

**Development of mouse models of hydrogen sulfide-induced neurotoxicity for
the evaluation of neuroprotective strategies**

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

H₂S is a colorless gas with a strong “rotten egg” odor. While at physiological concentrations, H₂S is a vital gasotransmitter; exposure to high concentrations can result in extreme intoxication, leading to acute death within minutes. H₂S poisoning is a leading cause of occupational deaths. Among toxic gases, H₂S follows only carbon monoxide poisoning as a leading cause of death. There is also concern for this chemical to be weaponized as it is easy to make from household chemicals found in local general stores. H₂S can cause severe neurodegeneration and neurological sequelae post exposure. There is no ideal treatment for acute H₂S-intoxication in humans. Our laboratory has characterized two translational mouse (C57) models to evaluate potential drugs for prevention and treatment of H₂S-induced toxicity, and potentially elucidate the molecular mechanisms involved in the pathophysiology of H₂S poisoning. We observed that H₂S consistently induced seizures and knockdown in the mice, and induced motor deficits, neurochemical alterations, neuroinflammation, oxidative stress and neuropathology. Our data demonstrated that Cobinamide dose and time dependently reduced the behavioral deficits, neuroinflammation, neurochemical changes, oxidative stress and the histopathological changes induced by H₂S. Additionally, our studies show promise for Midazolam as an antidote for treatment of H₂S-induced neurotoxicity as it reduced H₂S-induced mortality by 90% when given prior H₂S exposure, and reduced mortality induced by H₂S by 50% when given during the exposure. Midazolam administered pre-exposure prevented clinical signs, motor deficits, and histopathological lesions induced by H₂S. Collectively, we have validated and characterized a translational mouse model of H₂S-induced neurotoxicity, and used the model to demonstrate efficacy of cobinamide and midazolam as countermeasures against H₂S-induced neurotoxicity.

CHAPTER 1

GENERAL INTRODUCTION

Dissertation Organization

The alternative format was chosen for this dissertation and consists of manuscripts that have either been published or are in preparation for submission. There is a general introduction containing a literature review of hydrogen sulfide, three research chapters in the format of their corresponding journals, and a general conclusions/future directions section. Each research chapter contains references in the format of the journal it was or will be submitted to. The introduction, literature review, and conclusion sections have the references combined at the end of the thesis.

Chapter 1, The Background and Literature Review section provides an in-depth review of current literature regarding hydrogen sulfide-induced neurotoxicity; providing an overview of hydrogen sulfide, sources of hydrogen sulfide, human health effects, current treatments, animal models currently in use, molecular mechanism of action, and potential therapeutic strategies.

Chapter 2 is a research chapter describing the mouse model characterized and validated in our laboratory. This chapter has been accepted and published in the journal *Annals of New York Academy of Sciences*. Chapter 3 is a manuscript that is currently under revision for publication in *Annals of New York Academy of Sciences*. It reveals Cobinamide, a therapeutic compound that binds hydrogen sulfide, prevents neurotoxicity and neurodegeneration induced by hydrogen sulfide. Chapter 4 is currently being prepared to be submitted to the *Journal of Medical Toxicology*. This chapter outlines studies investigating

the therapeutic potential of an anti-seizure medication, Midazolam, as a therapeutic for treatment of hydrogen sulfide-induced neurotoxicity.

For this dissertation, the experimental data and results were obtained by the author during the course of her PhD study in the Interdepartmental Toxicology Graduate Program under the supervision of her major professor, Dr. Wilson K. Rumbleha at Iowa State University.

Introduction

Hydrogen Sulfide

Recently hydrogen sulfide (H_2S) has been identified as an endogenous signaling molecule^{1, 2}. However, historically, H_2S is well known as a toxic gas and as an environmental and occupational hazard³⁻⁷. H_2S is a clear, flammable gas that has a strong “rotten egg” odor. It is a lipophilic molecule that can readily pass through membranes without the need for specific transporters⁸. Increasing concentrations of H_2S exposure manifest diverse toxic effects in humans; these can range from mild irritability of the eyes and respiratory tract to death within minutes of exposure^{3, 9}. Due to its wide use as a by-product in many industries, most commonly the oil and gas industry, it is the second most common cause of fatal gas inhalation in an industrial setting^{5, 10}. It is also listed as a chemical of interest by the Department of Homeland Security, as these and H_2S storage facilities could be potential targets for terrorists, especially those located in highly populated areas^{11, 12}. Currently, there is no ideal treatment of H_2S -induced neurotoxicity, particularly in the field. This thesis summarizes extensive work on development and evaluation of mouse models of H_2S induced neurotoxicity and use of these models for evaluation of potential therapeutics for H_2S -induced intoxication.

Physiological Sources and Role

H₂S is an endogenously produced signaling molecule in human tissues, actively involved in neuromodulation^{8, 13, 14}. *In vivo*, H₂S can be produced via enzymatic and non-enzymatic pathways. There are two main enzymes actively involved in the synthesis of H₂S from amino acid L-Cysteine; cystathionine B-synthase (CBS) and cystathionine g-lyase (CSE), as shown in Figure 1^{8, 14}. Another third enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST) uses α -ketoglutarate and L-cysteine by metabolically interacting with cysteine aminotransferase. 3-mercaptopyruvate predominantly found in the mitochondria, while the other enzymes can be active in the mitochondria and cytosol. A study investigating the distributions of these enzymes in the liver, kidney and brain found CSE to be more abundant in the liver and kidney, while CBS was found to be in higher concentrations in the brain^{15, 16}.

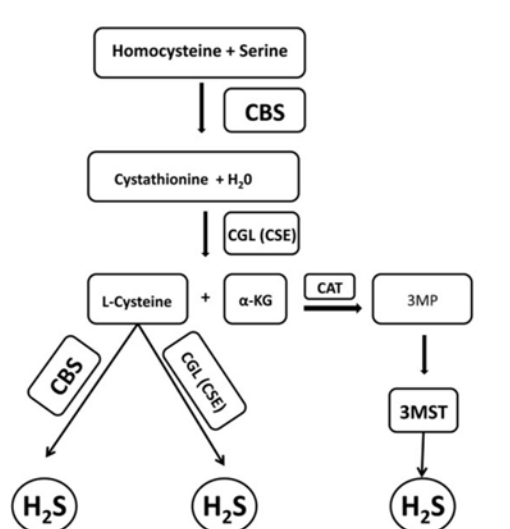


Figure 1. Synthesis of hydrogen sulfide *in vivo*. Adapted from Moody et al.

Recent studies have shown these enzymes to be tissue specific, some tissues require both main enzymes (CSE and CBS) to produce H₂S, while other require one or the other¹⁷. These enzymes have been identified globally in mammalian cells, including liver, kidney, fibroblasts, brain, and skin¹⁷. Metabolism of H₂S occurs via oxidation in the mitochondria or by methylation in the cytosol and is excreted predominantly by the kidney. H₂S can also be scavenged by methemoglobin or oxidized glutathione. H₂S is also produced in the gut by gut microbial flora.

The functional role of H₂S in the brain was uncovered in the early 1990s. H₂S concentrations in the brain have been reported to be 50-160 uM^{18, 19}. H₂S has been reported to be involved in the regulation of intracellular signaling molecules, ion channel function, and the release and function of amino acid neurotransmitters²⁰. It is possible that H₂S exerts neuro-modulatory effects via astrocyte and microglia in the brain, as H₂S has been shown to induce long-term potentiation (LTP) and an increase in calcium release from astrocytes. H₂S induces LTP via activation of N-methyl D-aspartate (NMDA) receptors, by activating cAMP/PKA pathway²¹. Importantly H₂S does not induct LTP on its own, but has strong enhancing effects, leading to a significant increase in NMDA current. NMDA receptors have been implicated in many neurological diseases, such as Huntington's Disease, Schizophrenia, and Alzheimer's disease; since H₂S can enhance these receptors, it may modulate these diseases²².

At physiological concentrations, H₂S also exerts an effect on the inhibitory neurotransmitter, gamma-aminobutyric acid (GABA). GABA is the major inhibitory neurotransmitters in the mammalian brain, about 20-30% of all synapses employ GABA as a neurotransmitter²⁰. GABA mediated inhibition is important for normal brain function, as the loss of this inhibition can lead to seizures and hyper-excitability^{20, 23}. H₂S has been reported to reduce injury of hippocampal damage from seizures by reversing the loss of GABA receptors via an increase in Ca²⁺.

H₂S has also been shown to modulate calmodulin kinase, an enzyme important in the regulation of Ca²⁺ homeostasis in neurons. It is believed that CBS, the enzyme responsibly for a majority of the H₂S production in the brain is controlled by calmodulin kinase, which indicates that CBS is regulated by intracellular Ca²⁺ concentration. H₂S is produced when

CBS is activated by Ca^{2+} which occurs during neuronal excitation, indicating a role in memory formation.

Exogenous Sources of H₂S and Exposure Scenarios

There are several anthropogenic sources of H₂S either by chemical or microbial action. Natural environmental sources of H₂S include volcanoes, marshes, hot springs, and underwater thermal vents²⁴. H₂S can be also naturally released by anaerobic bacteria decomposing organic material; hence, H₂S is also known as “sewer gas”^{25, 26}. Agriculturally, specifically in the Midwest, H₂S is responsible for many livestock and human deaths. Normally, H₂S levels are very low in animal farms; however high concentrations can be released when manure pits are agitated^{27, 28}. H₂S is also encountered in sewers and sewage treatment plants as bacteria breakdown organic material there as well²⁹.

In 2007, there were an estimated 1,134 documented acute exposure to H₂S³⁰. Although H₂S poisoning is considered uncommon, reported deaths due to H₂S are becoming more prevalent. From 2011-2013 there were a total of 43 victims of H₂S intoxication, including 15 suicide victims and 21 victims who were first responders^{31, 32}. In industry, most of the H₂S exposures occur in confined spaces. Confined spaces are particularly hazardous because H₂S is heavier than air, it is able to settle and accumulate in small, restricted areas. H₂S is a hazardous byproduct in crude oil and natural gas production, waste disposal and pulp and paper manufacturing, it is also widely used in rayon textile productions and chemical manufacturing. As this gas is stored in bulk at these industrial sites, there is potential for mass civilian exposure following catastrophic industrial accidents in any of these facilities^{33, 34}. For example, civilians were exposed to high concentrations of H₂S in Poza Rica, Mexico, where an accident led to 320 people hospitalized and 22 people dead from H₂S poisoning.

Another incident in Kaixan, China, led to the death of 191 people from a sour gas well blowout that caused the release of H₂S into the city.

In recent years, H₂S has been increasingly utilized as a tool for suicide^{32, 35, 36}. The gas can be generated from the mixing of common household chemicals found at the local general store to create fatal concentrations of H₂S, especially in confined spaces, such as a car or apartments. This process of suicide also increases the potential for secondary casualties, such as first responders, or family and friends of the victim.

The fact that this chemical can be generated easily, or is readily accessible in large quantities in industries, highlights the potential for misuse of this chemical in the form of chemical warfare.. H₂S was previously used in WWI as a chemical weapon, even though it was not very effective as it was used in an open area. The use of this chemical in a smaller confined spaces, such as a subway station would make it an effective chemical weapon. For this reason, there is a significant concern by the Department of Homeland Security about potential misuse of H₂S as a chemical weapon, and is listed as a chemical of interest^{11, 12}.

Hydrogen sulfide can also be produced via metabolic transformation of carbonyl sulfide (COS) *in vivo*. Carbonyl sulfide is a neurotoxic gas used as a grain fumigant. COS is metabolized physiologically via carbonic anhydrase to produce H₂S, which is ultimately believed to be the chemical responsible for COS-induced neurotoxicity and neurodegeneration³⁷⁻³⁹.

A majority of H₂S induced fatalities occur in an occupational setting, specifically in confined spaces^{40, 41}. Many workers and farmers in these settings are unaware of the hazard associated with H₂S. Renewed efforts are needed to educate individuals working under these conditions, including site specific procedures and protective equipment knowledge.. Personal

protective equipment should include respiratory equipment, a gas monitor, long sleeves, and safety glasses. Many of the deaths associated with H₂S-induced toxicity could have been prevented with the use of a safety monitor to alert the victims of H₂S gas in their occupational setting^{7,40}.

Human Health Effects and Clinical Toxicity

The major route of exposure to H₂S is via inhalation^{24, 42, 43}. The main targets of H₂S include

the central nervous system,

respiratory system, and the

cardiovascular system^{5, 7, 43}.

At physiological pH, two

thirds of H₂S exists as a

disassociated form (HS⁻) and

one-third exists in the un-

disassociated form⁴⁴. Detoxification of H₂S can occur via enzymatic or non-enzymatic

oxidation to sulfate and thiosulfate in the liver. In healthy individuals, there are large

amounts of sulfate and sulfite in the blood and urine, but low levels of thiosulfate. Because

thiosulfate is a major metabolite of H₂S, thiosulfate levels are reported to be a useful

biomarker of H₂S poisoning, especially in urine^{45, 46}.

Toxic effects from H₂S exposure can occur from both low and high concentrations of

H₂S, and is concentration dependent rather than time dependent. H₂S can be detected as low

as 0.025 ppm due to its very strong rotten egg odor. Low levels of H₂S (10-20 ppm) can

cause irritation to the eyes, while exposure to 50-100 ppm can cause eye damage such as

conjunctivitis. Levels around 100-150 ppm can damage the olfactory nerve, preventing

Table 1. Human Health effect of H ₂ S	
Concentration (ppm)	Effect
0.25	Detectable odor
2	Respiratory irritation to asthmatics
20	Eye irritation
50-100	Eye and respiratory irritation
150	Olfactory nerve paralysis
200-500	Pulmonary edema
	Seizures
750	Rapid loss of consciousness
Table 1.	Respiratory paralysis
	Death if not rescued
1000	Immediate collapse to death

Adapted from Fuller et al. Snyder et al., and Reiffenstein et al.

perception of the rotten egg odor, which allows for continued exposure to H₂S without the victim being aware. At higher concentrations, 200-500 ppm, H₂S causes pulmonary edema leading to respiratory distress, coughing, and shortness of breath. Levels ranging from 500-700 ppm can lead to severe toxicity, including the “knockdown”. Knockdown is the sudden loss of consciousness at high levels of H₂S exposure. Survivors have reported this phenomenon as a slightly euphoric. Many oil and petroleum industry workers have experienced some toxic effects of H₂S; 30% percent have experienced some symptom of H₂S toxicity, and 8% have become unconscious^{6, 44, 47}. If H₂S dissipates quickly, recovery from the knockdown can be instantaneous. At greater than 1000 ppm, just a few breaths of H₂S can cause immediate knockdown and death. These health effects of H₂S are summarized in Table 1.

Long-term consequences of acute exposure to high concentrations of H₂S in survivors can range from none to persistent neurological problems; victims of H₂S exposure are recommended to undergo neurological and neuropsychological testing for at least 5 years following H₂S exposure⁴¹. The duration of neurological sequelae in victims of H₂S toxicity can range from days to several months⁴². Neurological sequelae can include persistent headaches, nausea, convulsions, seizures, confusions, psychosis, memory impairment, ataxia, movement disorders, coma, sleeping disorders, and hearing impairment, among others^{7, 39, 42, 48}. Human victims of H₂S poisoning who have been in a knockdown for 5-30 min are reported to have many of these neurological sequelae. One of the main issues associated with H₂S intoxication is differentiating the primary effects of H₂S from effects of anoxia⁵. The neurological conditions associated with H₂S-induced toxicity can be a consequence of many effects including hypoxia from oxygen deprivation, direct interference with oxygen uptake,

trauma from falls, and potential direct mechanisms of H₂S-induced neurotoxicity⁵. Seizure activity observed in victims of H₂S poisoning can also interfere with breathing and may worsen hypoxia. Victims with pre-existing seizurogenic conditions appear to be vulnerable to H₂S intoxication. For example, in one case report, a patient with neurofibromatosis with a history of epilepsy since childhood, developed seizures post H₂S exposure. The patient had been seizure free for 5 years prior to H₂S exposure. This case is of considerable interest, since the H₂S or anoxia induced by H₂S may lower the seizure threshold³⁴. This case report is impactful as the general population includes individuals with epilepsy, making them potentially more susceptible to H₂S-induced toxicity.

H₂S poisoning typically occurs acutely, and the manifestation of clinical signs often varies between individuals⁴⁵. Factors that also could contribute to the variability in the toxic outcome include genetic variability, pre-existing conditions, and the duration of unconscious following a knockdown. Many victims of H₂S poisoning are seemingly normal immediately post recovery from unconsciousness, but can relapse and develop complications 3-5 days post exposure. Brain regions affected in victim of H₂S poisoning include the basal ganglia, thalamus, cortex, and brainstem^{7, 43, 49-52}. The deficits in motor function, memory, hearing and vision are attributed to dysfunction in these brain regions, but why these brain regions are specifically more vulnerable to H₂S induced toxicity is unclear. Fatal poisoning causes green discoloration of the brain, specifically in the basal ganglia and the cortex⁴⁶. The exact mechanism leading to the neurodegeneration in these regions is also unknown. Elucidating these mechanisms will provide further insight into the neurotoxicity of H₂S poisoning. .

Molecular Mechanisms of Hydrogen Sulfide Toxicity

The molecular mechanisms of H₂S toxicity are yet to be determined. As mentioned above, one of the broader systemic mechanisms implicated with H₂S toxicity is ischemic hypoxia that could be a culmination of the pulmonary edema, hypotension, and inhibition of cellular respiration via cytochrome c oxidase. Neurodegeneration induced by hypoxic ischemia has been known to occur in poisoning by cyanide, carbon monoxide, and following stroke among others^{53, 54}. While current literature supports this idea, there is no direct link between H₂S and the hypoxic-induced injury. The downstream molecular mechanisms leading to neurodegeneration have not been well characterized. Understanding the mechanism of toxicity will help elucidate potential therapeutic targets of H₂S-induced neurotoxicity.

Much like cyanide, H₂S acts directly by inhibiting the electron transport chain at complex IV (cytochrome c oxidase), the last enzyme prior the complex V, ATP synthase²⁴. The affinity of H₂S to cytochrome c oxidase is much higher than cyanide, making it a more potent inhibitor of this enzyme than cyanide. Cytochrome c oxidase inhibition has been shown in many cellular models of H₂S toxicity including human colon carcinoma cells, hepatocytes, liver mitochondria, human smooth muscle aortic cells, skeletal muscle cell, and cortical neurons⁵⁵⁻⁵⁹. While cytochrome c oxidase inhibition by H₂S is well known, its relevance to H₂S-induced neurotoxicity is uncertain. Since the brain demands high energy, the depletion of cellular energy could potentially interfere with vital cellular functions; but in cyanide models of toxicity, ATP depletion is not necessary for the development of lesions⁶⁰. This could be the case with H₂S as well. The brain potentially could switch to anaerobic metabolism.

Given that H₂S is a gasotransmitter involved in various cellular functions, the direct effects of H₂S on neuronal cellular mechanisms needs to be investigated. Other mechanisms of toxicity have been proposed, but have not been well studied. Reactive oxygen species (ROS) and mitochondrial depolarization have been implicated in H₂S cytotoxicity in hepatocytes^{61, 62}. Aside from cytochrome c oxidase inhibition, ROS formation can potentiate toxicity of H₂S. Glutathione depletion and P450 catalyzed H₂S oxidation contributes to ROS development⁶².

The ERK and caspase-3 dependent apoptosis pathway has also been implicated in the propagation of H₂S-induced cytotoxicity⁶³. While the role of this pathway has not been extensively studied in H₂S intoxication, activation of both ERK and caspase-3 have been shown in cortical neurons and smooth muscle aorta cells treated with H₂S^{56, 63}. These ERK kinases have important signal transduction functions involved in cell growth, differentiation, and programmed cell death⁵⁶. Investigating how H₂S modulates these signal transduction pathways may potentially lead to possible antidotes to treat H₂S-induced neurotoxicity.

H₂S also causes changes in the neurochemical synthesis, breakdown, and modulation of neurochemical receptors leading to cytotoxicity. Specifically, H₂S has been shown to inhibit monoamine oxidase, an important catabolic enzyme for monoamines¹⁸. Catecholamines play an important role in respiratory rhythm controlled by the brainstem; an increase of these neurotransmitters has been suggested to contribute to the toxicity of H₂S¹⁸. H₂S has shown to increase norepinephrine, epinephrine, and serotonin levels in different regions of the brain, specifically the brainstem. These changes can be a factor in increased respiratory frequency noted during the early stages of H₂S intoxication^{18, 43}.

Other neurotransmitters thought to be involved in H₂S toxicity are gamma-aminobutyric acid (GABA) and glutamate^{59, 64}. Modulation of these neurotransmitters has been attributed to the impairment of Na⁺/K⁺ ATPase. There is an efflux of L-glutamate after

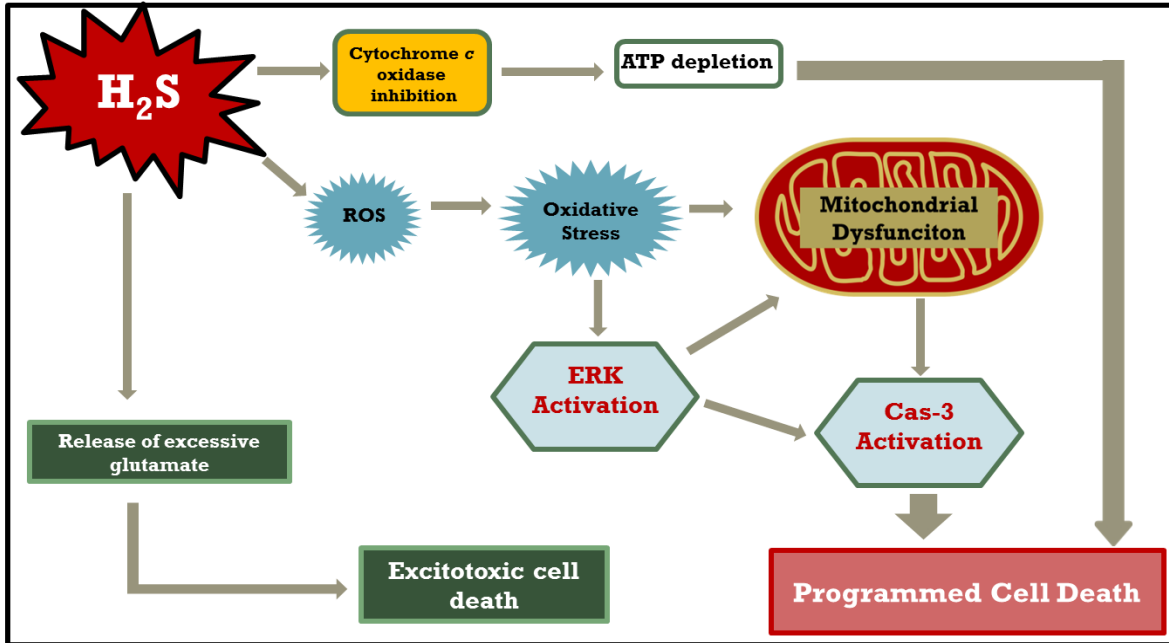


Figure 2. Molecular mechanisms of H₂S-induced neurotoxicity.

Adapted from Cheung et al., Kurokawa et al., Tan et al., and Wang et al.

exposure to H₂S, likely due to an inadequate supply of ATP to the Na⁺/K⁺-ATPase. Inhibition of the Na⁺/K⁺ ATPase reduces the efficiency of the glutamate transporter, leading to glutamate accumulation in the synapse¹⁶. Glutamate neurotoxicity was shown in acute H₂S exposure in rat cerebellar granule neurons, by activating L-type calcium channels. By using an N-methyl d-aspartate (NMDA) antagonist, H₂S-induced neurotoxicity was prevented⁶⁵. H₂S also has been shown to cause a reduction in the uptake of GABA, causing it to accumulate in the synapse as well, although the exact mechanism is unknown¹³. Further experimentation needs to be conducted on how exactly H₂S interferes with the breakdown of these neurotransmitters, but the alteration of these neurotransmitters could be important in the seizure activity observed in victims of H₂S exposure.

Although cytochrome c inhibition by H₂S has been shown time and time again, there are other mechanisms of cytotoxicity that could be contributing factors to H₂S-induced neurodegeneration^{18, 42, 61, 62, 66}. The mechanisms described above are depicted in Figure 2. Understanding these mechanisms can help identify novel therapeutic targets for treatment of H₂S toxicity.

Animal Model of H₂S-induced neurotoxicity

Research has been conducted in order to understand the neurotoxicology induced by H₂S using *in vitro* and *in vivo* models. Unfortunately, a knowledge gap still exists on the mechanisms and pathways of neurodegeneration leading to neurological sequelae. The challenges involved in developing a model have been demonstrated in many studies investigating H₂S induced neurotoxicity. Currently, there is no perfect model of H₂S-induced neurodegeneration.

A key study conducted by Lund et al. highlighted neurodegeneration in rhesus monkeys exposed to H₂S in inhalation chambers⁶⁷. Much like human case reports, the monkeys lost consciousness within 15 min of exposure and had many of the clinical signs of human exposure to H₂S. Knockdown, gasping of breath, shortage of breath, and death occurred during exposure. Following repeated, acute exposures, surviving monkeys lacked movement post exposure, but when they did move, they were disorganized and ataxic. The brains of surviving monkeys were analyzed for histological changes and exhibited necrotic lesions in the cerebrocortex and basal ganglia, specifically in the putamen and caudate nucleus. This study concluded that H₂S poisoning can cause brain damage very similar to that caused by acute lack of oxygen. Importantly, they were able to recapitulate many of the human clinical toxicity⁶⁷.

While only one study has been conducted with monkeys, most animal studies have utilized rodents. The main challenge documented with rodent models of H₂S-induced neurotoxicity is the difficulty in reproducing brain lesions to recapitulate the human condition. A study conducted by Baldelli et al., attempted to produce cerebral necrosis. They utilized a common source of H₂S, sodium hydrosulfide, because it is stable and much easier to work with than H₂S gas⁴⁴. The circulatory and systemic effects of NaSH are identical to those produced by H₂S⁶⁸. Rats were injected with NaSH intraperitoneally to simulate single, high exposure to H₂S. This study was characterized by high mortality, and only 25% of the unventilated rats injected with 120 mg/kg NaSH exhibited necrosis in the brain⁶⁸. Among the ventilated rats, only one rat that survived exhibited cerebrocortex necrosis. The investigators concluded that pulmonary edema and hypotension were contributing factors to the pathogenesis of neuronal necrosis⁶⁸.

A more recent study conducted by Sonobe et al. also injected rats with NaSH was characterized by high mortality, only 4 out of 12 rats survived injections⁶⁹. The investigators injected rats once with NaSH, which produced a coma in about 30% of the rats; they continued to inject the animals up to two more times. Two rats that exhibited persistent motor deficits had extensive neuronal necrosis in the cerebral cortex with more variable lesions in the thalamus, amygdala and the caudate putamen⁶⁹. This study also reiterates the challenge of producing neuronal lesions in rodent models of H₂S-poisoning.

In order to develop effective countermeasures against H₂S-induced neurotoxicity, it is important to develop animal models that recapitulate the human conditions. This includes exposure route, clinical signs, and pathology of the H₂S exposure. A significant effort in this thesis was directed at developing an ideal mouse model to recapitulate the human condition.

Current Treatments for H₂S-induced toxicity

There is no suitable antidote for treatment of acute H₂S toxicity. Developing an antidote that can be administered for treatment of mass civilian exposure to H₂S is particularly necessary. Challenges in developing these treatments include the knowledge gap of the pathologic mechanisms underlying H₂S induced neurotoxicity, lack of an animal model that recapitulates the toxicity observed in humans, the variability in toxic outcomes of victims of H₂S poisoning, and a steep dose-response curve resulting in a narrow range between no effect and toxic concentrations. Furthermore, H₂S disassociates rapidly systemically and can produce downstream effects quickly, causing an additional challenge of developing an antidote that will prevent these changes from occurring.

First, the immediate treatment is to remove the victim from exposure to H₂S as soon as possible, while ensuring rescuers and first responders are properly protected³². Decontamination protocols are followed as H₂S poisoning victims can be continually exposed if H₂S is on their skin and clothes⁷⁰. Once the victim has been removed from H₂S exposure, respiratory support is essential; they are provided with 100% oxygen therapy to focus on airway oxygenation⁴.

Because of some similarities in mechanisms between hydrogen sulfide and cyanide toxicity, treatments used in cyanide poisoning have been advocated. Therapeutic goals are to

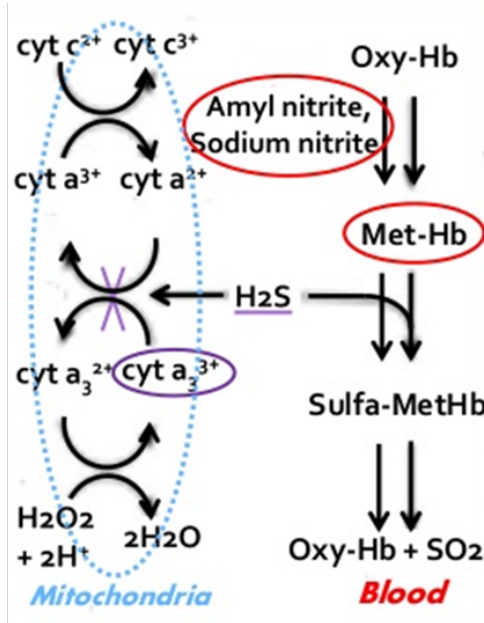


Figure 3. Mechanism of sodium nitrite treatment for H_2S -induced cytochrome c inhibition. Adapted from Smith et al.

inhibit H_2S binding to cytochrome c oxidase enzyme, much like cyanide, although there are different downstream biochemical effects of this binding from each chemical. Because of this, treatment with nitrites (sodium or amyl nitrite) has been used as an antidote for H_2S intoxication (Figure 3)⁷¹⁻⁷³. In a hospital setting, 3% sodium nitrite treatment is administered intravenously; this induces the production of methemoglobinemia, creating sulfmethemoglobin, which has a greater affinity for H_2S than cytochrome c oxidase but is less toxic, thus freeing cytochrome c oxidase allowing cellular respiration to resume^{48, 70, 71, 74}. This detoxifies

H_2S in the blood as well.

Using nitrite has its disadvantages; further studies with nitrite have shown nitrite to be ineffective if given more than a few minutes post H_2S exposure, and sometimes even slow detoxification thereafter⁴³. Given that nitrite needs to be given immediately after exposure, this might not be practically feasible as the route of administration (intravenous) requires medical expertise. If nitrites are given too aggressively, they can complicate and compound the toxicity of H_2S by contributing to hypotension^{4, 42}. Nitrite treatment can also cause vomiting, headaches, and excessive methemoglobin formation⁷⁵.

Another common treatment for H₂S intoxication, sometimes used in conjunction with nitrite therapy, is hyperbaric oxygen therapy⁷⁶. This involves breathing pure oxygen in a pressurized setting; the air pressure is increased to three times higher than normal air pressure. Under these conditions, lungs and other peripheral tissues are able to receive more oxygen than pure oxygen at regular pressure⁷⁷. Oxygen is also able to detoxify H₂S by oxidizing sulfide to sulfate and thiosulfate^{76, 78}. Along with any direct effects on H₂S toxicity, hyperbaric oxygen therapy also prompts vasoconstriction, inhibits an inflammatory response, increase nerve cell regeneration, and reduces cerebral edema^{79, 80}.

As with nitrite treatment for H₂S poisoning, there are disadvantages to hyperbaric oxygen therapy. Hyperbaric oxygen therapy needs to be administered in chambers or specialized room (Figure 4) requiring the victim to be transported to an emergency setting where this equipment is available. Toxicity associated with hyperbaric

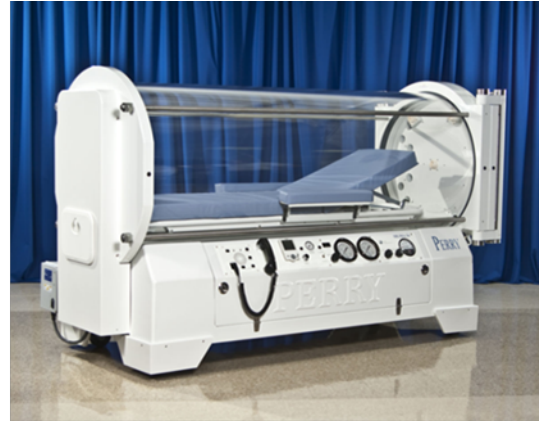


Figure 4. Hyperbaric oxygen chamber
<http://blog.medfriendly.com/2012/09/can-hyperbaric-oxygen-therapy-improve.html>

oxygen therapy have also been reported; patients can experience barotrauma due to the increased air pressure, oxygen toxicity, and general risk of transferring an unstable patient to the hyperbaric oxygen therapy chamber⁷⁵.

Normally, treatment in a hospital setting can involve both hyperbaric oxygen therapy administered with nitrite injection. Laboratory studies have found co-administration of these therapies are far more successful combined than either given alone⁸¹. Other antioxidants that generate methemoglobin that have been used include 4-dimethylaminophenolm and

thiosulfate, and can be used with combination hyperbaric oxygen therapy. Unfortunately, these treatment modalities require hospital based settings and cannot be used in the field for treatment of mass civilian casualties following acute H₂S exposure.

In the more recent years, an emerging antidote for cyanide and H₂S poisoning is vitamin B12 and analogs. Cobalt compounds, such as hydroxocobalamin can combine with free H₂S and catalyze its oxidation during H₂S exposure⁸². Hydroxocobalamin is an FDA approved antidote for treatment of cyanide poisoning⁸² and has to be administered intravenously due to the large volume required to treat cyanide poisoning. In experimental settings, hydroxocobalamin has been shown to reduce concentrations of soluble H₂S to almost zero during infusion of severe levels of H₂S⁸³. Alternatively, hydroxocobalamin also can exert anti-nitric oxide effects which could counteract some of the H₂S toxicity. One case report describes the use of hydroxocobalamin in a patient who committed suicide by combining household commercial products⁸⁴. The patient was given hydroxocobalamin upon arrival to the hospital. Importantly, thiosulfate and sulfate levels were measured before and after administration of hydroxocobalamin. The results showed hydroxocobalamin reduced serum sulfide and thiosulfate concentrations indicating hydroxocobalamin might be effective in treating H₂S intoxication⁸⁴. There are no studies evaluating and characterizing hydroxocobalamin for treatment of H₂S poisoning in clinically relevant large animal models. Other vitamin B12 analogs are being investigated for treatment for H₂S-induced neurotoxicity, specifically Cobinamide, a precursor to Cobalamin, will be discussed in further detail below.

The treatments described above are recommended to be given within 5-15 minutes of H₂S exposure⁸⁵. Given that these need to be given intravenously, starting an intravenous line

can take several minutes, making that window even narrower. Currently, there is no antidote for treatment of H₂S intoxication that can be utilized in the field in case of a mass civilian exposure, such as in the case of a catastrophic industrial accident or following terrorism. An antidote that can be given quickly and efficiently is much needed, preferably administered intramuscularly from a pre-filled syringe, or intranasal administration. This requires the antidote to be potent, administered in small volume, and rapidly absorbed post intramuscular injection and can be stored long term.

Potential Therapeutics

Binding Agents

Drugs that directly bind H₂S to enhance its excretion before it can exert its toxic effects may be helpful but this has been proven to be challenging as H₂S disassociates rapidly *in vivo*. The challenge with current binding agents is that they need to be administered during exposure or immediately after.

Cobinamide is the precursor to hydroxocobalamin and is currently being investigated as a cyanide antidote as well. Cobinamide is water-soluble and can be rapidly absorbed following intramuscular injection, making it ideal for intramuscular emergency injections in the field. It has been shown that cobinamide is a better H₂S antidote compared to hydroxocobalamin^{85, 86}. Given that it has a high affinity for the sulfide and it readily reacts with sulfide, cobinamide is able to bind 2 moles of sulfide and excrete it from the circulatory system to prevent further poisoning by H₂S^{44, 85}.

While cobinamide has been more extensively studied as treatment for cyanide poisoning, recent studies have been investigating cobinamide for treatment of H₂S-induced toxicity *in vitro* and *in vivo*. A study conducted by Jiang et al. investigated *in vitro* mechanisms of H₂S toxicity. In many cell types, Jiang et al. observed H₂S inhibited

cytochrome c oxidase activity and induced apoptosis. More importantly, cobinamide reversed the cellular toxicity of sulfide. In a lethality study, cobinamide rescued *Drosophila melanogaster* and mice from lethal exposures of H₂S. Limitations of this study include the route of administration, cobinamide was injected intraperitoneally, and the mice were anesthetized during exposure to H₂S, not allowing for assessment of clinical signs of toxicity during exposure.

A second study conducted by Bebarta et al., investigated the efficacy of cobinamide compared to hydroxocobalamin for treatment of H₂S toxicity in swine. This study administered lethal concentrations of H₂S via NaSH infusion, and injected the swine 1 min post exposure. While all of the cobinamide infused swine survived, none of the swine that received hydroxocobalamin and saline infusions survived. Cobinamide also prevented vital signs and arterial blood gases from deteriorating. The objective of the study was to show efficacy of cobinamide over hydroxocobalamin, but this does not address the use of cobinamide as an agent for mass civilian exposure; NaSH was used by infusion. This is a problem as this route of H₂S exposure does not recapitulate real life exposure scenarios.

Based on both these studies, further work needs to be done to evaluate cobinamide in a well characterized model of H₂S induced toxicity. Not only do we need to investigate if cobinamide prevents H₂S-induced lethality, but also if the compound can prevent clinical toxicity and reduce neurological sequelae that occur in human intoxication cases.

Anti-convulsants

Acute exposure to high levels of H₂S have been implicated in the pathology of seizure-like events, glutamate and GABA transmission, and involved in glutamate excitotoxicity^{59, 87, 88}. Preventing seizure activity and associated neurological sequelae is a

potential therapeutic strategy of H₂S intoxication. Our studies have indicated that the severity of seizure activity is correlated to mortality; anticonvulsants, a group of drugs used to treat epilepsy, could be potentially be used to treat this.

Common anti-convulsants include diazepam and midazolam, but midazolam is currently considered to be superior to diazepam in efficacy. Midazolam is a common benzodiazepine used for its powerful anxiolytic, sedative, anticonvulsant, and amnestic properties. Due to its short half-life and rapid onset, midazolam is being recommended as a drug of choice in anticonvulsant

kits⁸⁹. It has been approved for prehospital treatment of epileptics, and is listed as an essential medication according to the World Health Organization⁹⁰.

Midazolam is very water soluble, causing it to have better injection site tolerance and more readily

absorbed⁸⁹. The dual chemical structure of midazolam allows it to have different configurations based on the pH of the solution (Figure 5), allowing it be absorbed and to enter the brain rather easily. Maximum plasma concentration is reached in about 30 minutes post injection with 90% bioavailability^{89,91}. The shelf life of midazolam is also much longer; it can be stored for about 36 months at room temperature. Midazolam is currently being considered to replace diazepam as an anticonvulsant for nerve agent exposure⁸⁹.

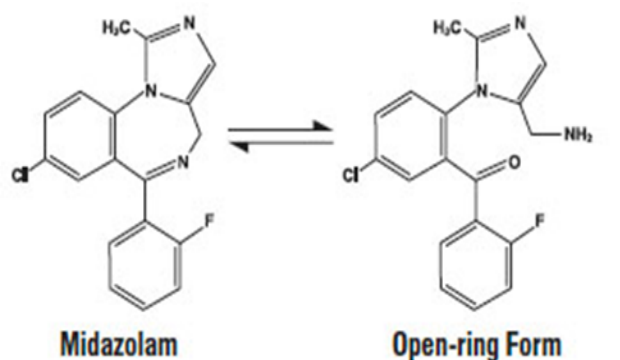


Figure 5. Configuration of Midazolam in physiologic pH
<https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl>.

Midazolam exerts its anticonvulsant effects by allosterically potentiating GABA_A receptors in the synapse^{89, 92}. It does not activate GABA_A directly, but allosterically modulates the effects of GABA, the primary inhibitory neurochemical in the brain. Midazolam binds to the gamma containing receptors which leads to allosteric potentiation of GABA gated hyperpolarization of the cell, inhibiting excitability⁸⁹.

Midazolam has also been used for critically ill patients suffering from pathologic effects of oxidative stress such as infection, hemodynamic instability, and hypoxia⁹³. There is evidence supporting the inverse correlation between midazolam and ROS. Midazolam has been shown to interfere with the synthesis and release of NO and TNF-alpha⁹³. Midazolam also exerts protective effects during oxidative stress through the activation of Akt via phosphorylation in neuronal cells. Akt phosphorylation plays an important role in cell proliferation and cell survival^{93,94}.

Given that midazolam possesses anti-seizure activity and inhibits the effects of ROS, midazolam could exert efficacy by interfering with the seizure activity induced by H₂S and by protecting the cells from ROS produced by H₂S intoxication.

Inflammation and H₂S-induced injury

Neuroinflammation is now recognized as a significant contributor to neurodegeneration⁹⁵. It is generally known that microglial and astroglial activation, secretion of pro-inflammatory cytokines and nitric oxide production have all been implicated in hypoxia-related injury, as is present following H₂S exposure. About 10% of the brains' glia are microglia which typically display a ramified morphology in their resting state. Microglia remove debris and toxins, help to establish synapses during development, and produce trophic factors^{96,97}. Upon stimulation by antigens, mis-folded proteins, or interleukins,

microglia can become activated. In the activated state, microglia morphology changes to a larger cell body with shorter, thickened processes^{98,99}. These classically activated microglia are referred to as having an amoeboid shape and are known to secrete pro-inflammatory factors. Another activation phenotype deemed ‘alternative’ activation is associated with wound healing and repair, where the cells instead produce protective factors such as IL-10 and arginase 1. Other intermediate stages of microglial activation have also been proposed¹⁰⁰. Microglia can also become classically activated by neuronal damage in a self-propelling process referred to as reactive microgliosis. Astrocytes are the most abundant glia in the brain. Astrocytes migrate to the site of injury and develop a hypertrophic morphology during neurodegenerative processes^{101,102}. These glia, when activated, produce cytokines to set off a cascade to mitigate the toxic effects of any insult. When reactive microgliosis occurs, it is possible that this process can become detrimental and lead to neurodegeneration.

Bebarta et al, found an increase in TNF- α , IL-1 β , IL-6 from exposure to NaSH in the serum¹⁰³. It is not known why this increase was observed, whether it was due to hypoxic conditions or another mechanism. Hypoxia is known to induce the expression of cytokines such as TNF- α , IL-1 β , IL-6, and IL-8¹⁰⁴, however at low, physiologic conditions H₂S has been shown to diminish the secretion of pro-inflammatory cytokines^{105,106}. Further studies would need to be done to evaluate the role of H₂S on neuroinflammation and whether persistent neuroinflammation leads to the neurodegeneration observed in H₂S intoxication.

CHAPTER 2

**CHARACTERIZING A MOUSE MODEL FOR EVALUATION OF
COUNTERMEASURES AGAINST HYDROGEN SULFIDE-INDUCED
NEUROTOXICITY AND NEUROLOGICAL SEQUELAE**

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Abstract

Hydrogen sulfide (H₂S) is a highly neurotoxic gas. It is the second most common cause of gas-induced deaths. Besides mortality; surviving victims of acute exposure may suffer long-term neurological sequelae. There is a need to develop countermeasures against H₂S poisoning. However, no translational animal model of H₂S-induced neurological sequelae exists. We hereby describe a novel mouse model of H₂S-induced neurotoxicity for translational research. Paradigm I: C57/BL6 mice were exposed to 765 ppm H₂S for 40 min on day 1, followed by 15 min daily exposures for periods ranging from 1 to 6 days. Paradigm II: mice were exposed once to 1000 ppm H₂S for 60 mins. Mice were assessed for behavioral, neurochemical, biochemical, and histopathological changes. H₂S intoxication caused seizures, dyspnea, respiratory depression, knockdowns, and death. H₂S-exposed mice showed significant impairment in locomotor and coordinated motor movement activity compared to the controls. Histopathology revealed neurodegenerative lesions in the collicular, thalamic, and cortical brain regions. H₂S significantly increased dopamine and serotonin concentration in several brain regions and caused time-dependent decrease in GABA and glutamate concentrations. Further, H₂S significantly suppressed cytochrome c oxidase activity and caused significant loss in body weight. Overall, male mice were more sensitive than females. This novel translational mouse model of H₂S-induced neurotoxicity is reliable, reproducible, and recapitulates acute H₂S poisoning in humans.

Keywords

Inhalation exposure, hydrogen sulfide, neurotoxicity, neurodegeneration, acute toxicity, translational model

Abbreviations

TIC, toxic industrial compounds; IC, Inferior colliculus; GABA, gamma-aminobutyric acid; DA, dopamine; DOPAC, 3,4 3,4-Dihydroxyphenylacetic acid; HVA, homovanillic acid; NE, norepinephrine; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; 4-HNE, 4-hydroxynonenol; Iba-1, inducible calcium binding adapter molecule 1; GFAP, glial fibrillary acidic protein; NeuN, Hexaribonucleotide Binding Protein-3; Glu-d5, L-glutamic-2,3,3,4,4-d5 acid; 4-aminobutyric-2,2,3,3,4,4-d6 acid-GABA-d6; PFA, paraformaldehyde; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; TBS, tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; EGTA, Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid; NaF, sodium fluoride; Na₃VO₄, sodium orthovanadate; Na₄P₂O₇, sodium pyrophosphate tetrabasic; HClO₄, perchloric acid; DAB, diaminobenzidine; COS, Carbonyl Sulfide; NaSH, Sodium Hydrosulfide.

Introduction

Hydrogen sulfide (H₂S) is typical of compounds which are beneficial at normal physiological concentrations, but toxic at high doses. Endogenously produced H₂S functions as a signaling molecule in the brain^{1, 2}. However, H₂S in high acute dose exposures is a severely toxic xenobiotic^{3, 4}. H₂S is a by-product of many industries, including the oil and gas industry, intensive animal farming operations, sewer and waste treatment plants, and is present in gas storage facilities⁵⁻⁸. It is also used as a raw material for several industrial applications⁸. It is classified as a highly toxic industrial compound (TIC) and is a second leading cause of gas-induced death after carbon monoxide^{9, 10}. H₂S is a colorless gas and has a distinctive rotten egg odor. It is sometimes referred to as sewer gas. It is heavier than air,

and therefore collects in low lying enclosed spaces such as manholes, sewer lines, and manure pits where it is a hazard^{11, 12}. It can be made easily and inexpensively at home from raw materials commonly found in local stores. For this reason, it is increasingly being used as suicidal agent^{13, 14}. H₂S was used in World War I as a chemical weapon¹⁵. For this reason, there is significant concern by the Department of Homeland Security about its potential misuse as a weapon of mass destruction, particularly in confined spaces such as underground transit facilities¹⁶. Mass civilian exposure to H₂S can also happen if terrorists target H₂S storage facilities, or industrial plants producing or using this gas in or near highly populated areas¹⁷. Industrial plant accidents can also lead to mass civilian population exposures and deaths, as occurred in the Poza Rica Mexico recycling¹⁸ or in sour gas well blow outs, as occurred in Kaixian district in China which resulted in 243 civilian deaths¹⁹.

Acute toxicity and lingering long-term consequences of acute H₂S exposure in humans have been reported for many years^{5, 20, 21-25}. Acute exposure to high levels of H₂S have been reported to cause acute neurotoxic, cardiovascular, and respiratory effects, as well as long-term neurological sequelae, including neurodegeneration, memory and motor impairment, neuropsychiatric disturbances, moribund state, and even death^{6, 12, 22-25}. It is a systemic toxicant, but the central nervous system, respiratory, and cardiovascular systems are most sensitive^{6, 26}. Clinical signs of acute H₂S intoxication include convulsions, respiratory distress, and acute death^{6, 26, 27}. H₂S is also known as the “knockdown” gas. A few breaths at a high concentration can cause immediate collapse without warning²⁴. Some survivors of acute H₂S exposure suffer lingering neurotoxic effects, including movement disorders, persistent headache, seizures, neurobehavioral and cognitive deficits, blindness, hearing impairment, and sometimes, permanent vegetative states^{6, 12, 22, 23, 25, 28, 29}. The toxic

mechanisms of H₂S poisoning are not well known. However, ischemia-hypoxia, cytochrome c oxidase enzyme inhibition, changes in brain amino acid profiles, changes in neurotransmitters, and oxidative stress have been proposed as some of the neurotoxic mechanisms^{30, 31}. Regardless of the trigger of H₂S-induced neurotoxicity, the downstream cellular molecular mechanisms leading to neurodegeneration are yet to be defined^{6, 23, 32}.

Currently, there is no antidote for treatment of H₂S poisoning, particularly for field treatment of mass civilian casualties in the event of terrorism or industrial accidents. Our lab is interested in treatment of acute neurotoxicity and neurological sequelae of H₂S-induced poisoning. The objective of this study was to develop a novel translational mouse model of H₂S neurotoxicity to recapitulate human inhalation exposure and subsequent health effects. This model will be used to study H₂S-induced neurotoxic mechanisms and as a benchmark to evaluate the efficacy of potential countermeasures for treatment of acute H₂S poisoning. Prior to commencement of this study, an extensive review of the literature revealed that existing animal models do not faithfully recapitulate the human exposure routes and or/focus on modeling H₂S-induced mortality. For example, the Jiang et al. mouse model used anesthetized mice in their H₂S inhalation exposure model³³. In rat models, rats were injected with sodium hydrosulfide (NaHS) intraperitoneally, which does not faithfully reflect the common route of exposure in humans, which is inhalation^{34, 35}. Other mouse models of inhalation exposure only reported metabolic effects and did not report behavioral changes or neurodegeneration^{36, 37}. Our mouse model is unique because it is comprehensive, uses freely moving mice exposed to H₂S by whole body inhalation exposure, recapitulating the typical human exposure route. Most importantly, this mouse model reproduced significant

neurological lesions in different parts of the brain and allows evaluation of drug candidates easily.

Materials and Methods

Chemicals and Reagents

Methanol (HPLC grade), acetonitrile (HPLC grade), MD-TM mobile phase, sodium dodecyl sulfate (SDS), Tris-Cl, phosphate buffered saline (PBS), Alexa Fluor 680 goat anti-mouse secondary antibody, and formic acid were obtained from Thermo Fisher Scientific (Waltham, MA). (D- and L-) Glutamic acid, γ -aminobutyric acid (GABA), dopamine (DA), 3,4 dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), paraformaldehyde (PFA), ethylenediaminetetraacetic acid (EDTA), Triton X-100, Sodium Chloride (NaCl), Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Sodium Fluoride (NaF), Sodium orthovanadate (Na_3VO_4), Sodium pyrophosphate tetrabasic ($\text{Na}_4\text{P}_2\text{O}_7$), sodium deoxycholate, and 60% perchloric acid (HClO_4) were purchased from Sigma Aldrich (St Louis, MO). L-glutamic-2,3,3,4,4-d5 acid (Glu-d5) and 4-aminobutyric-2,2,3,3,4,4-d6 acid (GABA-d6) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). IRDye 800 donkey anti-rabbit secondary antibody was purchased from Rockland Antibodies and Assays (Limerick, PA) Li-COR Blocking buffer was purchased from LI-COR (Lincoln, Nebraska). Tris- buffered saline (TBS) and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA) Fluoro Jade C was purchased from Histo-Chem, Inc. (Jefferson, AR). Vectastain Elite ABC HRP kit and diaminobenzidine (DAB) was purchased from Vectastain Laboratories (Burlingame, CA). NeuN, Iba-1, and GFAP antibody was

purchased from Abcam (Cambridge, UK). Aqueous solutions were prepared using 18.2 M Ω ·cm water (Aries Filter work, Berlin Township, NJ).

Animals

All animal studies were approved by the Iowa State University Institutional Animal Care and Use Committee. The 7-8 week old C57/BL6 mice used in these studies were purchased from The Jackson Laboratories (Bar Harbor, ME). In Paradigm I experiments both male and female mice were included in the experiment. In Paradigm II experiments only male mice were used. All mice weighed 20-25g at the beginning of the experiment. Mice were housed 5 per cage, for each sex, in the Laboratory Animal Resource Facility of the Iowa State University College of Veterinary Medicine (ISU CVM, Ames, IA). They were housed at a room temperature of 68-70°F, relative humidity of 35-50%, and a 12-hr light/dark cycle, and were provided 14% Protein Rodent maintenance diet (Teklad HSD Inc., WI, USA) and drinking water ad libitum. Mice were acclimated for 1 week prior to the start of the studies.

H₂S Inhalation Exposure

Fully conscious and freely moving mice were placed in a whole body exposure chamber (Fig 1A). The chamber is designed to hold up to 10 mice at a time. Hydrogen sulfide is a highly toxic gas and all experiments were conducted under a chemical fume hood certified by the Environmental Health & Safety at ISU. Gas access to the chamber was from two lines; one for breathing air tank under pressure, and the other from a tank containing H₂S under pressure. The two lines connect to a control panel that allowed separate regulation of breathing air and gas in flow (liters/min). Breathing air was introduced first, followed by H₂S 2 min later. The concentration of H₂S in the exposure chamber was constantly monitored

using a H₂S monitor (Environmental Equipment and Supply, Harrisburg, PA) that was custom designed to measure concentrations of up to 1000 ppm of H₂S.

Exposure Paradigm I

The exposure Paradigm I is summarized in Fig.1B. Mice were exposed to H₂S by inhalation either once or they received 2-7 short additional acute exposures. On the first day of H₂S exposure, mice in groups of 10 were exposed to 765 ppm H₂S for 40 min. On subsequent days, groups of mice were exposed to 765 ppm H₂S for 15 min, each day. Following each exposure, the chamber was flushed out with breathing air for 2 mins before mice could be safely removed. Separate groups of mice were euthanized after 1, 3, or 7 exposures, 2-10 min following removal from the H₂S exposure chamber. Mice in the negative control breathing air group were exposed following a similar paradigm but were euthanized after the 7th exposure. This experimental approach allowed progressive evaluation of clinical signs, behavioral deficits, and histological lesions in brains of mice following a single or repeated short-term exposure to H₂S over a 1 week period.

Clinical Assessment

To obtain baseline data, animals were clinically evaluated and weighed starting 3 days prior to H₂S exposure. Mice were weighed daily until euthanasia. Toxicity signs monitored during H₂S exposure were a modification of the McDaniel and Moser functional observational battery³⁸. Adopted endpoints included dyspnea, seizure activity, and latency to seizure activity. Knockdown, a clinical effect of H₂S poisoning, in mice presented as lateral recumbency following seizure activity was an endpoint that we added. The time latency to knockdown and number of mice in knockdown were recorded. Mice were evaluated for these

clinical signs during exposure in the chamber and again 2 hrs post-exposure. For post-exposure assessment, mice were removed from their home cages and placed individually in an empty chamber for 2 min to assess gait, posture, tremors or convulsions. The same trained observer assessed the mice throughout the entirety of the experiment.

Behavior Testing

Behavior assessments for open field activity were performed 3 hrs after mice were exposed to H₂S. This was performed after 2, 4, or 6 H₂S exposures using a protocol adapted from Gosh et al 39 Briefly, an automated computer-controlled device (Model RXYZCM-16; Accuscan, Columbus, OH, USA) was used to measure the spontaneous activity of mice in this open field test. The dimensions of the activity chamber were 40 × 40 × 30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. Data was collected and analyzed by a VersaMax Analyzer (Model CDA-8; AccuScan). Mice were acclimated to the chamber two days before H₂S exposure. On test days, mice were placed inside the infrared monitor for 2 min to acclimate to the chamber. Open field activities were recorded for 10-minute test sessions assessing multiple parameters including vertical activity, horizontal activity, and distance traveled. To test for coordination and balance, mice were subjected to the AccuRotor 4-Channel RotaRod test (rod diameter = 30 mm, height= 38 cm). Mice were also trained on the rotating rod for two consecutive days prior to H₂S exposure at 24 rpm for 20 min. Following H₂S exposure, mice were given 5 trials for 20 min at 24 rpm on test days post H₂S exposure.

Thiosulfate analysis

Thiosulfate is a good biomarker of H₂S exposure. Blood was allowed to clot at room temperature for 15 min, and then spun down at 2,900 x g for 10 min. The serum was analyzed for thiosulfate concentration according to Togawa et al⁴⁰. Briefly, a 50 µl serum sample was added to a 7 dram glass vial, along with 50 µl water and 100 µl 5mM monobromobimane. This was vortexed and incubated for 1 h at room temperature in the dark. A standard curve was prepared by adding using 100 µl water and 100 µl 5mM monobromobimane as a blank along with 3 standards of 55, 110, 220 ng of thiosulfate adjusting with water to a volume 100 µl. A 100 µl volume of 5 mM monobromobimane was added to all. After 1 hr, 100 µl of stopping reagent 0.05M KCl-HCl (pH 2.0) was added to all samples. All samples were analyzed by HPLC with fluorescence detection. The mobile phase consisted of 25 mM sodium perchlorate (pH 3.0) with acetic acid:acetonitrile, 70:30, V:V, with a flow rate of 1 mL/min on a Waters separation module 2695 HPLC system. A waters 2475 fluorescent detector was used with an Ex-396 nm and Em-476 nm. The thiosulfate was separated on a Dionex WAX-1 150 x 4.6 mm column; retention time was approximately 15 min.

Histopathology and Immunohistochemistry

Mice were euthanized 24 hrs after the last H₂S exposure. First, mice were anesthetized deeply with a cocktail of 100 mg/kg body weight ketamine and 10 mg/kg body weight xylazine given IM. Once in a surgical plane of anesthesia, the thoracic cavity was surgically opened to expose the heart. Fresh 4% paraformaldehyde solution (PFA, pH 7.4) was then injected through the left ventricle to perfuse the animal. After perfusion, brains

were post-fixed in 4% PFA for 24 hrs, paraffin embedded, sectioned at 5 microns, and stained with hematoxylin and eosin for routine histopathology. Additional brain sections were stained using fluoro jade C or stained using an indirect immunostaining protocol that employed primary antibodies directed against NeuN, GFAP, Iba-1 , or 4-HNE. Diaminobenzidine was the chromogen used. Stained sections were examined microscopically using a Nikon Eclipse Ci-L microscope with DS-Fi2 camera or EVOS FL fluorescence microscope. Routine histopathology, as well as immunohistopathology, was conducted by a board-certified pathologist blinded to the study design. The semi-quantitative scale used for scoring the severity of lesions is summarized in Supplemental Table 1.

Cytochrome c oxidase enzyme activity

Mice for all biochemical assays were euthanized by decapitation. Brains were immediately removed from the skull and micro dissected into different brain regions on ice. Brain tissue samples were stored at -80 °C until analysis. Cytochrome c oxidase enzyme was extracted from micro-dissected brain regions and enzyme activity determined using an assay kit (ab109909) from Abcam (Cambridge, MA, USA) according to manufacturer's protocol.

Neurochemical analysis

Monoamines

To determine H₂S-induced neurochemical changes, different brain regions were analyzed for changes in dopamine (DA) and its metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Samples from these brain regions were also analyzed for serotonin (5-HT), its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) as well as norepinephrine (NE). Samples were prepared and quantified as described previously⁴¹.

Briefly, neurotransmitters were extracted from different brain regions in 0.2 M perchloric acid solution containing 0.05% Na₂EDTA, 0.1% Na₂S₂O₅, and isoproterenol (internal standard).

DA, 5-HT, NE, and their respective metabolites were separated isocratically by a reverse-phase column with a flow rate of 0.6 ml/min mobile phase (10% acetonitrile, 1% sodium phosphate, 89% water) using a Dionex Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL) equipped with a refrigerated automatic sampler (model WPS-3000TSL). The electrochemical detection system included a CoulArray model 5600A coupled with an analytical cell (microdialysis cell 5014B) and a guard cell (model 5020). Data acquisition and analysis were performed using Chromeleon 7 and ESA Coularray 3.10 HPLC Software.

GABA/Glutamate

Micro-dissected mouse brain tissues were weighed and transferred to 1.5 mL Eppendorf tubes. All samples were spiked with 10 µl Glu-d5 (1400 µg/mL) and GABA-d6 (780 µg/ml) as internal standards and mixed thoroughly. This procedure was performed on ice. Subsequently, samples were extracted with 900 µl Methanol/water 85/15 (v/v) 42 and vortex-mixed for 5 min at 2500 rpm on a multi-tube vortexer for thorough mixing. The mixtures were then filtered through 0.45 µm filter discs and subject to LC-MS/MS analysis.

GABA and glutamate levels were measured by LC-MS/MS analysis on a Quattro Premier XE triple quadrupole mass spectrometer (Milford, MA), equipped with a Waters e2695 HPLC separation module. The separation of GABA and glutamic acid was performed by a reversed phase C18 HPLC column (Luna, 150×4.6 mm, 5 µm) obtained from

Phenomenex (Torrance, CA). The mobile phase consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B). The analysis was performed using an isocratic elution of 75%:25% mobile phase A:mobile phase B at a rate of 0.6 ml/min. Column temperature was 30°C, and autosampler temperature was 4°C. Analytes were detected in the positive electrospray ionization mode with the capillary voltage of 3.1 kV. The source temperature was 350°C. The desolvation gas flow rate was 800 l/hr. the cone gas flow rate was 20 l/hr. Data were acquired and analyzed using MassLynx version 4.1 (Waters, Milford, MA).

Western Blot

Micro-dissected brain tissues were lysed in modified RIPA lysis buffer (1% Triton X-100, 1 mM EDTA, 100mM NaCl, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 50 mM Tris-HCl, pH 7.4) via sonication. Brain homogenates were prepared as described previously⁴³. Protein concentration of samples was measured using the Bradford assay. Western blotting was performed as described previously 43. Briefly, the samples containing equal amounts of proteins were loaded and fractionated in a 10-12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in TBS supplemented with 0.1 % Tween-20 or Li-cor blocking buffer. Primary antibodies against specific proteins were incubated with the membrane overnight at 4°C. After rinsing thoroughly in PBS supplemented with 0.1% Tween-20, the membrane was incubated with Alexa Fluor 680 goat anti-mouse or IRDye 800 donkey anti-rabbit secondary antibodies. For the loading control, β -actin antibody was used. Immunoblot imaging was performed with an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA). ImageJ software (National Institutes of Health, Bethesda MD, USA) was used to quantify Western blot bands.

Data Analyses

Data are presented as mean and standard error of the mean. Biochemical and neurochemical endpoints were analyzed using a student's t-test comparing the H₂S exposed mice to the breathing air mice. Behavioral data was analyzed using 2-way ANOVA with a post-hoc test comparing the H₂S groups with the breathing air group. Endpoint data from male and female mice were compared using analyses of variance (ANOVA) models, with treatment, gender and their interaction and fixed effects. Behavioral data were analyzed using repeated measures ANOVA models, with treatment, day, gender and their interactions and fixed effects, and mouse as the subject of repeated measures. Statistical tests were performed on Prism 6 (GraphPad Prism Software) or SAS. A p value less than 0.05 was accepted as statistically significant.

Exposure Paradigm II

Exposure Paradigm II is summarized in Fig 7A. This paradigm reflects the typical human exposure scenario of a single acute exposure to high H₂S concentrations, followed by rescue. This experiment used only male mice because results of previous experiments indicated that males are more sensitive to H₂S than females. Separate groups of mice were exposed either to breathing air or 1000 ppm

H₂S by whole body inhalation for 60 min. Following exposure, the chamber was flushed out with breathing air for 2 min before mice were retrieved from the chamber. Mice were euthanized 7 days post-H₂S exposure for histopathological evaluation of brain lesions using the same protocol as in Paradigm I above.

Results

Paradigm I: H₂S induced seizures and knockdowns in mice

Both male and female mice exposed to H₂S showed similar clinical toxic effects.

The main clinical effects of H₂S exposure are summarized in Supplemental Table 2. Mice exposed to H₂S exhibited hunched posture, piloerection, and dyspnea during exposure. Neurologic effects observed during exposure included tonic-clonic seizures, knockdown, and ataxia (Suppl. Table 2). Prior to seizure activity, mice exhibited characteristic running fits followed by tonic-clonic seizures with paddling of limbs and urination. Salivation was frequently observed in mice in knockdown state. Upon termination of gas exposure, mice regained consciousness and activity within 5-10 min. For mice which received more than one exposure, a notable observation was that H₂S-exposed mice showed increased sensitivity with each subsequent exposure. For example, during the first exposure the seizures were not observed until after 20 min of H₂S exposure, whereas seizures were observed as early as 5 min in subsequent exposures (Fig.1C). The average time mice experienced the first seizures and knockdown was shorter in males than in females, indicating that males are more sensitive than females. In males, the average time at first seizure was 21±6 min while in females it was observed at 29±4 min. The first knockdown was observed at 25±9 min in males, compared with 27±4 min in females. Cumulative mortality at the end of the study was relatively low; 15% for male mice and 13% for female mice. H₂S also caused a statistically significant loss in body weight in both male and female mice, compared with controls. Negative control mice exposed to breathing air continued to gain weight during the course of the study (Fig.1D).

Evaluation of behavioral deficits caused by H₂S exposure was tested by performance on the RotaRod and VersaMax open field tests. Mice exposed to H₂S had a statistically significant reduction in locomotor activity in the open field test based on vertical activity, horizontal activity, and total distance traveled compared with negative controls. On the RotaRod, mice exposed to H₂S fell off the RotaRod at a significantly shorter time compared with negative controls. Statistically significant changes were observed on all days of behavioral assessment for males including following a single exposure. In female mice, however, changes were observed on days 4 and 6, again suggesting higher susceptibility of males than females (Fig. 1E).

Thiosulfate is an oxidation product of H₂S and is used as a biomarker of H₂S exposure 14, 44. In this model, thiosulfate levels were significantly increased in H₂S-exposed mice in both sexes compared with control mice (Suppl. Fig 1). Our data provides further confirmation over the validity of our model to assess H₂S-induced neurodegenerative changes. Collectively, these results indicate that H₂S caused significant motor impairment in mice exposed to H₂S relative to breathing air negative control, with the males being more sensitive than females.

H₂S-induced lesions in the brain

H₂S exposure induced morphological changes in select brain regions. Brain regions affected and the severity of neurological lesions evaluated are summarized in Fig 2. Neurodegeneration was observed in different brain regions but especially in the inferior colliculus (IC) and the thalamus. Severity of lesion increased with additional exposures and varied from activated glial cells and oxidative stress with single or two exposures to frank

bilateral or, much less commonly, unilateral, well-circumscribed necrosis in the IC and or thalamus on day 7 (Fig 2). Bilateral neurodegeneration and necrosis of the IC was consistently present following 7 days of H₂S exposure. This was the first region to be affected and lesions were noticeable as early as 3 days after H₂S exposure. The thalamus was the second regions most frequently affected after the IC. As in the IC, thalamic lesions were also well demarcated from adjacent, non-necrotic tissue. In both regions, lesions began as fine vacuolation of the neuropil, followed by gliosis, and degeneration and necrosis of neurons, ultimately with loss of all cellular components. Mild to moderate hemorrhage was also frequently observed, especially in the IC region. Late stage morphological changes were characterized by gliosis and Gitter cells phagocytosis of cellular debris, as well as proliferation of capillaries in necrotic areas. H₂S inhibited cytochrome c oxidase activity in mice

We characterized the effects of H₂S in different brain regions over time. Results are summarized in Fig 3. H₂S statistically significantly inhibited cytochrome c oxidase activity in all brain regions examined in this study, compared to negative control mice ($p < 0.05$). By day 7, H₂S exposure resulted in 70% inhibition of cytochrome c oxidase enzyme activity in the IC of both male and female mice, indicating effects of H₂S on this enzyme are cumulative. There were no statistically significant differences between male and female mice with respect to enzyme inhibition by H₂S. Statistically significant inhibition of cytochrome c enzyme activity was also observed in the thalamus and in other brain regions where histologic lesions were not prominent, such as the cortex (Fig. 3C).

H₂S induced changes in neurotransmitter levels in various brain regions

Because of the observed clinical, behavioral and morphologic changes in H₂S-exposed mice, we measured DA, 5-HT, NE and their metabolites in different brain regions. Results are summarized in Table 1. There were statistically significant differences among neurotransmitters, which were anatomically location-dependent. Interestingly, DA concentration, with a few exceptions, was significantly increased across all brain regions examined (Table 1). These results indicate H₂S exposure induced statistically significant changes in monoamine neurotransmitter content across brain regions studied, including those where neurodegeneration was not manifested (Suppl. Table 3).

Gamma-aminobutyric acid (GABA) and glutamate play a role in seizure activity, so we investigated the effect of H₂S on levels of these neurotransmitters. Results are summarized in Fig. 6. In male mice only, H₂S caused a significant decrease in GABA and glutamate following 3 or 7 exposure compared with breathing air negative control (Fig. 4A-B). These changes were not found in female mice.

H₂S-induced neuronal loss and oxidative stress

Immunohistochemical studies focused on the IC and thalamus. Immunohistochemical staining with antibody directed at NeuN, a marker of neurons, revealed loss of neurons in these regions (Fig 5A). Immunostaining with Fluoro jade C revealed degenerating neurons in the IC of H₂S-exposed mice (Fig 5B). Therefore, neuronal cell loss in these brain regions is a major effect of H₂S-induced neurotoxicity. We also examined the level of 4-hydroxynonenol (4-HNE), a by-product of lipid peroxidation, via Western blot analysis and immunohistochemistry. Results show that H₂S exposure produced

50% more 4-HNE in the IC on day 7 of H₂S exposure compared to control (Fig. 6A). Ionized calcium binding adapter molecule 1 (Iba-1), a biomarker of microglial activation, was significantly increased by more than 50% on day 3 (Fig. 6B). Expression of GFAP was also induced by H₂S exposure, reflecting activated astrocytes (Fig. 6C). These results indicate the roles of oxidative stress and neuroinflammation in H₂S-induced neurotoxicity.

Exposure Paradigm II

H₂S induced seizures and knockdowns in mice

A single acute exposure to a higher concentration of H₂S at 1000 ppm for 60 minutes resulted in mice exhibiting similar clinical signs as in Paradigm I but with high mortality. Mice started seizing within 6-9 min of H₂S exposure, and manifested knockdown by 10-16 min of exposure. Seizure activities were similar to those described in Paradigm I. Cumulative mortality at the end of the 7 day study was 40%.

H₂S-induced lesions in the brain

Approximately 40% of surviving mice manifested neurological lesions, compared to 100% of the mice in Paradigm I (Fig 7B). Control animals had no lesions. Exposure to a single high concentration of H₂S also resulted in vacuolar lesions with degeneration and loss of neurons and formation of a glial response, similar to those observed in animals in Exposure Paradigm I. Brain regions affected included the IC, thalamus, and additional foci of degeneration, necrosis and scarring in the cortex (one of 5 mice) and pons (two of five mice) in the single, acute exposure (Fig 7C). The animal that had a cortical lesion had a large, unilateral area of necrosis in the frontal and parietal region with marked vacuolization and neuron loss in layer 4 of the cortex, neuronal degeneration and necrosis in more

superficial layers, and moderate gliosis and endothelial hypertrophy and hyperplasia in all layers of the affected cortical regions.

Discussion

The goal of this study was to develop a rodent model of H₂S-induced neurotoxicity, with a relevant route of human exposure, for characterizing mechanisms of H₂S-induced neurotoxicity and for use in translational studies aimed at evaluating the efficacy of countermeasures against H₂S neurotoxicity². This study has led to the development of a novel inhalation mouse model of H₂S-induced neurotoxicity and neurodegeneration that recapitulates many of the features of the human condition², including clinical signs, behavioral responses, and neurodegeneration^{5, 45-48}. There have been some recent attempts at developing such models, but the routes of H₂S exposure employed were irrelevant to chemical terrorism or farm/industrial H₂S exposure. These animal models also failed to express reproducible brain lesions in an appreciable number of test subjects. For example, a rat model demonstrated neuronal necrotic lesions in the superficial and middle layers of the cerebral cortex and posterior thalamus, but the route of H₂S exposure was by intraperitoneal (IP) injection of NaHS³⁵. This study was also limited in scope, focusing on histopathological and behavioral endpoints, without evaluating neurochemical and biochemical changes. This model was characterized by high mortality, with only a small number of rats surviving for morphological evaluation of neuropathology. Another excellent study using a similar rat model of IP NaHS injection highlighted the difficulty of inducing brain lesions in this model³⁴. Only 1 in 3 surviving rats that received a higher dose demonstrated frank lesions 1 week post exposure³⁴. In an inhalation mouse model using anesthetized mice, the focus of the study was biochemical changes and neurodegeneration was not evaluated³³. None of

these alternative models examined sex differences in rats or mice. An additional advantage of our mouse model, besides using the appropriate route of exposure in conscious mice, is that multiple endpoints were evaluated, and neurotoxicity and neurodegeneration was assessed using various exposure paradigms to assess the progression and severity of H₂S-induced toxicity. Besides, gender-specific differences in susceptibility to H₂S-induced neuropathology were also evaluated, which was lacking in other models.

The mechanisms underlying acute toxicity and continued neurodegeneration of victims of H₂S toxicity are not well understood, but cytochrome c oxidase inhibition, ATP depletion, and ischemia/hypoxia are often cited as causal to H₂S-induced neurodegeneration^{30, 49-51}. H₂S exposure has been known to cause a wide array of neurological clinical effects, including persistent headaches, nausea, lethargy, seizures, dizziness, abnormal reflexes, sleeping disorders, hearing impairment, movement disorders, and loss of consciousness, sometimes leading to permanent vegetative states²¹. All mice in this study exhibited seizures and the “knockdown” effects during H₂S exposure, replicating what is commonly reported in humans. Humans have also been reported to have a lower threshold to H₂S induced neurotoxicity following additional H₂S exposure; as was observed in this mouse model⁵⁰. Mice receiving repeated H₂S exposure were more vulnerable to H₂S poisoning. In a pilot dose-range finding study preceding this detailed study, upon a second exposure to 765 ppm H₂S for 40 min, there was almost 100% mortality; yet on 1st exposure only about 5% mortality was observed at the end of a 40 min exposure. The earlier manifestations of seizures and knockdown in mice in this study upon subsequent second and third exposures, compared to a single naïve exposure further confirms the validity of our model and its relevance to human exposure. In a study conducted on female mice exposed

four times to 100 ppm at 4 day intervals, Savalaine et al. reported that biochemical effects of repeated H₂S exposure are cumulative³⁷. Collectively, these results suggest that the acute effects of H₂S-induced neurotoxicity manifested in mice in Paradigm I reflect cumulative effects of H₂S as was observed by Savalaine et al³⁷. Results of this study showed cumulative inhibition of cytochrome c oxidase activity, neuro-inflammatory response, and reduced GABA and glutamate with repeated short-term exposures. The mechanisms of these cumulative effects are beyond the scope of this study and should be the subject of future investigations. Cumulative effects are likely one of the factors contributing to enhanced sensitivity of mice to subsequent acute H₂S exposures.

The brain is a primary target organ of H₂S intoxication and many victims of acute H₂S poisoning report persistent neurological problems post-H₂S exposure, including movement disorders such as spastic gait^{22, 23, 25, 50}. Consistent with this observation, both open field test and RotaRod tests revealed impairments in locomotor activity and motor coordination in mice exposed to H₂S. Interestingly, male mice were more sensitive to these motor deficits than females, whereby males displayed these symptoms earlier than females. The notion that male mice are more sensitive than female mice is further supported by the observation that time to seizure induction was shorter in males than females and that only male mice manifested significant inhibition of GABA. The reasons for these qualitative sex differences in response to H₂S poisoning are currently not clear. Future studies to examine the role of hormones in these qualitative differences are recommended.

It is interesting that H₂S induced brain region-specific neurodegeneration and necrosis. Necrosis in the IC and thalamus of H₂S-exposed mice suggests these regions to be most vulnerable to H₂S-induced neurotoxicity. Severe neuronal damage and an intense glial

response induced by H₂S bears close resemblance to that induced by NaHS and carbonyl sulfide (COS) gas, a precursor of H₂S^{35, 52}. Unlike NaHS and COS, which cause severe cortical necrosis, H₂S exposure by inhalation did not result in severe cortical necrosis in this mouse model, though the cortex was also affected. The mechanisms underlying the selective sensitivity of the IC and thalamus to H₂S-induced neurodegeneration are not clear. However, the IC is a brain region known to receive the highest blood supply and to have a very high metabolic rate⁵³. The IC is part of the auditory pathway; it is interesting that hearing impairment is a frequently reported lingering effect of H₂S intoxication in humans⁵⁰. The thalamus is a key brain region which is at the cross roads of key neural tracts⁵⁴. Neurodegeneration in this region can virtually manifest in many of the clinical signs reported in human victims of H₂S poisoning, including migraines⁵⁵. It will be of interest to determine the reasons why other regions are not vulnerable to the same extent as the IC and thalamus. Considering cytochrome c oxidase enzyme activity was globally affected in the brain, inhibition of this enzyme by H₂S is unlikely to be the explanation for regional differential susceptibilities. Previous studies point to the idea that ischemia-hypoxia could be contributing factors to the neurodegeneration induced by H₂S^{30, 34, 36}, further studies would need to be conducted to elucidate the exact role hypoxia plays in H₂S induced neurodegeneration.

A key observation in this study is that histopathological and immunohistopathologic findings were readily reproducible and served as reliable endpoints to assess the severity of H₂S-induced neurotoxicity in this model. We therefore recommend histopathology and immunohistopathology as key endpoints of translational studies evaluating the efficacy of medical countermeasures for treatment of H₂S-induced neurodegeneration.

In human victims of acute H₂S exposure lesions are reported in various parts of the brain with the cortex, thalamus, and the basal ganglia being regions most commonly involved^{20, 22, 23}. Lesions in similar brain regions, including the collicular regions have also been reported in animals⁵⁶. Faithfully reproducing lesions in brains of laboratory animals, following a single H₂S exposure, has been a challenge^{34,35,57}. This may be a result of physiological differences between rodents and nonhuman primates and humans. Baldelli and Sonobe using injected NaHS showed that lesions were manifested only in a small number of surviving rats. Besides the brain, lungs and the cardiovascular system are other secondary target organs of H₂S exposure. H₂S-induced lung edema in particular may contribute to hypoxia and is best replicated in an inhalation model. A good inhalation model of H₂S-induced neurotoxicity was shown in an elegant study in Rhesus monkeys exposed either once or at least twice to H₂S. Using this primate model, Lund et al. reproduced lesions in the cortex and basal ganglia of monkeys exposed twice to 500 ppm with 3 days in between exposures for 25 mins initially and 17 mins on second exposure⁵⁷. Monkeys that died acutely lacked brain lesions. Those with at least 2 exposures that lived at least 3 days post exposure developed lesions. Results of this primate study are consistent with observations in the mouse model presented in this study. Although the non-human primate model is attractive because it yields lesions in similar brain regions as humans, facilities to support this kind of research are not widely available.

The mouse model reported in the current study followed two exposure paradigms. Though exposure Paradigm I is atypical of exposure scenario in humans, it has the advantage that it is associated with low mortality, and a high frequency of mice exhibiting brain lesions. This exposure paradigm is ideal for assessment of countermeasures against H₂S-induced

neurodegeneration with minimal numbers of mice to achieve meaningful statistical significance. Because the molecular mechanisms leading to cell death are yet to be defined, the window of opportunity for efficacy of such drugs is not known³⁰. From our observations in this study, neurodegeneration becomes morphologically manifested at least 3 days post H₂S-exposure, suggesting a wider intervention time for treatment of neurodegeneration. Paradigm II on the other hand, faithfully replicates the typical single human exposure, but it is associated with high mortality and is ideal for evaluating efficacy for countermeasures that increase survival. In our models, most deaths occurred during H₂S exposure, as is reported in human case reports of H₂S poisoning¹⁸, or other rodent models³³⁻³⁵. Therefore, rescue countermeasures must to maximize survival must be given during exposure. .

Neurochemical alterations in the brain have been correlated with many movement disorders^{58,59}, specifically lesions in the basal ganglia⁶⁰. In order to understand the origin of the motor deficits observed in this mouse model, the striatum was analyzed for monoamine neurotransmitter changes after H₂S exposure. A single or repeated short term exposures to H₂S resulted in increased DA concentration (Table 1). This is consistent with the literature demonstrating H₂S as a monoamine oxidase enzyme inhibitor⁶¹. Interestingly, mice exposed to H₂S also had lower norepinephrine levels compared to negative controls (Suppl. Table 3). A positive correlation in the levels of the aforementioned neurotransmitters were evidenced in the thalamus, cortex and striatum of mice exposed to H₂S (Table 1). It is notable that these neurochemical changes were observed in brain regions that did not manifest morphological lesions, suggesting neurodegeneration may not be an essential prerequisite for the manifestation of neurochemical changes.

The mechanisms of H₂S-induced seizures have not been characterized. However, neurotransmitter imbalances can contribute to seizures^{62,63}, and in this study we demonstrated significant changes in neurotransmitter levels in the brain. GABA and glutamate are important regulators of inhibitory and excitatory signals in the brain. H₂S exposure time-dependently decreased GABA and glutamate in the inferior colliculus of the brain. Dysregulation of glutamate and GABA may partially explain the seizurogenic and neurotoxic effects of H₂S.

A consistent hallmark of H₂S exposure that is well characterized is the inhibition of cytochrome c oxidase (complex IV) of the electron transport chain^{33,64,65}. Our model recapitulates this biochemical effect: cytochrome c oxidase activity was significantly decreased on all days of H₂S exposure in both male and female mice. Specifically, cytochrome c oxidase activity was reduced in the inferior colliculus, the thalamus, and cortex. This data suggests that inhibition of cytochrome c oxidase may not be the sole cause of H₂S-induced neurotoxicity and neurodegeneration.

A biomarker of H₂S exposure which has been reported in the literature is an increase of thiosulfate^{14, 44}. In this study we also show that H₂S exposure increases serum thiosulfate levels. It is anticipated that serum thiosulfate concentration will be a valuable biomarker for evaluation of efficacy of countermeasures, such as cobinamide, which bind H₂S to prevent it from interacting with critical sensitive molecules such as cytochrome c oxidase.

This study also evaluated gender differences in susceptibility to H₂S-induced neurotoxicity in Paradigm I. Some differences were found, and overall males were found to be more sensitive to H₂S-induced acute toxicity than females. This is supported by data of

body weight loss, average latency time to exhibit seizures and time taken to demonstrate knockdown, severity of brain injury, and performance in behavioral tests. It is for this reason that only male mice were used in Paradigm II of the study. The reason(s) for this apparent increased susceptibility of male mice to H₂S is/are unknown but could be hormonal or magnitude of locomotor activity could be a critical determinant. For this reason, we recommend using male mice with inclusion of a smaller cohort of female mice in future translational efficacy studies evaluating potential drug candidates for treatment of acute and lingering effects of H₂S-induced neurotoxicity.

In conclusion, we have developed a mouse model which recapitulates many of the hallmarks of H₂S-induced neurotoxicity and neurodegeneration using two exposure paradigms. In Paradigm I, all animals exposed to H₂S had a reduction in body weight, motor deficits, and exhibited clinical signs characteristic of H₂S poisoning, including seizure activity and the “knockdown” response. Male mice were more sensitive than females, determined using multiple endpoints. The animals exposed to H₂S also showed a cumulative toxicity to H₂S, a phenomenon also observed in human beings. These animals also had neurochemical imbalances in almost all of the brain regions investigated, which could be a contributing factor to seizure activity and persistent neurological sequelae observed in this model. Mortality was low in Paradigm I and consistently reproducible necrotic lesions were present in brain regions previously shown to be sensitive to H₂S toxicity, such as the inferior colliculus and the thalamus. We recommend this approach for testing the efficacy of countermeasures against neurodegeneration because it uses a small number of mice to achieve statistically significant and meaningful results. Paradigm II on the other hand is associated with high mortality, with a small percentage of surviving mice manifesting similar

lesions in same brain regions, albeit less severe. We recommend this approach for testing the efficacy of rescue countermeasures to increase survival rate. There is no perfect animal model of the H₂S-induced neurotoxicity and neurodegeneration. The mouse model presented in this study is novel and for the first time provides a tool for mechanistic and translational studies. Currently, no other animal model uses whole body H₂S inhalation exposure on fully awake animals. A wide variety endpoints to be evaluated in this model have been established, including mortality, clinical, biochemical, and neurochemical changes, which parallel those observed in H₂S toxicity in human beings and will provide metrics suitable for the evaluation and screening of antidotes of H₂S-induced neurotoxicity.

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Conflict of Interest

All authors declare no conflict of interest.

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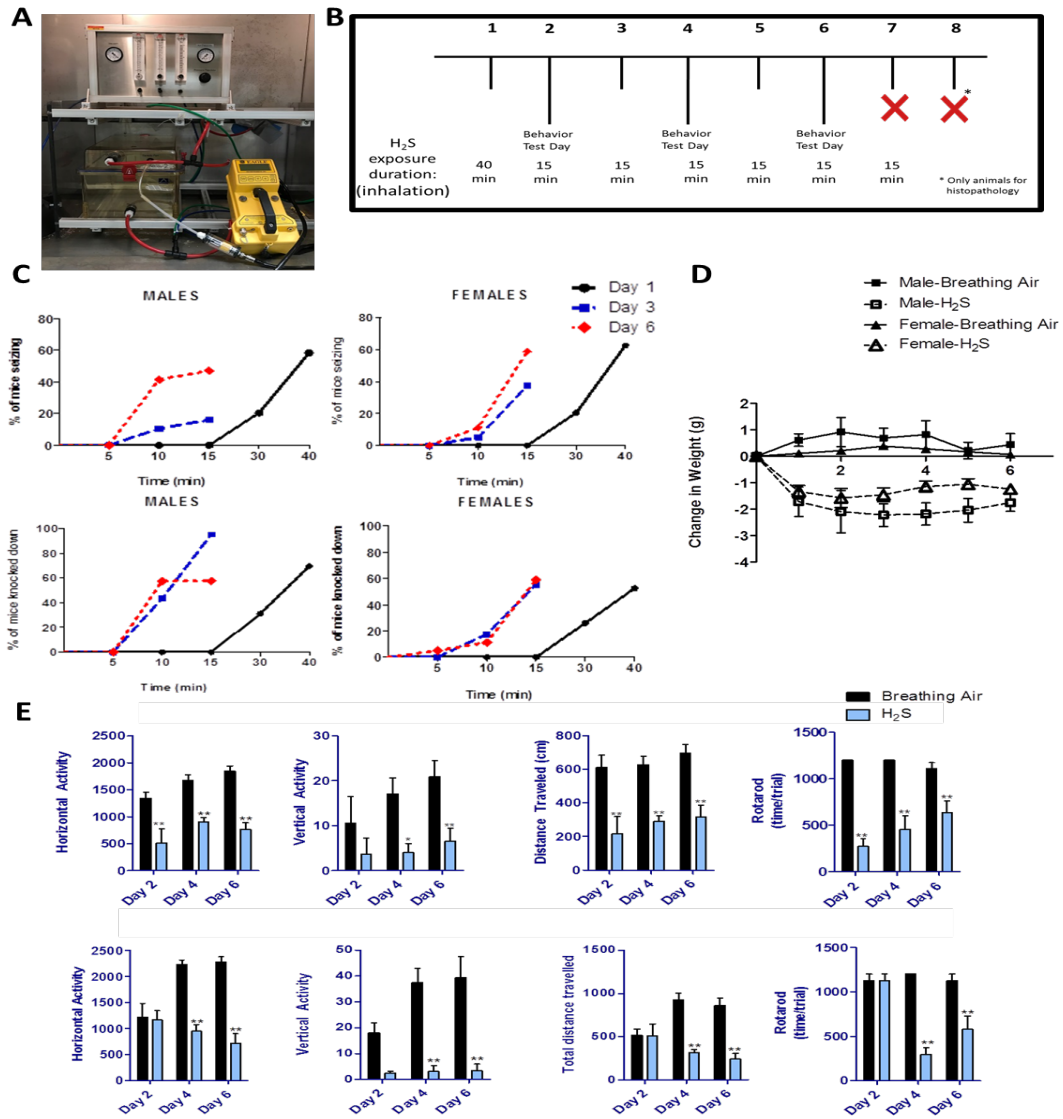


Figure 1. Whole body inhalation chamber and system used in the present studies (A) Treatment Paradigm 1 of H₂S induced neurodegeneration in mice (B). Seizures and knockdown induced by H₂S in male and female mice, n=8-10/ group (C) Weight loss in mice exposed to H₂S (765 ppm) n=8-10/group (D) Behavior deficits induced by H₂S (765 ppm) in male and female mice, n=8-10/group (E). *,**p<0.05-0.01 compared to breathing air controls of each sex.

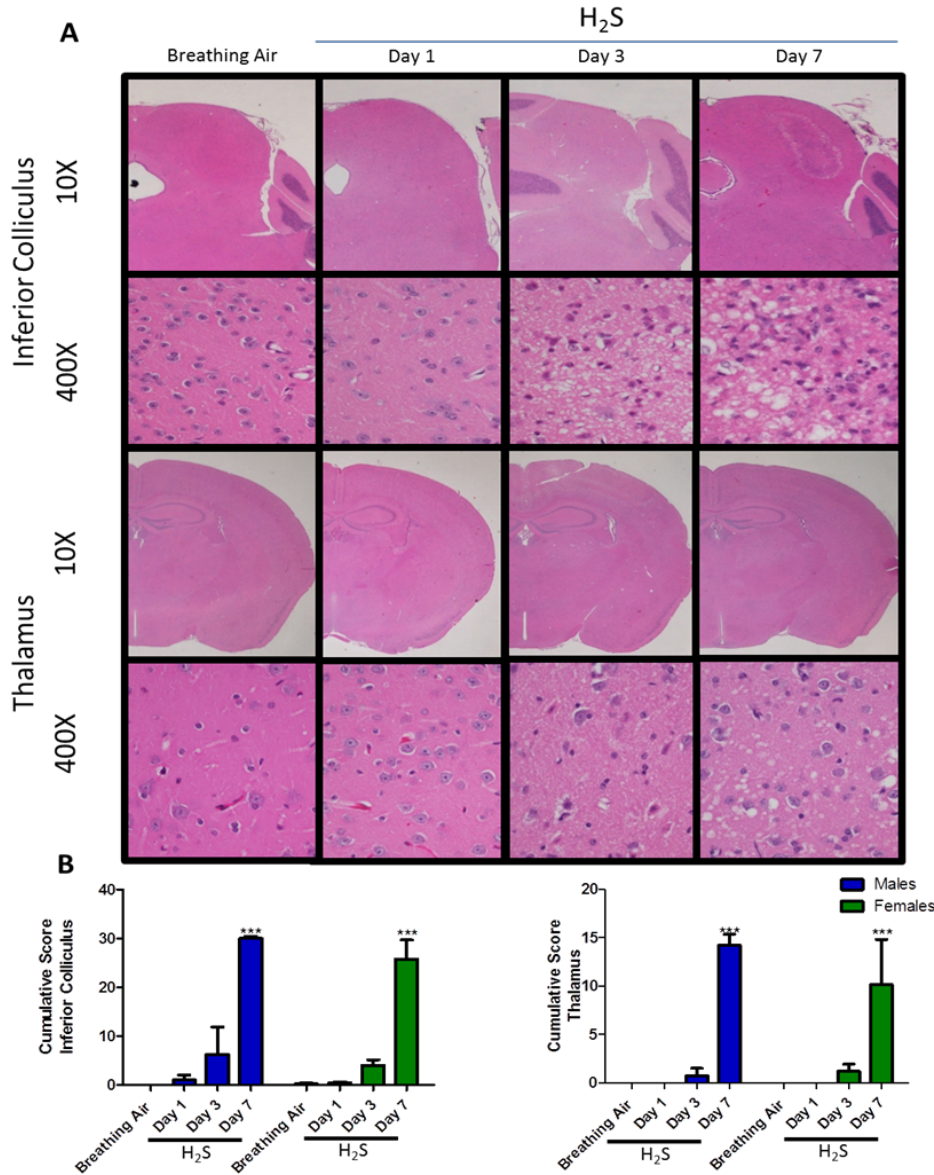


Figure 2. Vacuolization of the neuropil and neuronal death in the inferior colliculus and

thalamus of mice brains after 1, 3, and 7 exposures to H_2S (765 ppm). Severe and widespread necrosis and glial response in the IC (arrows) after 7 days of exposure to H_2S (A). Size bars =1000 microns for 10X and 100 microns for 400X. The attached graph summarizes changes in histopathology over time. Histopathology score determined from semi quantitative grading scheme in Supplemental Table 2 (B) n=5/group. *, **, ***p<0.05-0.001 compared to breathing air controls.

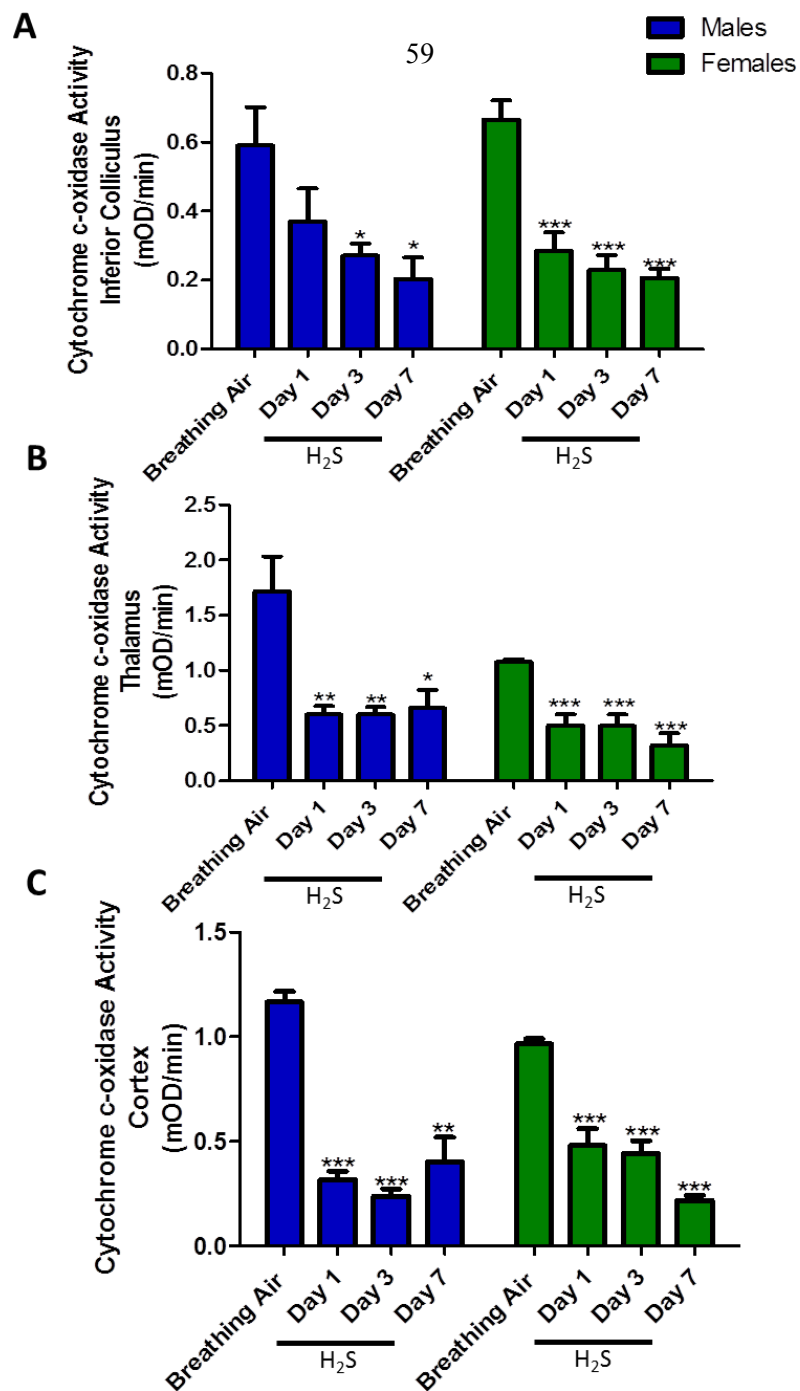


Figure 3. Decrease in cytochrome c oxidase activity in inferior colliculus (A), thalamus (B) and cortex (C) of male and female mice following exposure to H₂S (765 ppm). n=3-5/group *, **, ***p<0.05-0.001 compared with breathing air controls.

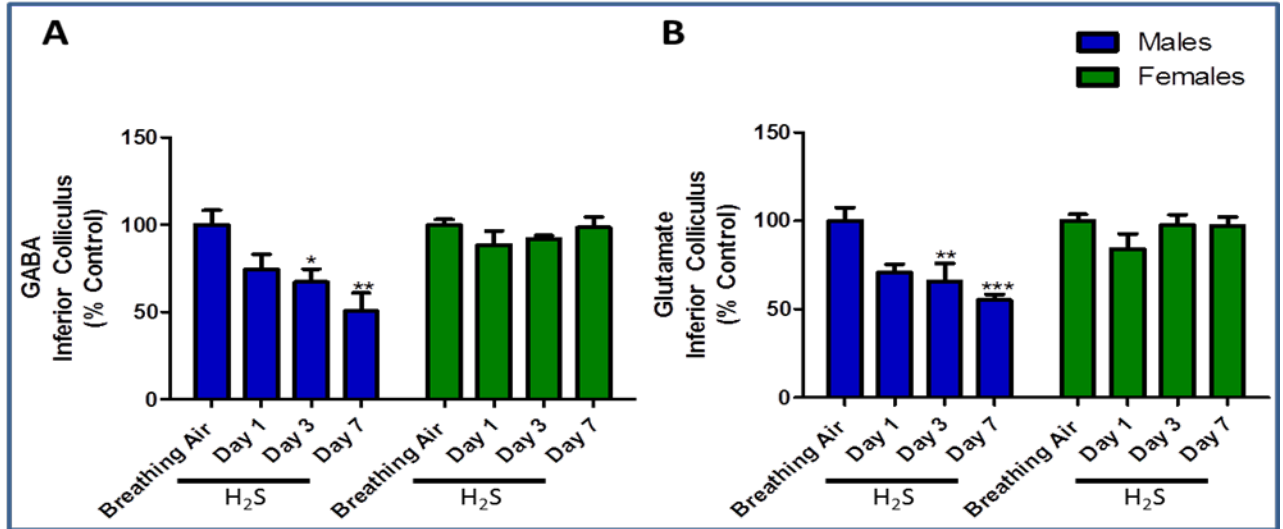


Figure 4. Changes in GABA (A) and glutamate levels (B) in the inferior colliculus of mice following exposure to H₂S (765 ppm). n=5/group. *, **, ***p<0.05-0.001 compared to breathing air controls.

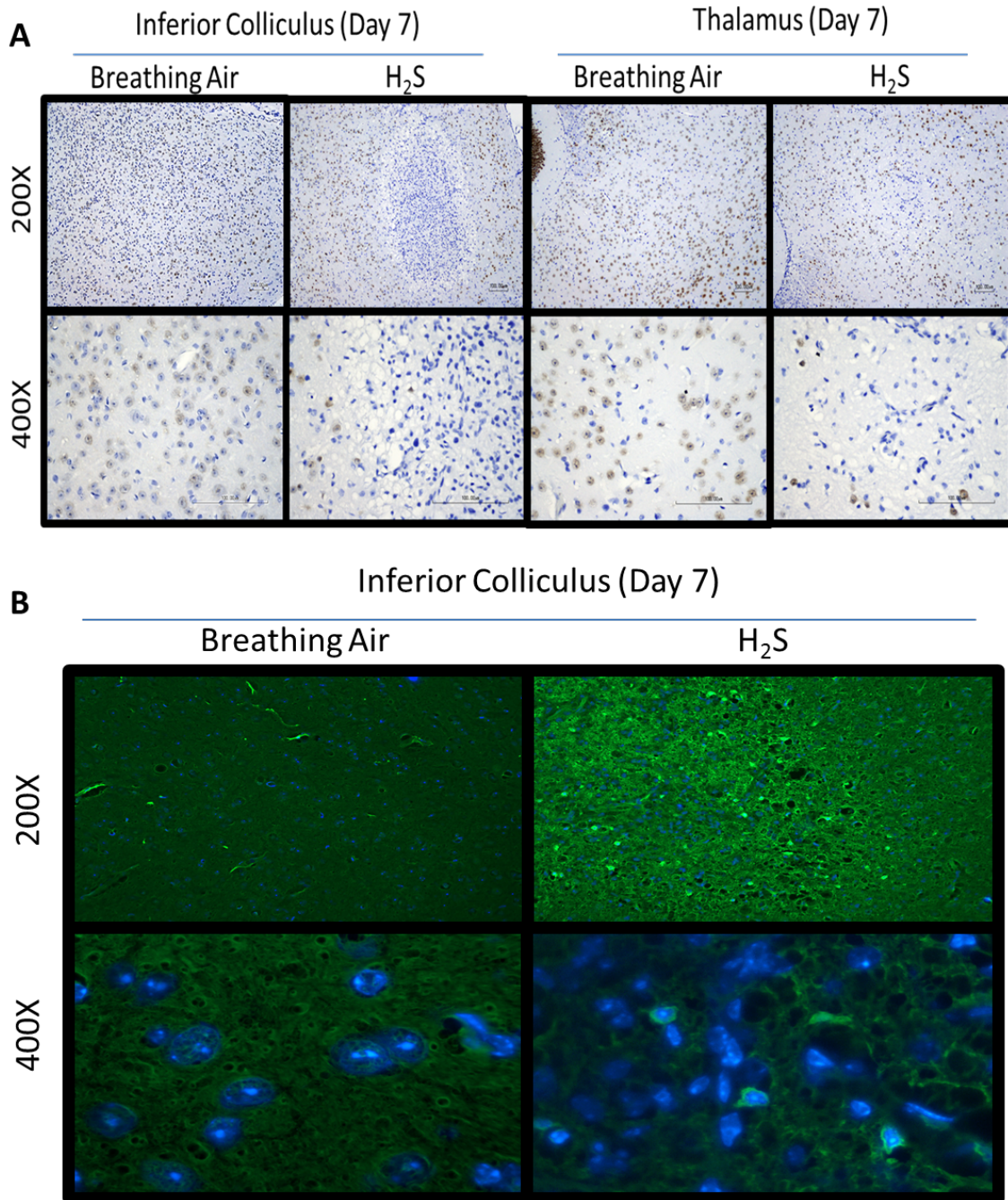


Figure 5. Loss of neurons (NeuN) in the inferior colliculus and thalamus of mice following exposure to H₂S (765 ppm) (A). Degenerating neurons, stained with fluoro jade c, (white arrows) within a representative inferior colliculus of mice following exposure of H₂S, n=6/group (B).

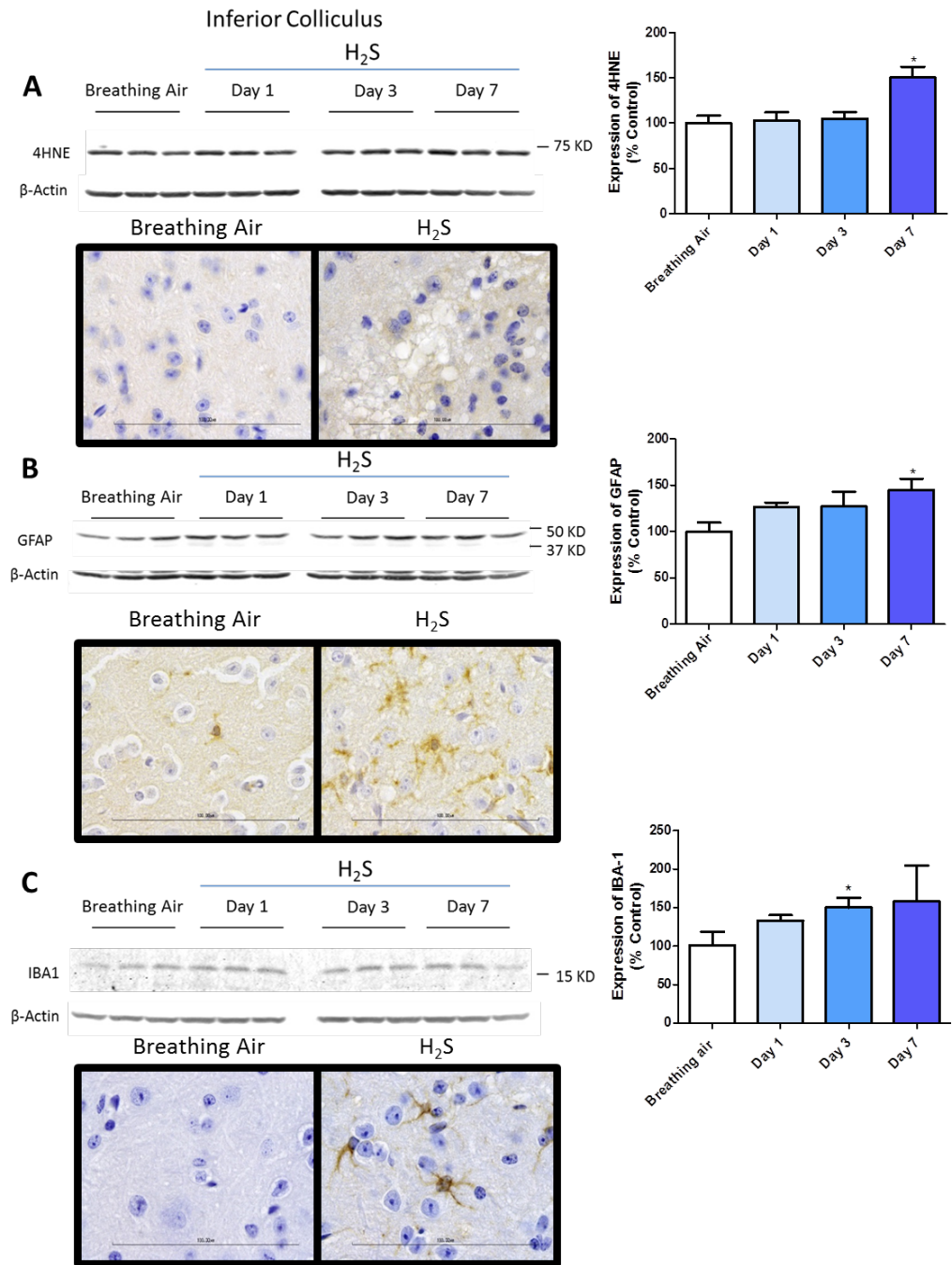
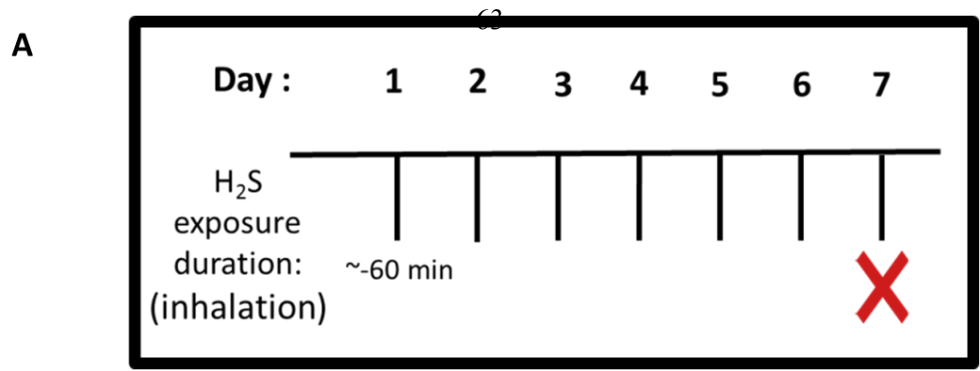


Figure 6. Oxidative stress in the inferior colliculus of male mice following exposure to H₂S (765 ppm). n=3/group. *p<0.05 compared with breathing air controls.



B

Prevalence of Lesions in Surviving Mice Exposed to H₂S

	Paradigm 1		Paradigm 2	
	Breathing Air	H ₂ S	Breathing Air	H ₂ S*
Inferior colliculus	0/5	5/5	0/5	2/5
Thalamus	0/5	5/5	0/5	2/5

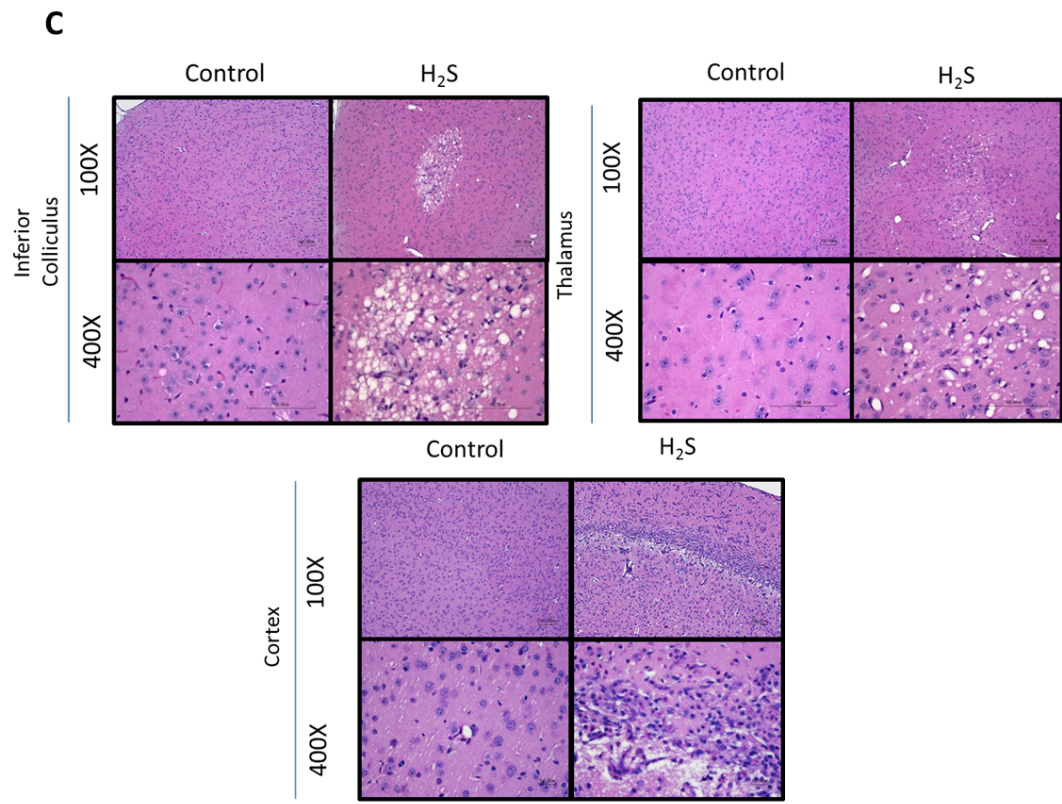
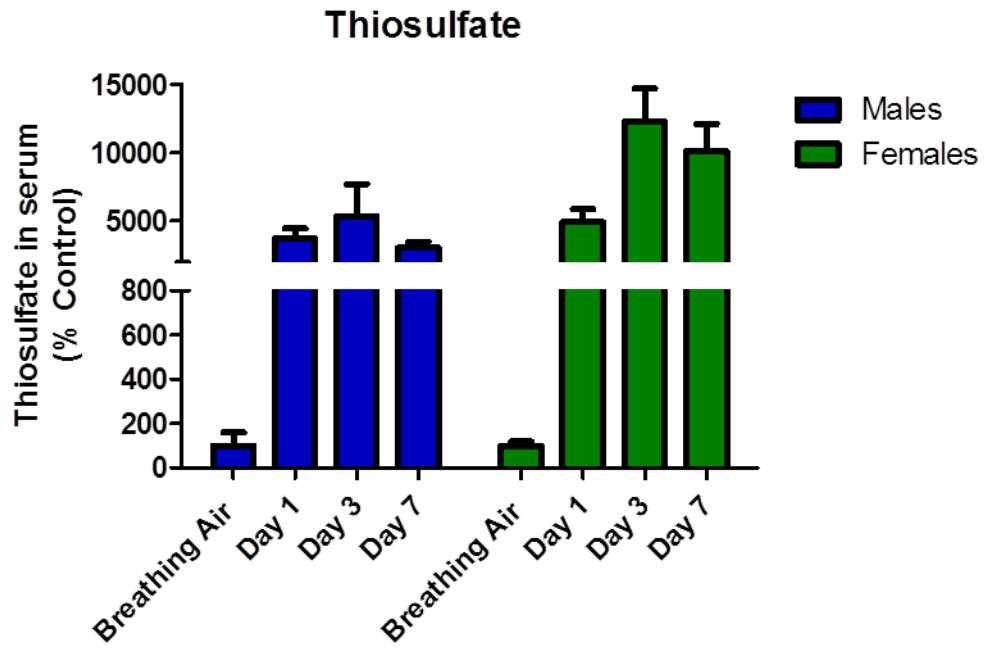


Figure 7. Treatment Paradigm II of H₂S induced neurodegeneration in mice (A). Prevalence of lesions in Paradigm I vs Paradigm II in the inferior colliculus and thalamus (B) Vacuolization of the neuropil and neuronal death in the inferior colliculus, thalamus, and cortex of mice exposed to H₂S (1000 ppm), n=5/group.*30% died in chamber during H₂S exposure. 100X(C)

Table 1. Dopamine and metabolite changes in various brain regions of mice following exposure to 765 ppm of H₂S. n=4-5/group. *,**p<0.05-0.01 compared to breathing air controls

Neurochemical changes in various brain regions		Breathing Air		H ₂ S						
				Day 1		Day 3		Day 7		
Inferior Colliculus	Dopamine									
	Male	100.0	±3.33	162.4	±20.29**	146.3	±9.87**	173.5	±26.45*	
	Female	100.0	±7.13	195.3	±23.00*	166	±15.97**	164	±11.99**	
	DOPAC									
	Male	100.0	±7.41	272.6	±44.19**	148.9	±9.56*	186.3	±38.42*	
	Female	100.0	±3.31	210.3	±25.85*	135	±20.43**	157.4	±11.75**	
Thalamus	HVA									
	Male	100.0	±3.26	129.1	±7.04**	139.1	±8.14**	150	±20.53*	
	Female	100.0	±13.05	155.1	±12.59*	74.07	±25.58	112.5	±8.82	
	Dopamine									
	Males	100.0	±31.89	214.7	±89.37	75.39	±58.87	138.5	±62.97	
	Females	100.0	±16.82	80.58	±15.67	62.35	±6.99	51.86	±20.70	
Cortex	DOPAC									
	Males	100.0	±16.82	80.58	±15.67	62.35	±6.99	51.86	±20.7	
	Females	100.0	±20.42	103.5	±15.74	38.57	±2.26	56.81	±18.02	
	HVA									
	Males	100.0	±20.99	180.3	±26.67	79.74	±48.29	218.7	±49.09	
	Females	100.0	±14.33	77.47	±13.55	64.54	±7.55	65.33	±24.21	
Striatum	Dopamine									
	Males	100.0	±19.07	192.9	±75.87	241.9	±35.64**	191.1	±42.53	
	Females	100.0	±9.05	127.1	±21.2	159.9	±41.83	104.6	±7.36	
	DOPAC									
	Males	95.6	±14.73	99.94	±32.47	217.2	±34.50*	126.8	±9.04	
	Females	95.6	±8.54	118.7	±18.99	186.9	±43.53	108	±7.35	
Striatum	HVA									
	Males	95.6	±10.97	116.1	±32.05	230.4	±30.91**	128.5	±11.12	
	Females	95.6	±8.58	113.3	±17.8	186.6	±42.63	114.1	±11.4	
	Dopamine									
	Males	95.6	±11.84	171	±28.32**	163.6	±52.26	104.2	±18.66	
	Females	100.0	±17.74	187.2	±44.11	100.1	±8.93	83.67	±6.426	
Striatum	DOPAC									
	Males	100.0	±10.5	131.9	±15.84	132	±10.79	152.7	±34.4	
	Females	100.0	±19.77	59.1	±41.65	15.17	±13.52**	2.16	±0.52**	
	HVA									
	Males	100.0	±8.19	142.8	±22.03	125.1	±6.838	131.2	±20.09	
	Females	100.0	±21.92	166.3	±36.74	97.2	±7.49	94.36	±6.114	



Supplemental Figure 1. Thiosulfate levels in serum of male and female mice following exposure to H₂S. n=5/group *, **, ***p<0.05-0.001 compared to breathing air controls.

Supplemental Table 1. Semi-quantitative grading scheme to assess histological lesions caused by H₂S in mouse brains.

Semi-Quantitative Grading Scheme for H₂S-induced Neuropathology							
Score	Neurons: Clumped Nissl or central chromatolysis	Neurons: Angular, shrunken neurons or early apoptotic neurons	Neurons: Loss of neurons	Glia: Astrocytes	Glia: Microglia/ macrophages	Apoptotic bodies	Neuropil: Pallor or Vacuoles
0	None	None	No loss	Normal morphology	Normal morphology	None	Solid, bright eosinophilic
1	Mild	<2/20X		Clumped chromatin		<2/20X	Slightly pale eosinophilic
2		2-5/20X			Rare amoeboid	2-5/20X	Finely vacuolated
3	Moderate	6-10/20X	~50% loss	Type II Alz cells	Many amoeboid	6-10/20X	Coarsely vacuolated
4		11-15/20X			Mixed amoeboid/Gitter	11-15/20X	Condensation of vacuoles
5	Severe	>15/20X	100% loss	Loss of astrocyte pop'ns	Gitter cells present	>15/20X	Loss of large areas of neuropil

Supplemental Table 2. Summary of functional observation of battery evaluation during H₂S exposure (765 ppm) of male and female mice, including seizure activity, knockdowns, and other clinical signs.

Clinical effects in mice during H ₂ S exposure					
				Breathing Air	H ₂ S
Autonomic					
Lacrimation				No	present
Salivation				No	present
Urination/Defecation				Normal	increased
Convulsions					
Tonic movements				No	mild
Clonic movements				No	severe
Paddling feet				No	severe
General Measures					
Piloerection				No	present
Body Posture				Normal	hunched
Dyspnea				No	present
Gait					
Hindlimbs splayed				No	present
Ataxia				No	severe
Unable to support weight				No	present

Supplemental Table 3. Serotonin, 5-HIAA, and norepinephrine levels in various brain regions of mice following exposure to 765 ppm of H₂S. n=4-5/group. *,**p<0.05-0.01 compared to breathing air controls.

Neurochemical changes in various brain regions		Breathing Air		H2S					
				Day 1		Day 3		Day 7	
Inferior Colliculus	Dopamine								
	Male	100.0	±3.33	162.4	±20.29**	146.3	±9.87**	173.5	±26.45*
	Female	100.0	±7.13	195.3	±23.00*	166	±15.97**	164	±11.99**
	DOPAC								
	Male	100.0	±7.41	272.6	±44.19**	148.9	±9.56*	186.3	±38.42*
	Female	100.0	±3.31	210.3	±25.85*	135	±20.43**	157.4	±11.75**
HVA									
	Male	100.0	±3.26	129.1	±7.04**	139.1	±8.14**	150	±20.53*
Female	100.0	±13.05	155.1	±12.59*	74.07	±25.58	112.5	±8.82	
Thalamus	Dopamine								
	Males	100.0	±31.89	214.7	±89.37	75.39	±58.87	138.5	±62.97
	Females	100.0	±16.82	80.58	±15.67	62.35	±6.99	51.86	±20.70
	DOPAC								
	Males	100.0	±16.82	80.58	±15.67	62.35	±6.99	51.86	±20.7
	Females	100.0	±20.42	103.5	±15.74	38.57	±2.26	56.81	±18.02
HVA									
	Males	100.0	±20.99	180.3	±26.67	79.74	±48.29	218.7	±49.09
Females	100.0	±14.33	77.47	±13.55	64.54	±7.55	65.33	±24.21	
Cortex	Dopamine								
	Males	100.0	±19.07	192.9	±75.87	241.9	±35.64**	191.1	±42.53
	Females	100.0	±9.05	127.1	±21.2	159.9	±41.83	104.6	±7.36
	DOPAC								
	Males	95.6	±14.73	99.94	±32.47	217.2	±34.50*	126.8	±9.04
	Females	95.6	±8.54	118.7	±18.99	186.9	±43.53	108	±7.35
HVA									
	Males	95.6	±10.97	116.1	±32.05	230.4	±30.91**	128.5	±11.12
Females	95.6	±8.58	113.3	±17.8	186.6	±42.63	114.1	±11.4	
Striatum	Dopamine								
	Males	95.6	±11.84	171	±28.32**	163.6	±52.26	104.2	±18.66
	Females	100.0	±17.74	187.2	±44.11	100.1	±8.93	83.67	±6.426
	DOPAC								
	Males	100.0	±10.5	131.9	±15.84	132	±10.79	152.7	±34.4
	Females	100.0	±19.77	59.1	±41.65	15.17	±13.52**	2.16	±0.52**
HVA									
	Males	100.0	±8.19	142.8	±22.03	125.1	±6.838	131.2	±20.09
Females	100.0	±21.92	166.3	±36.74	97.2	±7.49	94.36	±6.114	

CHAPTER 3

**COBINAMIDE IS EFFECTIVE FOR TREATMENT OF HYDROGEN SULFIDE-
INDUCED NEUROTOXICITY AND NEUROLOGICAL SEQUELAE**

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Abstract

Hydrogen sulfide (H₂S) is a highly neurotoxic gas. Acute exposure can lead to death and neurological sequelae among survivors. A drug for treating acute H₂S intoxication in the field is acutely needed. Using a novel mouse model, we evaluated the efficacy of Cobinamide (Cob) for this purpose. There were two objectives: 1) to determine the dose-response efficacy of Cob; and 2) to determine the effective therapeutic time window of Cob. We exposed mice to 765 ppm H₂S gas in a sealed chamber. In objective 1, mice were injected intramuscularly with Cob at 0, 50 or 100 mg/kg at 2 min post H₂S exposure. In objective 2, mice were injected intramuscularly with 100 mg/kg Cob at 2, 15, and 30 min post H₂S exposure. Control mice exposed to breathing air or to H₂S alone were injected with 0.9% saline. Cob significantly reduced H₂S-induced lethality in a dose-dependent manner ($p < 0.05$). Cob-treated mice exhibited significantly fewer seizures and “knockdowns” compared to the H₂S-exposed group. Cob also reversed H₂S-induced weight loss, behavioral deficits, neurochemical changes, cytochrome c oxidase enzyme inhibition, and neurodegeneration in a dose- and time-dependent manner ($p < 0.01$). Overall, our findings show that Cob increased survival and is neuroprotective against H₂S-induced toxicity.

Keywords

Cobinamide, hydrogen sulfide, neurotoxicity, neurodegeneration, neuroprotection

Introduction

Hydrogen sulfide (H_2S) is a highly toxic gas. High, acute exposures lead to severe toxic effects, with most deaths occurring at the scene of exposure¹. Among toxic gases, H_2S is the second most common cause of human death after carbon monoxide poisoning^{2, 3}. Most H_2S -induced deaths are occupational-related^{1, 4-6}, but suicide in confined spaces is a growing cause of death by H_2S exposure^{7, 8}. There are many sources of H_2S in the environment, including occupational settings and natural discharges from volcanic activities and hot springs^{9, 10}. Hydrogen sulfide is a contaminant of oil and natural gas and is a hazard to oil and petroleum industry workers^{2, 11}; in addition, sour oil pipeline explosion accidents have led to mass civilian casualties¹². Rotting animal and plant matter as in sewer systems, animal manure from intensive animal agriculture, and waste products of food processing plants have also been sources of acute H_2S poisoning^{13, 14}. In the Midwest, deaths of family members are reported due do acute H_2S exposure following livestock manure pit agitation and pumping^{15, 16}. Because H_2S is relatively easy to make at home from ingredients commonly available in retail stores, concerns exist about potential misuse of H_2S as a chemical weapon, particularly in confined spaces such as underground train stations and high-rise buildings^{17, 18}. Mass civilian exposure to H_2S has occurred following industrial accidents, as was the case in Poza Rica, Mexico¹², and following a sour oil pipeline explosion in Kaixian County, China: the Poza Rica accident led to 22 deaths and hundreds were affected, and the Kaixian accident led to hundreds of civilian deaths and affected several thousands¹⁹.

Acute inhalation exposure to high H_2S concentrations affects the nervous, cardiovascular, and the respiratory systems^{20, 21}. Death from acute H_2S exposure arises from

cessation of breathing, due to inhibition of the respiratory center in the brain stem^{22, 23}. During the Poza Riza and Kaixan industrial accidents, as well as during other occupational accidents, most deaths occur at the scene, but a substantial number of others die within 12 h after exposure^{12, 19}. Of survivors discharged from the hospital with apparent recovery, many return 3-7 days post exposure complaining of neurological complications^{4, 24, 25}. These can include movement disorders, persistent migraines, memory loss, cognitive dysfunction, fatigue, hearing impairment, blindness, and seizures^{20, 25}. Neurological sequelae usually occur in victims who have suffered a knockdown and have been on a coma for at least 5 min².

Currently, no specific treatment exists for H₂S poisoning, and because most deaths occur at the scene, there is an acute need for specific treatment in the field. Several approaches are used for treating patients in the hospital, including intravenous injections of sodium nitrite and hydroxocobalamin, oxygen supplementation, hyperbaric oxygen, and hypothermia^{15, 26-28}. These modalities require intravenous access and specialized equipment, and are not suitable for field use. The ideal drug for field treatment should be easy to administer by first responders such as via intramuscular, sublingual, or transnasal routes. Cobinamide (Cob), a vitamin B12 precursor, has a high affinity for H₂S and has shown promise in animal models of H₂S poisoning increasing overall survival³. However, no data have been published on the efficacy of Cob for treating H₂S-induced neurological sequelae.

This study had two specific objectives. Objective #1 was to conduct a dose-response study to identify an ideal therapeutic dose of Cob for treatment of acute H₂S poisoning. Objective #2 was to conduct a time-course study to determine the therapeutic window of

Cob, using a dosage chosen from Objective #1 above. We found that Cob markedly reduced H₂S-induced neurotoxicity, neurodegeneration, and neurological sequelae in mice.

Materials and Methods

Chemicals and Reagents

Methanol (HPLC grade), acetonitrile (HPLC grade), MD-TM mobile phase, and formic acid were obtained from Fisher Scientific (Waltham, MA). (D- and L-) Glutamic acid, γ -aminobutyric acid (GABA), dopamine (DA), 3,4 dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), paraformaldehyde, and 60% perchloric acid were purchased from Sigma Aldrich (St Louis, MO). All aqueous solutions were prepared using 18.2 M Ω ·cm water (Aries Filter Network, West Berlin, New Jersey, USA). Cobinamide was produced from hydroxocobalamin as described previously²⁹.

Animals

All animal studies were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). Only male mice were used in this study because previous studies showed that male are more sensitive to H₂S-induced neurotoxicity than female mice³⁰. The 7-8 week old C57/BL6 male mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and weighed 20-25g at the beginning of the experiment. Mice were housed 5 per cage, in the Laboratory Animal Resource (LAR) Facility, Iowa State University College of Veterinary Medicine (ISU CVM, Ames, IA). They were maintained at a controlled room temperature of 68-70°F, relative humidity of 35-50%, and a 12-hr

light/dark cycle. Mice were provided 14% Protein Rodent maintenance diet (Teklad HSD Inc, WI, USA), and drinking water ad libitum. These mice were acclimated to these environmental conditions for at least 1 week prior to the start of the experiments.

Experiment 1:

Determining an ideal efficacious cobinamide dosage for treatment of acute H₂S-induced neurotoxicity

In this dose-response experiment, we tested the hypothesis that Cob manifests dose response efficacy in a model of H₂S-induced neurotoxicity and neurodegeneration. To achieve this objective, we used a novel mouse model which was recently published³⁰. Briefly, fully conscious and freely moving mice were placed in a whole-body exposure chamber designed to hold up to 10 mice at a time. Mice were exposed by whole body inhalation exposure. Gas access to the chamber was from two lines; one for breathing air from a tank under pressure, and the other from a tank containing H₂S under pressure. The two lines connected to the chamber via a control panel that allowed regulation of both breathing air and H₂S flow (liters/min). The real-time concentration of H₂S in the exposure chamber was constantly monitored using a H₂S monitor (Environmental Equipment and Supply, Harrisburg, PA) that was custom designed to measure concentrations of up to 1000 ppm of H₂S. The H₂S exposure paradigm used in this experiment is summarized in Figure 1A. This exposure paradigm results in mice with behavioral, biochemical, neurochemical changes, and morphologic lesions recapitulating those of the human condition using a reasonably small number of mice to demonstrate the beneficial effects of countermeasures³⁰.

Mice were randomly divided into 4 different groups as follows: Group 1 mice were exposed to normal breathing air and injected with 0.9% saline; Group 2 mice were exposed to 765 ppm H₂S and injected with 0.9% normal saline (Cob 0 mg/kg bw); Group 3 mice were exposed to 765 ppm H₂S and injected with Cob at 50 mg/kg body weight; and Group 4 mice were exposed to 765 ppm H₂S and injected with Cob 100 mg/kg body weight. Cob or 0.9% saline were injected in the rear leg muscle in 50 µL of solution. Normal breathing air or H₂S were delivered from gas cylinders. On the first day, mice in groups of 10 were exposed to 765 ppm H₂S or breathing air for 40 min. On subsequent days, the same groups of mice were exposed either to 765 ppm H₂S or to normal breathing air for 15 min only, each day. Following each exposure, the chamber was flushed out with breathing air for 2 min and injected with Cob or saline. All mice were euthanized 1 h after the last saline or Cob injection after the 7th exposure, except for mice designated for histopathological analysis which were euthanized 24 h after the 7th H₂S exposure. End-points monitored included daily clinical assessment during and after H₂S exposure using a modified functional observational battery (FOB)³¹. Other tests included behavior tests, neurochemical, biochemical, molecular, and histopathology endpoints. Number of animals used for each endpoint is summarized in Supplemental Table 2.

Clinical Assessment

To obtain baseline data, animals were clinically evaluated and weighed daily starting 3 days prior to H₂S exposure. Following exposure, mice were weighed daily until euthanasia. A modified functional observational battery (FOB) was used for clinical evaluating during exposure in the chamber³¹. A clear advantage of this model employing freely moving

anaesthetized mice is that it allowed clinical assessment of mice during exposure. To assess the efficacy of Cob, clinical parameters evaluated included seizure activity and knockdown; which typically presented as lateral recumbency following seizure activity. Time to seizure and knockdown was recorded. Other assessed parameters included lacrimation, salivation, piloerection, dyspnea, body posture, and gait. For consistency, the same trained observer assessed the mice throughout the entire experiment.

Behavior Tests

Movement disorders are a common neurological sequela of acute H₂S exposure in human beings²⁵. To test the efficacy of Cob on coordination and balance, mice were subjected to the AccuRotor 4-Channel RotaRod test (rod diameter = 30 mm, height= 38 cm). Mice were trained on the rotating rod for two consecutive days prior to H₂S exposure at 24 rpm for 20 min. Following H₂S exposure, mice were given 5 trials for 20 min at 24 rpm on test days 2, 4, and 6.

Histopathology and Immunohistochemistry

In humans, neurodegeneration is a common sequela of acute H₂S poisoning. To evaluate this and the efficacy of Cob, designated mice were anesthetized deeply with a cocktail of 100 mg/kg bw ketamine and 10 mg/kg bw xylazine IM. Once in a surgical plane of anesthesia, the thoracic cavity was surgically opened to expose the heart. Fresh 4% paraformaldehyde solution (PFA, pH 7.4) was then injected through the left ventricle to perfuse the animal. After perfusion, brains were post-fixed in 4% PFA for 24 h, paraffin embedded, sectioned at 5 microns, and stained with hematoxylin and eosin for routine

histopathology. Additional brain sections were stained using an indirect immunostaining protocol (Vectastain Elite ABC kit, PK-6101, Vector Laboratories, Inc., Burlingame, CA) that employed primary antibodies directed at glial fibrillary acidic protein (GFAP, ab72600, Abcam), ionized calcium binding adaptor molecule 1 (Iba1, ab153696, Abcam), 4-hydroxynonenal (4HNE; ab46545, Abcam). Diaminobenzidine (DAB, SK-4100, Vector Laboratories, Inc.) was the chromogen used. Stained sections were examined microscopically using a Nikon Eclipse Ci-L microscope with DS-Fi2 camera or EVOS FL fluorescence microscope. Routine histopathology, as well as immunohistopathology, was conducted by a board-certified veterinary pathologist blinded to the study design. Lesion severity was assessed using semi-quantitative scale summarized in Supplemental Table 1.

Biochemical and Neurochemical Analysis

Hydrogen sulfide inhibits cytochrome c oxidase activity and induces neurochemical changes by inhibiting the monoamine oxidase enzyme. These were used to assess the efficacy of Cob. Mice were euthanized by decapitation 1 h after the last Cob injection. Brains were immediately removed from the skull, held on ice, and micro-dissected into different brain regions. Brain tissue samples were stored at -80 °C until analysis. Cytochrome c oxidase enzyme was extracted from micro-dissected brain regions (inferior colliculus, thalamus, and cortex) and enzyme activity determined using an assay kit (ab109909) from Abcam (Cambridge, MA, USA) according to manufacturer's protocol.

To determine H₂S-induced neurochemical changes, the striatum was analyzed for changes in dopamine (DA) and its metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The striatum was also analyzed for serotonin (5-HT), its

metabolite, 5-hydroxyindoleacetic acid (5-HIAA) as well as norepinephrine (NE). Samples were prepared and quantified as described previously³⁰. Briefly, neurotransmitters were extracted from different brain regions in 0.2 M perchloric acid solution containing 0.05% Na₂EDTA, 0.1% Na₂S₂O₅, and isoproterenol (internal standard). DA, 5-HT, NE, and their respective metabolites were analyzed by HPLC ECD consisting of a CoulArray model 5600A coupled with an analytical cell (microdialysis cell 5014B) and a guard cell (model 5020). Neurochemicals and metabolites were separated isocratically by a reverse-phase column with a flow rate of 0.6 ml/min mobile phase (10% acetonitrile, 1% sodium phosphate, 89% water) using a Dionex Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL) equipped with a refrigerated automatic sampler (model WPS-3000TSL). Data acquisition and analysis were performed using Chromeleon 7 and ESA Coularray 3.10 HPLC Software.

Molecular Targets

In order to elucidate the molecular effects of H₂S-induced neurotoxicity and to evaluate efficacy of Cob as a therapeutic agent, the inferior colliculi from mice in the breathing air-control group, H₂S-saline control, and H₂S-Cob (100 mg/kg) mice were analyzed for gene and protein changes. First, a total RNA isolation protocol was adapted from Seo et al³². Briefly, brain tissue was placed in TRIzol Reagent, homogenized, and incubated for 5 min. Then, 200 µl of chloroform was added and shaken vigorously for 15 s. This mixture was incubated for 3 min and then centrifuged at 12,000 x g for 15 min at 4°C. The upper, aqueous layer was transferred to a separate tube and 500 µl of isopropanol was added. This suspension was incubated at RT for 10 min before being centrifuged for 10 min

at 4°C for 12,000 x g 32. First strand cDNA synthesis was performed using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, #4368814) following the manufacturer's protocol. qRT-PCR was performed following previous protocols³³. The following validated mouse primers were purchased from Qiagen and used for performing qRT-PCR: TNF α , IL-1 β , IL-6, IL-12, and IL-18, TLR9, and MyD88. For normalization of each sample, the 18S rRNA gene (purchased from Qiagen) was used as the housekeeping gene. No-template controls (NTCs) and dissociation curves were run for all experiments to exclude cross-contamination³³.

With the aim of studying protein changes via Western Blot, the inferior colliculus samples were lysed in modified RIPA lysis buffer (1% Triton X-100, 1mM EDTA, 100mM NaCl, 1mM EGTA, 1mMNaF, 20mM Na₄P₂O₇, 2mM Na₃VO₄, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 50mM Tris-Cl, pH7.4) via sonication. Brain homogenates were prepared as described previously³⁴. Protein concentration of samples was measured using Bradford assay. Western blotting was performed as described previously³⁴. Briefly, the samples, containing equal amounts of proteins, were loaded and fractionated in a 10-12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in TBS supplemented with 0.1 % Tween-20 or Lico blocking buffer. Primary antibodies against specific proteins were incubated with the membrane overnight at 4°C. These included TNF- α receptor 2 (TNF- α R2, ab15563, Abcam), Caspase-3-cleavage (Santa Cruz Biotechnology), BCL2 associated death promoter (Bad, sc-943, Santa Cruz Biotechnology), and TNF α (R&D Biosystems). After rinsing thoroughly in PBS supplemented with 0.1% Tween-20, the membrane was incubated with Alexa Fluor 680 goat anti-mouse or IRDye 800 donkey anti-rabbit secondary antibodies. For the loading control, β -actin antibody was used.

Immunoblot imaging was performed with an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA). ImageJ software (National Institutes of Health, Bethesda MD, USA) was used to quantify Western blot bands.

Experiment 2:

Determining the efficacious time window of cobinamide given post-H₂S exposure

A key question of any drug showing efficacy for treatment of H₂S poisoning is how far out after rescue will the drug still benefit the patient. Under mass casualty conditions, some victims may not be reached for treatment 30-60 min post exposure. Using an effective Cob dosage chosen from Experiment 1 above, we tested the hypothesis that Cob at 100 mg/kg bw is efficacious for treatment of H₂S-induced toxicity given up to 30 mins post-H₂S exposure. The same H₂S exposure paradigm as Experiment 1 was used for this study.

Mice were randomly divided into 5 groups as follows: Group 1 mice were exposed to normal breathing air and injected with 0.9% normal saline 30 min post exposure; Group 2 mice were exposed to H₂S and injected with 0.9% normal saline 30 min post exposure; Group 3 mice were exposed to H₂S and injected with Cob (100 mg/kg) 2 min post-H₂S exposure; Group 4 mice were exposed to H₂S and injected with Cob (100 mg/kg) 15 min post-H₂S exposure; Group 5 mice were exposed to H₂S and injected with Cob (100 mg/kg) 30 min post-H₂S exposure. Measured endpoints included clinical assessments using a modified functional observation battery, behavioral tests, neurochemical, biochemical, and histopathological endpoints as described under Experiment 1. All mice were euthanized 1 h post saline or Cob injection after the 7th exposure.

Data Analysis

Data are presented as mean and standard error of the mean. Clinical toxicity during exposures was analyzed using linear regression. Biochemical and neurochemical endpoints were analyzed using a student's t-test comparing the H₂S-saline treated group to the H₂S-Cob treated group. Behavioral data was analyzed using 2-way ANOVA with a Bonferroni post-hoc test. Statistical tests were performed on Prism 6 (GraphPad Prism Software). Data was considered statistically significant at $p < 0.05$.

Results

Experiment 1: Dose-response Cobinamide study

Clinical assessment results

Results of clinical assessment are summarized in Table 1. Mice exposed to normal breathing were normal and healthy throughout the duration of the study. Mice in the group exposed to H₂S and treated with Cob were clinically better compared to mice exposed to H₂S and treated with saline (Table 1). For example, mice exposed to H₂S and injected saline exhibited typical clinical signs of H₂S poisoning such as lacrimation, salivation, ataxia, impaired righting reflex, and convulsions which were absent in mice exposed to H₂S and treated with Cob. However, all H₂S dosed mice exhibited dyspnea characterized by open mouth breathing and hunched body posture, regardless of whether they were treated with Cob or not. Mice exposed to H₂S and injected with higher dosage of Cob at 100 mg/kg lost significantly less weight than those injected with saline. The lower Cob dosage at 50 mg/kg

dose did not prevent the H₂S-induced weight loss (Fig 1B). However, Cob at 50 mg/kg and 100 mg/kg increased survival in this model (Fig 1C).

Over the course of the study, seizures were observed in about 80% of the mice exposed to H₂S treated with saline, whereas only 5% of the Cob treated mice exhibited seizure activity (Fig 1D). Mice treated with the high dose of Cob (100 mg/kg) experienced significantly fewer knockdowns compared to the H₂S-saline treated group, while the lower dosage Cob (50 mg/kg bw) did not protect against seizures as efficiently (Fig 1E). Overall, Cob at the 100 mg/kg dosage was more effective in countering clinical signs induced by H₂S.

Behavior Tests

Results of the Rotarod test are summarized in Fig 1F. Cob at the higher dosage (100 mg/kg bw), but not at the lower dosage (50 mg/kg bw), significantly protected mice against H₂S-induced motor deficits.

Histopathology and Immunohistochemistry

The inferior colliculus and the thalamus were the most consistently affected brain regions in this model. Hydrogen sulfide-induced microscopic lesions consisted of vacuolar degeneration with formation of coalescing vacuoles in the neuropil, neuronal degeneration and cell death, and infiltration by activated neuroglia (Fig 2A). In severely affected animals, there was complete loss of neurons in affected regions at 7 days, with increased numbers of astrocytes, microglia, and the presence of microglial phagocytic (Gitter) cells. Capillaries within injured regions had prominent endothelial cells and were mildly tortuous, consistent with glial scar formation. Cob at the highest dosage (100 mg/kg) significantly reduced the

severity of lesions in the inferior colliculus and thalamus, resembling control animals, while several of the animals treated with the lower dosage (50 mg/kg) developed degenerative lesions. These results showed that Cob at the higher dose was effective in reducing lesion severity and incidence than the lower dosage (Fig 2A-B).

Biochemical and Neurochemical Analysis

Previous work in our lab has found H₂S to inhibit cytochrome c oxidase in multiple brain regions. We investigated cytochrome c oxidase activity in H₂S-poisoned mice treated with saline versus those treated with Cob. Cytochrome c oxidase activity was significantly diminished in mice exposed to H₂S and injected with saline, but was rescued in mice injected with Cob at 100 mg/kg. However, Cob at 50 mg/kg bw failed to rescue H₂S-induced suppression of H₂S-induced cytochrome c oxidase activity. This observation was observed in multiple brain regions including the inferior colliculus, thalamus, and the cortex (Fig 2C).

Mice exposed to H₂S and injected with saline showed a statistically significant increase in dopamine (DA) and its respective metabolites DOPAC and HVA. Cob at the highest dose (100 mg/kg bw) prevented the H₂S-induced increase in DA, DOPAC, and HVA (Fig 2D).

Molecular Targets

We have previously reported that neuroinflammation is one of the mechanisms we hypothesize to be involved in H₂S-induced neurotoxicity²⁰. Since Cob was most effective at 100 mg/kg bw, in this portion of the study, we only investigated this dosage. Also, we focused on the inferior colliculus, the most consistently affected brain region in this model.

We found that TLR9 and MyD88 mRNA expression was significantly increased in the inferior colliculus of the H₂S exposed mice treated with saline (Fig 3A). Cob at 100 mg/kg bw prevented increased expression of TLR9 and MyD88 mRNA expression. Activation of MyD88 by TLR9 can lead to NFκB activation, causing increased expression of pro-inflammatory cytokines. Therefore, we investigated the effect of H₂S on cytokines and the impact of Cob. Mice exposed to H₂S and injected saline showed a significant increase in mRNA expression of TNF-α, IL-1β, IL-6, and IL-12. Cob at 100 mg/kg significantly lowered the expressions of these pro-inflammatory cytokines (Fig 3B). We also found upregulation of TNF-α receptor and TNF-α protein in mice exposed to H₂S, but these TNF-related H₂S-induced changes were prevented in mice exposed to H₂S and treated with 100 mg/kg Cob (Fig 3C). Increase in GFAP and Iba-1 protein typically reflects activated astrocytes and microglia indicating a neuroinflammatory response. H₂S significantly increased the protein expression of GFAP and Iba-1 in the inferior colliculus, surrounding the lesion. This GFAP and Iba-1 H₂S-induced increase was reduced by Cob (Fig 3D-E).

Oxidative stress is another common mechanism of toxic induced neurodegeneration and is often coupled to inflammation. In this study, using Western Blot and immunohistochemistry analysis, we observed increased 4-HNE, a biomarker of lipid peroxidation in mice exposed to H₂S and injected with normal saline (Fig 4A). Cob at 100 mg/kg prevented the generation of 4-HNE, implying reduced oxidative stress. H₂S also induced an increase of Bad protein, a pro-apoptotic protein, in the inferior colliculus; while Cob (100 mg/kg) reduced Bad expression (Fig 5B). We also found caspase-3 cleavage in mice exposed to H₂S, which is indicative of apoptosis. This cleavage was prevented in mice exposed to H₂S treated with Cob (Fig 4B).

Collectively, results of this mechanistic data show that Cob is neuroprotective against H₂S-induced inflammation, oxidative stress, and mitochondrial dysfunction. Overall, results of the dose response Cob study showed that Cob at 100 mg/kg bw was effective in treatment of H₂S-induced neurotoxicity and neurodegeneration.

We have previously reported that neuroinflammation is one of the mechanisms we hypothesize to be involved in H₂S-induced neurotoxicity²⁰. Considering that Cob was most efficacious at the 100 mg/kg bw, in this portion of the study, we investigated the efficacy of Cob at this dosage. Also, we focused on the inferior colliculus, the most consistently affected brain region in this model. In these experiments, TLR9 and MyD88 mRNA expression was significantly increased in the inferior colliculus of the H₂S-exposed mice treated with saline (Fig 3A), while Cob at 100 mg/kg bw prevented this increased expression of TLR9 and MyD88 mRNA expression. Activation of MyD88 by TLR9 can lead to NFκB activation, causing increased expression of pro-inflammatory cytokines. Therefore, we investigated the effect of H₂S on cytokines and the impact of Cob. Mice exposed to H₂S and injected saline showed a significant increase in mRNA expression of TNF-α, IL-1β, IL-6, and IL-12. Cob at 100 mg/kg significantly lowered the expressions of these pro-inflammatory cytokines (Fig 3B). We also found upregulation of TNF-α receptor and TNF-α protein in mice exposed to H₂S, but these TNF-related H₂S-induced changes were prevented in mice exposed to H₂S and treated with 100 mg/kg Cob (Fig 3C). Increase in GFAP and Iba-1 protein typically reflects activated astrocytes and microglia indicating a neuroinflammatory response. H₂S significantly increased the protein expression of GFAP and Iba-1 in the inferior colliculus, surrounding the lesion. This GFAP and Iba-1 H₂S-induced increase was reduced by Cob (Fig 3D-E).

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Collectively, results of this mechanistic data show that Cob is neuroprotective against H₂S-induced inflammation, oxidative stress, and mitochondrial dysfunction. Overall, results of the dose response Cob study showed that Cob at 100 mg/kg bw was efficacious for treatment of H₂S-induced neurotoxicity and neurodegeneration.

Experiment 2: Therapeutic time window of cobinamide

Clinical Assessment results

In this portion of study, we used only the high dose of Cob (100 mg/kg bw). As observed in Experiment 1, mice treated with 100 mg/kg Cob injected 2 min post exposure, lost significantly less weight than the groups of mice injected with saline post H₂S exposure throughout the 7 days of exposure. Body weights of the group of mice injected with Cob 15 min or 30 min post H₂S exposure were not significantly different from the group of mice exposed to H₂S and injected with saline (Figure 5A). However, H₂S exposed mice injected

with Cob at 2 min and 15 min post exposure had a significantly greater cumulative survival rate compared to the mice injected with saline (Fig 5B). All mice that died during the experiment died during H₂S exposure or within 5 min of termination of H₂S exposure.

Cob was most protective against seizure activity and knockdowns induced by H₂S when given 2 min post exposure as opposed to at 15 or 30 min post-exposure. Cumulatively, by the 7th exposure, only about 10% of mice given Cob 2 min post exposure had seizures and knockdowns, which were significantly lower than that of H₂S exposed mice injected with saline (60%). With respect to seizure activity, H₂S-exposed mice given Cob 15 or 30 min post H₂S exposure were not protected and were not different than the H₂S-saline treated group (Figure 5C-D).

Behavior Tests

Behavioral studies showed that motor deficits induced by H₂S were significantly prevented by injecting Cob injected 2 min post exposure compared to the H₂S group of mice injected with saline. Mice injected with Cob 15 and 30 min post H₂S exposure did not perform significantly better than H₂S-dosed mice injected with saline on the Rotarod test (Figure 5E). Overall, these behavioral results show that Cob is most efficacious against H₂S-induced toxicity if given 2 min post H₂S exposure rather than 15 or 30 min post H₂S exposure.

Histopathology

Control animals had no lesions. Animals exposed to H₂S consistently developed severe lesions in the inferior colliculus and mild lesions in the thalamus. None of the mice

given Cob 2 min post H₂S exposure developed moderate or severe lesions. Twenty percent of mice treated with Cob at 15 min post H₂S developed inferior colliculus injury with a histopathology score of >15 while 40 % of mice treated with Cob at 30 min post H₂S developed inferior colliculus damage. Cob treatment at 15 or 30 min resulted in protection from neuronal degeneration and death, and reduced glial responses though best results were observed at 2 min. Treatment with Cob at either 15 or 30 min post H₂S exposure strongly protected against thalamic injury as did treating at 2 min post-H₂S exposure. Results of the histopathology study suggest that injecting Cob 30 min post exposure was still beneficial in some mice, but best neuroprotection was achieved when treatment was given 2 min post-H₂S exposure (Fig 6A-B).

Biochemical and Neurochemical Analysis

Here we investigated the effects of Cob on the H₂S-induced inhibition of cytochrome C oxidase when the drug is administered at 2, 15 or 30 min post H₂S exposure. Results indicate that Cob was most effective in preventing cytochrome C oxidase inhibition when given 2 min post exposure. Cob injected 15 or 30 min post exposure did not prevent H₂S-induced inhibition of cytochrome c oxidase enzyme in any of the brain regions examined, including the inferior colliculus, thalamus, and cortex (Fig 6D).

As observed in Experiment 1, H₂S induced an increase in dopamine in the striatum. Injecting Cob (100 mg/kg) 2, 15 and 30 min post H₂S exposure prevented an increase in dopamine levels. Hydrogen sulfide DA-induced changes were most responsive to Cob therapy and giving Cob even 30 min out was still beneficial (Fig 6C).

Discussion

This is the first study to evaluate the efficacy of Cob for treatment of H₂S-induced neurodegeneration and other sequelae, and extends the work of others which focused on efficacy of Cob on enhancing survival³. We found that Cob is efficacious for treatment of H₂S-induced neurodegeneration, and that 100 mg/kg Cob (Human equivalent dose = 8.1 mg/kg) was most effective when given immediately after rescue from H₂S exposure. At 50 mg/kg Cob was not consistently effective across the suite of endpoints monitored in this study. This observation is borne out by results of percent survival, behavioral changes including prevention of body weight changes, rotarod tests, protection against neurochemical changes, protection against cytochrome C oxidase inhibition, preventing neural inflammation and oxidative stress, and reduction in neural lesion frequency and severity. As might be expected, delaying Cob injection post H₂S exposure reduced its efficacy.

Cobinamide is attractive for field treatment of H₂S because it can be easily given by the IM route. Nitrite and hydroxocobalamin, the other drugs with reported efficacy for treatment of H₂S poisoning require IV administration^{20, 35}, which is cumbersome and inefficient for treating mass casualties. Besides, nitrite is associated with side effects such as hypotension, confounding H₂S-induced hypotension^{20, 26}. Hydroxocobalamin, though effective as a cyanide antidote, is not as effective as Cob in H₂S animal models. Cob is 3-10 times more potent than hydroxocobalamin in binding H₂S³⁵. In this study we used dinitrocobinamide. This dissociates in vivo yielding Cob. One molecule of Cob binds 2 molecules of sulfide unlike hydroxocobalamin which binds only one molecule³⁵. It is also

possible that nitrite from dinitrocobinamide generates NO, with added benefit as does nitrite³⁵.

H₂S dissociates into different sulfide species within a few minutes in vivo. Excellent studies by Haouzi et al., Jiang et al., Salnikov et al., and Cronican et al. suggest that following H₂S exposure, < 50% of H₂S exists in the parent form^{27, 28, 36}. Considering this observation, it makes sense that Cob therapy would be most beneficial given during or a few minutes after termination of H₂S exposure. It is during this time that Cob would combine with H₂S before it transforms into other sulfide species. It is interesting however, that results from the time-course study (Experiment 2) showed Cob had some efficacy at 15 and even at 30 min post H₂S exposure. This suggests that Cob may harbor other mechanisms, which collectively contribute to its efficacy. Indeed H₂S has been shown to induce oxidative stress via generation of superoxide anions and Cob has been shown to neutralize these free radicals³. Free radicals beget free radicals, triggering a chain reaction of oxidative stress and giving Cob as late as 15 min post-exposure may be beneficial by interrupting this process. We hypothesize that by binding H₂S and neutralizing free radicles Cob protects critical molecular targets, resulted in reducing injury and associated inflammation. A recent case report of a human suicide victim of H₂S by inhalation found the victim expired H₂S for at least 7 h³⁷. If this is confirmed, the implication is that the window of opportunity for Cob therapy may actually be wider in some human cases.

Seizure activity was significantly reduced or prevented by Cob. The mechanisms of H₂S-induced seizures are unknown, but changes in neurotransmitters such as GABA and glutamate, and neural inflammation have been associated with seizures³⁸. We did not

measure GABA and glutamate in this study, but we observed neuroinflammation. Cob at 100 mg/kg reduced neuroinflammation and this potentially may have reduced seizure activity. Seizure severity has been linked to neural injury in other models of chemical-induced neurotoxicity such as nerve agents³⁹. Reducing seizure activity could be one way Cob reduced lesion severity. Regardless of the mechanisms involved, results of this study have clearly shown that Cob reduced H₂S-induced neurodegeneration.

The role of H₂S in inflammation is controversial both in vitro and in vivo⁴⁰⁻⁴³. H₂S has been shown to increase pro-inflammatory cytokine expression in human monocytes via the ERK-NF-kappaB pathway⁴⁴. We showed that Cob ameliorates neuroinflammatory effects of H₂S in the inferior colliculus of mice. Furthermore, we showed that H₂S caused an increase in TLR9 expression that caused increased MyD88 expression leading to transcription of pro-inflammatory cytokines via NFκ-B pathway. We also found an increase in IL-6 expression. Increased IL-6 is a vital factor in vascular inflammation⁴⁵, which is caused by monocyte infiltration in the injured vessel wall. In our model of H₂S-induced neurodegeneration, H₂S caused hemorrhage, in areas of the lesion, indicative of vascular damage; this was prevented by Cob.

Frequently, TNFα is implicated in inflammation⁴⁶. We found that both mRNA and protein expression of TNFα were increased in the H₂S/saline group. This was significantly reduced by Cob. Under toxic conditions, microglia can release large amounts of TNFα⁴⁶. Increased TNFα has been implicated in excitotoxicity by inhibiting glutamate transporters on astrocytes, increasing AMPA and NMDA receptors, while also decreasing GABA-A receptors on neurons^{38, 46}. These mechanisms could be involved in the seizure-like activity

that has been reported in human H₂S exposure cases⁴⁷. Interestingly, we also found an increase in TNF α R2 that was significantly diminished by Cob. TNF α R2 is found predominantly in microglia, and found to mediate both pro-inflammatory and pro-survival signaling⁴⁸.

Release of pro-inflammatory cytokines (IL-1 β , IL-6, IL-18, TNF α , and IL-12) from microglia can be toxic to neurons as they are rapidly upregulated when reacting to a toxic insult⁴⁹. The pro-inflammatory cytokines propagate the inflammatory response and can cause neuronal and synaptic dysfunction⁴⁹. The neurons themselves can produce cytokines in response to the peripheral cytokines⁵⁰, which then sustains the neuro-inflammatory response (Scheme 1). Damaged neurons can also lead to the transformation of microglia into phagocytic cells that remove cellular debris⁵¹. It is important to note that Cob (100 mg/kg) injected 2 min post H₂S exposure prevented these neuro-inflammatory changes from occurring.

Oxidative stress has been implicated in neuronal injury. In this model of H₂S-induced neurodegeneration, oxidative stress can be caused by both the activated microglia⁵¹, but also directly from H₂S. 4HNE is a biomarker of oxidative stress and has been implicated as a factor in mitochondrial dysfunction⁵². We found H₂S increased the production of 4HNE, and Cob prevented this increase.

We found that H₂S inhibited cytochrome c oxidase activity, a known hallmark of H₂S-induced toxicity^{53, 54}. Low cytochrome c oxidase activity can result in a loss of membrane potential leading to production of oxidative stress^{55, 56} and mitochondrial dysfunction. Cob (100 mg/kg) prevented the inhibition of cytochrome c oxidase by H₂S. We

found that Bad was upregulated in H₂S exposed brain tissue and Cob prevented the upregulation. Bad is a pro-apoptotic protein in the Bcl-2 family, a group of proteins that mediate pro- and anti-apoptotic signaling in the mitochondria^{56,57}. These results cumulatively show that Cob mitigates H₂S-induced mitochondrial dysfunction.

Mitochondrial dysfunction has been shown to lead to neurodegeneration⁵⁸. We found that H₂S induces mitochondrial dependent apoptosis. Specifically, we found H₂S induced caspase-3 cleavage in the inferior colliculus of H₂S exposed mice and that Cob prevented this reaction.

Collectively our data show proposed novel mechanisms of action for H₂S-induced neurotoxicity. We have shown that H₂S induced neuroinflammation resulting in production of many pro-inflammatory cytokines. This neuroinflammation leads to neuronal dysfunction in many ways, including excitotoxicity and oxidative stress inducing mitochondrial dysfunction, which can then propagate inflammation and also lead to neuronal cell death (Scheme 1). Importantly, Cob (100 mg/kg) injected 2 min post exposure prevented these changes. We speculate since Cob binds directly to H₂S preventing it from interacting with key molecular targets, thus preventing the clinical, pathological, neurochemical, and biochemical changes induced by H₂S.

Overall, results of this study show that Cob is protective against H₂S induced neurotoxicity and neurodegeneration. Importantly, our data has revealed some of the mechanistic effects induced by H₂S in a novel in vivo murine model, further identifying potential targets for therapeutic strategies. The inhalation model of H₂S poisoning used here for evaluation of efficacy of Cob is not perfect. It has advantages as well as limitations. A

major advantage is that the route of H₂S exposure is consistent with the route of exposure in humans. This model therefore replicates the pathophysiology implicated in human cases of inhalation H₂S toxicity. With the convenient NaHS intraperitoneal injection model, death occurs within 5 min of injection and pulmonary edema is generally absent. In human H₂S-induced deaths, about 50% of victims die at the scene, but while most others die 12-24 h later¹². This death pattern is not replicated by the NaHS. A limitation of the model is that mice do not readily develop H₂S-induced neurological lesions as humans do³⁰. Single, acute high dose H₂S causes high mortality during exposure, with only a few survivors developing neurodegeneration. Using a single, high dose exposure to mimic the typical human exposure scenario would have required an unreasonably large number of mice to test the efficacy of Cob. The approach used recapitulates H₂S-induced neurodegeneration³⁰. In conclusion, we have shown that Cob at 100 mg/kg bw is effective in treating H₂S-induced neurodegeneration; it is best if given within 2 min of rescue, but still showed some efficacy up to 30 min post exposure.

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Conflict of Interest

All authors declare no conflict of interest.

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Table 1. Modified functional observational battery for evaluation of clinical signs during H₂S exposure in the inhalation chamber.

Table 1. Modified Functional Observational Battery			
Measure	Breathing Air + Saline	H ₂ S + Saline	H ₂ S + Cobinamide (100 mg/kg)
Autonomic			
Lacrimation	No	present	No
Salivation	No	present	No
Urination/Defecation	Normal	increased	No
Convulsions			
Tonic movements	No	mild	No
Clonic movements	No	severe	Mild
Paddling feet	No	severe	Mild
General Measures			
Piloerection	No	present	No
Body Posture	Normal	hunched	hunched
Dyspnea	No	present	present
Gait			
Hindlimbs splayed	No	present	No
Ataxia	No	severe	Mild
Unable to support	No	present	No
Righting reflex	No	affected	Mild
Knockdown	No	severe	Mild

A H₂S Inhalation Exposure Paradigm

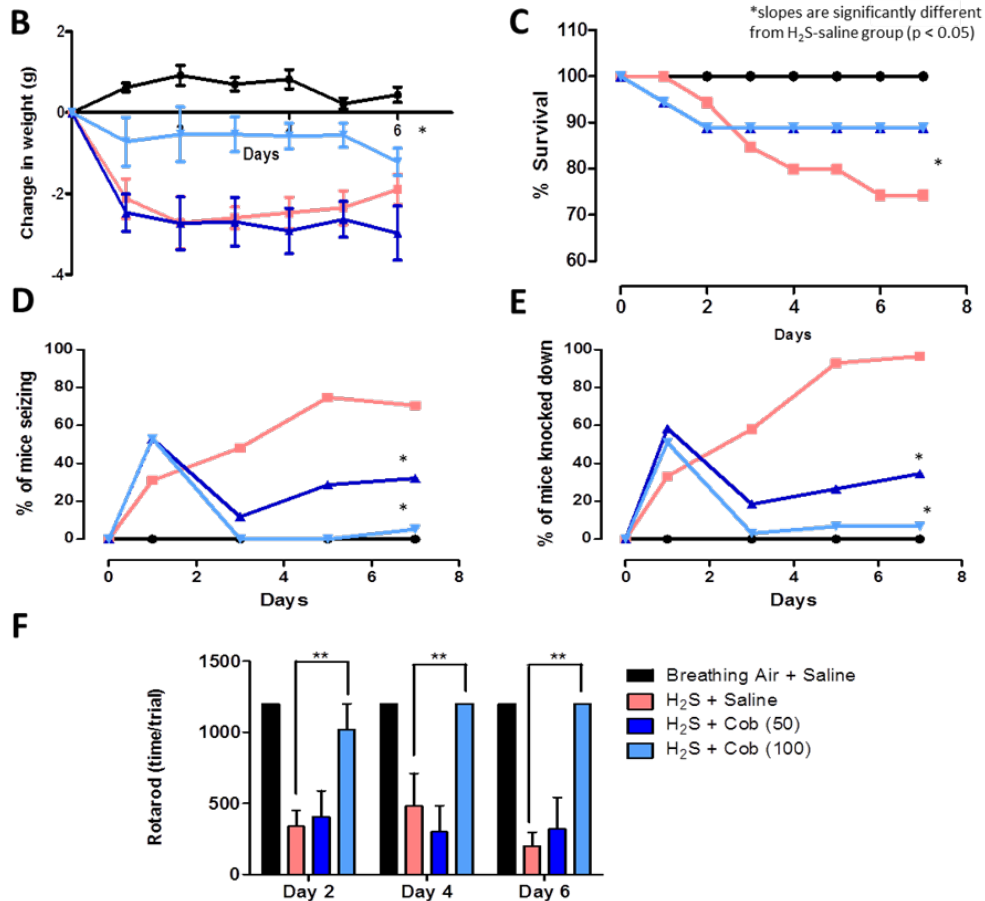
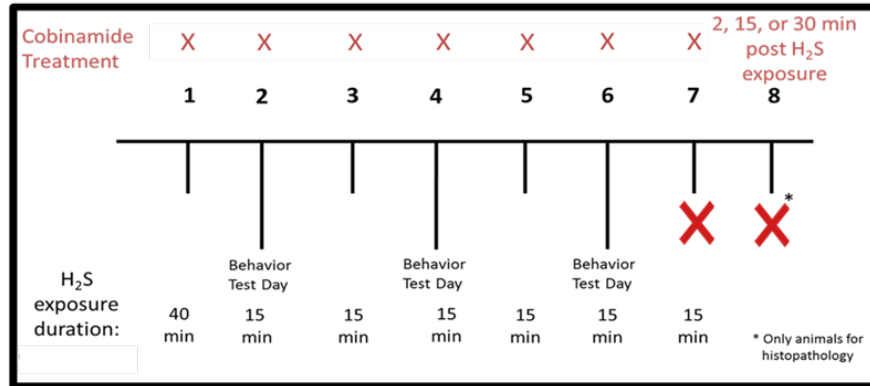


Figure 1. Cobinamide dose-dependently prevented H₂S intoxication. H₂S exposure paradigm in whole body inhalation chamber for 7 days (A). Cobinamide treatment prevented weight loss (B), mortality (C), seizure activity (D), and knockdown (E) following H₂S exposure. * $p < 0.05$, slopes are significantly different from H₂S-saline group. Rotarod behavior testing post 2, 4, and 6 H₂S exposure (F) ** $p < 0.01$ versus H₂S-saline group.

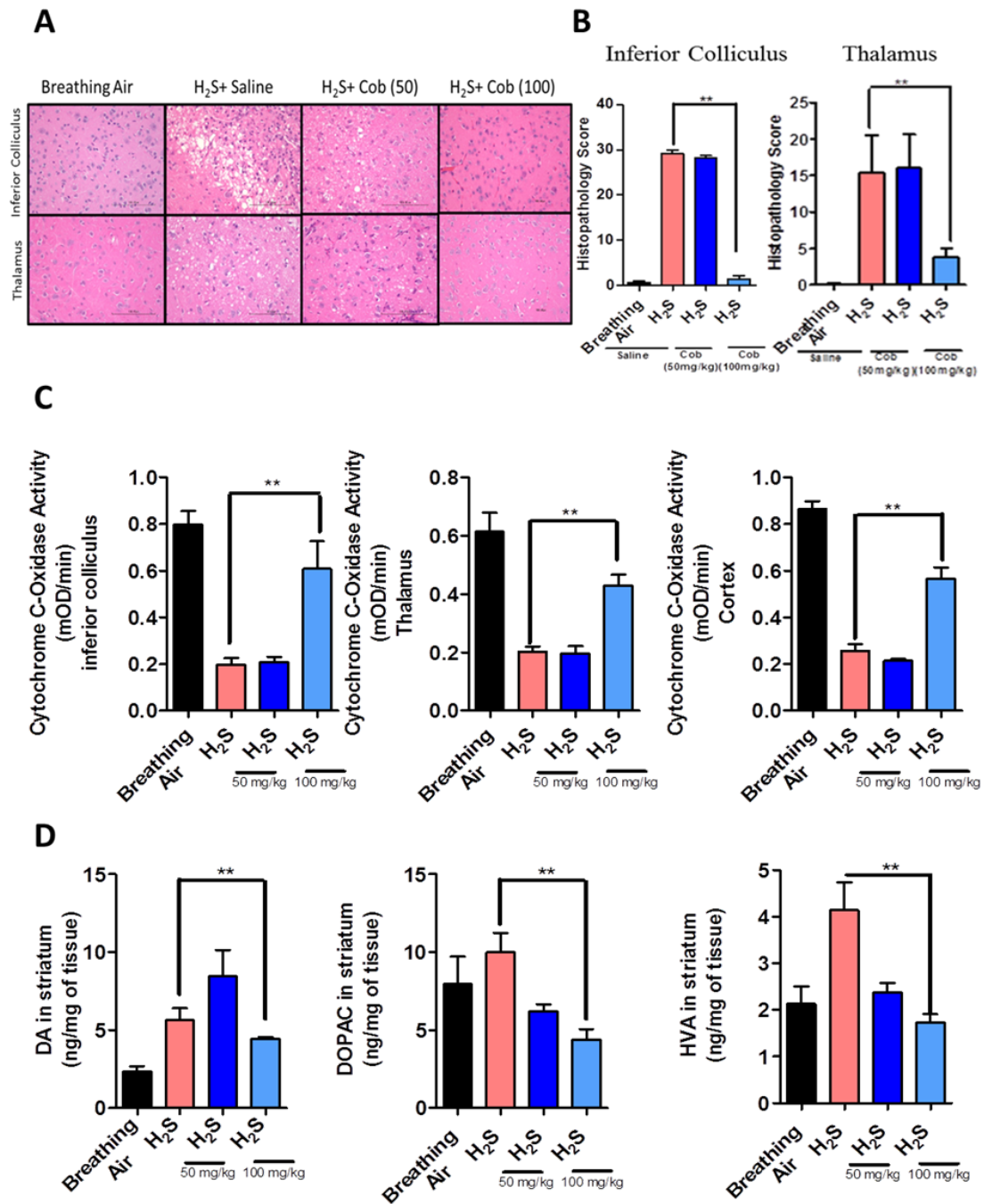


Figure 2. Cobinamide was dose-dependently effective in preventing H₂S neurotoxicity. Lesion severity was reduced following H₂S exposure with treatment of Cob (50 mg/kg and 100 mg/kg) (A-B). Prevention of cytochrome c oxidase activity inhibition (C) and neurochemical alterations (D) induced by H₂S utilizing Cob (100 mg/kg) ** p < 0.01 versus H₂S-saline group.

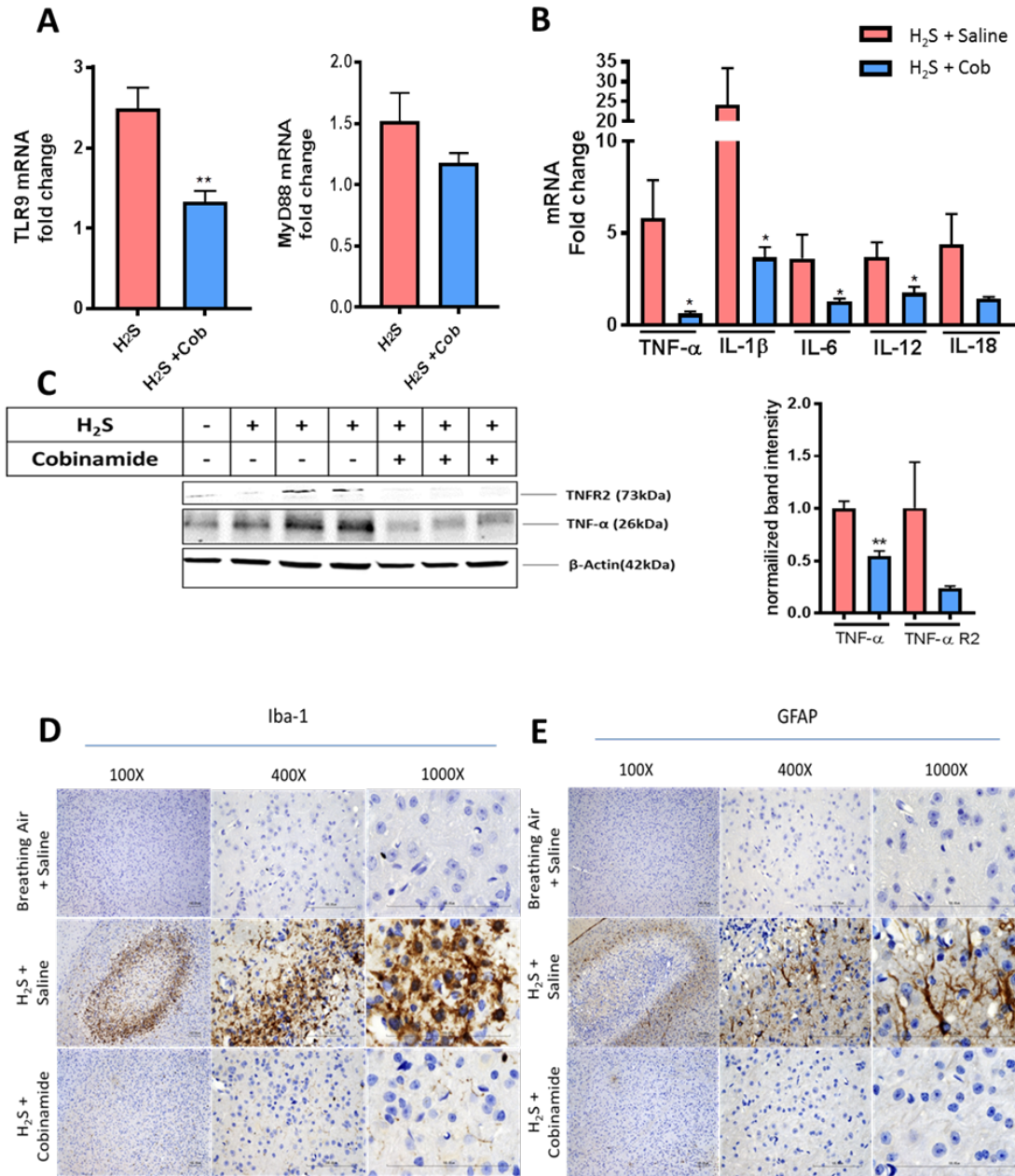


Figure 3. Cobinamide (100 mg/kg) prevented H₂S-induced neuroinflammation in the inferior colliculus. Decrease in TLR9, MyD88, and pro-inflammatory cytokine mRNA expression with treatment of Cob post H₂S exposure (A-B). Downregulation of TNFαR2 and TNF-α protein levels with Cob treatment post H₂S exposure (C). Reduction in activated microglia (D) and astrocytes (E) following treatment with Cob in H₂S exposed mice. * p < 0.05, ** p < 0.01 versus H₂S-saline group.

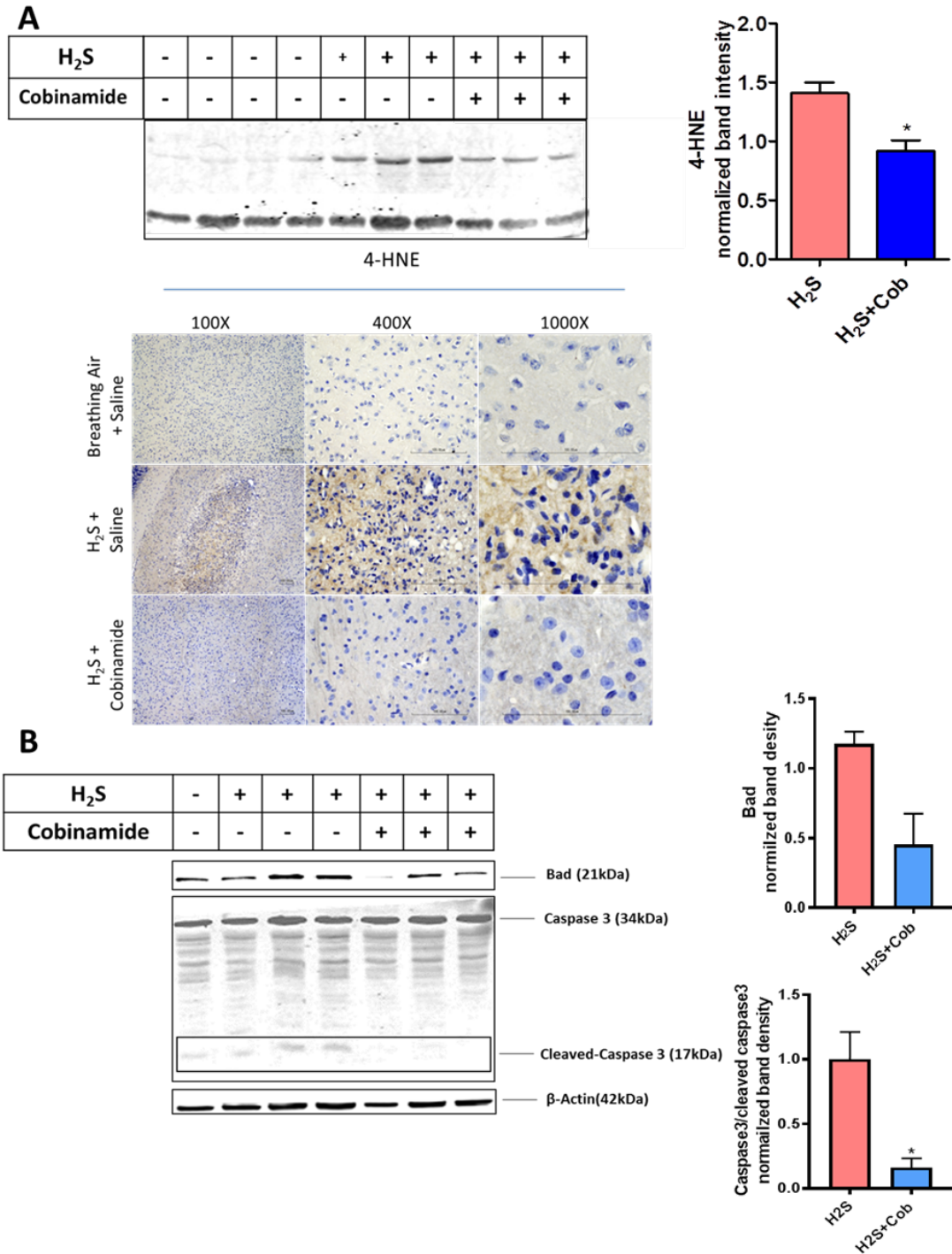


Figure 4. Cobinamide (100 mg/kg) prevented oxidative stress and mitochondrial dysfunction induced by H₂S exposure. Reduced 4-HNE production by cobinamide in the inferior colliculus post H₂S exposure (A). Cobinamide prevented Bad upregulation and caspase-3 cleavage induced by H₂S (B). * p < 0.05 versus H₂S saline group.

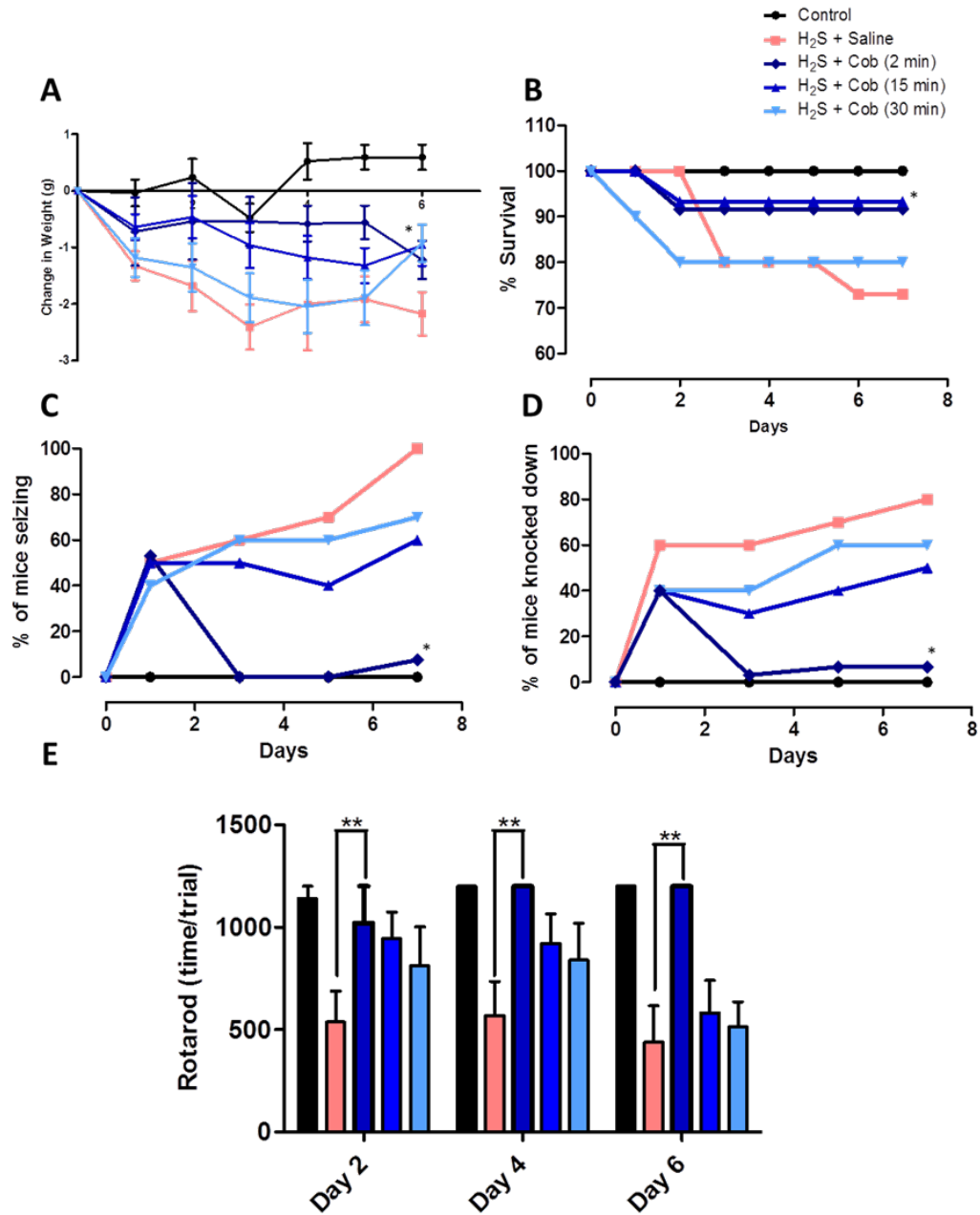


Figure 5. Cobinamide was effective in preventing clinical intoxication given 2 min post H₂S exposure. Weight loss (A), survival (B), seizure activity (C), and knockdown (D) during H₂S exposure. * $p < 0.05$, slopes are significantly different from H₂S saline group. Rotarod behavior testing post 2, 4, and 6 H₂S exposure (E) ** $p < 0.01$ versus H₂S-saline group.

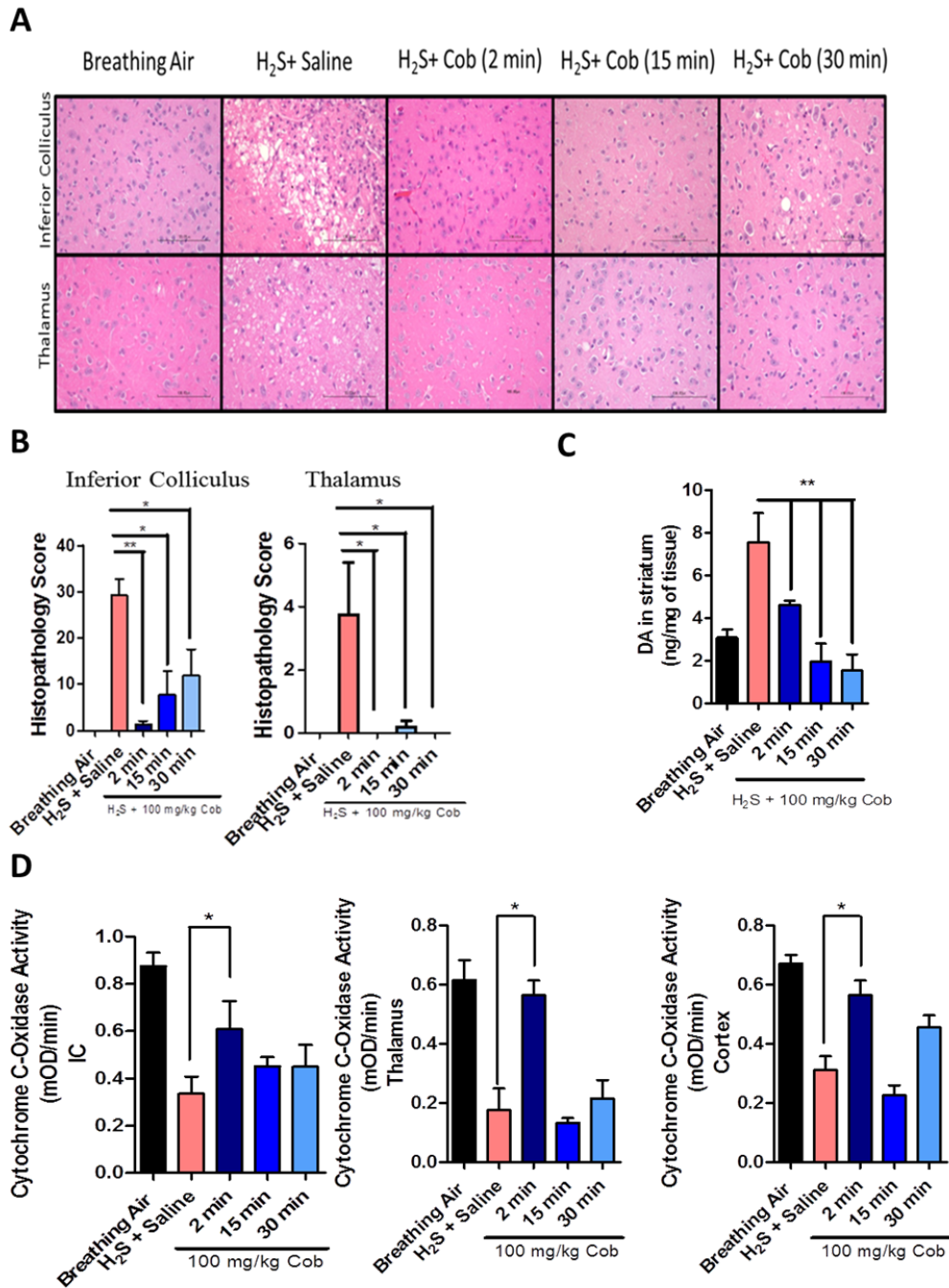
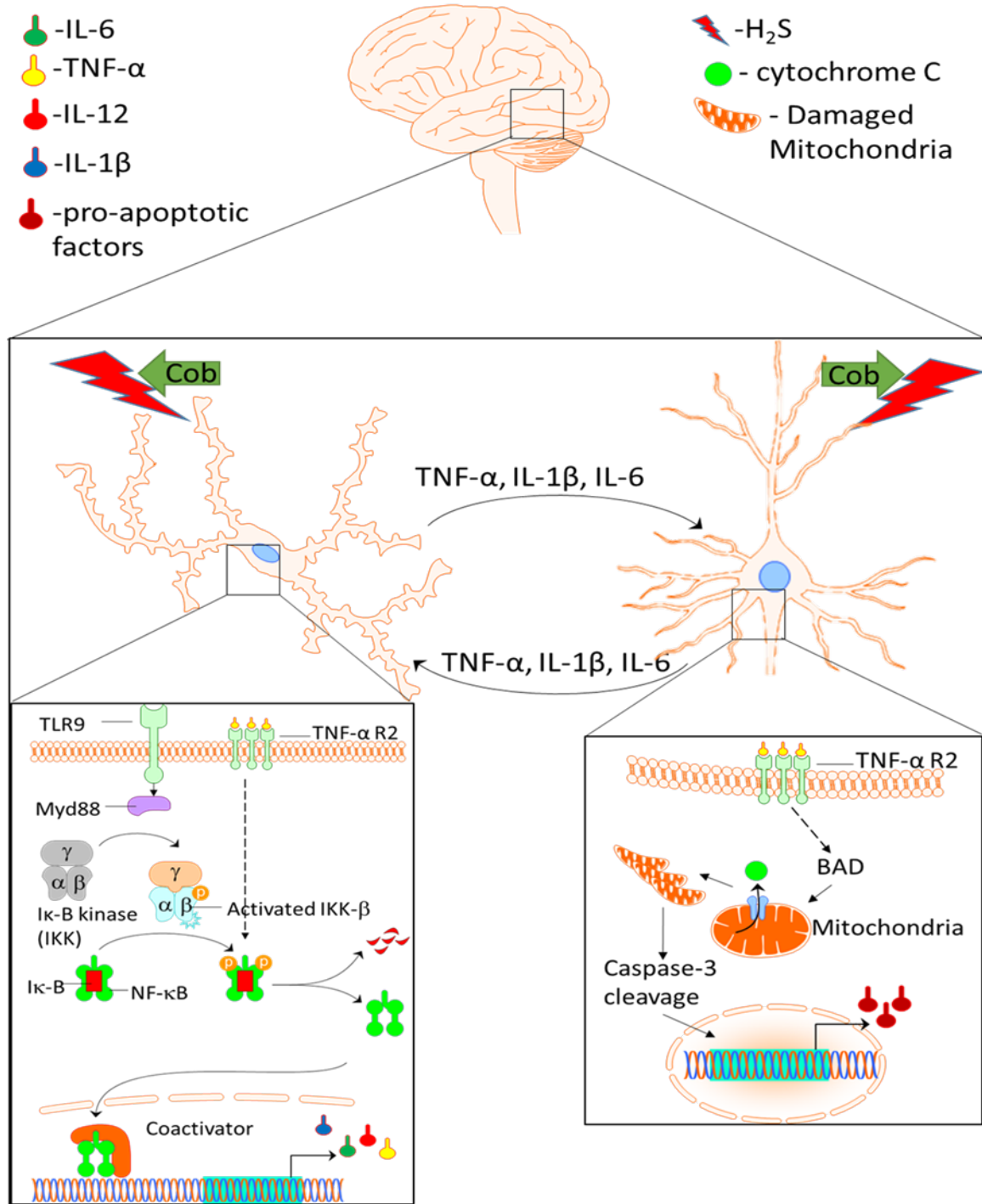


Figure 6. Overall, Cobinamide was most efficacious in preventing neurotoxicity given up to 30 min post H₂S exposure. Amelioration of H₂S-induced lesion severity with Cob treatment up to 30 min post H₂S exposure (A-B). Cobinamide prevented increased DA levels induced by H₂S up to 30 min post exposure (C). Inhibition of cytochrome c oxidase activity is prevented by administration of Cob 2 min post H₂S exposure (D). * $p < 0.05$, ** $p < 0.01$ versus H₂S-saline group.



Scheme 1. A Proposed Mechanism: Cobinamide prevents the downstream effects of H₂S – induced neurodegeneration by binding to H₂S to attenuate neuroinflammation, oxidative stress, and mitochondrial dysfunction.

Supplemental Table 1. Grading scheme for evaluation of histopathological lesions in the brain following H₂S exposure in mice.

Supplemental Table 1. Semi-Quantitative Grading Scheme for H₂S-induced Neuropathology							
Score	Neurons: Clumped Nissl or central chromatolysis	Neurons: Angular, shrunken neurons or early apoptotic neurons	Neurons: Loss of neurons	Glia: Astrocytes	Glia: Microglia/ macrophages	Apoptotic bodies	Neuropil: Pallor or Vacuoles
0	None	None	No loss	Normal morphology	Normal morphology	None	Solid, bright eosinophilic
1	Mild	<2/20X		Clumped chromatin		<2/20X	Slightly pale eosinophilic
2		2-5/20X			Rare amoeboid	2-5/20X	Finely vacuolated
3	Moderate	6-10/20X	~50% loss	Type II Alz cells	Many amoeboid	6-10/20X	Coarsely vacuolated
4		11-15/20X			Mixed amoeboid/Gitter	11-15/20X	Condensation of vacuoles
5	Severe	>15/20X	100% loss	Loss of astrocyte pop'ns	Gitter cells present	>15/20X	Loss of large areas of neuropil

Supplemental Table 2. Summary of number of mice used per endpoint in experiments.

Supplemental Table 2. Number of Mice/endpoint		
Endpoints	Experiment 1	Experiment 2
	n/group	n/group
Clinical Assesment	10	10
Behavior Tests	8-10	7-10
Histopathology	4-5	4-5
Biochemical Analysis	4-5	4-5
Neurochemical Analysis	4-5	4-5
Molecular Targets	6	N/A

CHAPTER 4

**MIDAZOLAM EFFICACY AGAINST ACUTE HYDROGEN SULFIDE-INDUCED
MORTALITY AND NEUROTOXICITY**

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Abstract

Introduction: Hydrogen sulfide (H₂S), previously used as a chemical weapon, is a colorless, highly neurotoxic gas with a rotten egg odor. It is an occupational and an environmental hazard. Acute high dose exposure causes peracute death, while survivors develop neurological sequelae. Currently, there is no suitable antidote for treatment of H₂S-induced neurotoxicity in the field. Midazolam (MDZ) is an anticonvulsant. Using a novel mouse model we tested the hypothesis that MDZ is effective in preventing and/or treating acute H₂S-induced neurotoxicity.

Methods: This proof-of-concept study had 2 main objectives: to determine whether MDZ prevents/reduces H₂S-induced mortality when given pre-or post during H₂S exposure; and to test whether MDZ prevents H₂S-induced neurological sequelae. MDZ (4 mg/kg) was administered IM, 5 min pre exposure to one high concentration of H₂S at 1000 ppm; or after 12 mins after exposure to 1000 ppm H₂S followed by a further 30 min exposure. A separate study tested the efficacy of MDZ pretreatment using a mouse model of neurological sequelae. Breathing air and H₂S controls were included in the study. Endpoints monitored included a functional observational battery used to assess clinical signs during H₂S exposure, mortality, behavior changes, and histopathological changes.

Results: Results indicated that MDZ given prophylactically or during H₂S exposure significantly reduced H₂S-induced lethality, seizures, knockdown, and behavioral deficits (p<0.01). MDZ also prevented H₂S-induced weight loss, behavior deficits,

neuroinflammation, and histopathologic lesions ($p < 0.01$) in the thalamus and inferior colliculus.

Conclusion: Overall, our findings show that MDZ is a promising drug for reducing H₂S-induced acute mortality, neurotoxicity, and neurological sequelae.

Introduction

Hydrogen sulfide (H₂S) is an extremely toxic gas and is only second to carbon monoxide as a leading cause of gas-induced deaths. It is a hazard in many occupational settings where accidental acute high dose exposure may occur during regular work, following industrial malfunction, or because of malice. Mass civilian casualties of acute H₂S poisoning have occurred in the past [1, 2]. Because of its history as a chemical weapon before, there is concern about potential misuse, especially in confined spaces such as the massive underground railroad system [3, 4]. At high concentrations, H₂S rapidly exerts its toxic effects not only on the central nervous system, but also on the respiratory and cardiovascular systems [5, 6]. Clinical signs of acute H₂S poisoning include dyspnea, anxiety, restlessness, and ocular and upper respiratory tract irritations in moderate concentrations. Sudden collapse ("knockdown") accompanied by seizures and breathing difficulty from pulmonary edema, arrhythmia and hypotension are signs of acute exposure at higher concentrations.

Hydrogen sulfide, which is characterized by a steep dose-response curve, causes high acute mortality. At least 50% of H₂S-induced deaths occurring during exposure, while the remainder of the mortality of intoxicated victims occurs within 48 h of rescue [2]. A unique characteristic of this toxic gas is that the knockdown associated with sudden exposure to high concentrations is incapacitating, rendering the victims unable to escape [7]. Despite the high

mortality, some victims of acute H₂S poisoning survive with or without supportive treatment. However, some of the survivors of acute intoxication develop long-term neurological sequelae characterized by psychiatric disturbances, persistent headaches, sleep disorders, anxiety, memory loss, learning disorders, hearing impairment, and movement disorders such as ataxia [5, 6, 8-10]. Neurological sequelae typically develop in victims, who succumb to knock down and coma, for as low as 5 mins but typically for 10-15 mins. These neurological complications may or may not be permanent but can be incapacitating, leading to work disability.

Because most deaths occur at the scene, there is a critical need for a drug or drugs that can be used in the field for treatment of victims of acute H₂S poisoning at the site. Currently recommended treatments of acute H₂S-poisoning are of questionable efficacy and cannot be effectively in the field for treatment of mass casualties. For example, treatment recommendations include nitrite and hydroxocobalamin, both of which require intravenous (IV) injections [11-14]. Intravenous injections can be challenging to use in mass victims in the field. Besides, IV nitrite injections are associated with hypotension, a limiting side effect¹. Hydroxocobalamin binds H₂S, but large volumes of IV hydroxocobalamin are recommended. Cobinamide (Cob) is a promising H₂S countermeasure that has shown efficacy in experimental animal models following intramuscular injection [14, 15] and can be delivered in the field. Nitrite, hydroxocobalamin, and cobinamide all largely work by binding H₂S *in vivo*. Because H₂S rapidly transmutes to other sulfide species, the therapeutic window for drugs that bind sulfide is very narrow. Consequently, there is a need to develop countermeasures with different mechanisms of action that can easily be used in the field for treatment of mass casualties.

Midazolam (MDZ), a common benzodiazepine and an anti-seizure medication, is on the list of The World Health Organization most essential drugs [16]. Available world-wide for treatment for epilepsy and seizures, this drug has recently shown promise as a countermeasure against nerve agent-induced neurotoxicity [16]. Midazolam is also a powerful anxiolytic, and has sedative and amnesic properties. Due to its rapid onset and relatively short half-life, MDZ is currently being considered to replace diazepam in the strategic defense stockpile as an anticonvulsant for nerve agent exposure [16]. It is very water soluble and therefore readily absorbed IM [16]. Maximum plasma concentration is reached in about 30 minutes post IM injection with > 90% bioavailability [16, 17]. Because of these desirable properties, we hypothesized that MDZ is effective for treatment of acute H₂S-induced neurotoxicity. The objective of this proof-of-concept study was to conduct a series of experiments to test the hypothesis that MDZ is efficacious for treatment of acute H₂S-induced mortality and neurotoxicity.

Materials and Methods

Animals

All animal studies were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). The 7-8 week old C57/BL6 male mice used in these studies were purchased from The Jackson Laboratories (Bar Harbor, ME) and weighed 20-25g at the beginning of the experiment. Mice were housed 5 per cage, in the Laboratory Animal Resource (LAR) Facility of the Iowa State University College of Veterinary Medicine (ISU CVM, Ames, IA). They were housed at a room temperature of 68-70°F, relative humidity of 35-50%, and a 12-hr light/dark cycle, and were provided 14% Protein

Rodent maintenance diet (Teklad HSD Inc, WI, USA), and drinking water *ad libitum*. Mice were acclimated for 1 week prior to the start of the studies.

Experimental approach

In this proof-of-concept study we conducted a series of experiments to evaluate the efficacy of MDZ for prophylactic treatment (pre-H₂S exposure) and for treatment of acute H₂S exposure (during exposure). Fully conscious and freely moving mice were utilized. The mice were exposed to H₂S by whole body inhalation exposure, details of which have previously been published[18]. Briefly, the experiments were conducted under a chemical fume hood approved by the Environmental Health & Safety at ISU. H₂S was introduced to the chamber and the desired concentration achieved by dilution with normal breathing air from a gas cylinder. The concentration of H₂S in the exposure chamber was constantly monitored using a H₂S monitor (Environmental Equipment and Supply, Harrisburg, PA) that was custom designed to measure concentrations of up to 1000 ppm of H₂S.

Objective 1: To test the efficacy of midazolam for reducing H₂S-induced acute mortality

Experiment 1: In this experiment we tested the hypothesis that injecting MDZ prophylactically before a single high dose H₂S exposure reduced mortality. Mice were injected once, either with 0.9% saline or MDZ(4 mg/kg), IM 5 min prior exposure to 1000 ppm H₂S for 120 minutes (Fig1A).

Experiment 2: In this experiment we tested the hypothesis that MDZ given once during acute high H₂S exposure reduces H₂S-induced mortality. Mice were exposed to 1000 ppm H₂S for 12 min in the inhalation chamber, after which mice were removed for injection of MDZ (4 mg/kg bw) or saline (0.9%) IM. All IM injections were 50 µL in the gastrocnemius muscle. Immediately after MDZ or saline injection, mice were returned to the

inhalation chamber for additional exposure to H₂S (1000 ppm) for 30 min. Mice were constantly observed during exposure for clinical signs of intoxication using a modified functional observation battery (FOB) [18, 19]. Specifically seizure activity and time to death were noted (Fig 2A). This exposure paradigm was done to simulate rescue from underground confined spaces or from high-rise buildings where victim will be treated upon arrival of first responders, which was estimated to take about 10 mins; but complete evacuation may last another half an hour. The difference is that in our model, we removed the mice from the chamber to inject them because our exposure chamber is not designed to allow safe injections to be done while the mice are in the chamber. Mice were immediately returned to the chamber and H₂S exposure immediately resumed. This procedure was completed within 5 mins.

Objective 2: To test the efficacy of midazolam for preventing neurological sequelae

In this proof-of-concept experiment we used a MDZ/H₂S exposure paradigm summarized in Fig. 3A. Briefly, we tested the hypothesis that MDZ administered prophylactically 5 mins prior to H₂S exposure prevents H₂S-induced neurological sequelae. The justification for repeated short-term exposures has been provided in a prior publication [18]. Briefly, some of the human survivors of single acute high dose H₂S poisoning develop neurodegeneration and other neurological sequelae. Whereas single high dose H₂S exposures result in neurodegeneration in this mouse model, this approach is characterized by very high acute mortality during exposure, with only a few of the surviving mice developing neurodegeneration. Taking this single exposure approach, as occurs in humans, would require an unreasonably a large number of mice to test the hypothesis. We found repeated

short term acute H₂S exposure to be more humane because it is associated with low mortality, and yet results in brain lesions recapitulating the human condition[18].

Mice were divided into 3 different groups of 5 male mice as follows: Group 1 mice were injected with 0.9% saline 5 min before exposure to normal breathing air from a cylinder; mice in Group 2 were injected with 0.9% saline 5 min prior to exposure to 765 ppm H₂S; Group 3 mice were injected with MDZ (4 mg/kg bw) 5 min prior to exposure to 765 ppm H₂S. MDZ or 0.9% saline were injected in the rear leg (gastrocnemius) muscle in 50 µL of solution. Normal breathing air and H₂S were delivered from gas cylinders. In this H₂S exposure paradigm, on day 0 mice in groups of 10 were exposed to 765 ppm H₂S or breathing air for 40 min post injection of saline or MDZ as described above. On subsequent days, the same groups of mice were exposed either to 765 ppm H₂S or to normal breathing air for 15 min only post injection with 0.9% normal saline, each day for 6 days.

Clinical Assessment

To obtain baseline data in both experiments, animals were evaluated clinically and weighed starting 3 days prior to H₂S exposure. Mice were weighed daily until euthanasia. In addition, a modified FOB was used to evaluate clinical signs during H₂S exposure, including knockdown, seizure activity, abnormal gait, and autonomic function such as urination and defecation. The same trained observer assessed the mice throughout the entirety of the experiment.

Behavioral Testing

For behavioral assessment we used the VersaMax open field test. Behavior assessments for open field activity were performed 3 hours after mice were exposed to H₂S. This was performed on days 2, 4, or 6 using a protocol adapted from Gosh et al [18, 20].

Briefly, an automated computer-controlled device (Model RXYZCM-16; Accuscan, Columbus, OH, USA) was used to measure the spontaneous activity of mice in this open field test. The dimensions of the activity chamber were 40 × 40 × 30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. Data was collected and analyzed by a VersaMax Analyzer (Model CDA-8; AccuScan). Mice were acclimated to the chamber two days before H₂S exposure. On test days, mice were placed inside the infrared monitor for 2 minutes to acclimate to the chamber. Open field activities were recorded for 10-minute test sessions assessing multiple parameters including vertical activity and horizontal activity.

Histopathology and Immunohistochemistry

Mice designated for histopathology were euthanized 24 hours after the last H₂S exposure using a previously published procedure that employed a cocktail of 100 mg/kg bw ketamine and 10 mg/kg bw xylazine given intraperitoneally [18]. Briefly, once the mice were in a surgical plane of anesthesia, the thorax was opened and fresh 4% paraformaldehyde solution (PFA, pH 7.4) was injected through the left ventricle to perfuse the animal. Thereafter, brains were post-fixed in 4% PFA for 24 hours, processed routinely, paraffin embedded, sectioned at 5 microns, and stained with hematoxylin and eosin for routine histopathology. Additional brain sections were stained using an indirect immunostaining protocol (Vectastain Elite ABC kit, PK-6101, Vector Laboratories, Inc., Burlingame, CA) that employed primary antibodies directed against glial fibrillary acidic protein (GFAP, ab72600, Abcam) or inducible nitric oxide synthase (iNOS, ab15323, Abcam). Diaminobenzidine (DAB, SK-4100, Vector Laboratories, Inc.) was the chromogen used. Stained sections were examined microscopically using a Nikon Eclipse Ci-L microscope

with DS-Fi2 camera. Routine histopathology was conducted by a board-certified veterinary pathologist blinded to the study design. The semi-quantitative scale used for scoring the severity of lesions has been previously published [5].

Objective 3: To test the effect of H₂S on brain midazolam concentrations

During preliminary studies we observed clinical differences between mice injected MDZ with or without exposure H₂S. Specifically, given equivalent dosages of MDZ, the sleeping time mice exposed to H₂S was longer than that of mice without exposure to H₂S. We hypothesized that high acute H₂S exposure causes higher MDZ levels in brains mice exposed to H₂S. In order to test this hypothesis two groups mice were exposed to 1000 ppm H₂S for 20 min. They were then removed from the inhalation for chamber for 5 min during which mice were injected with 4 mg/kg bw midazolam. Mice were then placed back in the inhalation chamber for another 95 min (Fig 6A). A breathing air group of mice injected with saline was used as a negative control. Upon termination of H₂S/breathing air exposure, mice were removed from the chamber, immediately decapitated and brain rapidly removed and placed on ice. After necropsy, brain tissues were subsequently stored at -80°C until ready for analysis. For this proof-of-concept experiment, only brain tissue was analyzed.

Analysis of brain midazolam

Whole brain tissue samples were individually minced uniformly with scissors. A 0.1g brain tissue sample was weighed for extraction. A matrix standard curve was also prepared using 4-0.1g control brain tissue samples containing 0, 0.1, 1, and 10 ng Midazolam. Midazolam was extracted according to Orkman et al., by adding 0.4 ml of 0.01 N hydrochloric acid (HCl) to each sample. Each sample was then vortexed for 10 seconds, and sonicated for 5 minutes. A 100 µL of 0.5N NaOH was subsequently added to each

sample and then vortexed for 10 seconds. Samples were further extracted with 0.5 ml ethyl acetate and vortexed for another 10 seconds. Samples were then centrifuged at 20000 x g for 5 minutes. The top layer of ethyl acetate was removed and placed into clean glass vials[21]. The ethyl acetate extraction was performed twice and the extracts combined. The combined extracts were then dried down under nitrogen, re-solvated in 200 μ L methanol and vortexed for 10 seconds, before being quantified by LC-MS/MS, by injection of 20 μ L out of the 200 μ L extract. This analysis was performed on a Varian 310 LCMS triple quadrupole instrument using positive ESI with a needle voltage of (+) 3500, a shield voltage of (+) 600, drying gas temperature of 325°C, nebulizer gas at 50 psi and drying gas at 30 psi. Detection ion used was 326-290.9 with a capillary voltage of 132 and collision energy of 21.5V. Confirmatory ion used was 326-244 with a capillary voltage of 132 and collision energy of 20V. Separation was performed on two Varian Prostar pumps equipped with a Varian 410 autosampler using a Polaris 5 micron C-18A column (150 x 2.0 mm) at a flow rate of 0.25 ml/min. The mobile phase contained 60% 10 mM ammonium acetate and 0.1% formic acid in methanol and 40% 0.1 % formic acid. Retention time of the Midazolam was 3.5 minutes [22, 23]. All samples were quantified against the matrix standard curve.

Data Analyses

Data are presented as mean and standard error of the mean. Clinical toxicity during exposures was analyzed using linear regression. Biochemical endpoints were analyzed using a student's t-test comparing the H₂S and saline treated mice to the H₂S and midazolam treated mice. Behavioral data was analyzed using 2-way ANOVA with a post-hoc test. Statistical tests were performed on Prism 6 (GraphPad Prism Software).

Results

Objective 1: Midazolam prevented H₂S-induced mortality.

Experiment 1: This experiment evaluated the efficacy of midazolam given pre-exposure to H₂S. In this study 100 % of mice injected with saline and exposed to H₂S experienced seizures and died (Fig 1B-C). In contrast, in the group of mice pretreated with midazolam, only 10% mortality was observed at the 2 hour time point when the experiment was terminated, with none of these mice experiencing seizures (Fig 1B-C).

Experiment 2: This study evaluated the efficacy of MDZ given during exposure to a single acute high dose of H₂S. All of the mice exposed to breathing air and injected with 0.9% normal saline survived. Compared to this group, only 25% of mice exposed to H₂S and injected 0.9% normal saline survived (Fig 2B). Hydrogen sulfide-induced mortality was time- and concentration-dependent. However, in the group of mice exposed to H₂S and treated with MDZ the survival rate was 100%, meaning none of the mice injected MDZ died (Fig 2A). Furthermore, none of the H₂S exposed mice treated with MDZ manifested seizure activity compared to 40% in H₂S /Saline group (Fig 2C).

Objective 2: Midazolam prevented H₂S-induced neurodegeneration and neurotoxicity

During H₂S-exposure, mice pretreated with MDZ and exposed to H₂S were clinically healthy compared to mice treated with saline (Table 1). Specifically, saline pre-treated mice and exposed to H₂S exhibited lacrimation, salivation, ataxia, impaired righting reflex and convulsions which were absent in mice pre-treated with MDZ. None of the mice pre-treated with MDZ manifested any seizures or knockdowns (Fig 3B-C). Midazolam also significantly

prevented H₂S-induced weight loss (Fig 3D). The weights of mice from H₂S/MDZ group were statistically similar to those of saline/H₂S group.

In the open-field test, mice pre-treated with MDZ performed statistically significantly better overall than mice in the saline/H₂S group on all days of testing. The vertical and horizontal activity of mice pre-treated with MDZ was better by 55% or greater compared to the H₂S and saline group (Fig 3E).

Without MDZ pre-treatment, exposure to H₂S consistently induced severe necrotic lesions in the inferior colliculus and thalamus, often with mild or moderate hemorrhage (Fig. 4A-B). Microscopically, the inferior colliculus of H₂S-exposed mice showed extensive vacuolization of the neuropil, degeneration or loss of neurons, scattered apoptotic cell debris, influx and activation of microglia and astrocytes, and foci of hemorrhage in some animals. Pre-treatment with MDZ markedly reduced the incidence and severity of these neurologic lesions. The most consistent changes observed in the inferior colliculus or thalami of MDZ-treated mice were minimal to mild enlargement and prominence of microglial nuclei and vacuolization of the neuropil. Lesions were not observed in animals exposed to breathing air. Subjective assessment of intensity and distribution of immunopositivity in GFAP- and iNOS-immunostained sections revealed moderately increased expression of GFAP and iNOS in untreated, H₂S-exposed mice and minimal to mildly increased GFAP and iNOS in MDZ-treated animals (Fig. 5)

Objective 3: H₂S affects brain midazolam concentration

This experiment evaluated the effect of H₂S on MDZ brain concentration. By measuring MDZ in brain tissue, we found that mice exposed to breathing air and injected

with MDZ had significantly less MDZ concentration in the brain than mice which were injected with MDZ and exposed to H₂S (Fig 6).

Discussion

H₂S is a rapidly acting, highly neurotoxic gas, with high acute mortality, usually at the scene of exposure. Currently, there is a need for drugs for treatment of victims of acute H₂S intoxication in the field [7, 24]. This seminal proof-of-concept study has shown that prophylactic treatment with MDZ before H₂S exposure and treatment with MDZ during H₂S exposure significantly increases survival rate in mice exposed to lethal concentrations of H₂S. The study also shows that prophylactic treatment with MDZ prevents H₂S-induced neurodegeneration and neurological sequelae. These preliminary findings are significant considering there is no drug with such properties now currently on the market. The mechanism by which MDZ was able to do this are not known and are beyond this concept study. MDZ, an anticonvulsant drug, likely works by quieting neuronal activity. Until now, other therapeutics being evaluated or recommended for treatment of acute H₂S poisoning, including nitrite and hydroxocobalamin work by binding H₂S *in vivo*. Treatments that bind sulfides have the disadvantage that they are most efficacious if given during H₂S exposure, because H₂S rapidly dissociates into daughter sulfide species almost instantaneously *in vivo*. Optimal efficacy of such drugs occurs before H₂S dissociates. It is not surprising, therefore, that the efficacy of nitrite for treatment of sulfide toxicity is questioned [5]. Midazolam, which is well absorbed IM and acts rapidly [16], also has the advantage that it is currently approved as an anticonvulsant and is currently being considered for inclusion in the strategic defense stockpile for treatment of chemical induced seizures. Given the promising

preliminary results, repurposing MDZ for acute treatment of acute H₂S poisoning is attractive.

This preliminary data is encouraging because MDZ has significant potential for field application. For example, prophylactic treatment with MDZ could be an option for first responders before attempted rescue, as an added layer of security. Currently first responders use self-contained breathing apparatus in rescue missions to avoid intoxication. Sometimes these get dislodged and first responders get exposed to H₂S [25, 26]. MDZ, in appropriate doses, MDZ could potentially serve as an added layer of protection.

Its rapid absorption by IM route makes it particularly appealing, especially for treatment of mass civilian casualties during accidents or terrorist acts. Since convulsions are reported in severely affected victims of acute H₂S poisoning, even post-H₂S exposure, MDZ may potentially be useful for post-H₂S exposure as an anticonvulsant. Besides increasing survival, also significantly reduced H₂S-induced neurodegeneration and resulted in improved behavioral performance. We also found MDZ pre-treatment consistently prevented knockdown and seizures induced by high acute exposures to H₂S. The fact that MDZ pretreatment prophylactically prevented loss in body weight suggests these mice were clinically better than saline treated control mice.

Histologic lesions observed in the brains of untreated animals exposed to H₂S are consistent with those observed in our previous studies using a mouse inhalation model of H₂S exposure that generates severe lesions and are similar to those reported in human patients [18]. Pre-treatment with MDZ reduced the development and severity of histologic lesions, reinforcing the clinical and behavioral observations in these mice. Reduced induction of GFAP and iNOS, markers of astrocyte activation and inflammation, respectively, in animals

that were prophylactically pre-treated with MDZ supports the notion that MDZ prevents the induction of an astroglial response and activation of inflammatory pathways. We have previously shown that inflammation plays a role in H₂S-induced neurotoxicity[18]. The mechanism of action by which prophylactic treatment with MDZ reduced mortality and neurodegeneration is not known and cannot be ascertained from this limited proof-of concept study. However, it has been reported that MDZ reduces seizure activity by binding to the gamma containing receptors leading to allosteric potentiation of GABA gated hyperpolarization of the cell, inhibiting excitability [16]. Although not determined for H₂S, seizure activity has been linked to neurodegeneration following nerve agent exposure [27]. Reduced seizure activity is potentially one of the mechanisms by which MDZ was prophylactically neuroprotective in this study.

Midazolam has also been used for treatment of critically ill patients suffering from pathologic effects of oxidative stress such as infection, hemodynamic instability, and hypoxia [28]. H₂S-induced neurotoxicity is characterized by hemodynamic instability (hypotension) and hypoxia [29, 30]. H₂S-induced neurotoxicity is also characterized by oxidative stress [5, 15]. There is evidence supporting the inverse correlation between midazolam and reactive oxygen species [28]. Midazolam has been shown to interfere with the synthesis and release of nitric oxide and tumor necrosis factor-alpha [28]. Midazolam also exerts protective effects during oxidative stress through the activation of Protein Kinase B (Akt) via phosphorylation in neuronal cells. Akt phosphorylation plays an important role in cell proliferation and cell survival [28, 31]. Potentially these are some of the mechanisms worthy of investigating in future experiments.

Another interesting finding from this study is the potential interaction between H₂S and MDZ. Exposure to lethal concentration of H₂S increased brain MDZ concentration. The reasons(s) for this finding are not clear and cannot be determined from this study; but either H₂S increased penetration of MDZ in the brain or it impaired MDZ metabolism. Whatever the reason, this finding has practical implications. Dose-response MDZ studies are needed to identify an ideal therapeutic dose. In this study we chose to use 4 mg/kg bw basing on results of preliminary studies and because it is almost equivalent to the human dosage corrected for surface area (ref).

This proof-of-concept study has some limitations. For example, data was collected using a small number of animals and only male mice were used. It will be helpful to repeat this study with a large number of mice of both sexes. Also, although results of prophylactic pretreatment with MZD have relevance for first responders, a major need is to rigorously evaluate the efficacy of MDZ for treatment of victims of H₂S in the field. To this end, preliminary results showing increased survival, reduced seizure activity and reduced knockdown in mice injected MDZ during H₂S exposure are very encouraging. Hydrogen sulfide is uniquely characterized by a steep dose response curve with high mortality during or soon after exposure as a major outcome. More research is needed to conclusively determine the efficacy of MDZ given during exposure; and to evaluate its efficacy post H₂S-exposure. Appropriate dose-response MDZ studies are also need. Considering that the toxicity of H₂S is uniquely characterized by high mortality during exposure, reducing mortality by injecting MDZ during exposure will be a significant contribution towards treatment of acute H₂S poisoning.

In summary, in this mouse model, MDZ treatment reduced mortality, seizure activity, behavioral deficits, and was neuroprotective against H₂S-induced neurotoxicity. Results also indicated potential interaction between acute H₂S exposure and MDZ as brain MDZ concentrations were significantly higher in H₂S-exposed mice than those that were not. MDZ is a promising novel drug for treatment of acute H₂S-induced neurotoxicity and neurodegeneration. Considering that it reduced acute mortality, reduced seizures and knock down given during H₂S exposure is very appealing and further research is recommended to test the efficacy of MDZ for this purpose.

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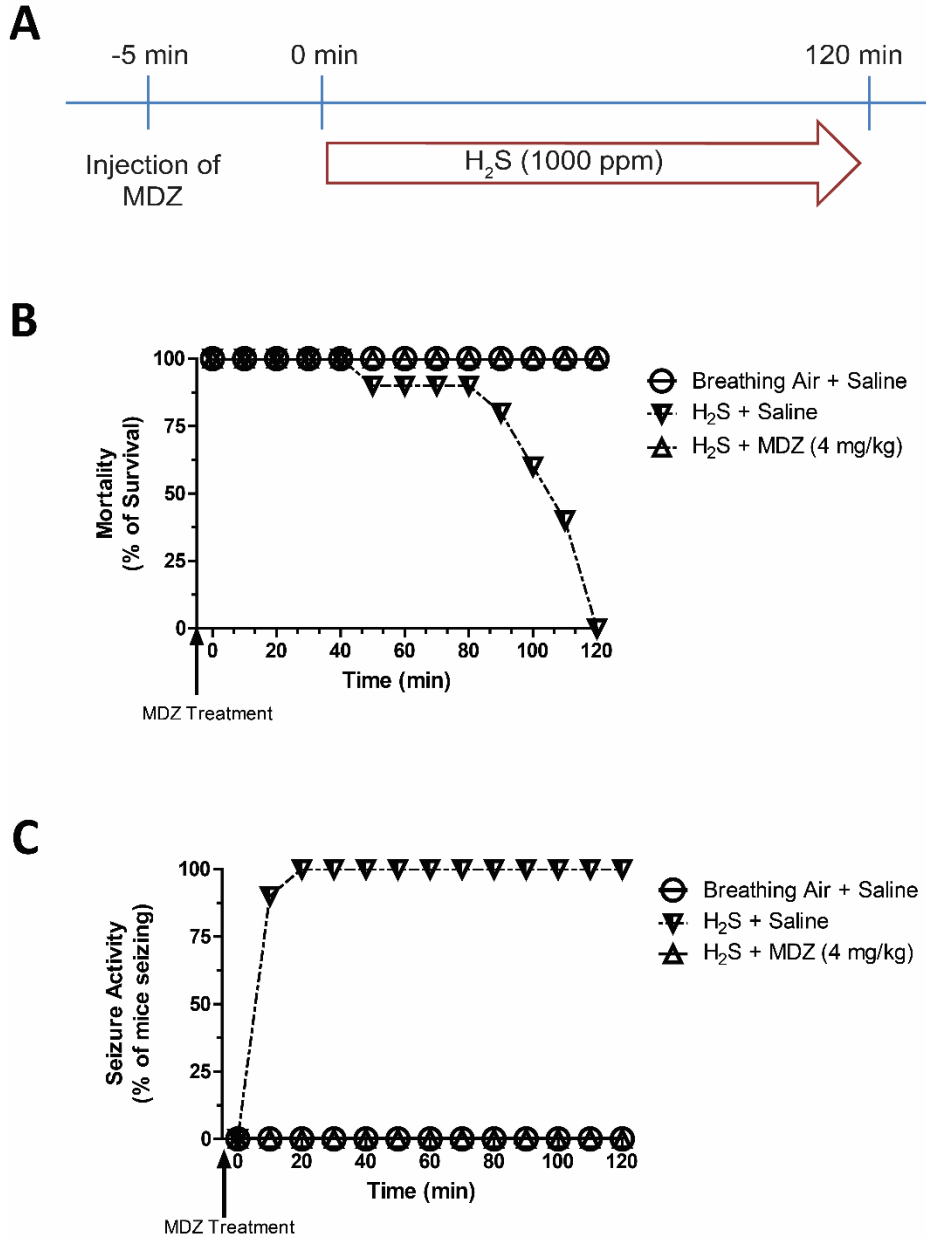


Figure 1. A) Treatment paradigm to determine the prophylactic efficacy of midazolam. B) Midazolam prevented H₂S-induced mortality by 90%. n=10. C) Midazolam prevented H₂S induced seizure activity in mice. n=10.

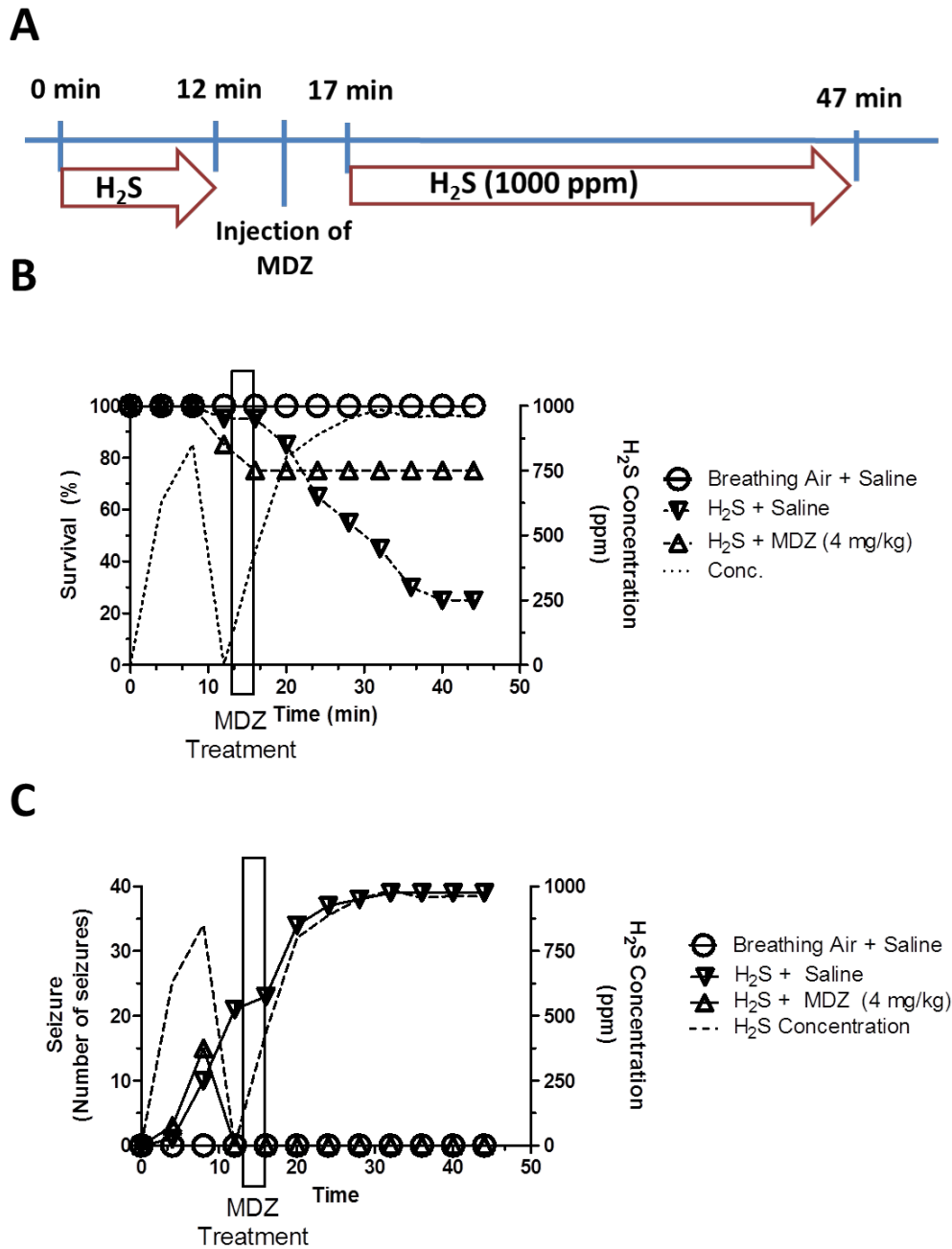
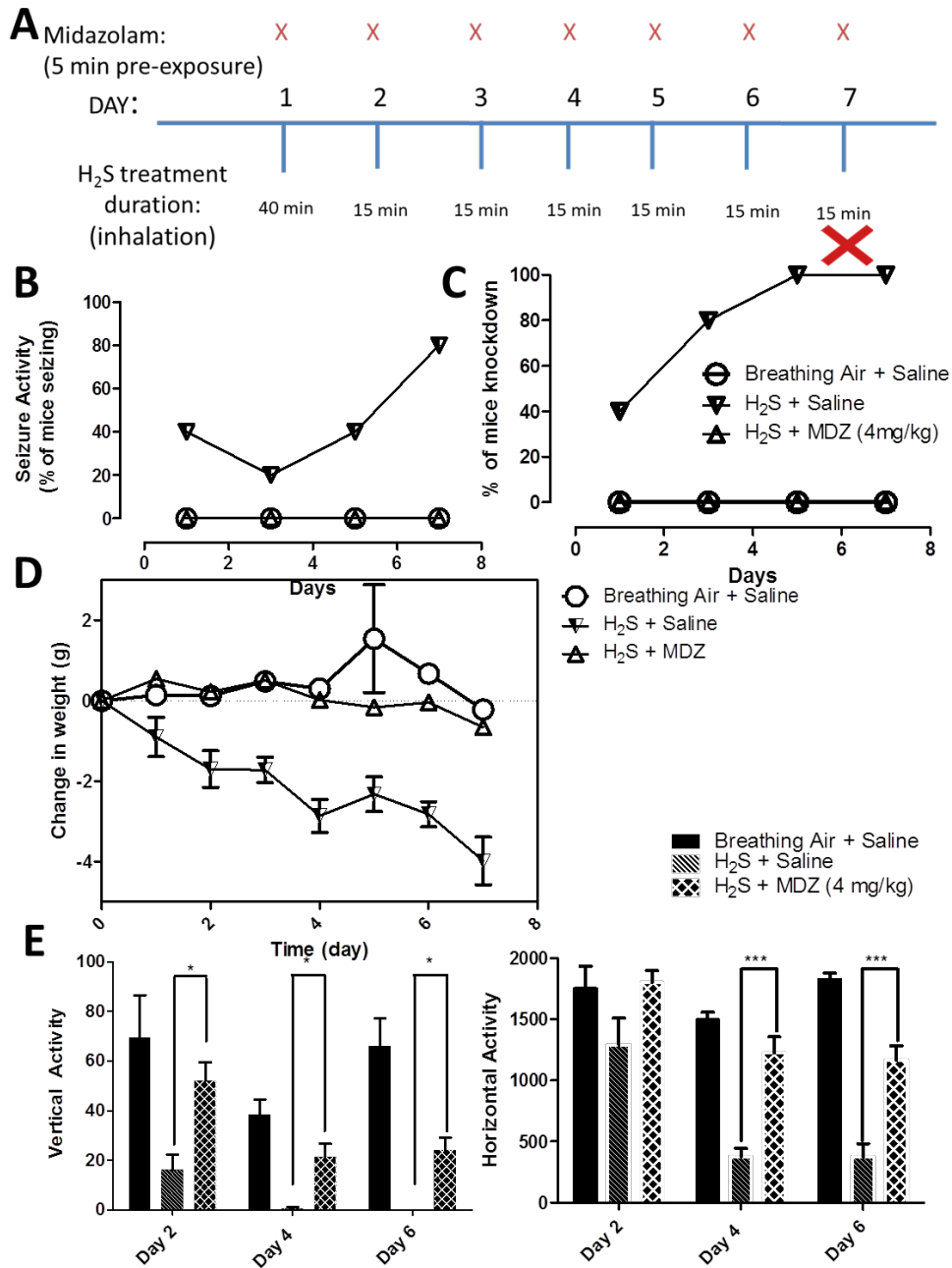


Figure 2. A) Treatment paradigm to determine the efficacy of midazolam for treatment of H₂S-induced neurotoxicity given during H₂S exposure. B) Following Midazolam treatment no more mice died compared to about 20% survival for saline treated mice n=20. C) Midazolam abolished H₂S induced seizure activity in mice. n=20

Table 1. Functional Observational Battery comparing mice injected with saline or midazolam prior exposure to H₂S.

Measure	Effect in mice during H ₂ S exposure		
	H ₂ S + Saline	H ₂ S + Midazolam	Breathing Air + Saline
Autonomic			
Lacrimation	present	No	No
Salivation	present	present	No
Urination/Defecation	increased	No	Normal
Convulsions			
Tonic movements	mild	No	No
Clonic movements	severe	No	No
Paddling feet	severe	No	No
General Measures			
Piloerection	present	No	No
Body Posture	hunched	hunched	Normal
Dyspnea	present	mild	No
Gait			
Hindlimbs splayed	present	No	No
Ataxia	severe	Mild	No
Unable to support weight	present	No	No
Righting reflex	affected	Not affected	No
Knockdown	severe	No	No



Figure

3. A) Summary treatment paradigm H₂S-induced neurological sequelae in mice prophylactically treated with MDZ. B) Midazolam completely prevented seizure activity and knockdown consistently during the entire exposure period n=5. C) Mice exposed to H₂S and injected with saline lost statistically significant more weight compared to the breathing air controls injected with saline. MDZ prophylactically prevented H₂S induced weight loss n=5. D) MDZ prevented H₂S induced motor deficits n=5.

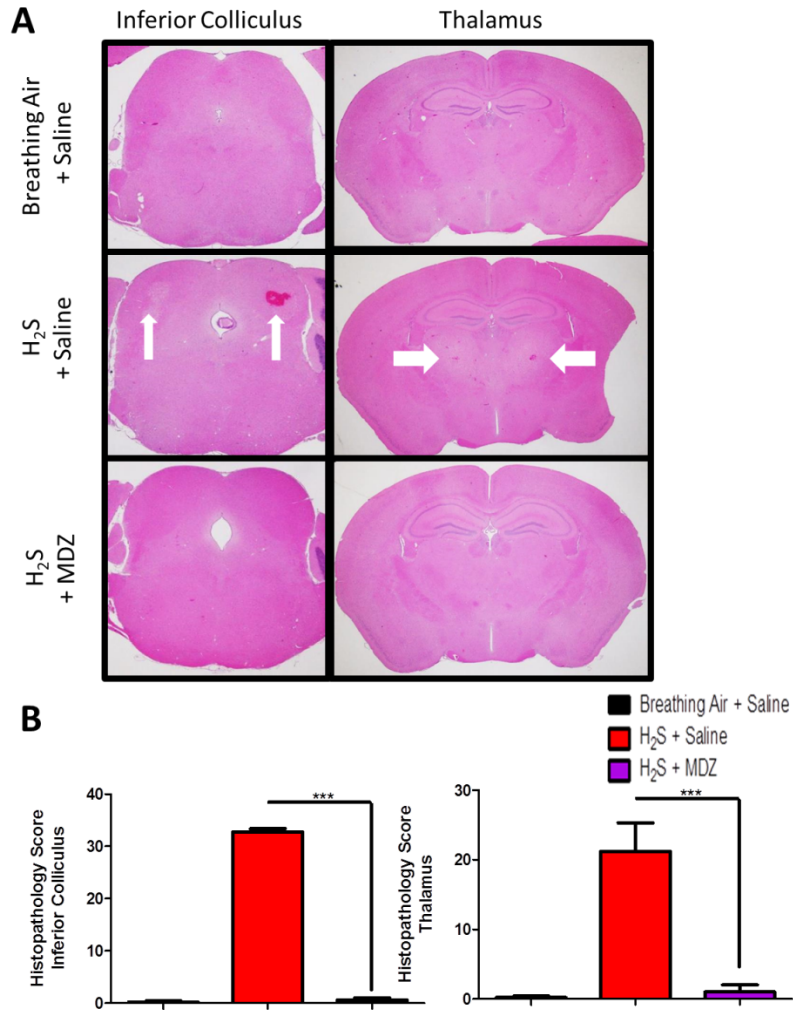


Figure 4. Photomicrographs of lesions in the thalamus and inferior colliculus of mice prophylactically treated with MDZ prior to H₂S. Note the pallor and loss of neurons in the thalamus of the saline/H₂S-exposed mouse. Note that brain tissue of the MDZ/H₂S is similar to that of the saline/breathing air group. Severe changes in the inferior colliculus of the saline/H₂S-exposed mice include marked vacuolization of the neuropil, degeneration and loss of neurons, and prominent glial response.

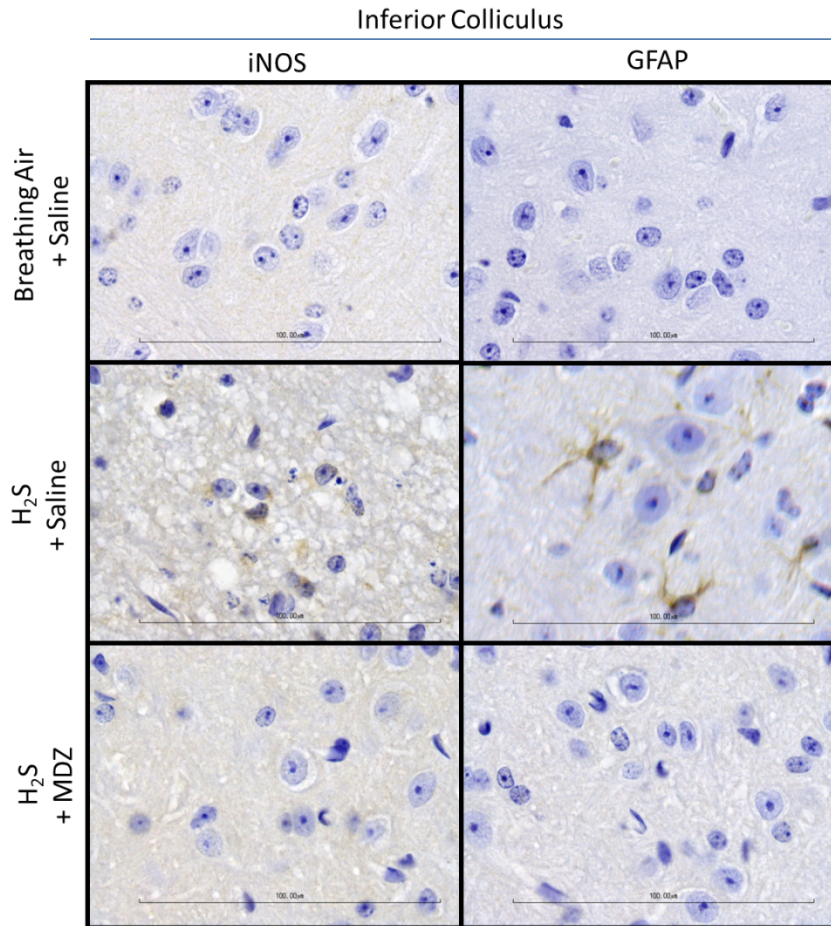


Figure 5. Representative photomicrographs of immunohistochemical staining of the inferior colliculus demonstrating expression of glial fibrillary acidic protein (GFAP), a marker of astrocyte activation, and inducible nitric oxide synthase (iNOS), a marker for neuroinflammation. Note the increased expression of GFAP and iNOS (brown chromogen deposition) in the brain of the Saline/H₂S group, while levels of these markers in the brains of MDZ-treated animals have less immunostaining, suggesting less inflammation the MDZ-treated group.

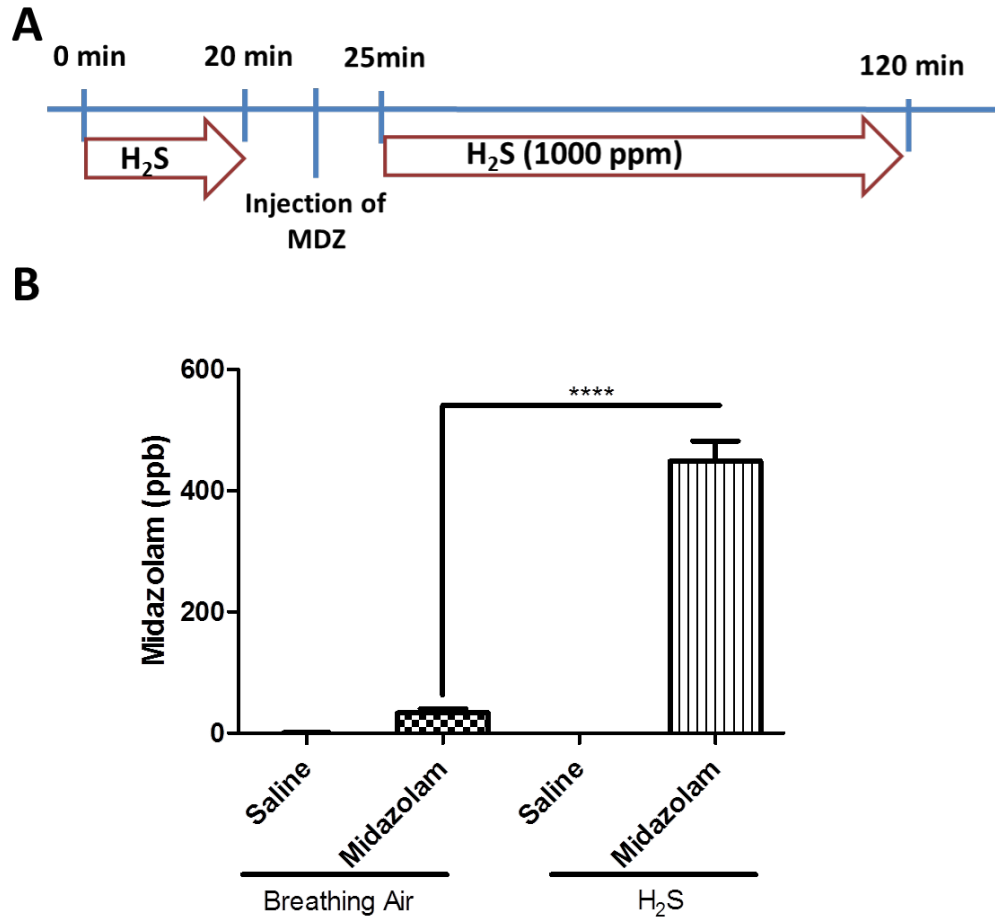


Figure 6. Midazolam concentration in the brain. Note the significantly higher MDZ concentration in mice exposed to high concentration of H₂S compared to those without H₂S exposure.

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In this section, there is an overview of the main results as described within the dissertation with an emphasis on future directions and impact of these conclusions. Key findings pertaining to the individual research chapters can be found in the “results” and “discussion” sections of the corresponding chapter.

A Mouse Model for H₂S-induced Neurotoxicity

The first chapter highlights the many challenges associated with studying H₂S-induced neurotoxicity. At physiological concentrations, H₂S is a gasotransmitter much like carbon monoxide and nitric oxide and can play a role in many normal signal transduction pathways in the brain^{4, 8, 107, 108}. At high, toxic concentrations, H₂S is acutely toxic and can cause neurodegeneration and other neurological sequelae^{71, 109-111}.

Acute exposure to toxic levels of H₂S can occur in many exposure scenarios. H₂S can be released from a natural source such as thermal hot springs or volcanoes, or it can be man-made²⁴. Bacteria also have the ability to produce toxic concentrations of this noxious gas under anaerobic conditions, causing the release of H₂S in environments such as a sewer system or in an agricultural settings²⁵. H₂S is utilized and released in many industrial settings including petroleum refineries, paper mills, rayon textile productions, chemical manufacturing, and waste disposal, posing an enormous occupational hazard^{9, 112}. H₂S is a leading cause of acute death, second only to carbon monoxide among toxic gas induced

deaths^{5, 44}. The use of H₂S in industrial settings creates the potential for sudden release following industrial accidents and/or a targeted attack. Given that H₂S can be easily manufactured from house hold chemicals, there is a significant concern for the use of H₂S as a chemical weapon¹¹. It is currently listed as a chemical of interest by the Department of Homeland Security²⁸. H₂S is heavier than air, and has the potential to settle and accumulate in confined, low spaces, such as a subway stations⁴.

The central nervous system is a major target for H₂S-induced toxicity. At high levels (ranging from 500-1000 ppm), H₂S can cause victims to rapidly collapse, commonly known as “knockdown”⁵. This prevents the victims from escaping the scene of exposure. At levels of 1000 ppm or above, a couple breaths of H₂S can cause immediate collapse and death. Victims exposed to high levels of H₂S, especially those who have experienced a knockdown, can experience neurological problems including persistent headaches, nausea, seizures, memory impairment, motor deficits, sleep disorders, vision and hearing impairment, among other symptoms^{48, 49, 52}. However, the molecular mechanisms of H₂S-induced neurotoxicity remain poorly understood.

H₂S is known to inhibit cytochrome c oxidase, the fourth complex of the electron transport chain⁴². Although it was earlier assumed that this inhibition led to ATP depletion causing cell death, more recent studies speculate that while this mechanism may contribute to H₂S-induced neurotoxicity, it might not be the only mechanism of action⁴².

Currently, there is no suitable antidote for treatment of H₂S-induced neurotoxicity, particularly one that can be administered in a mass civilian casualty setting such as following an industrial accident or a chemical attack. Such an antidote, which is easy to

administer by IM injection, is badly needed. Current treatments for H₂S intoxication include sodium nitrite administration, hyperbaric oxygen therapy, and hypothermia^{71, 77}. These treatments are not ideal for field use in a mass civilian exposure setting as they require administration by experienced medical staff. The relevant equipment is also only available within hospital settings. Furthermore, currently, there is no antidote for treatment of H₂S-induced neurotoxicity and neurological sequelae.

In order to develop an antidote and elucidate the molecular mechanisms underlying H₂S-induced neurotoxicity, it is vital to establish and validate animal models of H₂S toxicity. In Chapter 2, we characterized a mouse model of H₂S-induced neurotoxicity. As supported by previous studies, it is very challenging to produce neurological lesions that recapitulate the human condition following a single exposure. In order to tackle this challenge, we developed two mouse models; one using lethality as an endpoint to screen potential antidotes to reduce mortality; a second on short term repeated acute exposures to recapitulate H₂S-induced neurodegeneration for evaluation of countermeasures against neurodegeneration and other sequelae.

Our model, for the first time, has characterized the effects of H₂S-induced neurotoxicity utilizing a whole body inhalation model, on unanesthetized mice. By doing so, we were able to recapitulate realistic human exposure scenarios. This is an advantage over previously described models that used NaHS, a source of H₂S, intraperitoneally to study the effects of H₂S⁸². Limitation of other inhalation models include the requirement of anesthetizing the animals before administration of the toxin, because the anesthesia may also modulate the response to H₂S⁴⁴. This prevents monitoring of clinical signs such as seizures and knockdowns (two major clinical consequences of human H₂S intoxication) in their

animal models. Using our mouse models of H₂S inhalation exposure, we have substantially contributed to the understanding of major molecular mechanisms of H₂S-induced neurotoxicity, including the presence of neuroinflammation and oxidative stress. We have also discovered the neurochemical changes induced by H₂S, which could be contributing factors to many of the symptoms described in human cases of H₂S poisoning, including the seizure activity, dysphoria, motor impairment, and personality changes.

Although we have characterized a mouse model of H₂S-induced neurotoxicity, some disadvantages to this model do exist. In order to fully recapitulate the human exposure scenarios described above, a one exposure model would be ideal. One exposure to H₂S did cause neurodegeneration in some mice, but this was also accompanied by a high mortality rate, thus requiring us to use a large number of mice for use in translational studies to test the efficacy of drugs that will prevent neurodegeneration. Our repeated short-term acute exposures model consistently manifested lesions in the same regions as a single exposure, but with significantly less mortality.

Cobinamide as an antidote for H₂S-induced neurotoxicity

The main objective of Chapter 3 was to use the mouse model developed and characterized in Chapter 2 to test the efficacy of cobinamide (Cob) as an antidote for H₂S-induced neurotoxicity. Importantly, we have shown that cob is effective in preventing clinical signs, neurochemical, biochemical, and histopathological changes induced by H₂S. Furthermore, this study allowed us to identify novel key mechanistic changes that have not been implicated in H₂S-induced neurotoxicity.

Other groups have investigated the use of Cob as an antidote to cyanide toxicity⁸⁶. Due to some similarities between cyanide and H₂S, we investigated this precursor to cobalamin as a potential H₂S antidote. Studies done thus far have shown Cob to reduce mortality induced by H₂S⁴⁴, here we revealed that Cob treatment post H₂S exposure was protective in preventing the neurological damage caused by H₂S.

In chapter 3, we aimed to optimize the dose of Cob as well as the time window when Cob can be given. For example, an existing treatment for H₂S intoxication, sodium nitrite, has been shown to be efficacious when administered 2 min post exposure. This is a problem. Since it needs to be given intravenously this window of opportunity is not feasible for a mass civilian casualty scenario. Cob has the advantage that it can be administered intramuscularly; and is suitable for rapid treatment of mass civilian casualties.

Our studies demonstrated that Cob given 2 min post exposure, at the 100 mg/kg dose is the most efficacious at preventing H₂S-induced neurotoxicity in mice. While injecting Cob 2 min post exposure is the most efficacious, we noted that Cob offered some protection even as far out as 30 min post exposure. We also found that 50 mg/kg dose was not efficacious. Mice exposed to H₂S displayed clinical signs such as dyspnea, lacrimation, salivation, seizure activity, ataxia, and knockdown. When given Cob, very few of the mice exhibited seizure activity and knockdown, and only displayed mild ataxia.

In this study, we also further evaluated the mechanisms of H₂S-induced neurotoxicity. The hallmark of H₂S intoxication is cytochrome c oxidase inhibition. We showed that Cob significantly prevented this inhibition when administered 2 min post H₂S exposure. However, given 15 min or later, Cob did not prevent this specific inhibition. Further more, we

found that Cob prevented Casp-3 cleavage and upregulation of pro-apoptotic proteins induced by H₂S. This not only demonstrates that H₂S may have other mechanisms of action for the potentiation of cell death, but that Cob prevents this from occurring.

Seizure activity has been linked to neural injury in other models of chemical-induced neurotoxicity, and neuroinflammation. Cob reduced seizure activity via mechanisms that are yet to be investigated, could be a contributing factor in Cob's efficacy. Another pathway that Cob could be involved in is as an anti-inflammatory. While the role of H₂S in neuroinflammation is controversial, at these toxic levels, we demonstrated that H₂S caused an increase in pro-inflammatory cytokine expression, dependent on the ERK-NFκB pathway. The pro-inflammatory cytokines can potentiate the neuroinflammatory response by causing neuronal and synaptic dysfunction, which can lead to neuronal cytokine secretion, this response can sustain inflammation leading to chronic neuroinflammation causing neurodegeneration. More importantly, our studies showed that Cob attenuates the neuroinflammation induced by H₂S. Together, our studies have extended our understanding of mechanisms of action of H₂S, and novel potential mechanism of Cob as an antidote. Prior to our studies, Cob was thought only to act by binding H₂S. Using our well characterized model, we were able to conclude that Cob is effective in preventing the neurodegeneration associated with H₂S-induced toxicity. It is most efficacious when administered 2 min post exposure at a dose of 100 mg/kg, but was somewhat efficacious when administered 15 min or later post H₂S-administration.

Midazolam prevents H₂S-induced neurotoxicity

The objective of Chapter 4 was to again use the model we characterized in Chapter 2 to evaluate Midazolam as an antidote for H₂S-induced neurotoxicity. Given our observation that the severity of seizures correlated with mortality, we hypothesized that an anticonvulsant, such as Midazolam, will prevent H₂S induced death and potentially treat neurological problems associated with H₂S exposure. Midazolam, which has a rapid onset makes it more appealing as an antidote. Midazolam is also very water-soluble, making it ideal for intramuscular injections⁷⁵. The use of a midazolam to prevent H₂S-induced neurotoxicity is novel, as midazolam would act via different mechanisms than simply binding H₂S to excrete it.

This study had multiple objectives; to test the hypothesis that midazolam prevents H₂S-induced mortality when given prior to exposure, and reduces mortality given during exposure. This seminal study showed midazolam not only prevented mortality induced by H₂S, but also the neurodegeneration. Midazolam prevented knockdowns and seizure activity induced by high H₂S exposure, but also significantly prevented the neurological lesions induced by H₂S. Reduction of GFAP and iNOS, markers of inflammation, suggest that midazolam prevents the induction of inflammatory pathways induced by H₂S.

While we have demonstrated that midazolam prevents H₂S-induced toxicity, the mechanism by which it does this is yet unknown. Given that midazolam drastically reduced seizure activity induced by H₂S, and neurodegeneration, there might be a potential of seizure activity observed in H₂S victims and the neurodegeneration observed in the brain.

While this study demonstrates the efficacy of midazolam, it does have some limitations. Further studies would need to be conducted to show its efficacy post-H₂S exposure. This study is still beneficial as first responders responding to a scene where they may encounter H₂S could add this to the existing protocol for avoiding intoxication. We have also shown its potential as a life saving drug given during H₂S exposure. A major advantage of using midazolam is the fact that it has already been FDA approved⁸⁹. The drug does not need to undergo extensive safety testing, which is the case with the previously mentioned cobinamide. Midazolam can be repurposed for treating H₂S-induced neurotoxicity.

Future studies to investigate the potential combination of midazolam and Cob given concurrently for treatment of H₂S-induced neurotoxicity are recommended. Given that both these drugs would have different mechanisms of actions, combining these drugs would likely increase the efficacy compared to either drug given alone. A limitation to using cobinamide alone at the 100 mg/kg bw dose is that it can be toxic at 2-4 times this dose⁴⁴, combining the drug with midazolam can create a dose sparing effect that would make cobinamide safer to use as well.

Overall, we have characterized a mouse model of H₂S-induced neurotoxicity that can be used to screen and validate potential antidotes for treatment of the neurotoxicity, neurodegeneration, and neurological sequelae associated with H₂S exposure. We have successfully utilized this model to screen two promising drug candidates, cobinamide and midazolam, which were efficacious in preventing neurodegeneration induced by H₂S. The validated model is available for evaluation of other potential drug candidates. This novel model also allows exploration of mechanisms of H₂S-induced toxicity.

NOMENCLATURE

4-HNE= 4-hydroxynonenal

AKT = Protein kinase B

ANOVA = Analysis of variance

cAMP = cyclic adenosine monophosphate

Cob = Cobinamide

CNS= central nervous system

DAB = 3,3'-Diaminobenzidine

DOPAC = 3,4-dihydroxyphenylacetic acid

ERK= extracellular-signal-regulated kinase

GABA = gamma-aminobutyric acid

GFAP = glial fibrillary acidic protein

H₂S = hydrogen sulfide

HPLC= high performance liquid chromatography

HVA= homovanillic acid

IBA1= ionized calcium-binding adapter molecule 1

IHC= immunohistochemistry

IL = Interleukin

ISU= Iowa State University

iNOS= inducible nitric oxide synthase

KD= knockdown

L-DOPA= L-3,4-dihydroxyphenylalanine

MDZ = Midazolam

NMDA= N-methyl D Aspartate

NO = Nitric Oxide

PKA = protein kinase A

qPCR= quantitative polymerase chain reaction

ROS= reactive oxygen species

RNS = Reactive nitrogen species

TNF α = Tumor necrosis factor α

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