Title

Evaluating the Severity of Hydrogen Sulfide (H2S) Posioning on Cytochrome C Oxidase Activity in Mouse Tissues

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Evaluating the Severity of Hydrogen Sulfide (H_2S) Poisoning on Cytochrome C Oxidase Activity in Mouse Tissues

By

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Abstract:

Hydrogen Sulfide (H_2S) is a colorless, flammable, water-soluble, rotten egg-smelling gas placed on Homeland Security's chemicals of concern list. Exposure to H_2S can be either intentional or accidental. Some accidental exposures include industrial accidents such as petroleum refinery malfunction, or natural exposures such as volcanic emissions. Intentional exposures may occur as in suicide or terrorism. This study aims to test the hypothesis that inhibition of cytochrome C oxidase (COX) activity can be used to diagnose acute H_2S poisoning. To do this, we examined various tissues from mice including blood platelets, and select brain regions, and cardiac and skeletal muscles. Mice were exposed to 1000ppm of H_2S for 15, 30, or 60 minutes. The blood and tissues from each group were collected immediately after exposure following euthanasia by decapitation. The blood platelets were isolated and analyzed immediately after collection. The tissues were stored at -80°C until they were analyzed. The COX activity was measured spectrophotometrically in both the platelets and tissues using a Complex IV Rodent Enzyme Microplate Assay Kit. COX activity in the brain varied by region, with the cortex being the most sensitive and most inhibited at 60 minutes and the cerebellum the least inhibited. We determined that the COX activity in the heart muscle was not significantly affected by H_2S poisoning. The diaphragm was more sensitive than the heart muscle, with maximum inhibition observed at thirty minutes following H_2S exposure at 1000 ppm.

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Chapter 1: Introduction

1.1 Introduction

Hydrogen Sulfide (H_2S) is a colorless, flammable, water-soluble, rotten egg-smelling gas and placed on the chemicals of interest list by the Department of Homeland Security (DHS). H_2S targets the mitochondrial electron transport chain (ETC). It inhibits mitochondrial cytochrome C oxidase (COX) activity, resulting in decreased levels of ATP and eventual death. Exposure to toxic H_2S gas can occur via several sources, including industrial accidents, environmental exposure in swamps, sinkholes, manure pits and volcanic emissions, and as an agent of suicide [26]. Depending on the concentration of H_2S, it can either competitively or non-competitively bind to cytochrome C oxidase, preventing oxygen utilization and ultimately blocking the production of cellular ATP. At the cellular level, H_2S decreases cellular ATP by inhibiting cytochrome C oxidase enzyme. It also damages DNA, promotes cell apoptosis, and causes vasodilation and vasoconstriction, among other effects [26].

1.2 Background

Cytochrome C oxidase coupled with complex IV is an essential component of the mitochondria ETC, producing more than 98% cellular ATP [16]. Several metal prosthetic sites and 14 protein subunits compromise cytochrome C oxidase which all contribute to the overall function of COX. Complex IV contains two hemes, cytochrome a and cytochrome a3, and two copper centers, CuA and CuB [26]. In the ETC process, complex IV receives four electrons from cytochrome C. The electrons are then transferred within four proteins from the inner aqueous phase to an oxygen molecule, producing two water molecules [26]. H_2S specifically targets cytochrome C a component of complex IV in the mitochondrial ETC. H_2S binds to cytochrome C at two different sites, cytochrome a3, and CuB, which results in a ferric sulfide-bound state

[22]. This binding prevents oxygen from binding to Fe(II) thus preventing the conversion of oxygen to ATP [26]. H_2S, even at low concentrations, is a non-competitive inhibitor of COX and is reversible. Therefore, it is imperative to identify H_2S poisoning earlier to prevent any harmful effects of long-term exposure.

Although high concentrations of H_2S can be lethal, it can be beneficial in low concentrations, as it promotes oxygen consumption and increases membrane potential [5]. This phenomenon is part of the reason H_2S is considered a gasotransmitter, along with carbon monoxide (CO) and nitric oxide (NO) [7]. Gasotransmitters are a class of neurotransmitters that affect the function of all cells that express target proteins [6]. Previous studies have shown a link between H_2S and vascular tone, myocardial contractility, neurotransmission, insulin secretion, sleep, and hibernation [5]. However, there is a fine line, between beneficial and adverse effects, with higher concentrations causing inhibition of COX. H_2S toxicosis is evident at 50 ppm with eye irritation, then at 100-200 ppm with upper airway irritation. At higher concentrations (250-500ppm), a person begins to experience pulmonary edema. At even higher concentrations (500-1000 ppm), a person starts to have respiratory paralysis, CNS, and cardiovascular depression, ultimately leading to death [26].

1.3 Measurement of Cytochrome C Oxidase Activity Using Blood

Diagnosing of H_2S intoxication is very challenging. When testing for H_2S toxicosis, physicians will typically test the urine, cerebrospinal fluid (CSF), or pleural effusion samples for analysis of metabolites of H_2S [17]. The collection of CSF and pleural effusion samples is more invasive than blood collection and, thus, has a higher risk for complications. In addition, urine contains low concentrations of H_2S metabolites and is not very accurate [17]. Besides, metabolites like sulfate are non-specific and can be derived from dietary sources.

Previous studies investigated analysis of H_2S metabolites in arterial blood after intravenous administration of sodium hydrosulfide (NaHS) a H_2S donor [13]. Direct analysis of H_2S can be challenging due to the rapid metabolism of unbound H_2S. Previous researchers determined that relatively low H_2S concentrations were found in the blood when signs of toxicosis occurred [13]. This research concluded that using metabolites of H_2S in the blood is not an effective technique for diagnosing H_2S toxicosis. Also, arterial blood (compared to venous blood) is a very invasive technique in determining H_2S toxicity due to increased rates of infection and difficulty obtaining it. The study used an HPLC fluorescence technique to detect the reaction of the H_2S metabolites with monobromobrimane (MBB) [13]. This approach also requires a high-cost instrument which is only available in some labs, making it difficult for this protocol to be implemented in a clinical setting.

Use of blood platelets collected from venous blood is a far less invasive method of assessing COX inhibition. A previous study on 96 septic patients found that surviving patients showed higher COX activity than those who did not survive [16]. The researchers measured the COX activity by using mitoprofile human complex IV activity from Mitosciences (Eugene, OR). The kit is used to determine the activity colormetrically by following the decrease in COX activity due to oxidation by measuring the decrease in absorbance at 550nm. This study showed promise for assessing COX activity in a clinical setting, but some modifications will need to be made to assess H_2S toxicity, such as the amount of sample required for this mouse current study.

In a previous study in rats, COX activity in blood platelets was used as an indicator of serum copper status [12]. Although this previous research focused on copper levels, their methods for obtaining platelets provide insight into properly isolating platelets from whole blood. The researchers obtained 6-8mL of blood from the vena cava of the rats and centrifuged

the whole blood at 15°C for 20 minutes at 160 x g [12]. This isolated the platelets to the middle layer of the supernatant. They removed the platelets and washed them to remove any remaining red blood cells and plasma. The platelets were then used for a COX assay, where they correlated the results with levels of copper in the rats [12]. This study highlighted the ability to use platelets from rats to determine COX activity. Rats are a more ideal animal model as compared to mice due to their larger blood volume.

In this study, we explored the use of blood platelets for assessing COX activity following H_2S exposure. The quantity of blood in mice is small, therefore it is vital to have the most efficient protocol to isolate the platelets from whole blood. There are other previously researched protocols for isolating platelets focusing on obtaining enough platelets in order to measure the levels of COX activity accurately [12, 21]. Some investigators use gly-pro-arg-pro to prevent coagulation, others use only sodium citrate and sodium chloride, and others use prostaglandin E1 (PGE1) [12, 21]. The most common anticoagulant used is heparin whether that is a liquid heparin or the use of heparin treated tubes. The effect of these different anticoagulants on COX activity has not been established.

1.4 Measurement of Cytochrome C Oxidase Activity Using Tissue Samples

There has been limited research on the effects of H_2S toxicosis on COX activity on different tissue types. Previous research has focused primarily on brain and lung tissue. Research by Dorman et al. (2002) focused on brain, liver, lung and nasal epithelial tissue from rats. They determined that the hindbrain was not affected by H_2S exposure by inhalation, but the lung, liver, olfactory, and nasal epithelium were affected. This research suggested that in the brain different brain regions are not equally affected by H_2S poisoning.

H_2S can cause not only inhibition of COX activity in the brain but also cause brain lesions. The lesions are signs of lasting effects of H_2S toxicosis long after the exposure and metabolism have occurred. A previous study compared the chronic and acute effects of H_2S toxicosis and how this may affect the COX activity and subsequent neurodegeneration in the brain [2]. The study focused on the effects on the inferior colliculus, thalamus, and cortex. Lesions were only found in the thalamus and inferior colliculus in acute and chronic H_2S exposure groups [2]. Yet, COX activity was inhibited in all the brain regions they studied. There was more inhibition in the thalamus and cortex than in the inferior colliculus [2]. This study exemplified how H_2S does not target different brain regions equally and that more COX inhibition does not necessarily mean a lesion will form in that area. Further research can be done to understand effects of H_2S effects on COX activity in different brain regions and how that may relate to neurodegeneration.

Much of the previous research has investigated COX activity in the brain and lungs, but much less is known about the effects of H_2S on COX activity in the heart or diaphragm. Examining such other tissues can provide a clearer insight into how H_2S toxicosis affects the body and target sites. There are some practical challenges processing heart and diaphragm samples. Unlike the brain that can be easily homogenized, the heart and diaphragm must be sonicated to achieve homogenization. A study by Hsu et al. (2006) processed heart by dicing it followed by sonication. This protocol for homogenizing heart tissue can easily be applied to the diaphragm to achieve an optimal sample for analyzing the levels of COX activity.

The brain is currently known to be the most sensitive organ to H_2S-induced toxicity. Although a COX activity has been measured in some brain regions, there has not been a systemic evaluation of the activity of this enzyme in other brain regions. No studies were found that

compared brain, heart, and skeletal muscle COX activity following acute H_2S exposure. Other studies have focused on studying COX activity in the heart and diaphragm, but only as it relates to H_2S generated endogenously [15]. I posit there are differences in H_2S-induced inhibition of COX activity because H_2S toxicosis is not uniform in different brain regions. For example, studies have shown that H_2S preferentially accumulates in the brainstem [2]. Therefore, I posit that COX activity will be most inhibited in the brain stem where H_2S accumulates the most.

Evaluating COX activity in the blood of exposed patients or victims of acute H_2S toxicosis would be less invasive and therefore more desirable. In addition, it has yet to be studied whether the inhibition of COX activity in platelets is an effective way to diagnose H_2S.

I hypothesize that H_2S exposure will significantly inhibit COX activity in blood platelets of mice. I also hypothesize that H_2S exposure will cause a significantly higher level of inhibition of COX activity in the heart than the diaphragm. Finally, I hypothesize that H_2S will cause a significantly higher level of inhibition of COX activity in the brainstem than the olfactory bulb due to the brainstem regulating breathing and heart rate. The goals of this study are: 1) determine normal COX activity in mouse blood platelets, brainstem, olfactory bulb, cortex, cerebellum, heart, and diaphragm; and 2) conduct a dose-response study of H_2S on COX activity in the blood platelet, brainstem, olfactory bulb, cortex, cerebellum, heart and diaphragm. The goal is to identify the most ideal tissue that can be used to diagnose acute H_2S toxicosis.

Chapter 2: Experimental

2.1 Chemicals and Materials

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis (IACUC-23662). C57BL/6J mice were

purchased from Jackson Laboratories (Sacramento, CA, USA). All animals were treated with according to IACUC guidelines.

Heparin was purchased from Neta Scientific (Hainesport, NJ, USA), and a standard solution was made by dissolving 50mg of heparin in 1mL of deionized water. The solution was stirred until all heparin was dissolved. The heparin was stored in a refrigerator at 4°C. Sodium citrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). A 0.1M sodium citrate standard solution was obtained by weighing 1.47g of sodium citrate and slowly combining it with 50mL of deionized water. The solution was stored in a refrigerator at 4°C. The mixture was stirred to ensure all sodium citrate was dissolved. Phosphate buffered saline (PBS) was purchased from Life Technologies Corporation (Carlsbad, CA, USA). A diluted solution was made using 5mL 10x PBS and adding 45mL of deionized water to make a 1x PBS solution. The solution was stored in a refrigerator at 4°C.

Complex IV Rodent Enzyme Microplate Assay Kit (cytochrome C oxidase assay kit) was purchased from Abcam (Waltham, MA, USA). The lysis buffer, detergent, plate, and wells were stored in a refrigerator at 4°C. The reagent c was stored in a freezer at -80°C.

2.2 H_2S Exposure

Twenty 6-7 week-old C57BL/6J male mice were split into four different groups. The control group consisted of five mice kept at room air for sixty-minutes in the same chamber as those exposed to H_2S gas. The experimental groups included four mice exposed to 1000ppm for 15 minutes, five mice exposed to 1000ppm for 30 minutes, and six mice exposed to 1000ppm for 60 minutes. The H_2S gas was obtained from PraxAir and the target concentration of 1000 ppm was confirmed via a gas sample draw monitor manufactured by RKI instruments (Union City, CA).

2.3 Blood and Tissue Collection

After H_2S exposure, the blood samples were collected following decapitation. The blood collection tubes contained 50µl of sodium citrate and 10µl of heparin to prevent coagulation. Once the blood was collected, an additional 10µl of sodium citrate and 10µl of heparin was added. The samples were then inverted multiple times to allow for proper mixing.

After blood collection from each mouse, the heart, diaphragm, olfactory bulb, brainstem, cerebellum, and cortex were collected. The heart was fully excised from the mouse, and the left atrium was removed and placed in a polypropylene tube. The diaphragm was also entirely excised, and about one-third of the diaphragm was placed into a polypropylene tube. The olfactory bulb, brainstem, cortex, and cerebellum were dissected from the brain on ice. Once all samples were collected from one mouse they were immediately placed in a -20°C freezer. Once all samples were collected from the group of mice the samples were transferred to a -80°C freezer for storage until analysis.

2.4 Isolation of Platelets

Once all the blood was collected from each mouse in the group, the tubes were centrifuged at 400 x g for twenty minutes at 12°C. Following centrifugation, the top layer of plasma was removed carefully so as not to disturb the layer of red blood cells. The blood plasma from each sample was then slowly pipetted and pooled into a new polypropylene tube. The pooled sample was centrifuged at 1500 x g for twenty minutes at 15°C. At this point, the supernatant was removed and discarded from the platelet pellet at the bottom of the tube. The pellet was then re-suspended in 1mL of PBS and centrifuged at 1500 x g for twenty minutes at 15°C. After centrifuging the platelets, they were in a pellet, and the supernatant was removed and discarded. The pellet was then re-suspended in 200µl of PBS, and 30µl was then removed and

placed into a polypropylene tube and saved for later for platelet counting. The remaining platelets are then centrifuged at 1500 x g for twenty minutes at 15°C to pellet the platelets for further analysis.

2.5 Tissue Sample Preparation

The heart, diaphragm, olfactory bulb, cortex, cerebellum, and brainstem samples were removed from the -80°C freezer and thawed on ice. The heart and diaphragm samples was diced on ice into 7-13 pieces and placed back into their respective tubes. Approximately 30µl of the cerebellum and brainstem were removed from the original sample for further analysis. The entire olfactory bulb and cortex samples were used further analysis. Based on the approximate volume of the sample, five times the volume of lysis buffer from the cytochrome C oxidase assay kit was added (i.e. a 30µl sample equals 150µl of lysis buffer). The diaphragm and heart samples were then sonicated on ice. The sonicator was set at 50% power and each sample was sonicated on ice for five seconds, three times. The brain regions were homogenized using a homogenizer. Homogenized samples were kept on ice until the samples were ready to be further analyzed.

2.6 Cytochrome C Oxidase Assay

After the samples were properly homogenized as previously described, the COX activity was measured following the Complex IV Rodent Enzyme Microplate Assay Kit manufacturer's instructions.

2.7 Statistical Analysis

The data is represented by the mean and standard deviation of the mean. ANOVA with Tukey's multiple comparison tests was preformed to analyze the activity of COX using the

statistical models in GraphPad (version 5.0 and 9.0). A p value of <0.05 was considered statistically significant.

Chapter 3: Results

3.1 Clinical Signs in Mice

During the sixty-minute exposure time, one mouse died in the exposure chamber at approximately 56 minutes. H_2S exposure was immediately stopped at this point. We could not collect blood from this mouse because coagulation had already started. We were able to collect the other tissues from the mouse for further analysis. One other mouse died in the sixty-minute group before tissue collection. We were able to collect blood from this mouse via decapitation and we collected the other tissues for further analysis. There were no other deaths prior to euthanasia in the other two experimental groups. So, the mortality rate in the highest dose group was 33%.

3.2 Platelets

Results are summaries in **Figure 1**. The platelets COX activity was inconclusive. Reasons for this are not clear.

3.3 Brain Regions

As can be seen in **Figure 2**, there was a significant decrease in the COX activity in the olfactory bulb in the three experimental H_2S exposure groups. This decrease in COX activity indicates more H_2S inhibition of COX. The cortex was the most significantly affected. There was a significant difference in COX activity in the cortex between the room air group and all three H_2S exposure groups (**Figure 3**), which indicates H_2S inhibition of COX. There was a significant of inhibition in the group exposed to H_2S for one hour, depicting a dose-dependent effect. There

was also a significant decrease in COX activity in the brainstem between the room air group and all three experiment H_2S exposure groups, as seen in **Figure 4**. The inhibition of COX reached a maximum at thirty minutes. This maximum effect can be seen in **Figure 4**, with the thirty and sixty-minute groups being nearly identical in activity. There is a significant decrease in COX activity in the cerebellum between the room air group and the thirty-minute and sixty-minute H_2S exposure groups (**Figure 5**). However, there is no significant decrease in the COX activity in the cerebellum between the room air group and the fifteen-minute H_2S exposure group, as seen in (**Figure 5**). The most severe COX inhibition occurred in the sixty-minute group.

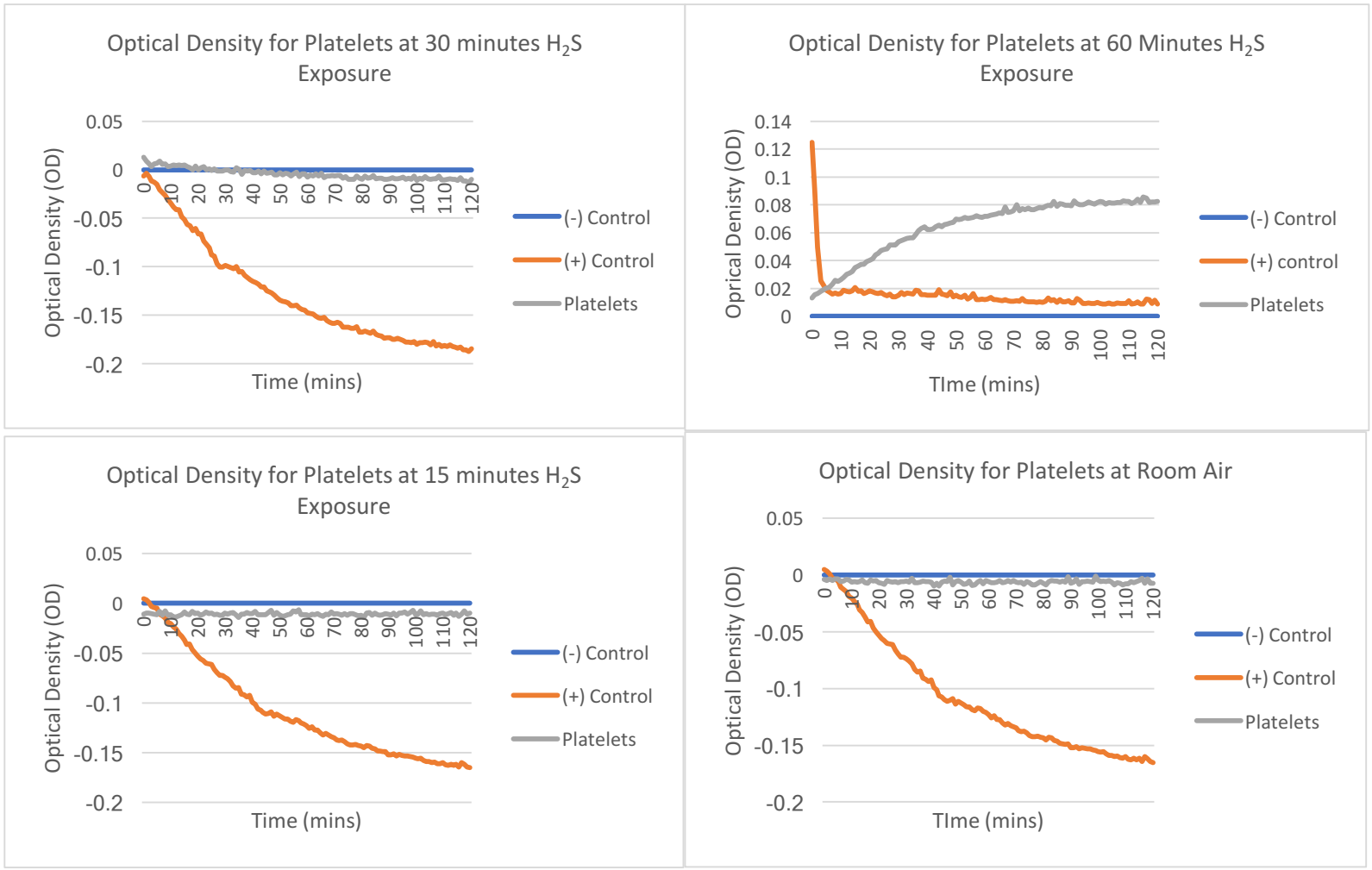

Figure 1. The change in COX activity over a two-hour period of the platelets at room air, 15, 30, and 60 minutes of exposure. Each chart has been normalized to the negative control.

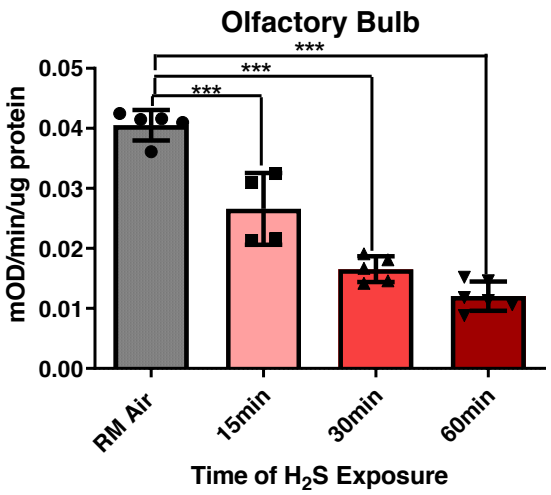

Figure 2. The mean ± the standard deviation of COX activity at room air [RM] (n=5), fifteen minutes H_2S at 1000ppm(n=4), thirty minutes H_2S at 1000ppm(n=5), and sixty minutes H_2S at 1000ppm(n=6). ANOVA with Tukey Multiple Comparison test preformed to determine significance. Asterisks indicate significance compared to RM air group. *** $p < 0.001$

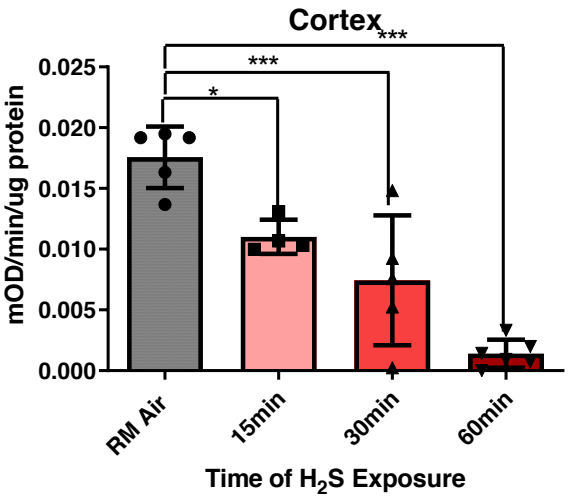

Figure 3. The mean ± the standard deviation of COX activity at room air (n=5), fifteen minutes H_2S at 1000ppm(n=4), thirty minutes H_2S at 1000ppm(n=5), and sixty minutes H_2S at 1000ppm(n=6). ANOVA with Tukey Multiple Comparison test preformed to determine significance. Asterisks indicate significance compared to RM air group. * $p < 0.05$ *** $p < 0.001$

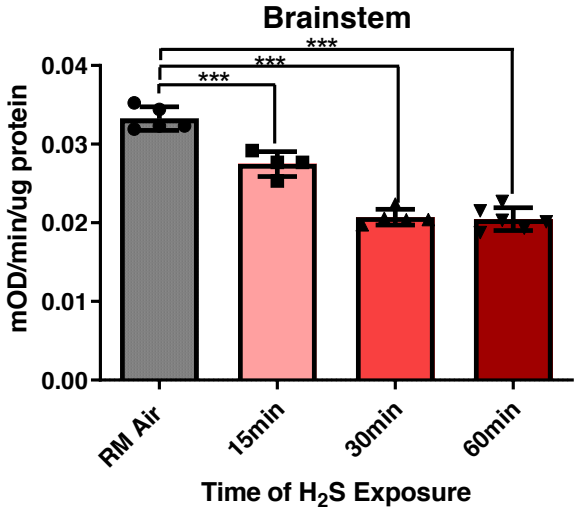

Figure 4. The mean ± the standard deviation of COX activity at room air (n=5), fifteen minutes H_2S at 1000ppm(n=4), thirty minutes H_2S at 1000ppm(n=5), and sixty minutes H_2S at 1000ppm(n=6). ANOVA with Tukey Multiple Comparison test preformed to determine significance. Asterisks indicate significance compared to RM air group. *** $p < 0.001$.

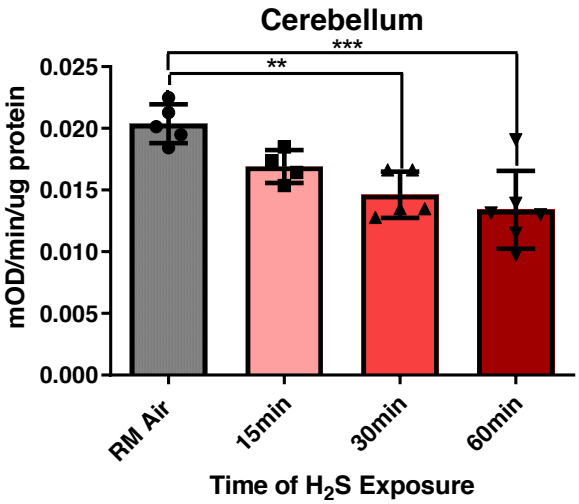

Figure 5. The mean ± the standard deviation of COX activity at room air (n=5), fifteen minutes H_2S at 1000ppm(n=4), thirty minutes H_2S at 1000ppm(n=5), and sixty minutes H_2S at 1000ppm(n=6). ANOVA with Tukey Multiple Comparison test preformed to determine significance. Asterisks indicate significance compared to RM air group. ** $p < 0.01$ *** $p < 0.001$

3.4 Heart and Diaphragm

Figure 6 demonstrates that there is no significant difference in the COX activity in cardiac tissue between the room air group and the fifteen, thirty, and sixty-minute H_2S exposure groups, indicating no significant inhibition of COX by H_2S in the heart. As can be seen in **Figure 7**, there is a significant dose-dependent decrease in the COX activity between the room air group and the experimental H_2S exposure groups in the diaphragm. Maximum inhibition was reached at thirty minutes of H_2S exposure because the thirty and sixty-minute groups had nearly the similar COX activity.

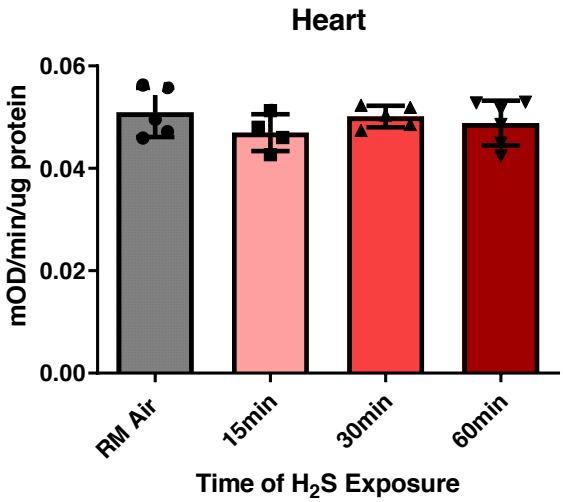

Figure 6. The mean ± the standard deviation of COX activity at room air (n=5), fifteen minutes H_2S at 1000ppm(n=4), thirty minutes H_2S at 1000ppm(n=5), and sixty minutes H_2S at 1000ppm(n=6). ANOVA with Tukey Multiple Comparison test preformed to determine significance. No significance when compared to RM air group.

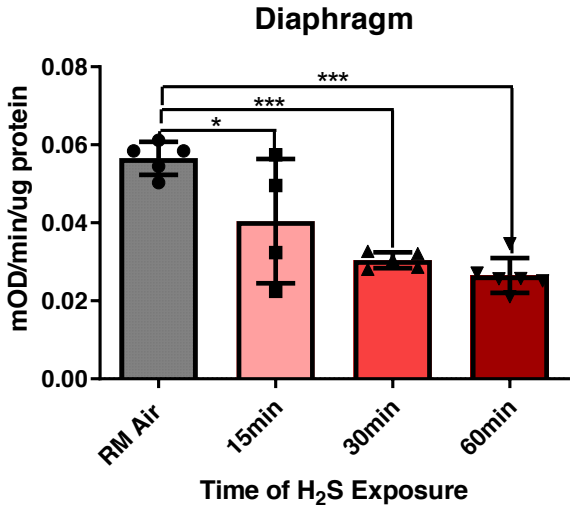

Figure 7. The mean ± the standard deviation of COX activity at room air (n=5), fifteen minutes H_2S at 1000ppm(n=4), thirty minutes H_2S at 1000ppm(n=5), and sixty minutes H_2S at 1000ppm(n=6). ANOVA with Tukey Multiple Comparison test preformed to determine significance. Asterisks indicate significance compared to RM air group. * $p < 0.05$ *** $p < 0.001$

Tissue	Room Air	H_2S at 1000ppm 15 Minutes	H_2S at 1000ppm 30 Minutes	H_2S at 1000ppm 60 Minutes
Cortex	100%	62.7%	42.3%	8.0%
Olfactory Bulb	100%	65.6%	40.8%	29.7%
Brainstem	100%	82.6%	62.3%	61.5%
Cerebellum	100%	83.0%	71.7%	65.7%
Diaphragm	100%	71.2%	53.7%	46.9%
Heart	100%	92.2%	98.4%	95.9%

Table 1. The ratio of the mean ± the standard deviation of COX activity of each exposure group compared to the room air group for each type of tissue. Mice were exposed to room air or to 1000ppm H_2S for 15-60mins.

Chapter 4: Discussion and Conclusion

H_2S is a chemical of significant interest to the DHS. There is no FDA approved drug for treating H_2S toxicoses and the toxic mechanisms are not clear. H_2S exposure can either occur

accidentally or intentionally. Some of the accidental exposures include volcanic emissions, industrial accidents, manure pits and sinkholes. H_2S is known to inhibit COX activity. This study examined the enzymatic activity of COX following acute exposure to different doses of H_2S. The current study highlights the dose-dependent effect of acute exposure to H_2S on COX activity. At different time points we observed different clinical signs in mice, such as dyspnea, seizures, or unconsciousness. How these clinical signs correlate to the COX activity is unknown. In this study, we examined the effect of H_2S on COX activity in different types of tissues with the goal of utilizing this knowledge for diagnosing H_2S poisoning.

4.1 Platelets

In this study, even though we had developed a protocol over multiple months of trial and error, the platelets still proved challenging to isolate and adequately measure COX activity. We were unable to determine the effects of H_2S on the platelets. Based on the data we have collected; we do not know if platelets can be used in diagnosing H_2S toxicosis in mice. Additional work is needed to determine whether mouse platelets can be used for this purpose. The alternative may be to use white blood cells. The main reason is that sample volume for blood platelets is not adequate to yield enough protein for this test. In future studies, a possible solution for sample volume is to use a larger animal such as rats.

4.2 Tissues

There is currently minimal research on of how H_2S effects COX activity in different tissues in the body of mice at different time points. This information is needed so we can better understand the mechanisms of H_2S toxicity and how it affects different types of tissues.

One key setback to studying tissues, such as heart and diaphragm tissue, is that they are more difficult to homogenize than the brain. The brain requires simple homogenization, while

the heart and diaphragm require sonication for proper tissue homogenization. Hsu et al. (2006) found that dividing the heart into smaller pieces and homogenizing the tissue on ice with a sonicator at 50% power three times for five seconds each allowed for optimal homogenization. While the previous research only used the sonication method on hearts, lungs, liver, and brain, this study was able to use this approach to homogenize the diaphragm. This study diced each heart and diaphragm sample into about 7-13 pieces to homogenize the sample for further analysis.

One study compared COX inhibition in the olfactory bulb, hindbrain, lung, and liver by measuring COX activity (Dorman et al 2002). Dorman et al. (2002) found that in rats exposed to H_2S at 400ppm, there was a higher level of inhibition in the olfactory bulb. A previous study by Santana et al. (2023) also determined that the brainstem COX activity is primarily affected by H_2S poisoning, which is confirmed in this study. One key difference between those two studies and this current one is that we found COX activity in the cortex to be the most severely inhibited of the four brain regions studied (**Table 1)**. By comparing the different brain regions, it can be noted that the cortex was the most inhibited overall compared to the olfactory bulb, brainstem, or cerebellum (**Table 1).** COX activity was almost zero in the sixty-minute H_2S exposure group of the cortex. The olfactory bulb was next in sensitivity. The brainstem and cerebellum have nearly the same level of inhibition in each exposure group. The results from this study indicate that the effects of H_2S on COX activity are different between different brain regions. Therefore, from this study, the olfactory bulb and the cortex are the best indicators of acute H_2S poisoning and would be ideal to sample for diagnostic testing.

It was surprising to see that cardiac COX activity was not as sensitive as the brain (**Table 1**). It is possible that the cardiac tissue may have an overall higher amount of cytochrome C

oxidase enzyme present, which would require more H_2S to be present before it starts to affect the overall COX activity. If confirmed this would be an important finding as the heart and brain are considered to be the most sensitive organs to H_2S intoxication through inhibition of COX activity.

In contrast to the cardiac muscle, the diaphragm (skeletal muscle) is sensitive to H_2S. Maximum inhibition was reached at approximately thirty minutes (about 50% of normal). These results suggest that the diaphragm may play a more critical role in H_2S poisoning than previously thought. If the cells in the diaphragm can no longer make ATP, breathing would be dramatically reduced and would quickly contribute to death of the animal. Among all tissues examined the diaphragm was the 3rd most sensitive tissues behind the cortex, and the olfactory bulb.

4.3 Conclusion

Overall, this study has yielded new information. Though we were not able to determine an efficient protocol for detecting the effects of H_2S toxicity on the platelets of mice by assessing COX activity, we were able to identify other tissues that could potentially be used for the diagnosis of H_2S poisoning. We determined that the cardiac COX activity was the least inhibited by acute H_2S poisoning. We further determined that H_2S does not uniformly inhibit COX activity in the four different brain regions examined. The brain regions from most inhibition to least inhibition were cortex, olfactory bulb, brainstem, and cerebellum. We also showed that the diaphragm is also as sensitive as parts of the brain to acute H_2S poisoning.

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