced coma were 1) mortality within 10 min; 2) in surviving animals, the presence of clinical deficits within 24 h; 3) behavior in the Morris water maze; and 4) the presence of brain lesions (Sonobe et al., 2015).

For the MWM analysis, the following parameters were analyzed: Latency to reach the hidden platform, distance traveled to the platform, and path efficiency (ratio of the actual swimming distance to the ideal path that the animal could take to locate the platform). For the probe trial, percent of total time that animals spent in the platform quadrant and number of times crossing the platform location were also analyzed. In addition, swimming patterns to locate the platform were analyzed following the protocol previously described (Sonobe et al., 2015), originally established for mice (Brody and Holtzman, 2006; Garthe and Kempermann, 2013). Different "searching" strategies were identified based on visual criteria, from "ineffective" searching patterns (thigmotaxis, random search, and scanning) to spatially precise efficient strategies (directed search, focal search, and direct swimming) (Sonobe et al., 2015). Two observers reviewed all swimming patterns, and a third observer was involved if there was a disagreement. Data obtained in this study were compared to those obtained in healthy control rats (n = 21) which we use as reference values for MWM studies (Sonobe et al., 2015).

2.6. Statistical analysis

All results are presented as mean ± SD. Variables of interest were analyzed using Chi square (survival), one-way repeated-measured or a two-way repeated ANOVA (for other variables), followed by Bonferroni's post-hoc comparisons. $P < 0.05$ was regarded as significant for any of these comparisons. All statistical analyses were conducted using GraphPad Prism 5 (Graphpad.

Software, La Jolla, CA).

3. Results

3.1. Protocol 1

3.1.1. H_2S-induced coma in control rats (saline group)

The characteristics of the H_2S-induced coma were similar to those previously described (Sonobe et al., 2015). The number of H_2S injections necessary to produce a coma averaged 2.1 ± 1.0: three rats became comatose after one injection, 11 rats required two injections, and 6 rats needed 3 injections. Of the 20 animals studied, all became comatose within 2 min of the last injection. Sixteen animals died 10 min into the coma with no relationship with the number of injections (90 % of the rats were found pulselessness within 5–7 min and the rest within 10 min). The 4 rats that survived the acute phase of coma remained alive for the duration of the study (Fig. 4). The time course of the animal's behavior (time to coma, duration of coma) was the same regardless of the number of H_2S injections.

3.1.2. Immediate outcome of H_2S-induced coma treated with TNCbi

The number of injections necessary to produce coma in the TNCbi group was the same as in the control group (2.3 ± 0.9). Of 20 sulfide-intoxicated rats that received TNCbi in protocol 1, 13 (65 %, p = 0.0039) recovered from the coma and remained alive, while 7 did not recover and died within 10 min following NaHS administration (Fig. 4). Of note, 2 of the 7 rats that did not recover had extremely weak cardiac pulses before the injection of TNCbi. All 13 rats that recovered from coma behaved normally and presented no clinical deficit after 24 h. One surviving rat died at 48 h, while another surviving animal did not gain weight within 3 days and was euthanized. Examination of the

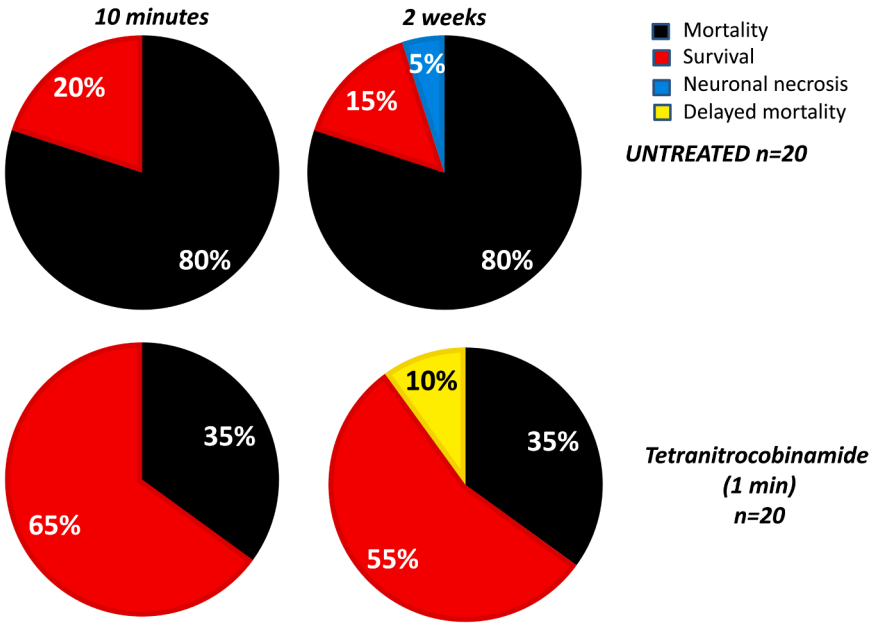

Fig. 4. Early and late mortally and morbidity in sulfide intoxicated animals that received saline or tetranitrocobinamide in protocol 1. There was a significantly lower immediate mortality in the group treated by tetranitrocobinamide (chi-square statistic was 8.2864, a p-value is 0.004). At 2 weeks, the difference in survival was reduced but was still significant (The chi-square statistic is 5.226, *p*-value is 0.02). None of the surviving rats in the tetranitrocobinamide group had neuronal lesions, while one rat in the saline group displayed diffuse neural necrosis, but without obvious clinical signs of deficit (see Fig. 9).

brain, heart, and lungs of these 2 animals did not reveal any lesions. Thus, immediate survival of TNCbi-treated animals was 65 %, while overall survival at 2 weeks was 55 % (11/20, $p < 0.05$ compared to the 20 % survival in saline-treated animals).

3.2. Morris water maze

After 2 weeks of recovery, all surviving rats were able to swim over 2 min. No animal showed unusual swimming patterns, such as continuous "looping" that we have previously observed in severely H_2S-intoxicated animals (Sonobe et al., 2015). The daily averaged swimming distance, latency and path efficiency to find the hidden platform are shown in Fig. 5 and was not different from our reference values in non-intoxicated rats in either group. Probe test data are shown in Fig. 6. There were no significant differences between the rats that received

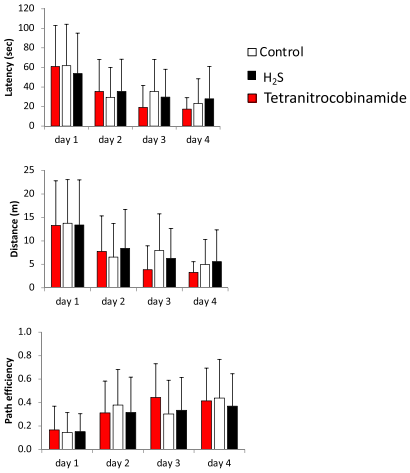

Fig. 5. Latency to reach the platform, distance, and path efficiency during Morris Water Maze testing. Note that the tests were conducted 2 weeks after the episode of sulfide-induced coma Data obtained in all surviving rats in both groups from protocol 1. Control data were obtained from 21 rats in a previous study (Sonobe et al., 2015). All rats were able to swim and to find the platform. All rats decreased the latency and distance to locate the platform and increased the path efficiency throughout the 4 days of training with no difference among groups. Values are shown as mean ± SD.

TNCbi, those that recovered spontaneously, and non-intoxicated rats.

3.3. Strategy used to find the platform

The frequency distribution of search strategies in TNCbi-treated animals was similar to that used by control/non-intoxicated animals, whereas saline-treated animals displayed direct search patterns less frequently ($p < 0.05$, Fig. 7).

3.4. Protocol 2

The number of sulfide injections to produce a coma was not different between the 2 groups (2.0 ± 0.5 vs 2.1 ± 0.3 in the TNCbi group). . Immediate mortality reached 50 % in the saline group, and remained the same within 2 weeks. Survival was not significantly different from the group of animals that received TNCbi after epinephrine (Fig. 8).

3.5. Histopathology

In protocol 1, one animal in the saline-treated group displayed neuronal necrosis in the cortex and the thalamus. More specifically, there was diffuse neuronal necrosis of the middle and superficial laminae of the parietal and orbital cerebral cortex and cingulate gyrus. There was asymmetric necrosis of the frontal cortex, diffusely affecting the superficial and middle laminae on one side and multifocally affecting the middle lamina of the other. There was also extensive but asymmetric neuronal necrosis in the thalamus (Fig. 9). These lesions were similar to those we have previously reported (Sonobe et al., 2015). None of the TNCbi-treated animals in either protocol or animals that received epinephrine only in protocol 2 displayed any neuronal necrosis. Small foci of cardiomyocyte loss with plump fibroblasts were found in hearts of many intoxicated animals regardless of treatment. These findings were not considered to be related to sulfide exposure.

4. Discussion

We found that TNCbi rescued 55 % of rats (65 % immediately) when administered early in a rapidly lethal form of H_2S-induced coma, while only 20 % of saline-treated animals survived. TNCbi-treated animals displayed normal behavior during the MWM testing, and with no histological evidence of neurological lesions. When administered later

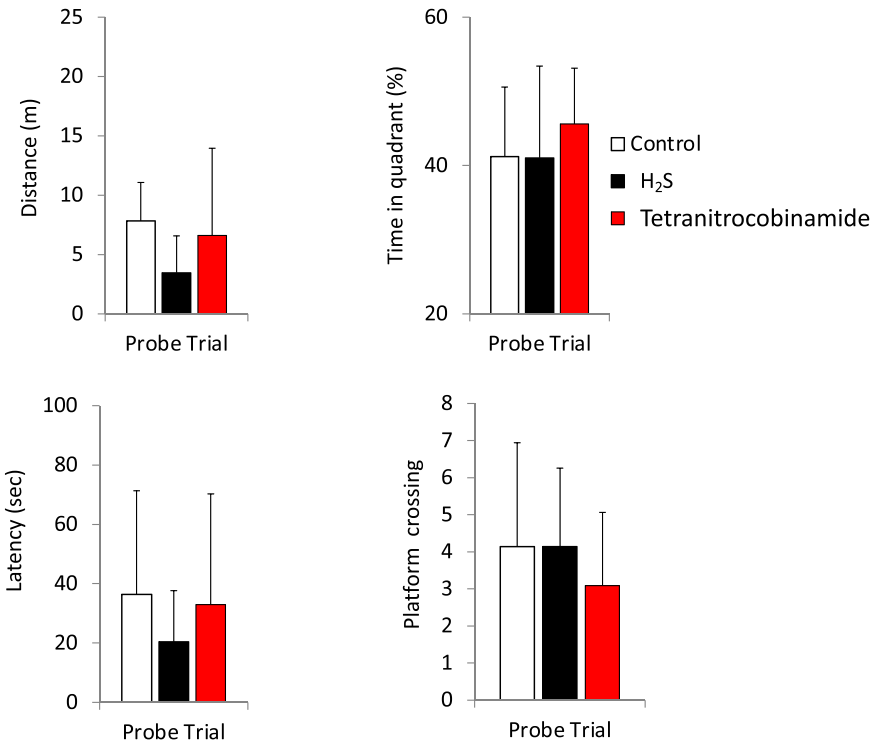

Fig. 6. Probe trials: The average latency, distance, time spent in the platform quadrant, and number of times the rats crossed the platform zone at D4 during the probe trial are displayed. No significant difference was observed among the 3 groups. Values are shown as mean ± SD. (Cobi: tetranitrocobinamide). The tests were conducted 2 weeks after the episode of sulfide-induced coma.

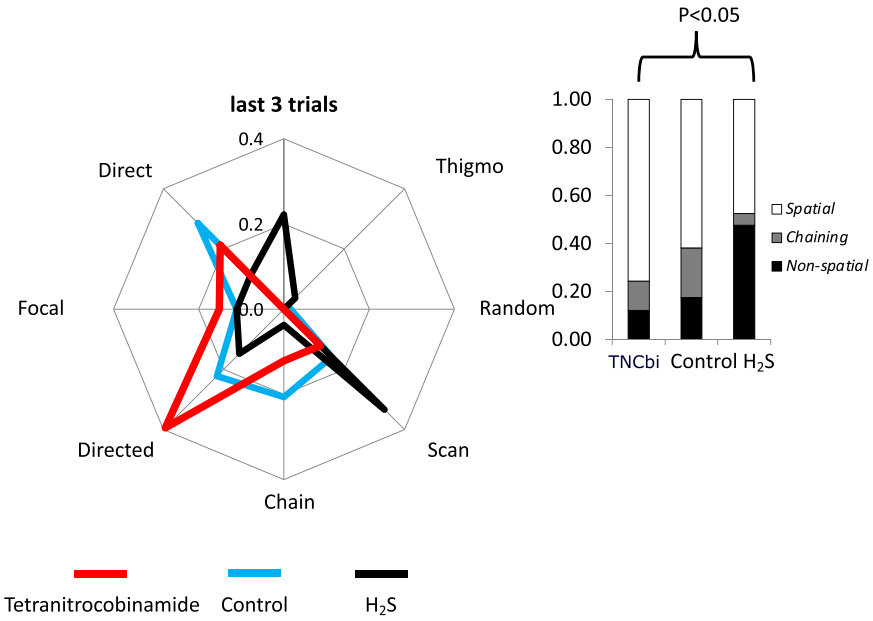

Fig. 7. Radar chart showing the relative occurrence of searching strategies at day 4. The tests were conducted 2 weeks after the episode of sulfide-induced coma. All animals able to find the platform used a significantly less effective pattern in the H_2S group that received saline, consisting in higher occurrence of scanning strategy when compared to the tetranitrocobinamide-treated group and the control group. The frequency distribution of the strategies used during the MWM test are displayed in the right upper corner.

(5 min), at a time when essentially free sulfide concentrations have decreased from the blood, TNCbi, after epinephrine did not produce any additional benefit when compared to epinephrine alone.

4.1. *Relevance of the model of H_2S intoxication*

This mode of H_2S administration, which we have already used (Sonobe et al., 2015), recapitulates the symptoms of H_2S intoxication reported in humans, i.e. rapid loss of consciousness, breathing depression, cardiac arrest, and rare neurological lesions. Mooyaart et al. (2016). reviewed clinical outcomes of 56 victims of life-threatening sulfide intoxication, most of them related to exposure that took place in a manure pit: 45 % of the victims were found dead at the scene, and 14 % of victims needed CPR. All surviving victims were admitted to a hospital, some exhibiting persistent circulatory and respiratory failure or neurological complications. Of note, only 25 % of the victims who required cardio-pulmonary resuscitation recovered with neurological deficits. The lack of neurological lesions in victims surviving even a relatively severe form of intoxication supports the conclusions of Burnett et al. (1977) who looked at much lesser forms of H_2S intoxication in the

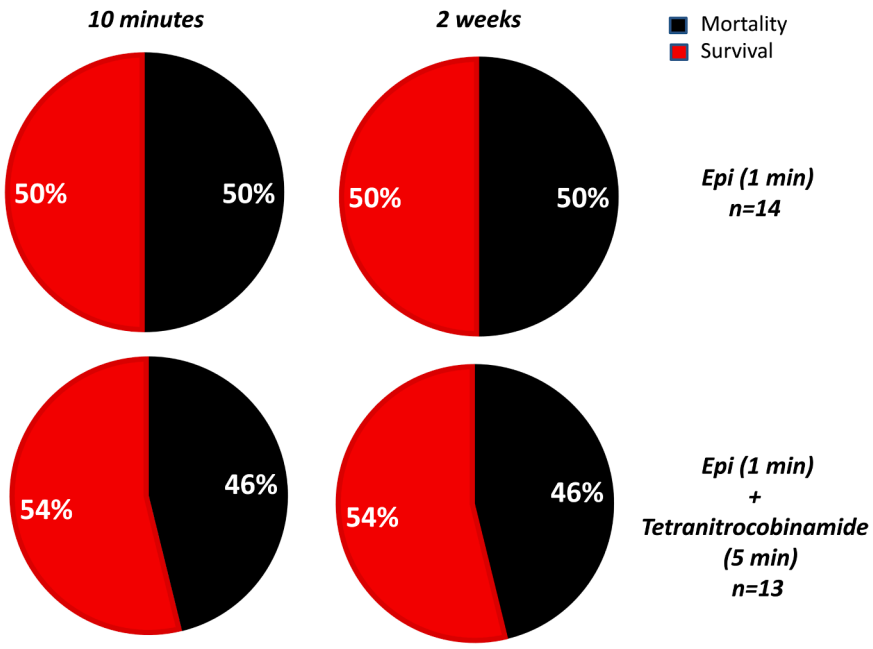

Fig. 8. Early and late mortality and morbidity in NaHS intoxicated animals that received saline or tetranitrocobinamide, 5 min into coma (~ 7 min following the injection) after receiving epinephrine (protocol 2, see text for further details). There was no difference in immediate mortality in the tetranitrocobinamide/epinephrine-treated and epinephrine group groups. At 2 weeks, the difference in survival was unchanged. None of the surviving rats in the tetranitrocobinamide/epinephrine and in the epinephrine group had neuronal lesions.

oil, gas and petrochemical industries. These authors reported that 65 % of 221 victims of H_2S exposure had loss of consciousness with an overall mortality of 6 %, in major contrast to the manure pit exposure study (Mooyaart et al., 2016). Most victims recovered from their coma spontaneously with no sequelae. Isolated case reports have found abnormal brain imaging following H_2S intoxication, including basal ganglia lesions (Gaitonde et al., 1987; Matsuo et al., 1979), cortical atrophy (Tvedt et al., 1991a, 1991b), and decreased metabolism in thalamus, basal ganglia, temporal and inferior parietal lobe by PET Scan (Schneider et al., 1998). Neuronal lesions affecting the motor cortex (Nam et al., 2004) fit well with the lesions we found (Sonobe et al., 2015). All these lesions remain rare (Sonobe et al., 2015), as long-term neurological deficits are usually absent even in victims presenting in coma (Burnett et al., 1977). Although the mode of H_2S administration we used is different from inhalation, which does produce lung lesions, uncommon in our model, we feel that our conclusions remain clinically relevant due to the similarities of the systemic symptoms with those reported following exposure in victims of H_2S intoxication.

We have more recently explored the kinetics of the changes in circulatory status occurring in a rat model, similar to the one used in the present study, using echocardiography measurements during the period of coma (Judenherc-Haouzi et al., 2016) and found that a very early and profound deterioration in cardiac contractility is present as soon as animals become unconscious. This effect on the heart has been confirmed in anesthetized animals, including sheep (Haouzi et al., 2019), and is the cause of immediate death. Although H_2S at high concentrations can be toxic for neurons, a persistent depression in cardiac contractility seems to be an important factor leading to neurological deficits, via brain ischemia (see (Haouzi et al., 2020) for discussion). The substratum of this decrease in cardiac contractility has been more recently explored (Judenherc-Haouzi et al., 2016; Cheung et al., 2018b): it is in large part mediated by a very rapid inhibition of L-type Ca^{2+} currents, which can persist well beyond the period of H_2S exposure (Haouzi et al., 2019).

4.2. *Effects of TNCbi, early versus delayed injection*

The framework that we are currently using to understand the effects and the treatment of H_2S intoxication relies on the kinetics of the different pools of H_2S (Sonobe and Haouzi, 2016a; Haouzi et al., 2014; Klingerman et al., 2013). As soon as H_2S diffuses into the blood and tissues, it almost completely and instantly disappears in its free/-diffusible forms due to immediate "combination" of H_2S with various proteins and its very rapid rate of oxidation (see (Haouzi et al., 2016) for review). As a result, only a very small portion of sulfide can be found in "soluble" form even during the exposure, comprising gaseous H_2S and the sulfhydryl anion HS^-. The rate of oxidation of H_2S is so high and rapid that the pool of "free/soluble" H_2S spontaneously vanishes from the tissue as soon as the exposure to H_2S ceases (Sonobe and Haouzi, 2016b). However, while the pool of free H_2S disappears, the various pools of combined H_2S remain fixed on metalloenzymes for a much longer period (Haouzi et al., 2014). Similarly, the consequences of H_2S induced mitochondrial impediments (production of ROS, decrease in ATP production) have a much longer kinetics than the pool of free/-diffusible H_2S and could also explain the persistence of symptoms of toxicity beyond the phase of exposure (Cheung et al., 2018a). These kinetics have been established during non-lethal intoxication (Sonobe and Haouzi, 2016a; Haouzi et al., 2014; Klingerman et al., 2013) and the reduction in cardiac output may certainly decouple the fraction of sulfide expired from the level of free/diffusible sulfide in the blood.

We have previously found that a very high dose of hydroxocobalamin (5 g), injected early *after* the cessation of an acute H_2S exposure (within few minutes), improved cardiac contractility and prevented death in a sheep model (Haouzi et al., 2015). In the rats we also found that hydroxocobalamin (70 mg/kg) decreased the concentrations of free/soluble H_2S in the blood to almost zero during a constant infusion of H_2S, suggesting that free H_2S is trapped and/or oxidized in the presence of this cobalt compound, supporting other recent reports in the rabbit (Brenner et al., 2014) or in swine (Ng et al., 2019b; Hendry-Hofer et al., 2020) with TNCbi. It is important to consider that in these models of sulfide intoxication, the antidote was administered while free H_2S was still present in the blood of the animals.

TNCbi injected after 5 min of coma (~ 7 min after NaHS injection) reaches the blood at a time when free sulfide is certainly at much lower levels in the blood than at the cessation of exposure (Fig. 1). The difference in efficacy between an early vs late administration of in the blood could be accounted for by this rapid decrease in the pool of free H_2S, in turn impacting our ability to offset the consequences of H_2S induced cardiac dysfunction by the antidote. Fig. 10.

The fact that many animals displayed severe cardiac dysfunction

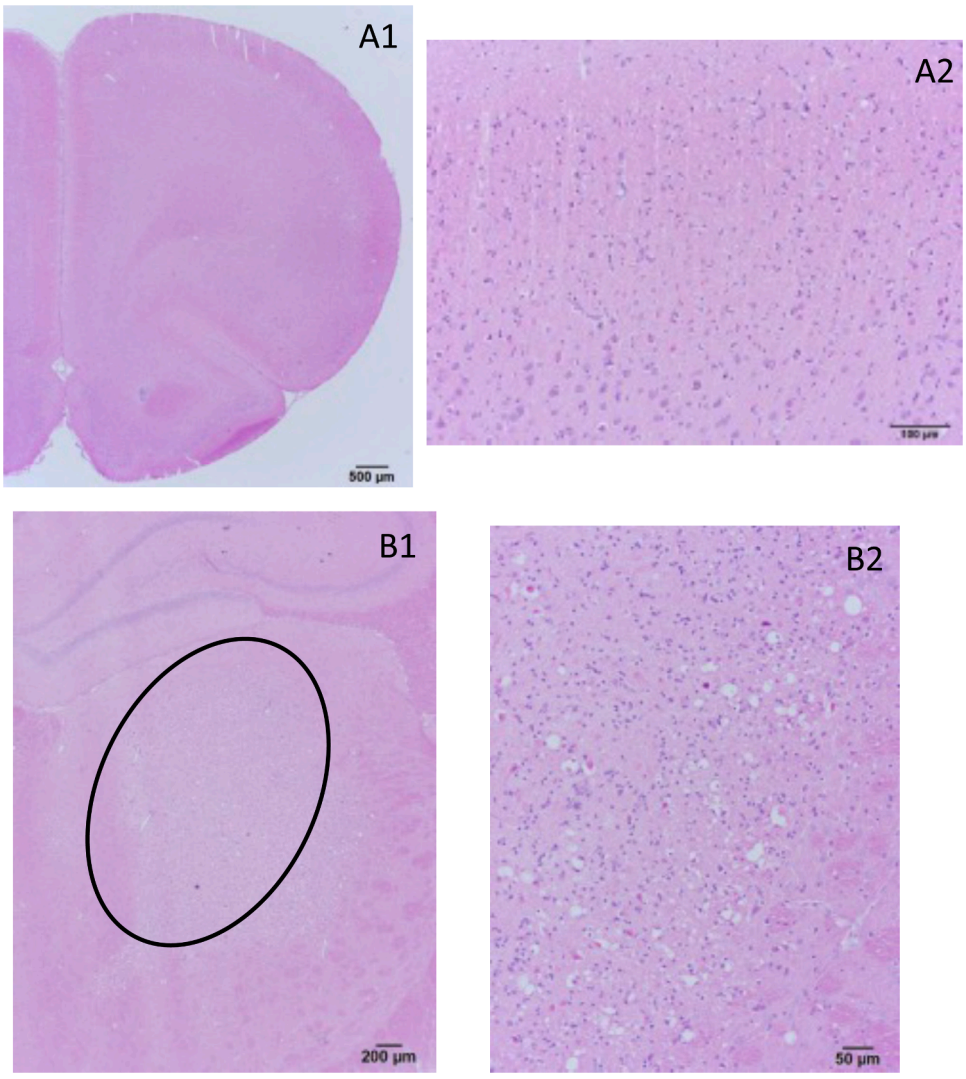

Fig. 9. Coronal sections (Panel A1 and A2) of the brain including the frontoparietal and orbital cerebral cortex and olfactory bulb of the only rat from the non- TNCbi treated group that showed neuronal lesions (protocol 1). Note the diffuse neuronal necrosis of the middle and superficial laminae of the parietal and orbital cerebral cortex and cingulate gyrus. Panels B1 and B2 show extensive and neuronal necrosis in the thalamus.

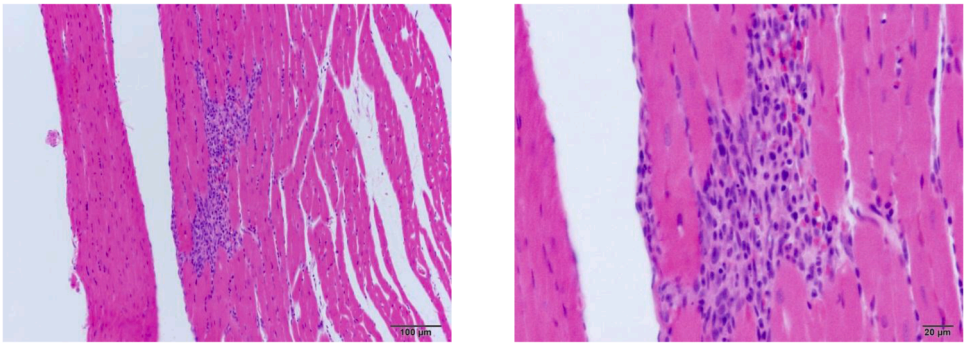

Fig. 10. Longitudinal section of heart in one animal including left atria and ventricle, showing area of cardiomyocyte loss within the subendocardial myocardium, with replacement by organizing immature fibrosis containing low numbers of macrophages and scattered apoptotic cells. Total area of myocardium affected was < 2 %. Of note, since these lesions are also frequently observed in non-exposed rats, they were not considered to be related to H_2S intoxication.

when TNCbi was administered, could also contribute to the lack of efficacy of the drug. Delayed TNCbi administration did not bring any additional benefit in animals pre-treated with epinephrine. Whether or not, in a less severe form of intoxication, a delayed injection of TNCbi could affect the outcome remains an outstanding question. Answering this question poses an experimental challenge, since in less severe forms of sulfide intoxication, even when a coma is present, spontaneous recovery is usually the rule with no long-term consequences, making it very difficult to demonstrate the benefit of any antidote (Mooyaart et al., 2016).

4.3. Limitations

Certainly, the modality of sulfide exposure used in the present study (IP) is not faithful to human intoxication. Since the exposure to H_2S is only via inhalation and H_2S produces, after a period of hyperventilation, a central breathing depression with apnea, exposure to this toxic agent ceases during apnea only resuming during gasping (Haouzi, 2012). Obviously, this phenomenon cannot occur during systemic administration of sulfide; on the contrary, part of sulfide removal by the lungs is impeded during an apnea. However, this difference is less relevant *after* exposure, since during recovery all the compartments (alveolar blood tissues) reach a rapid equilibrium in H_2S partial pressure (Haouzi et al., 2014; Klingerman et al., 2013), regardless of the modality of exposure. It should be kept in mind however that following an IP infusion, sulfide may continue to diffuse into the blood for a longer period than following inhalation.

Another potential problem is that the cobinamide formulation (TNCbi) that was used in this study can release molecules of sodium nitrite. As reviewed and discussed by Chan et al. (2015), based on data obtained in sedated rabbits, TNCbi at doses ~ 4 micromol / kg or ~ 5 mg/kg did not produce any methemoglobinemia or decrease in blood pressure, since the amount of nitrite present would not be sufficient to produce any measurable pharmacological effect (Kohn et al., 2002). In the present study, we used higher concentrations of TNCbi, to create a powerful sink for sulfide, in keeping with doses of hydroxocobalamin (Haouzi et al., 2015; Van de Louw and Haouzi, 2012a) previously used to treat severe H_2S intoxication. We used ~ 60 micromol/kg (~70 mg/kg) tetranitrocobinamide, which could correspond to a dose of sodium nitrite potentially available of 15 micromol/kg or (~ 0.9 mg/kg), assuming that sodium nitrite comprises ~ 20 % of the molecular mass of tetranitrocobinamide (Chan et al., 2015). Yet, doses of sodium nitrite of at least one order of magnitude higher would be required to produce a meaningful methemoglobinemia (10 %) in the rat (Kohn et al., 2002). In addition, we found that in the rat the presence of a methemoglobinemia (~ 6 %), which has remarkable trapping effects on free H_2S, has no measurable antidotal effect during the recovery phase of an acute H_2S intoxication (Chenuel et al., 2015). Since this lack of effect was observed when the level methemoglobinemia would have been able to effectively decrease the concentration of free H_2S in the blood *during* a period of exposure to H_2S (Haouzi et al., 2014, 2011; Van de Louw and Haouzi, 2012a, 2012b), we postulated that the very rapid disappearance of free/soluble H_2S in the blood at the time of infusion of methemoglobin or nitrite administration was the most likely explanation for this observation. In addition, the action of methemoglobin would have been limited to trapping free suflide in the intravascular compartment (Chenuel et al., 2015), in contrast to tetranitrocobinamide, which can diffuse into most tissues. In other words, while an increased capacity of the blood to trap H_2S cannot be totally discarded since the level of methemoglobinemia was not determined in the present study, this effect, if any, should have remained very moderate. Of note, TNCbi (Broderick et al., 2005) shares with hydroxocobalamin (Roderique et al., 2014) scavenging properties against nitric oxide, which should oppose some of the effects of nitrite on vascular smooth muscles, in turn preventing or limiting nitrite-induced hypotension (Chan et al., 2015). Finally, the use of larger animals models to confirm the present results may be of importance as rodents display unique physiological response to an anoxic injury, specific to small-sized mammals (see (Haouzi, 2011) for discussion).

4.4. Practical implications

As previously discussed (Haouzi et al., 2015), the steepness of the dose-toxicity relationship for H_2S makes the development of countermeasures very challenging. Such an endeavor requires creating very specific experimental conditions allowing animals to live long enough to receive an antidote in an otherwise lethal intoxication. How these models translate into real-life exposure certainly represents the main challenge of any study on sulfide toxicity. Based on the present experimental rodent model, the window of opportunity to treat patients with a potent trapping agent, when withdrawn from a source of exposure, would be very short. An effective strategy must allow antidotes that are trapping sulfide to be immediately available and administered if lethality is to be expected within a few minutes (Mooyaart et al., 2016). Yet, one can imagine that in specific scenarios of sulfide intoxication – comatose victims impossible to remove from a source of H_2S or as a prophylaxis for instance – trapping agents such as TNCbi would be of interest. As discussed above, the study of 56 victims of life-threatening sulfide intoxication by Mooyaart et al., 2016, along with the 221 victims reported by Burnett et al., 1977. do not suggest that this is the most common scenario. The ideal antidotes or "mixture" of antidotes should be able to combine great trapping properties while still being effective on sulfide-induced cellular toxicity when most free sulfide is gone. The search for molecules acting directly on the metabolic consequences of sulfide induced mitochondrial dysfunction must therefore be pursued (Haouzi et al., 2016).

In conclusion, administration of TNCbi early in the period of coma following a potentially lethal dose of H_2S increases survival. Injection of TNCbi five minutes after the onset of coma has no additional benefit to epinephrine alone in these very severe forms of intoxication. These results illustrate the main challenges of treating exposure to H_2S, where the free pool rapidly decreases in the blood after the end of exposure.

CRediT authorship contribution statement

Philippe Haouzi: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Marissa MacCann:** Writing – review & editing. **Matthew Brenner:** Conceptualization Writing – review & editing. **Sari Mahon:** Conceptualization, Writing – review & editing. **Vikhyat S. Bebarta:** Conceptualization, Writing – review & editing. **Adriano Chan:** Conceptualization, Writing – review & editing. **Annick Judenherc-Haouzi:** Conceptualization, Writing – review & editing. **Gerry R. Boss:** Conceptualization, Writing – review & editing. **Nicole Tubbs**: Methodolgy, Review and editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Philippe Haouzi reports financial support was provided by National Institutes of Health. Marissa MacCann, Matthew Brenner, Sari Mahon, Vikhyat S. Bebarta, Adriano Chan, Annick Judenherc-Haouzi, Nicole Tubbs and Gerry R. Boss reports financial support was provided by National Institutes of Health. This work was supported by the NIH CounterACT program, grant # U01 NS087964.

Data Availability

Data will be made available on request.

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