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**HYDROXOCOBALAMIN TREATMENT FOR CARBON MONOXIDE  
EXPOSURES: CHARACTERIZING HEMOGLOBIN CHANGES AND  
TESTING FOR NEUROLOGICAL SEQUELAE**

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
at Virginia Commonwealth University.

by

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position of the United States Air Force, Department of Defense, or the U.S. Government



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## List of Abbreviations

AA – ascorbic acid or Ascorbate. The reducing agent used on Vitamin B12

B12 – Vitamin B12, or Hydroxocobalamin (OHCbl)

B12r – Reduced Vitamin B12. The “antidote” is prepared with AA.

CO – Carbon Monoxide

CO<sub>2</sub> – Carbon Dioxide

DNMTP – Delayed Non-Matching to Place

DPTP – Delayed Placement to Position

Hb - Hemoglobin

HbCO – Carboxyhemoglobin. A form of hemoglobin bonded with CO.

HbO<sub>2</sub> – Oxyhemoglobin. A form of hemoglobin bonded with O<sub>2</sub>

MWM – Morris Water Maze

O<sub>2</sub>Ct – Oxygen Content, % vol O<sub>2</sub> gas in the blood. Includes Hb bound O<sub>2</sub> and dissolved O<sub>2</sub>

OHCbl – Hydroxocobalamin, or Vitamin B12

PPM – parts per million

RBC – red blood cells

WTM – Water T-Maze

## Abstract

# HYDROXOCOBALAMIN TREATMENT FOR CARBON MONOXIDE EXPOSURES: CHARACTERIZING CARBOXYHEMOGLOBIN CHANGES AND TESTING FOR NEUROLOGICAL SEQUELAE

By Leonardo Gamueda Somera, III, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Advisor: Bruce D. Spiess, MD, FAHA  
Professor, Department of Anesthesiology  
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Prior work in our lab has indicated that reduced Hydroxocobalamin (B12r) can be added to human blood and is able to convert carbon monoxide (CO) into carbon dioxide. This has great potential as a direct antidote to mitigate the toxic effects of CO poisoning which is a public health risk. In the first part of our work, we use highly specific wavelengths of light and Raman spectroscopy to study changes in carboxyhemoglobin (COHb) between blood treated with oxygen and blood treated with oxygen and B12r in a flowing circuit of blood. Using Raman spectroscopy, we found that the addition of B12r hastens the conversion of the COHb Raman signals to oxyhemoglobin (HbO<sub>2</sub>) Raman signals. In addition, the B12r absorbance of light energy within the Raman spectrum is an exploitable relationship that can be used to measure B12r presence in the blood. In part two of our study we focused on the neurobehavioral testing of rats injured by CO exposure, however, we were not able to find statistical differences in the behavioral tests between exposed and unexposed rats.

## **Introduction**

### *Carbon Monoxide Exposure and Toxicity*

Carbon Monoxide (CO) results from the incomplete combustion of carbon materials due to inadequate concentrations of available O<sub>2</sub> to facilitate full oxidation of carbon into CO<sub>2</sub>. Common sources of concern for CO are structure fires, poorly ventilated or malfunctioning gas heaters or stoves, exhaust from fuel combustion vehicles and generators. Inhalation exposures are a common public health concern resulting in over 15,000 exposures and 500 deaths per year for non-fire incidents alone.<sup>1</sup> Exposures are known to cause acute neurological sequelae and delayed neurological sequelae (DNS) in about 10% of victims.<sup>2-5</sup> Neurological sequelae, or encephalopathy, manifests as impaired cognitive behaviors. Generally, it is due to injury or disease of the nervous system. In CO injury, this may last hours or days depending on the severity of exposure. In humans, the encephalopathy typically associated with CO exposure is dizziness, confusion, memory loss, disorientation, difficulty in coordinating, and loss of consciousness.<sup>4,5</sup> DNS due to CO exposures occurs days to weeks later and may reappear after recovery from the initial encephalopathy. DNS symptoms associated with CO exposures include memory disturbances, depression, anxiety, personality changes, deficiencies in higher cognitive abilities, as well as motor dysfunctions. DNS can be a chronic, unyielding condition. The complex mechanism for neurological sequelae from inhalation exposure starts with CO transport via the circulatory system to affect the nervous system at the cellular level. Classically, hypoxia-induced toxicity due to CO binding hemoglobin (Hb) to form carboxyhemoglobin (COHb) has been the primary explanation of encephalopathy. More recent discoveries show the relationship to be more complex with CO binding to proteins such as guanylyl cyclase, cytochrome c, and

perhaps signaling molecules. The effects of guanylyl binding by CO lead to cerebral vasodilation potentially with loss of consciousness. The binding of cytochrome c impairs cellular metabolic pathways affecting the production of essential energy.<sup>6</sup> Studies in this field continue to show CO directly affecting normal cellular functions. DNS's longer evolution time led to evidence that DNS may have immune-mediated mechanisms on rats. The study showed an immune signature alteration of myelin causing an autoimmune reaction with neural tissue after exposure to CO.<sup>3</sup>

The treatment for victims of CO exposure is breathing 100% O<sub>2</sub> up to several hours to provide oxygen delivery. CO removal is a secondary effect of the treatment. This treatment reduces the body's half-life of CO from 5-8 hours down to 1-2 hours. In more severe cases, and if the capability is present, administration of O<sub>2</sub> is conducted in a hyperbaric chamber at 1.5 to 2 atmospheres. Even with hyperbaric capabilities, the treatment can take hours to remove sufficient CO; treatment that seems to have the best outcomes involves rotating between normal pressure O<sub>2</sub> and hyperbaric O<sub>2</sub> in a 48-hour treatment.<sup>5,7</sup> At the time of this study, there were no treatments that directly removed CO from the body.

Here we study the first intravenous antidote to directly affect CO. The treatment, B12r, is a reduced form of Vitamin B12, or Hydroxocobalamin (OHCbl), that catalyzes a conversion of CO to CO<sub>2</sub> thus removing the CO toxicity threat directly.<sup>8,9</sup> This injectable treatment should be faster and more efficient than ventilation with 100% O<sub>2</sub> alone. It could be combined with oxygen delivery therapies to enhance removal of CO.

## *Hemoglobin and Gas Transport*

In this study, blood refers to whole human blood obtained from donors. Hemoglobin-bound O<sub>2</sub> accounts for 98% of the O<sub>2</sub> content in blood, and the remaining 2% is dissolved in plasma and the intracellular fluid of red blood cells. Similar to O<sub>2</sub>, CO is transported between the lungs and tissues via the circulatory system. CO is also primarily transported bound to hemoglobin. Less than 1% may be found freely dissolved in plasma or interstitial fluid.<sup>10</sup> Hemoglobin, a globular protein, has two alpha-chain and two beta-chain subunits. Each of the four subunits contain a single porphyrin ring with an iron center that can bind O<sub>2</sub> or CO. Hemoglobin with iron in the ferrous state (Fe<sup>2+</sup>) can bind to O<sub>2</sub> and CO. Methemoglobin contains iron in the ferric state (Fe<sup>3+</sup>) and cannot bind either O<sub>2</sub> or CO. Deoxyhemoglobin is ferrous hemoglobin unbound to any gas molecule.<sup>11</sup>

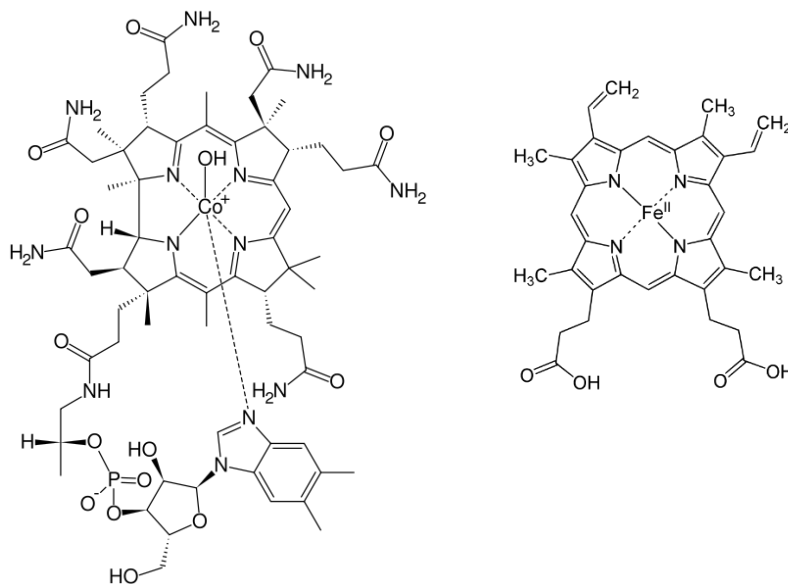
As hemoglobin changes from one form to another, its spectral absorption properties change. When blood is exposed to oxygen, a bright red color is observed; with nitrogen gas, a dark red appears as Deoxyhemoglobin forms. Absorption spectroscopy has been used in clinical settings to measure percentages of various hemoglobin forms and calculate oxygen saturation—vital metrics to diagnose the status of a patient. Blood/Gas machines used in clinical settings contain absorption-spectroscopy technology to measure relative amounts of hemoglobin forms. Some pharmaceutical treatments, however, interfere with the machines' abilities to differentiate absorption spectra due to the treatments' pigment similarities to Hb. Cyanokit™, a B12 treatment for cyanide poisoning is one, and the proposed B12 treatment in this study, naturally, would be another. Raman spectroscopy is used in this study to overcome this interference.



## *B12r: a Potential Carbon Monoxide Exposure Treatment*

Due to the long half-life of CO in the body, blood can continue to deliver CO to tissue and organs for several hours even after removal of the victim from the site of exposure. Elimination of the CO benefits victims. B12r, a reduced form of Hydroxocobalamin (B12), may catalyze the conversion of CO to CO<sub>2</sub>.<sup>8,9</sup> We propose that an injection of B12r into the circulatory system may enhance elimination of CO from the blood and block delivery to tissues.

B12 consists of a corrinoid ring with a cobalt center resembling a porphyrin ring. The core molecular structure of B12 resembles the heme ring of a hemoglobin subunit. Figure 1 below shows the B12 moiety, Hydroxocobalamin, alongside a porphyrin ring with an iron center (Heme B).



**Figure 1 - Hydroxocobalamin (Vit B12 moiety) on the left and Heme B on the right. This shows structural similarities between the two molecules.**

Buffered ascorbic acid (AA), was used to reduce the B12 to B12r.<sup>8</sup> This reduction modifies the Cobalt center from Co<sup>+3</sup> to Co<sup>+2</sup> and thus making it reactive to CO. Raman spectroscopy was

used to validate the reduction of B12r with AA (Figure 8). The Raman spectra of reduced and unreduced B12 are well documented.<sup>12-14</sup>

B12r's structure and its affinity for CO are not the only similarities to hemoglobin. As mentioned, spectral absorption properties of B12 molecules (porphyrin-like corrin rings) are similar to Hb molecules (containing four porphyrin rings), and analyzers used to measure oxygen saturation and concentrations of Hb forms are confounded by this spectral similarity.<sup>12,15</sup> Raman spectroscopy techniques have been used to measure Hb forms as well as B12 moieties. Our earlier work indicates that some forms of Hb may be measureable in spite of B12 interference.<sup>8,12,13</sup>

### *Raman Spectroscopy*

The Raman Effect shifts the energy of the light scattered from a molecule that has Raman active vibrational bonds. This is in contrast to Rayleigh scattering or reflection, where the energy being absorbed is the energy being emitted. The change in energy is slight, but measurable with sensitive spectrometers. For example, our study uses a 406.7 nm wavelength laser for the excitation energy. Much of this energy is scattered by the blood being analyzed with no change in wavelength and remains 406.7 nm. However, a tiny percentage of this light is scattered with different shifted wavelengths above and below 406.7 nm (termed stokes and anti-stokes). These shifted wavelengths of light are unique to the material and give insight to the vibrational modes of the molecule's bonds. Two forms of the same molecule that differ in their molecular bonds can be distinguished by Raman spectroscopy. Hemoglobin forms are good examples.

Figure 2 depicts Raman spectra taken from oxygenated and deoxygenated blood. Since blood contains a high concentration of Hb, the Raman spectra of hemoglobin is intense, and the two

forms are readily distinguishable. The most intense peak (tallest amplitude) in both Hb forms is referred to as the  $\nu_4$  (pronounced *nu-four*) band which is associated with heme-ligand binding. This band shifts frequencies depending on whether the heme ligand is bound or unbound, oxygenated or deoxygenated. This property has been used to quantify Hb characteristics such as oxygen saturation.<sup>16</sup> The  $\nu_4$  band also shifts slightly depending on which ligand is bound to it, O<sub>2</sub> or CO. Figure 3 shows Raman spectra of oxygenated blood and blood exposed to CO until >90% COHb levels were reached. Depicted here are peaks related to either O<sub>2</sub> or CO association with Hb. In HbCO<sub>2</sub>, CO<sub>2</sub> is not a heme-protein ligand, and the Raman spectrum of CO<sub>2</sub> exposed hemoglobin resembles that of deoxygenated blood (deoxygenated with N<sub>2</sub> gas).

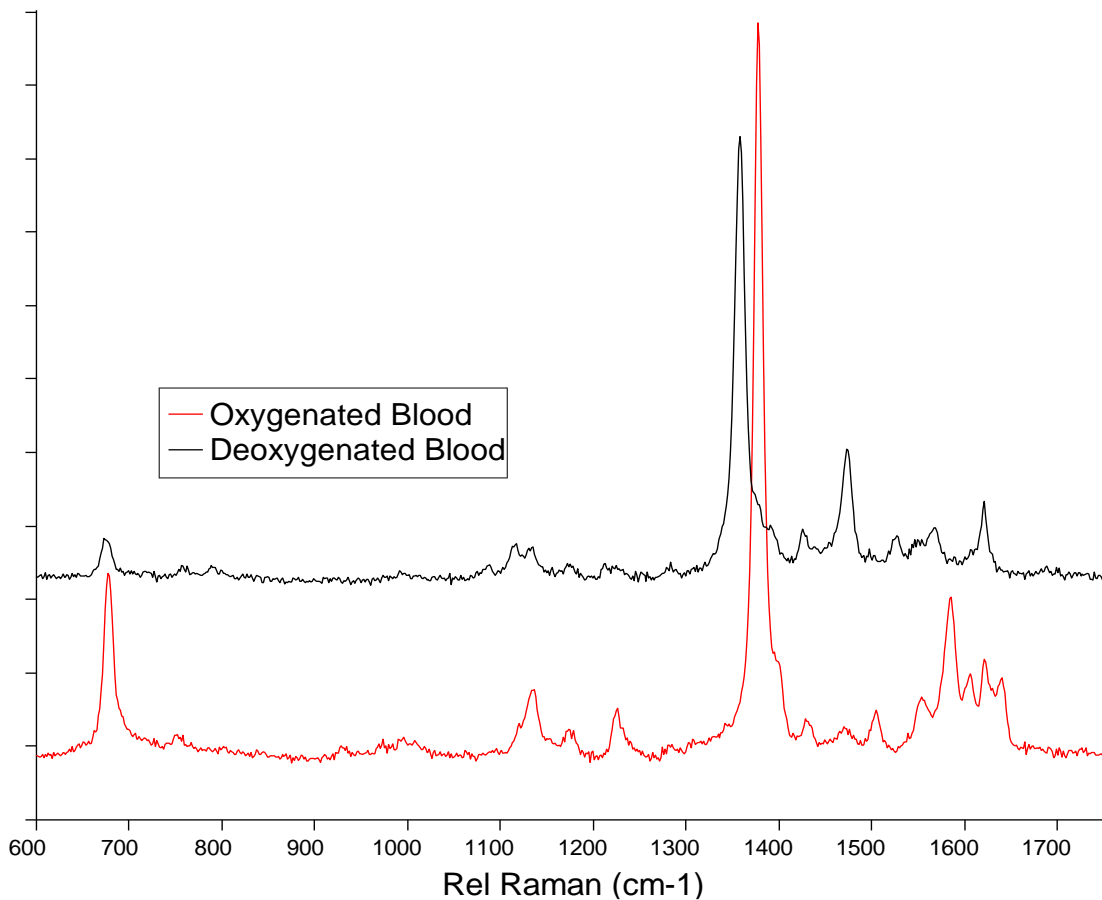


Figure 2 – 407.6 nm Raman spectra of oxygenated hemoglobin (Red) and deoxygenated hemoglobin (black).

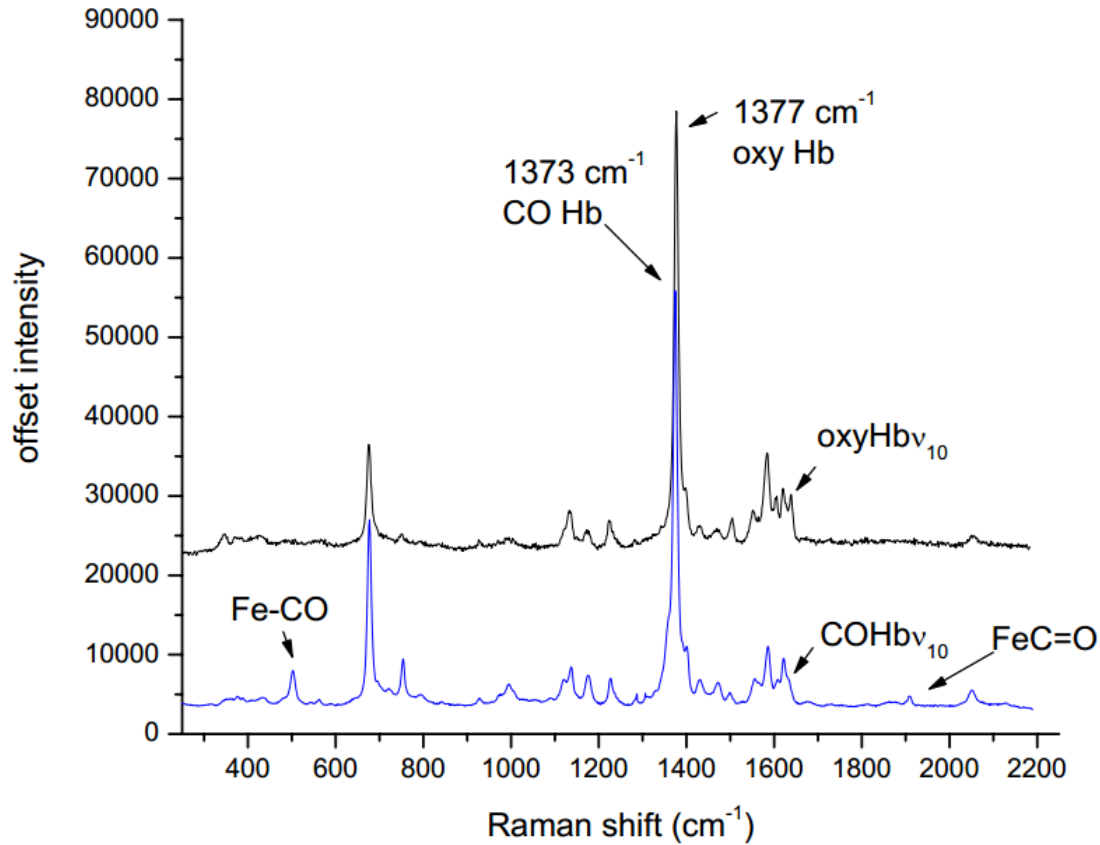


Figure 3 – 406.7 nm Raman Spectra of Oxygenated Blood (top, black) and CO-Exposed Blood (>90% COHb, bottom, blue). This figure depicts the major peaks of interest for this study. Note the  $\nu_{10}$  band at 1640 is attenuated in CO Blood. 1373 and 1377 peaks represent the positions for the  $\nu_4$  band for CO and Oxy blood respectively. The CO Blood spectrum shows the Fe-CO and FeC=O vibrational bonds.

### *Cognitive Behavioral Tests*

Impairment in memory and cognitive abilities are associated with CO injury, and both manifest post exposure and after a delay with no symptoms. In rats impairment has also been demonstrated with various CO injury protocols and with various behavioral tests such as radial arm mazes, Morris water mazes, and open field tests.<sup>3,17-22</sup> In this study, the Morris Water Maze

(MWM) was chosen as a test for learning and use of working memory by the rat. The MWM utilizes a pool in which the rat is placed. A hidden and submerged platform is the rat's only escape from the pool. Escape is the key motivation, and various protocols can be used for repetitive exposures to the pool. Rats use visual cues to remember the location of the hidden platform between trials. This spatial memory is combined with adaptation induced by the protocol such as delays between trials and relocation of the hidden platform. The MWM and protocol used is further described in the Methods section.

Novel to CO injury testing is the Water T-Maze (WTM) which seems to be more sensitive than the MWM.<sup>23</sup> Instead of a circular pool, the rat swims a T-shaped maze. Starting from the stem of the T, they must swim to an escape platform located either in the left or right arm of the T. Each trial has two swim runs, and the rat must remember the Non-Matching rule: the escape platform in the second swim is in the *opposite* arm found in the first swim. The rat must use working memory to be successful in escaping. This working memory is taxed by the protocol with increasing delays between swim runs. The WTM is further described in the Methods section. In this study, we explore the difference between Exposed and Unexposed groups in the WTM.

### *Hypothesis and Objectives for Study*

The first part of this study uses Raman spectroscopy, and hypothesizes that as CO in CO-poisoned blood is removed from plasma and intracellular fluid (within red blood cells) by B12r catalyzing it to CO<sub>2</sub>, a local pressure gradient promotes removal of CO from HbCO. HbCO becomes deoxyhemoglobin (Hb) momentarily, and then it may form oxyhemoglobin (HbO<sub>2</sub>) with the O<sub>2</sub> already present in the blood. Our objective was to investigate the relationships between HbCO, HbO<sub>2</sub>, Hb, and B12r as CO poisoned blood is treated with B12r via an *in vitro*

circulating gas exchange system, and using Raman spectroscopy as the accurate measurement technique. This includes qualitative and quantitative observations of the potential interference B12r may have with the Raman spectrum of hemoglobin, and how we may be able to use that interference to our advantage.

Specifically:

1. Is the quality of the Raman spectrum of hemoglobin with B12r presence unhindered enough to measure hemoglobin signals?
2. Comparing the Raman spectrum of CO-poisoned treated and untreated blood, can a measurable difference be observed in COHb Raman signal reduction that shows the impact of B12r?
3. Comparing the Raman spectrum of blood before and after B12r introduction, can the differences in the spectra be used to quantify B12r concentration in blood?

The second part of this study used behavioral tests and hypothesizes that with timely removal of CO in blood shortly after CO exposure, neurological sequelae is mitigated. This study uses CO injury in rats followed by treatment and testing with cognitive behavior mazes to observe changes in the working memory of rats. The Morris Water Maze (MWM) Delayed Placement to Position test is used. In addition, an exploration of a Water T-Maze Delayed Non-Matching to Position test is explored.

Specifically:

1. Using the a CO Injury Protocol and the MWM, can we detect a difference between Exposed, Exposed Treated, and Unexposed groups in MWM parameters that indicate cognitive impairment due to injury.

2. Is a sufficient difference in WTM performance detectable between exposed and unexposed groups of rats such that it may be used for future tests of CO injury?

## Materials and Methods

### *Blood*

Fresh whole human blood was obtained from anonymous donors via the VCU Health System Apheresis Clinic's phlebotomy program. Blood was restricted to non-sickle cell and non-HIV donors. The blood was treated with 1000 units of Heparin per 100 ml of blood in sterile blood donation bags, stored in the laboratory refrigerator and used within 5 days. Stored blood bags were turned over to unsettle blood daily and prior to use. Prior to experiments, blood bags were brought to room temperature and filtered using transfusion lines with 100 um clot filters as a precaution against blood clots; little to no clotting was observed, and blood with significant clotting activity were discarded. The *ex-vivo* blood was circulated in our unique gas-exchange system to obtain target COHb percent concentration levels (apparatus described below). Blood samples were obtained from the experimental apparatus, and analyzed with one of two blood gas machines. The Radiometer™ ABL 800 clinical blood gas analyzer was available in the Sanger Hall facility where antidote preparation and initial blood preparation occurs. The Radiometer™ OSM3 research blood gas analyzer was available in the Oliver Hall facility where the Raman spectroscopy measurements were made. The ABL 800 measures pO<sub>2</sub>, pCO<sub>2</sub>, %HbO<sub>2</sub>, %HbCO, %MetHb, pH, and O<sub>2</sub> Content. The OSM3 measures %HbO<sub>2</sub>, %HbCO, %MetHb and O<sub>2</sub> Content. When possible, blood samples from the Raman spectroscopy facility were brought back to Sanger Hall for blood gas analysis via the ABL 800.

### *Preparation of Reduced Hydroxocobalamin*

Hydroxocobalamin (OHCbl), or Vitamin B12, in powdered form and pharmaceutical grade buffered L-Ascorbic Acid (AA) were obtained from Sigma-Aldrich. Both were refrigerated at 4 °C and sealed away from light in brown glass bottles (from manufacturer) or in clear vials



wrapped in foil as needed. The AA was also maintained in an O<sub>2</sub> free environment, and diluted with normal saline. The normal saline used was deoxygenated with several cycles of vacuum and N<sub>2</sub> gas exposure, then stored in laboratory glass containers filled with N<sub>2</sub>. All mention of normal saline in this study will refer to this deoxygenated saline source unless otherwise noted.

Anaerobic preparation of the antidote was in a room temperature, positive pressure, N<sub>2</sub> glove box by mixing B12 with the AA in syringes, and taking the solution out of the glove box in the stoppered syringe with needle. Great care was taken to ensure preparation and handling in an O<sub>2</sub>-devoid environment prior to use. Target concentrations for most uses ranged from 1 mg of B12 per ml of blood to 10 mg/ml B12r/blood.

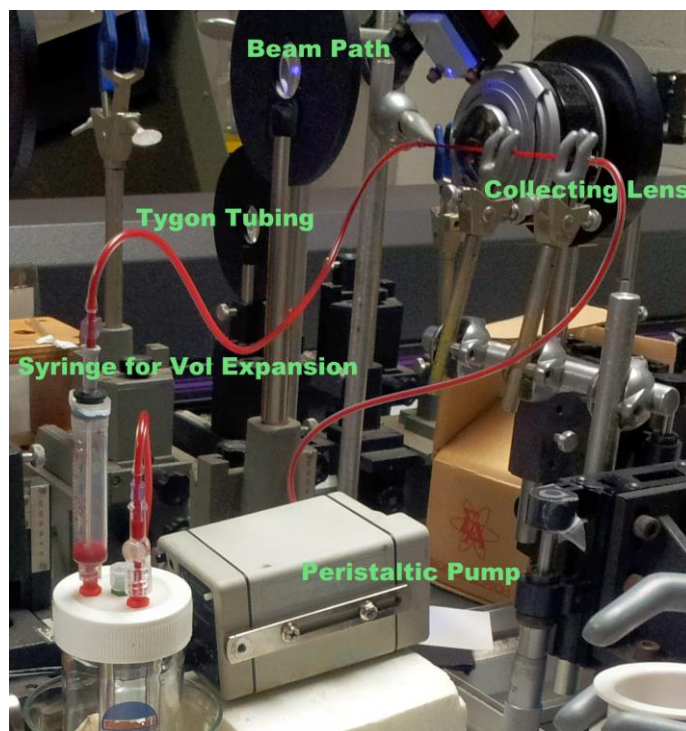
#### *Raman Spectroscopy System*

The Raman apparatus included: a 406.7 nm krypton laser excitation source (Coherent Saber), various optics to optimize the excitation beam onto the sample flowing through a capillary tube, more optics to collect the emission light, a spectrometer fitted with a 600 mm grating, and a CCD camera (Princeton Instruments Python CCD) connected to a computer running spectroscopy recording software (Princeton Instrument WinSpec32). The excitation laser beam hits the flowing blood through the capillary tube orthogonal to the emission light being collected. The apparatus is mounted on a laboratory table along with the blood circulating systems described below. The laser output power was 0.7 mW to 08 mW, but was attenuated by a neutral density filter to 0.07 mW or 0.08 mW prior to hitting the sample. Spectra of hemoglobin was collected for 3 to 5 minutes (20 seconds exposures, for 9 to 15 exposures). The low power ensured minimal effect of photolysis of CO and O<sub>2</sub> from Hemoglobin.

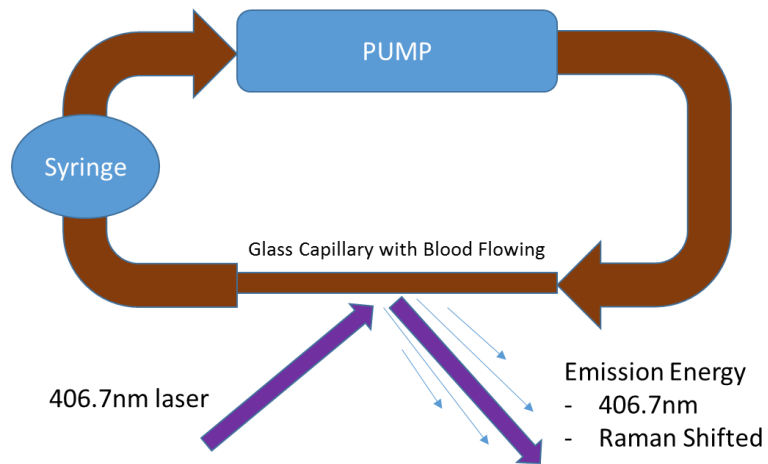
## *Gas Exchange Systems*

Raman measurements were taken via three methods: blood circulating in a non-gas exchange setup or in a gas exchange setup (both described below), or via capillary tubes of non-flowing fluids. The experiments occurred between 22 and 24 °C. The circulating system for blood minimized photolysis of the gases bound to hemoglobin, and ensured thorough mixing of B12r with blood.

The non-gas exchange system (Figure 4 and Figure 5) used a peristaltic pump to facilitate the flow of blood through Tygon tubing and a capillary tube. A syringe allowed volume changes to prevent pressure changes in the system. The internal volume of the setup was 5 ml. The syringe allowed for up to 8 ml; however, minimal fluid was used to prevent areas of stagnant blood. This non-gas exchange system observed anaerobic B12r effects on Hb with the trapped gases in blood with no or minimal gas-exchange, or respiration.



**Figure 4 - Non-Gas Exchange Circulating Apparatus**



**Figure 5 - Schematic of Non-Gas Exchange Apparatus**

The gas exchange setup (Figure 6 and Figure 7) used a Maquet™ QUADROX-ID Pediatric Oxygenator with Tygon™ tubing. A shunt was made to divert a portion of the blood flow through smaller Tygon tubing and a capillary tube. The Gas Out port was attached to tubing, the end of which was placed into a lab hood. The Gas-In port was used to flow gases into the gas-exchanger. Air-tight syringes were used to inject 100% CO gas into the Gas-In ports until desired COHb levels were reached. The gas ports were either left open to allow flow, or clamped to trap the gases in the Maquet unit as needed. The circulating fluid volume of the system was approximately 135 ml.

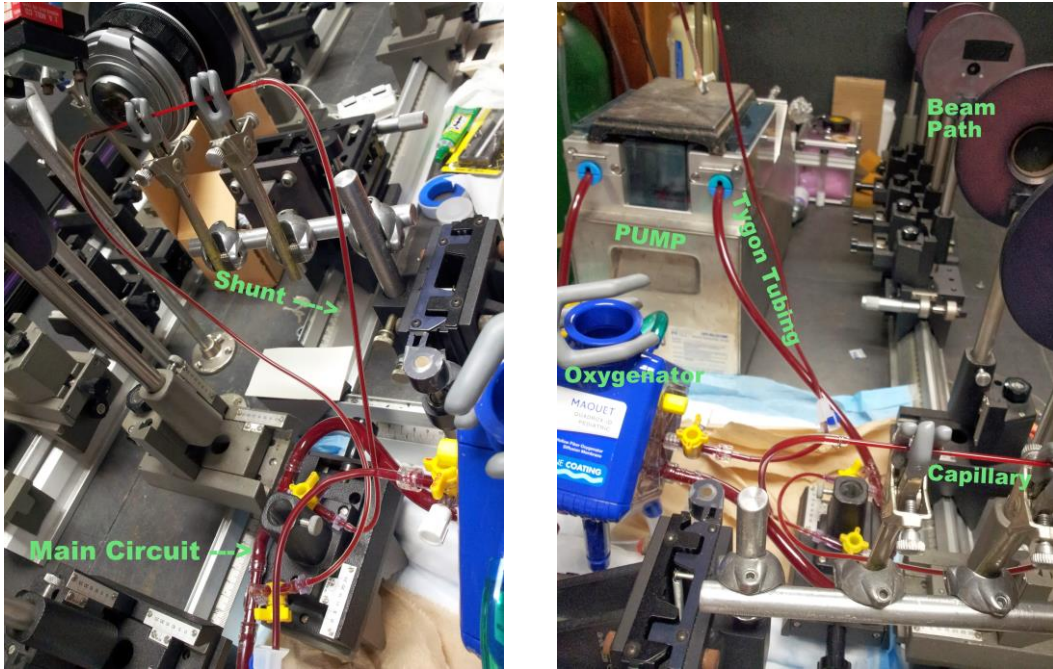


Figure 6 - Gas-Exchanging Apparatus

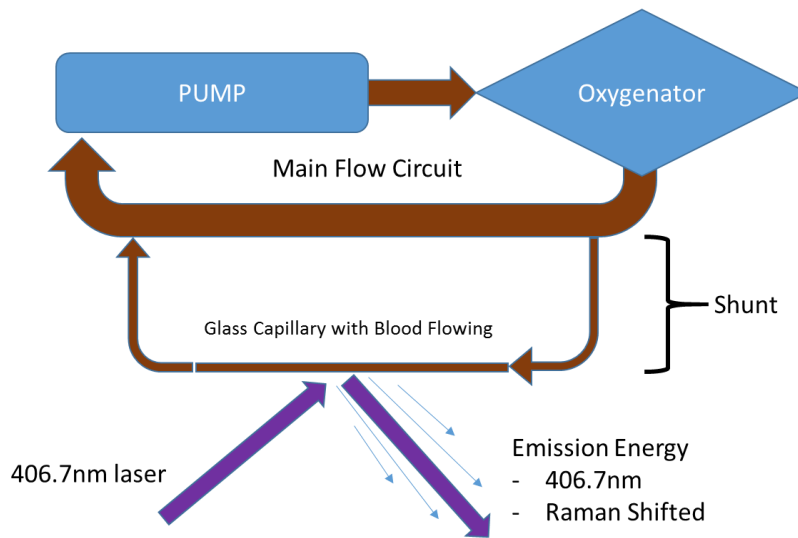


Figure 7 - Schematic of Gas Exchange Apparatus with Shunt

The blood was sampled periodically for %COHb and %HbO<sub>2</sub> concentration depending on the gas analyzer used, and sampled for Raman spectra.

### *Animals and Injury (CO and Air Insult)*

All procedures followed the guidelines established in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services) and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (Protocol Number AD10000569). Long Evans Rats (LE rats) and Sprague Dawley Rats (SD rats) were used in the neurological testing. LE Rats were used in the Morris Water Maze test. SD rats were used in T-Maze pilot testing. Rats were obtained from Harlan Laboratories, Inc, and weighed  $211\pm 6$  grams (LE Rats) and  $203\pm 41$  grams (SD Rats) upon arrival. All rats were housed two per cage, and maintained by VCU Department of Animal Resources veterinarian staff. Rats were acclimated and their weights were monitored for a minimum 5 days prior to insult with CO or with Medical Grade Air (Air) as the control. During acclimation, rats were habituated to handling by experimenters. Rats undergoing T-Maze tests were habituated to the T-Maze environment on Day 3, and trained starting on Day 4 post-delivery. After insult animal weights were monitored for at least 3 days, and on each day of neurological behavior testing. After behavior testing, rats were euthanized with an intra-peritoneal injection of Sodium Barbital and monitored until their heart beats were no longer detectable. Rats from each group were randomly selected for brain histology comparisons for a separate study (results in progress as of this writing).

Rats were divided into Control, Exposed Treated, and Exposed Untreated groups. Morris Water Maze rats underwent insult, and were tested in blocks of 12. T-Maze Pilot rats were tested in three batches of 8 and 12. Rats were exposed to either CO or Air in 2 L air-tight chambers at 0.5 L/min after an initial two minutes at 2 L/min.<sup>3,17,18,20,21</sup> For the CO exposure, rats were exposed to CO at 2500 ppm for 60 min, and then at 6000 ppm for a maximum 10 min

or until the animal was no longer responsive to stimuli. To decrease mortality, CO flow was interrupted for a few minutes with air as needed near the 60-minute point prior to the 6000 ppm exposure phase, but total exposure time was maintained at 60 minutes. After insult rats were administered treatment per their group assignment. Treatment was given via intra-peritoneal injection. Rats were placed in a separate holding cage until they regained normal response to stimuli, and then placed into their primary housing cage thereafter.

### *Morris Water Maze Behavioral Tests*

In the Morris Water Maze (MWM) test, a platform was submerged in a 1.8 m diameter pool filled with water to approximately 0.5 m. An escape platform was submerged 2 – 2.5 cm below the surface of the water. The water was made opaque with white paint so that the platform was not visible, and the temperature was maintained between 25-27 degrees Celsius with a heat exchange pump. The pump was removed during the swim trials. There were 4 Stages of MWM testing. The location of the escape platform was changed each Stage as follows: Stage 1 in the NE Quadrant, Stage 2 in the NW Quadrant, Stage 3 in the SE Quadrant, and Stage 4 in the center of the pool. A Stage of the MWM was conducted in a day. Within each Stage rats performed four swim trials. For each trial the start position of the swim was changed between North, South, East and West starting points. Within each Stage, the start position was constant between Trials, and randomized between Stages. The pool room had visual cues on the walls and other visual features that remained unchanged during the entire MWM experiments for consistent cues. The rat was placed gently, keeping their heads from submerging, at the start position facing the wall; and the software tracking was started. The AnyMaze™ system tracked the rat via a camera centered about 10 feet above the pool. AnyMaze marks the end of the trial at 60 seconds or when the rat has successfully climbed onto the escape platform, whichever comes first. The

software recorded escape latency and other parameters. After each swim trial, the rat is placed into a holding cage under a heat lamp with towels for 10 minutes prior to the next swim trial. LE Rats underwent MWM tests on Days 1, 3, 6 and 8 post-exposure; these days correspond to Stages 1 to 4 respectively. The animal handler was blinded to the treatment group assignments.

An independent reviewer watched the video recordings of the trials to correct automation recording errors. The tracking software tracked the head of the animal rather than the body. At times, the rat's head would stick out beyond the boundaries of the platform, and the tracking software would not mark a correct end-time. The independent reviewer adjusted the *Escape Latency* times as necessary.

#### *Water T-Maze Behavioral Tests*

In the Delayed Non-Matching to Position (DNMP) Water T-maze the rat needs to learn to swim to the *opposite* platform from which it was previously *informed* (or forced) to go, and retain working memory of this rule with increasing delays between the information phase and the choice phase. For example, in the “T” shaped maze where the base of the T is the start, a platform is located at the end of the right arm during the Information Phase, and passage to the left arm is blocked. The rat is forced to swim to the right arm and find the platform and thus is *informed* of the platform and its location. The rat is removed from the maze—this break from the T-maze is the Delay Phase and the Delay Time is a controlled variable. Passage to the left arm is cleared such that both arms are open, the right arm platform is removed, and a left arm (opposite side from first swim run) platform is inserted. After the Delay Time passes, the rat is then placed back at the start. A rat who has retained the rules of the test will swim to the left, or opposite, arm to find the platform versus the right as informed. As Delay Time increases, the rat's working memory is taxed, and failure to swim to the correct side increases.

The same pool and room from the MWM is filled with water and made opaque with non-toxic tempera paint and heated to 25-27 °C; all visual cues were maintained consistent for all trials. Inside the pool a custom built T-Maze structure was placed and had slots for escape platforms at the end of the right and left arms, and a slide divider at the entrance to each arm to separate each arm independently from the central arm. The T-Maze is submerged to a water level about 2 – 2.5 cm above the escape platforms. The *start location* for the rats was at the end of the central arm furthest from both platforms.

Prior to injury, rats were habituated to the WTM by freely wading through the maze from the start location to one of the platforms at a water depth where they could still walk on all four legs; during habituation day, the water level was increased until they were forced to swim by the last trial. Partitions were never placed to block the arms. The goal during habituation is for the rat to be familiar with the T-Maze and most importantly, realize that there are platforms for escape. Handlers encouraged the rats to find both platforms. Rats are removed from the maze only via the platform to enforce the need to find the platform. Rats perform this swim 10 times on Habituation Day. Prior to Injury, rats were trained the rules of the Water T-Maze during Acquisition Days. The goal during Acquisition Days was for each rat to learn the rules of the DNMP test. There were three phases to each trial: 1) *Forced (or Information)* – a divider will be placed in one arm (the Forced Arm) forcing the rat to swim to the platform in that arm; 2) *Delay* – rats will rest for 7s – 10s in pool-side holding box while observer sets up for the next swim; 3) *Choice* – dividers are removed and platform from *Forced* phase is removed, leaving only a platform in the Forced Arm. The arm opposite the Force Arm is the Choice Arm. *Forced* direction was randomized and pre-determined prior to trials with no more than 2 consecutive trials in the same direction (example: R, R,L, L, R, L, L, R, R, L). Work was conducted in



groups of 3 to 4 rats to ensure at least 4 – 5 minutes of rest between trials per rat. Entering the Forced Arm was considered “Incorrect.” If the rat entered the Force Arm, the divider placed to trap the rat in that arm. This was the punishment for the wrong choice, and the rat would be forced to swim in the trapped arm for 10 sec after which the divider was removed to allow the rat to find the platform in the Choice Arm. The rat was removed only after it firmly finds the platform. Entering the Choice Arm was considered “Correct.” If the rat entered the Choice Arm, it was removed immediately after it climbed onto the escape platform and then it was placed in the holding cage to dry and rest. Post injury days utilized increasing delay times between the Forced and Choice swims to tax the animals’ working memory. SD rats were used for the pilots and were separated in to Exposed and Unexposed groups.

### *Analysis and Statistics*

For the MWM studies, histogram plots are presented for visual and exploratory purposes. Statistical analyses were conducted in JMP Pro v10 software, or Microsoft Excel for simple means and standard deviations. The MWM subjects were divided into three primary treatment groups: Unexposed, Exposed + Saline Treatment, and Exposed + B12r. Predictor factors are as follows: *subjects*, *trials*, *stages*, and *treatment*. The *trials* are nested factors within *stages*. The most important response factor was *escape latency*, and a mixed model time-to-event analysis (Proportional Hazards Model) was conducted due to the upper time limit of the trials. *Mean speed* response factor checked for motor skills differences, and a repeated measure analysis of variance was conducted for this factor.

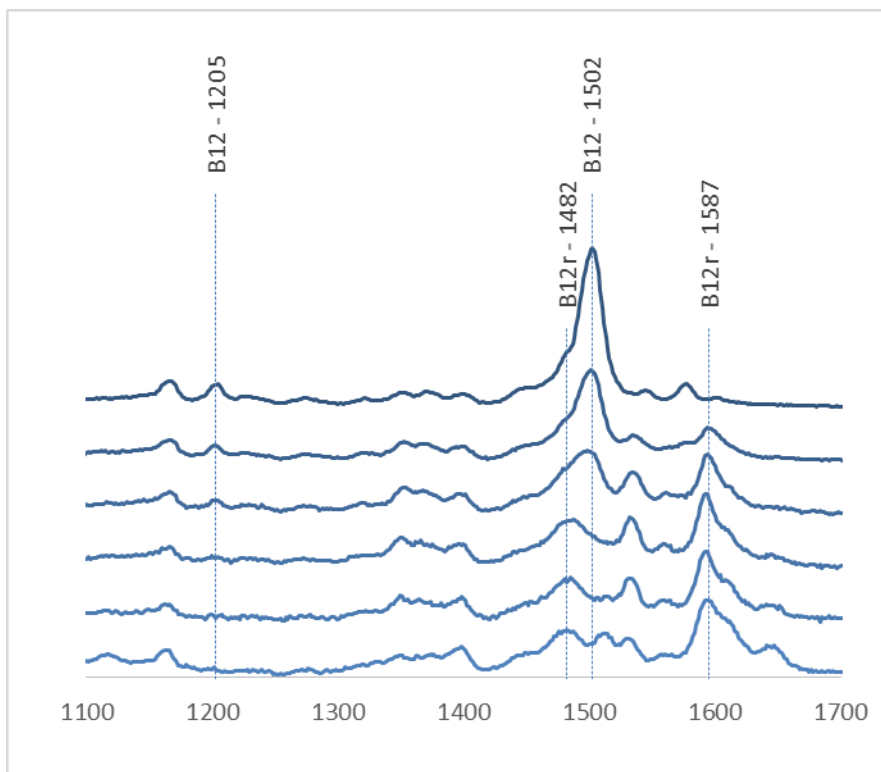
Two WTM pilots with SD Rats were conducted. Each pilot had two groups: exposed and unexposed. Predictor factors were: *subject*, *treatment*, and *delay*. The response factor is *score*. Post-injury scores were subtracted from Pre-injury scores to normalize the results. Line plots of

mean and standard error were presented for visual and exploratory purposes, and non-parametric T-tests were conducted at each delay.

## Results

### *Preparation of Reduced Hydroxocobalamin*

For the reduction of B12r, various AA:B12 mass ratios were explored from 1:1 to 0.08:1. Molar calculations showed that to completely reduce B12 to B12r, a 0.08:1 mass ratio was sufficient; however, analysis with Raman spectroscopy showed significant signals for B12 and weak B12r signals in the 0.08:1 solution produced (Figure 8, second spectrum from top). The 1:1 mixture showed a Raman spectra with strong B12r signals and undetectable B12 signals from the raw spectrum; therefore, to demonstrate proof of concept, the 1:1 mixture was chosen in the Raman spectroscopy studies. Since the Raman studies occurred after the MWM studies, the B12r used in the MWM used the calculated mass ratio of 0.08:1 AA to B12. For the Raman studies, a 1:1 mass ratio was used; as an example, if “10 mg B12r” is stated, it means 10 mg B12 mixed with 10 mg AA. Fine tuning the formula and optimal B12 reduction methodology may be explored in later research.

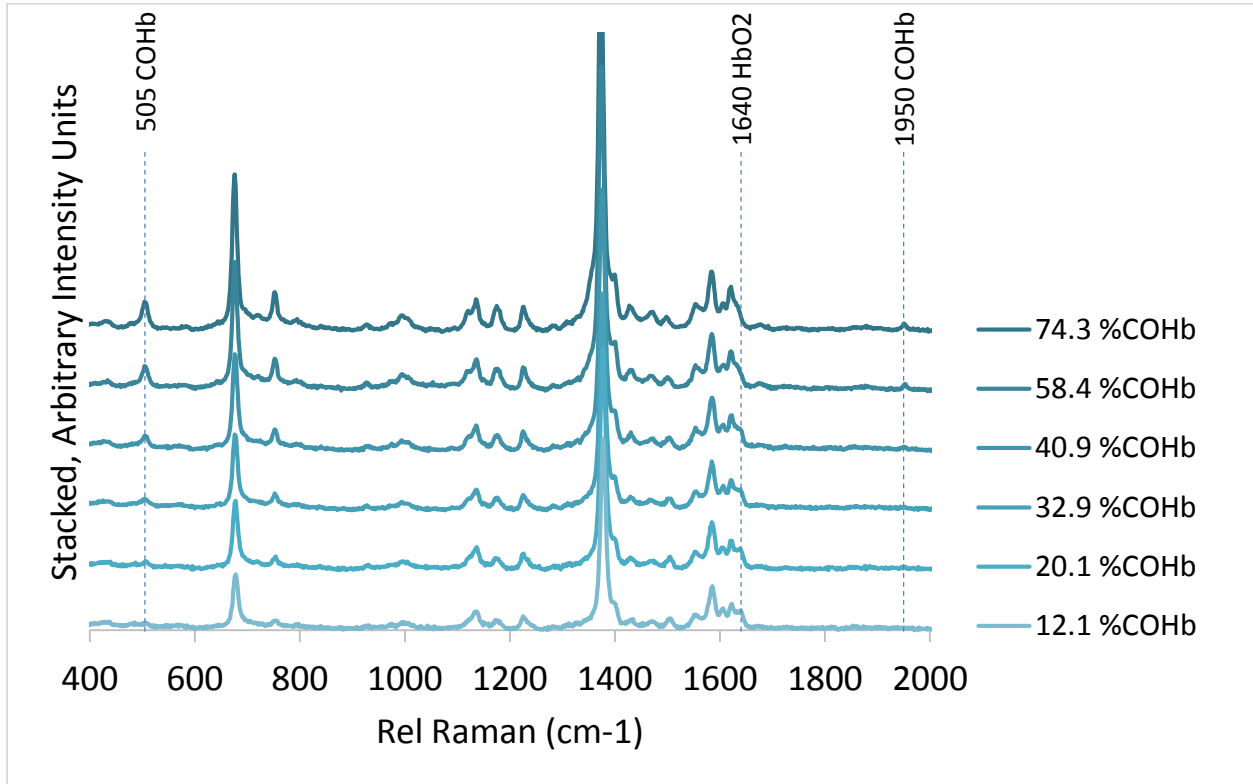


**Figure 8 - Raman spectrum of B12:B12r mixtures with Rel Raman ( $\text{cm}^{-1}$ ) on the horizontal and arbitrary intensity on the vertical axis. As increasing amounts of AA are added to B12 (keeping B12 concentration constant), the B12 Raman Signal (top) decreases in intensity and B12r Raman Signal (bottom) is more pronounced. Prominent peaks that identify each chemical have been labeled.<sup>12,13</sup>**

### *Raman Spectroscopy of Hemoglobin Changes*

Figure 9 to Figure 11 below shows Raman spectra of poisoned blood being exposed to 100%  $\text{O}_2$  in the Maquet oxygenator. Figure 9 is the spectra from frequencies between 400 and 2000, and Figure 10 is the same but zoomed in between 1350 and 1400. Figure 9 shows a visual decrease in peak heights for COHb (505 and 1950) as blood decreases from 74.3% COHb to 12.1% COHb over 60 minutes while exposed to a flow of 100%  $\text{O}_2$ . In addition, the figure shows an increase in the  $\text{HbO}_2$  related peak at 1640 as %COHb decreases. Figure 10 is a zoomed in view between 1350 and 1400 frequencies and shows the drift of the  $\nu_4$  band from its known COHb position to its known  $\text{HbO}_2$  position. Figure 11 shows the relationship between the Raman peak height changes at 505 and OSM3 measured %COHb. Both the 505-peak height

changes and the  $\nu_4$  band movement from 1373 to 1377 can be use to measure %COHb via Raman. At 12.1% COHb, the  $\nu_4$  band is solidly in the HbO<sub>2</sub> position at 1377.



**Figure 9 – Oxygen only treatment of poisoned blood. Transitioning Raman spectra of Hb from 74% COHb to 12% COHb (top to bottom). Arbitrary Intensity on the Vertical Axis.**

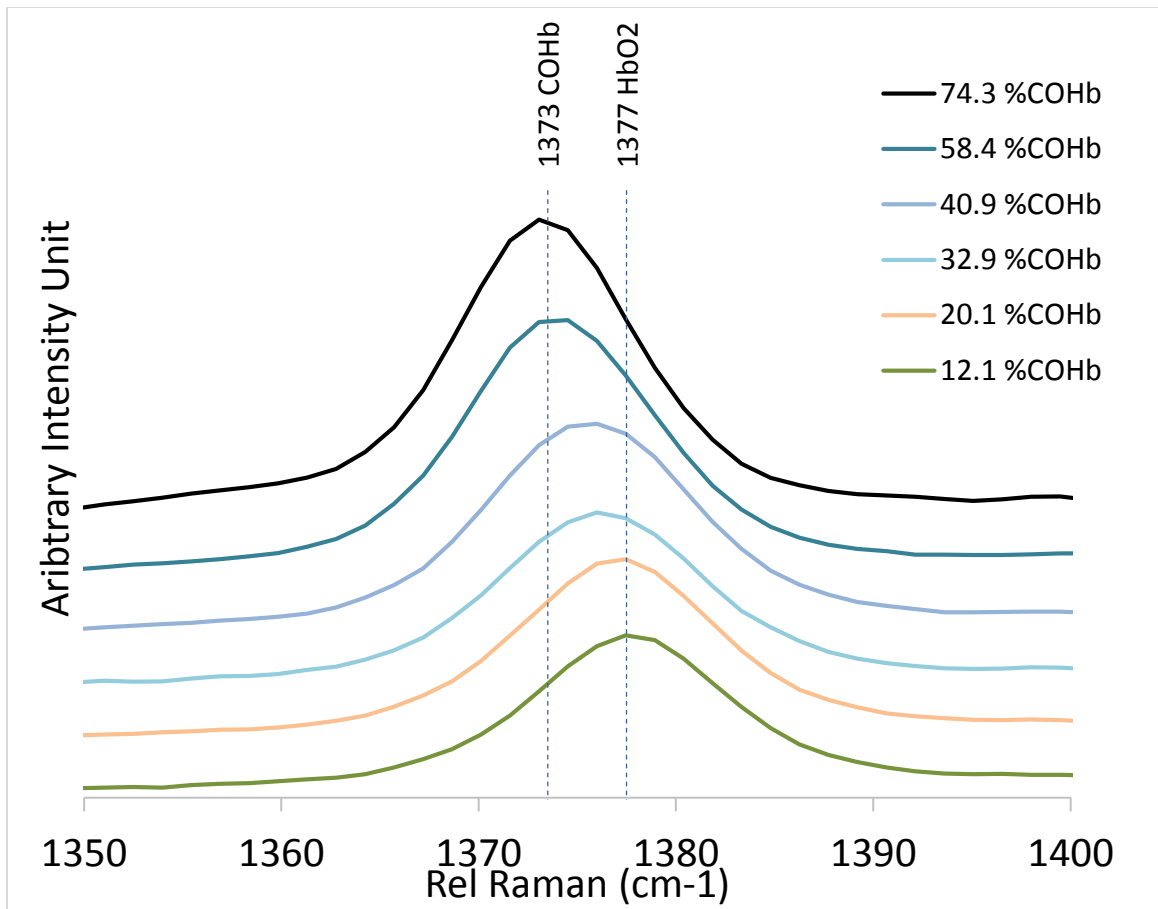
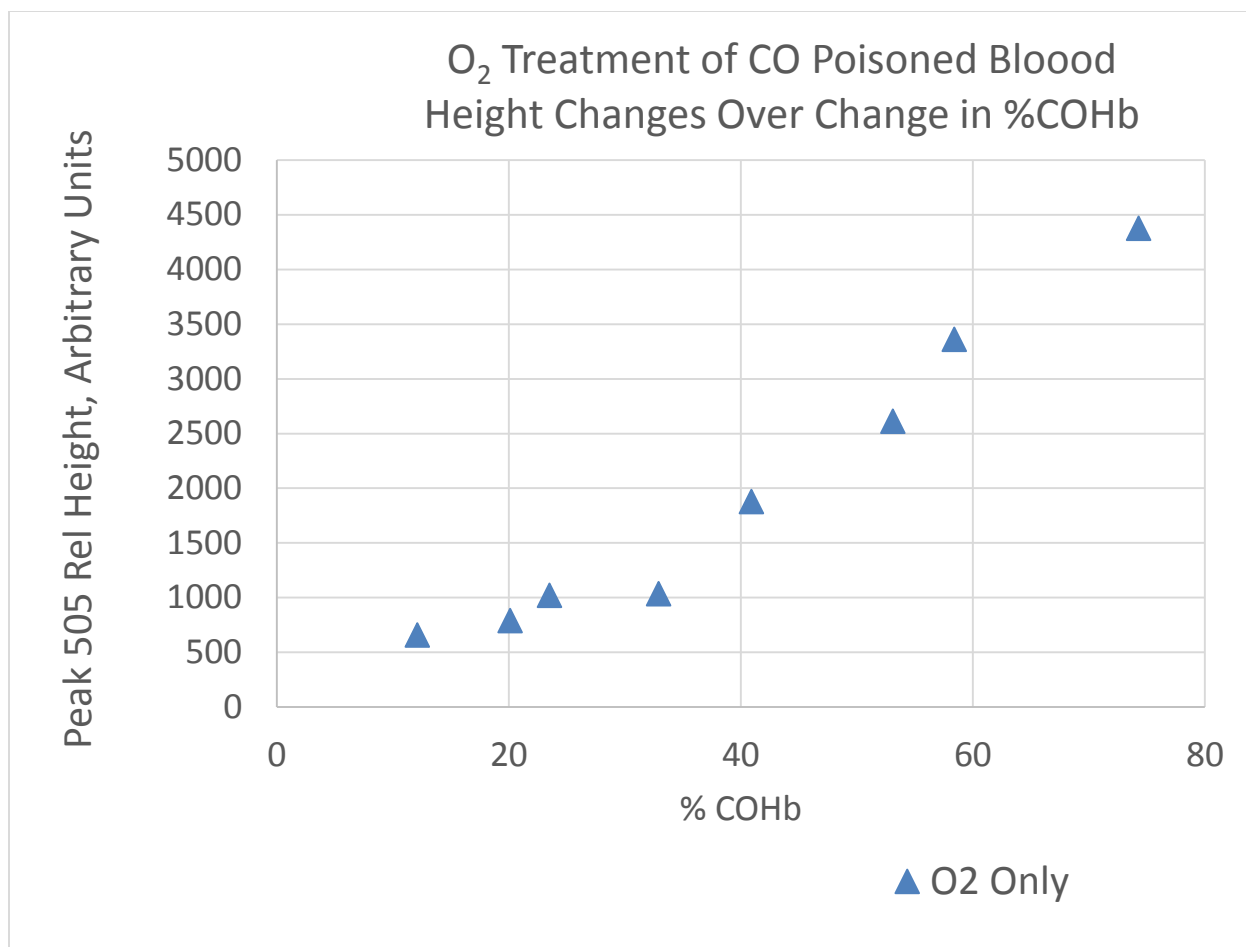


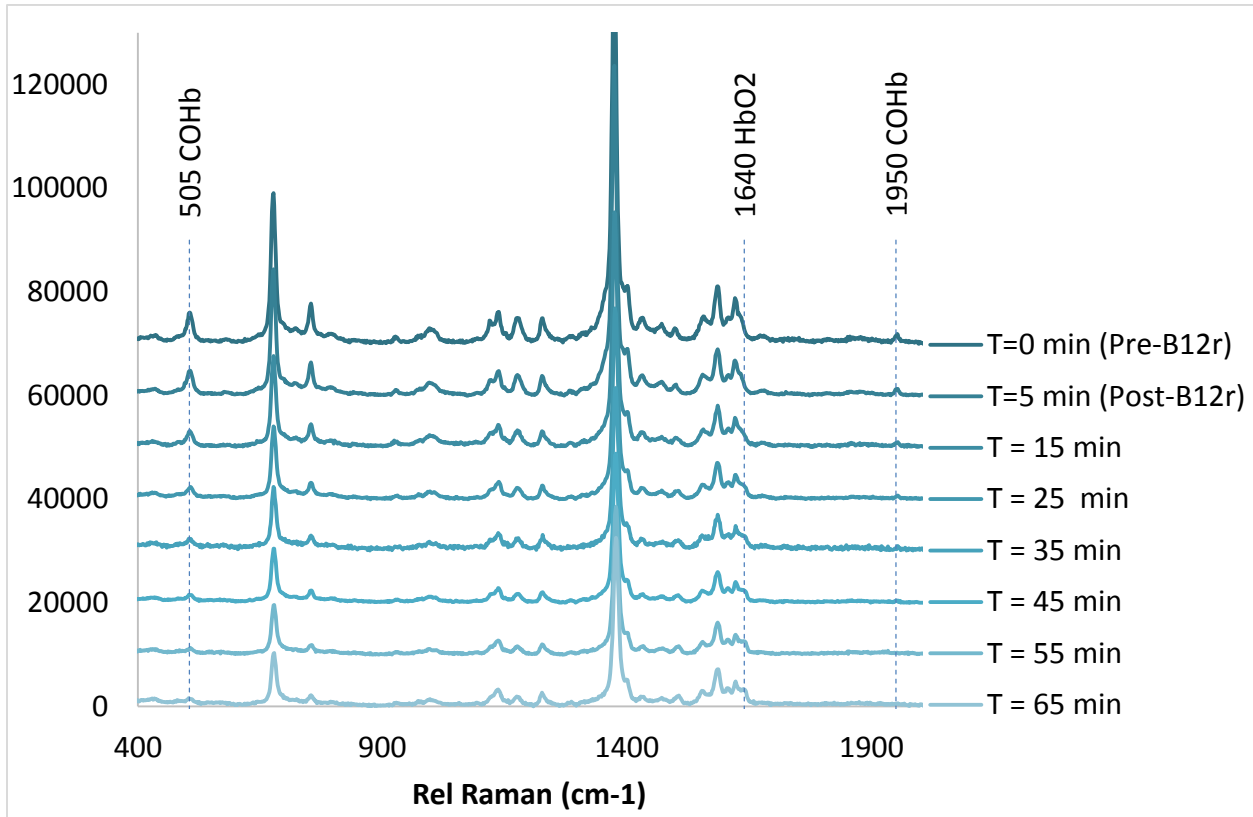
Figure 10 - Oxygen only treatment of poisoned blood. Transitioning Raman spectra of Hb from 74% COHb to 12% COHb (top to bottom) zoomed in on the  $\nu_4$  bands (1350 – 1400 frequencies). Arbitrary Intensity on the Vertical Axis.



**Figure 11 - Plot of Raman Peak 505 Height vs %COHb. Poisoned Blood (74% COHb) treated with a flow of 100% O<sub>2</sub>**

Figure 12, Figure 13 and Figure 14 below show a repeat of the above experiment but adding 250 mg of B12r (2 mg of B12r per ml of blood). The same blood was used and re-exposed to CO until 89% COHb was reached (marked as T = 0). Despite the interference from B12r, the same changes can be observed in the Raman spectra. Except for the pre-B12r sample, there is no measurement of %COHb; the OSM3 displayed error warnings with all values due to the B12r presence. The  $\nu_4$  band is solidly at the 1377 HbO<sub>2</sub> position at T = 55 min, and this may be associated with a COHb percentage of about 12% based on the preceding results. Figure 14 shows a comparison between O<sub>2</sub> and O<sub>2</sub>+B12r treatments in this oxygenation experiment. Coupled with Figure 13, Peak-505 height measurements versus time show an advantage of the

O<sub>2</sub>+B12r treatment to reduce the COHb signal to baseline; the  $\nu_4$  band position (Figure 13) confirms a strong HbO<sub>2</sub> signal has been reached. This occurs at about the 55 min point for the O<sub>2</sub> + B12r experiment while with the O<sub>2</sub> only trial, it occurs at 112 minutes.



**Figure 12 - B12r+O<sub>2</sub> on Blood. Transitioning Raman spectra of Hb from 89% COHb to an equivalent 12% COHb (top to bottom).**



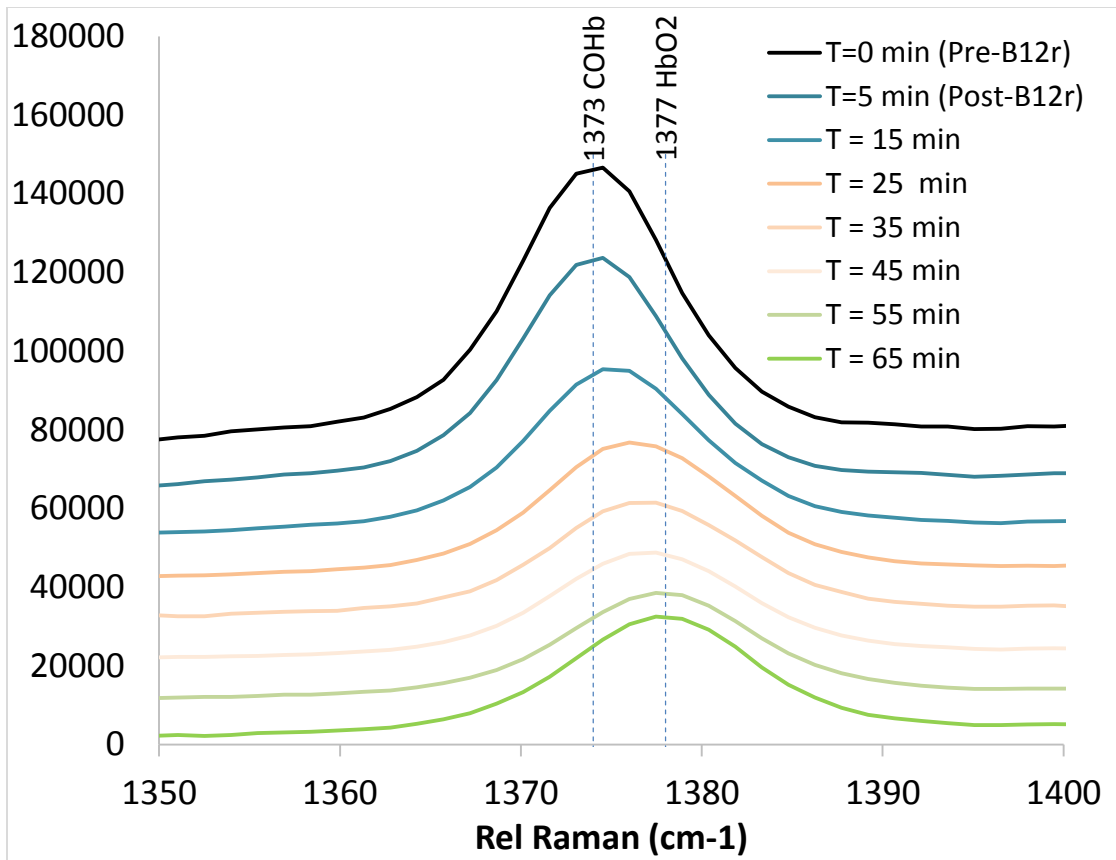
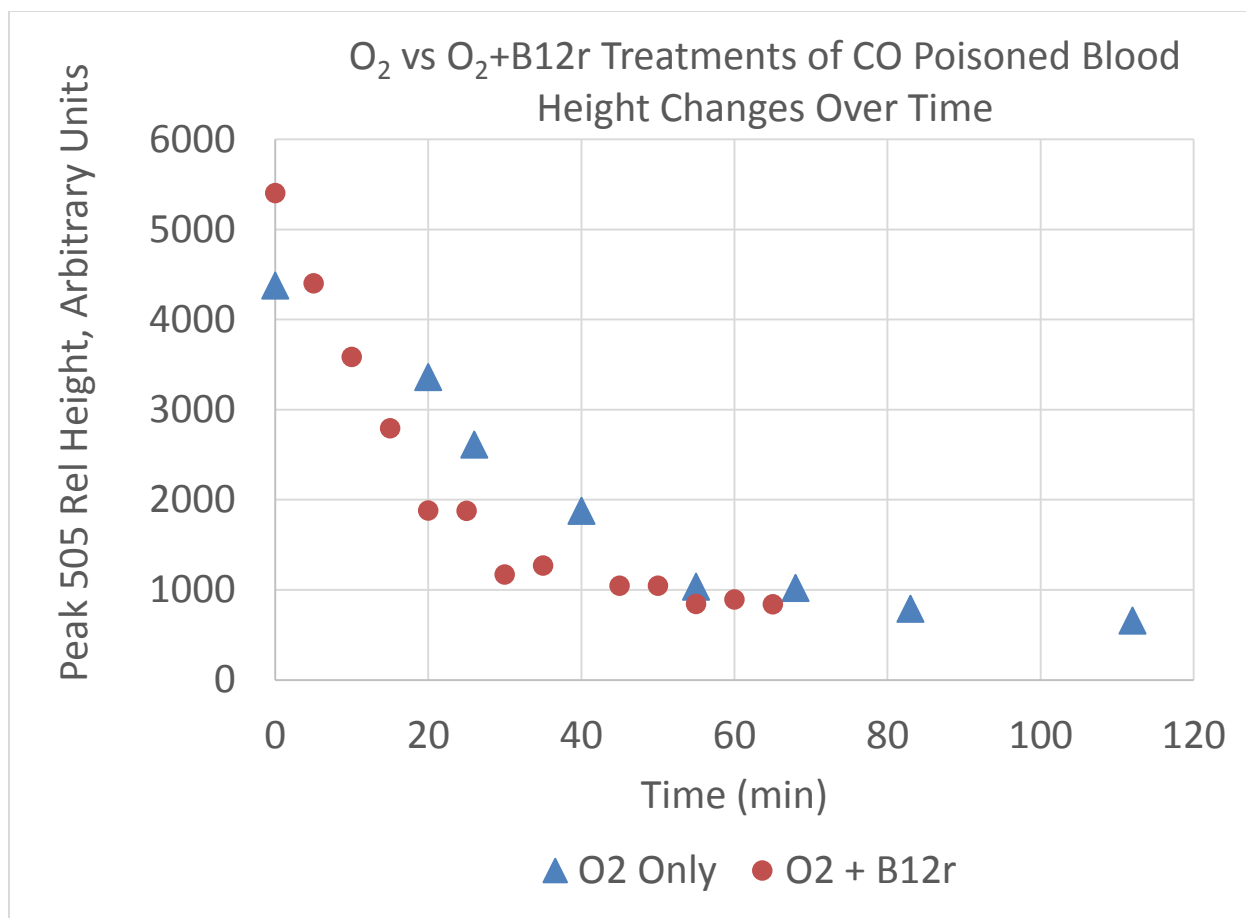
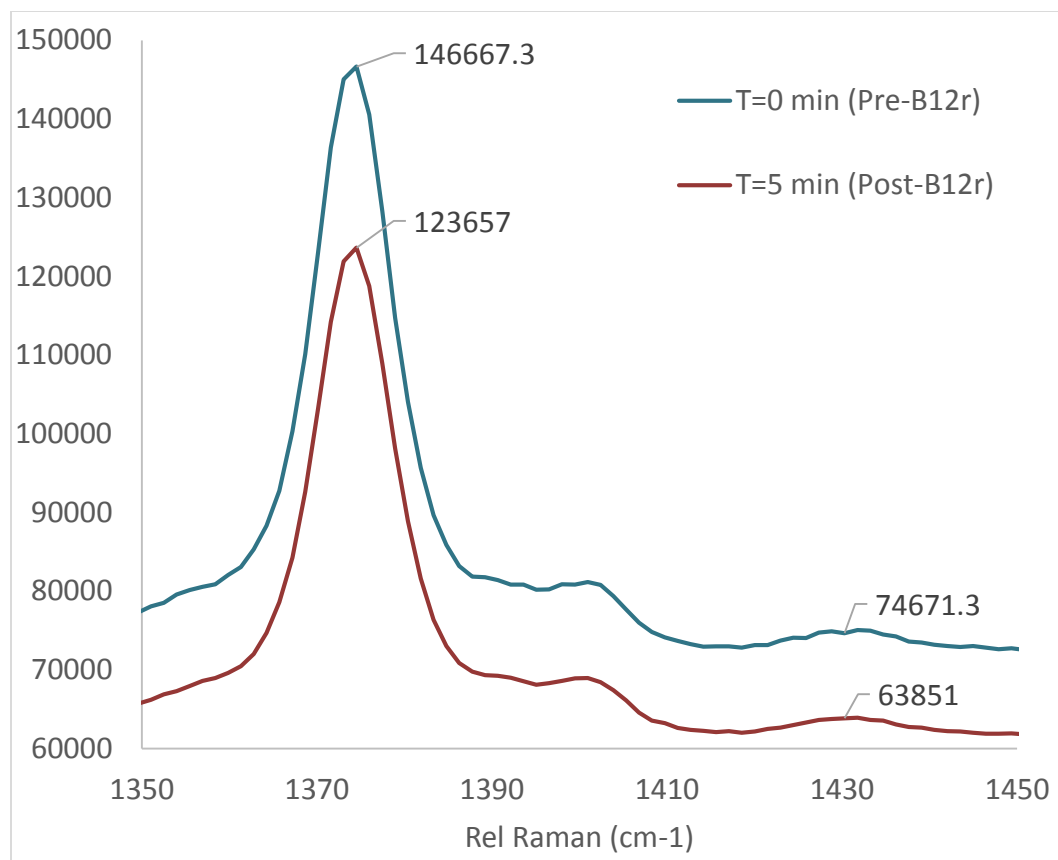


Figure 13 - B12r+O<sub>2</sub> on Blood. Transitioning Raman spectra of Hb from 89% COHb to an equivalent 12% COHb (top to bottom) focusing on the  $\nu_4$  band.



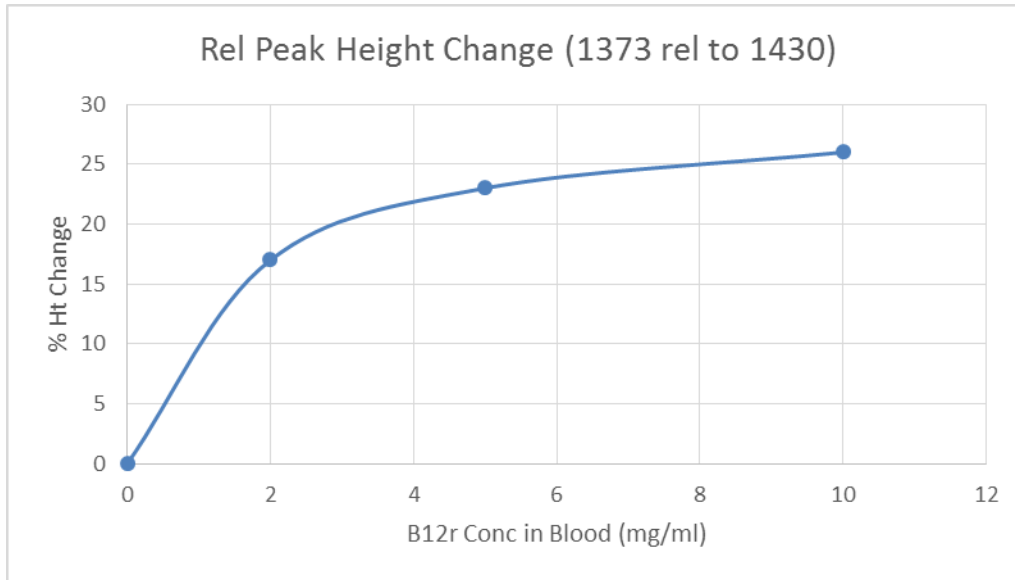
**Figure 14 - Peak-505 Height Changes over Time for both O<sub>2</sub> and O<sub>2</sub>+B12r Treatments. O<sub>2</sub> treatment started at 74% COHb and the O<sub>2</sub>+B12r treatment started at 89%COHb. Raman sampling stopped when Peak 505 became indistinguishable from background (112min for O<sub>2</sub>, 65min for O<sub>2</sub>+B12r).**

The below three figures show changes in the Raman intensity of the 1373 frequency peak due to the B12r's presence. Here we try to find an exploitable relationship between changes in the Raman spectrum and the concentration of B12r used. The non-gas exchange apparatus was used to minimize/slow down changes in COHb concentration. Relative height of the 1373 peak is used: height of 1373 (affected by B12r) is subtracted from the height of peak 1430 (not affected by B12r). Figure 16 shows a pre and post B12r spectrum of the  $\nu_4$  band. Peak height of the band will be taken relative to the 1430 peak (subtracting the height of 1430 from  $\nu_4$ ).



**Figure 15 - Effect of B12r on Raman Signals of CO-Exposed Blood.**

Figure 16 and Table 1 include data from another pre/post blood sample measurements at high COHb concentrations but with varying concentrations of B12r used. The plot indicates a relationship that can be used to instrument Raman for B12r measurements alongside Hb form measurements upon treatment.

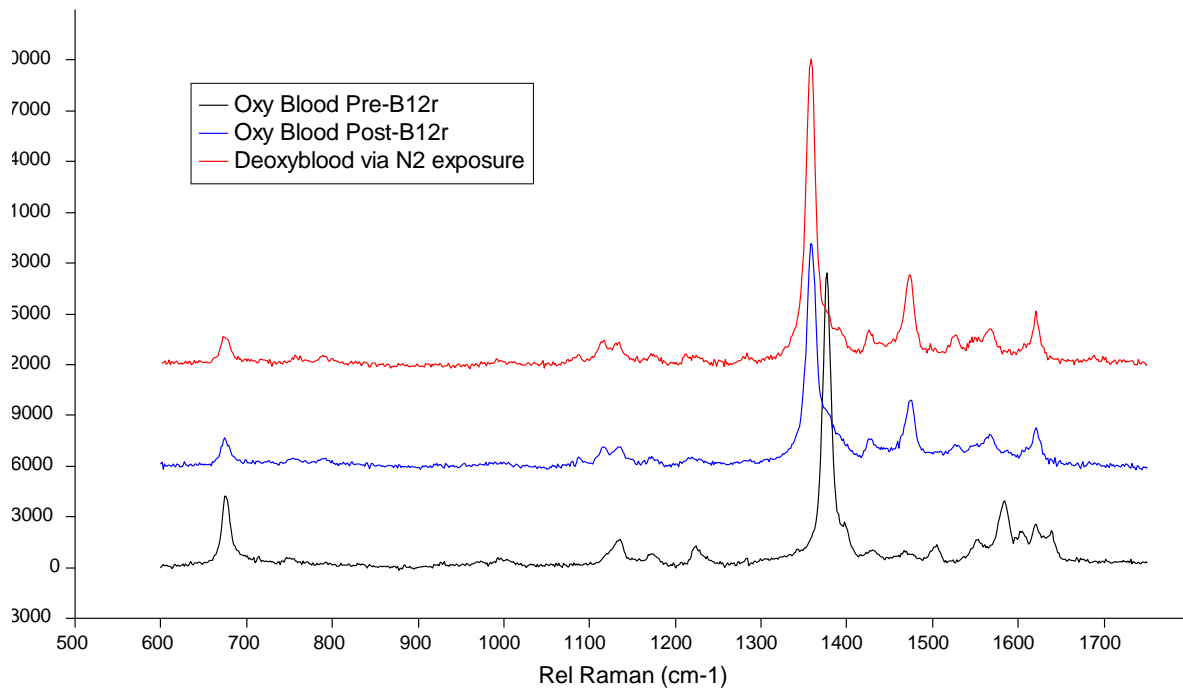


**Figure 16 - Relationship between Raman Peak Height at 1373 (normalized to peak height at 1430) vs B12r Concentration in Blood.**

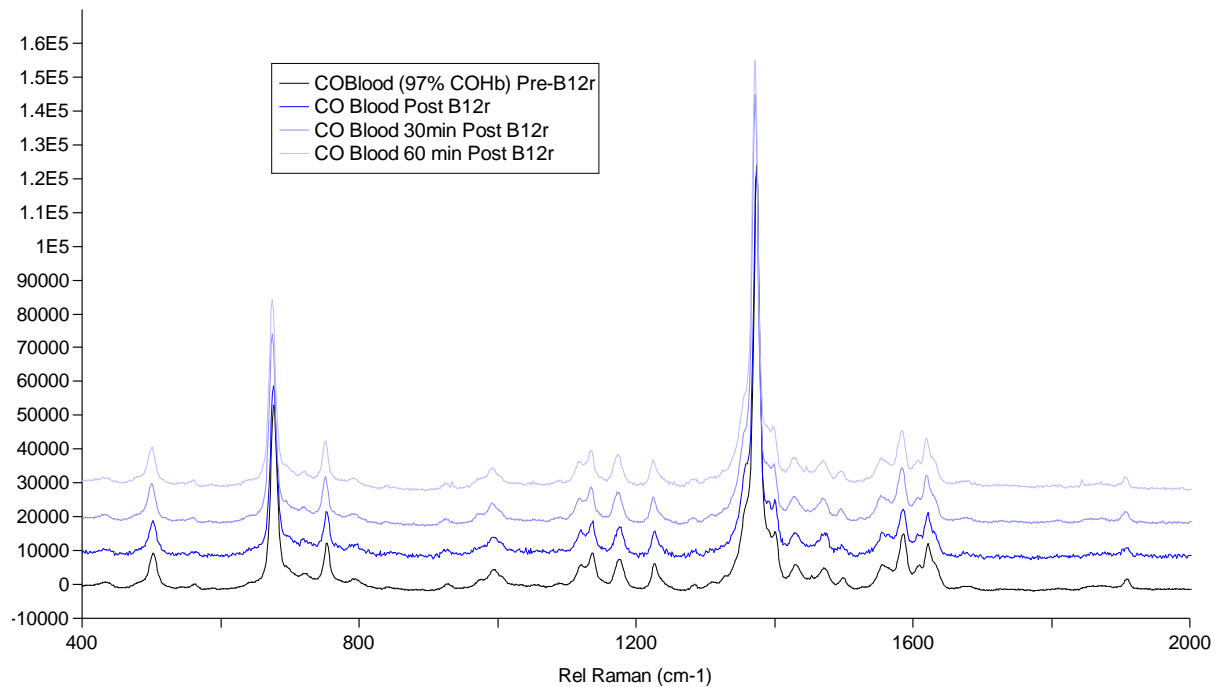
**Table 1- Table of Data Used in Figure 16**

%COHb	B12r (mg per ml of Blood)	Rel Pk Ht Change (%)
87.5	0	0
89.9	2	17
87.6	5	23
87.4	10	26

Figure 17 and Figure 18 show mixing of B12r with either Oxygenated Blood or CO-Exposed blood in the non-gas exchange circulator. Figure 17 results show Deoxy formation immediately after B12r introduction. However, after 10 minutes of circulating in the non-gas exchange system, a slow return to an Oxy spectrum started, and cumulated in a near return to the original spectrum after 25 minutes. A spectrum of Deoxy-blood is placed in this figure for reference (obtained several days later using N<sub>2</sub> exposed blood). The Post-B12r Oxy-blood spectrum is practically identical to Deoxy. Figure 18 shows no change in the CO Blood spectrum post B12r mixing; however, the blood was poisoned to a high level of CO (89.9% COHb). The 250 mg of B12r used was insufficient for such a high amount of CO molecules.



**Figure 17 - Anaerobic Mixing of B12r with Oxygenated Blood. From Bottom to Top, Oxygenated Blood (99% O<sub>2</sub>Sat), 1 minute after B12r mixing, and (Top) a reference spectrum of Deoxygenated Blood.**



**Figure 18 - Anaerobic mixing of B12r with CO-Exposed Blood (97% COHb). From the bottom: CO Blood Pre-B12r, CO Blood 2 min Post-B12r, 30min Post B12r, and (Top) 60min Post B12r.**

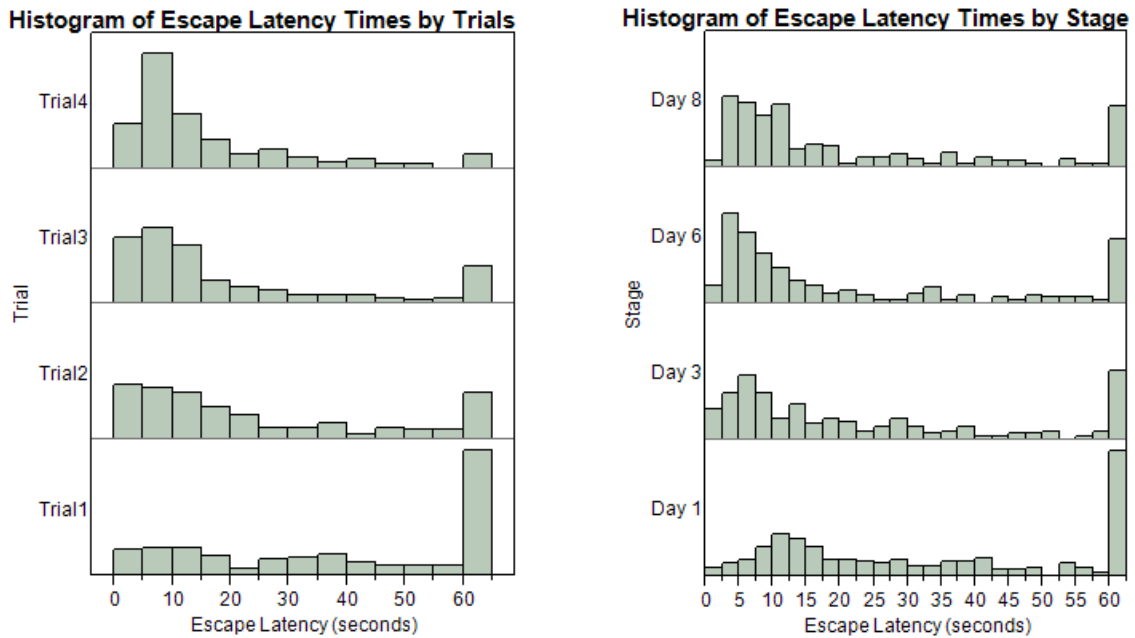
### *Animal Manipulations*

Rat behavior was unaltered during the first 30 minutes of exposure at 2500 ppm. Between 30 and 45 minutes rats exhibited lethargy, but still reacted to stimuli: sharply tapping on the exposure chamber wall would cause their ears to twitch and their heads oriented towards the noise. Between 45 and 60 minutes there was decreased reaction to stimuli: head orientation decreased or was slow, but ear twitching response was still immediate. After the 60-minute mark, the transition from 2500 ppm to 6000 ppm CO, animals became agitated and large movements were observed for 1 – 2 minutes: 180 degree body reorientations, deeper and/or increased breathing. Gradually, response to stimuli decreased, and the large movements desisted. Sharp tapping or jostling of their chamber was used to test for syncope. Most rats needed to be evacuated from their chamber before the 10 minute exposure period ended. A few rats ceased breathing and died. One rat died during post-exposure recovery. This injury protocol resulted in a 14.7% mortality.

### *Morris Water Maze*

Figure 19 below shows the histogram plots of the MWM Escape Latency times by trial and by stage showing learning and memory usage of the Unexposed, Exposed + Saline, and Exposed + B12r groups as a single population. Stage Day 1, Trial 1 (one day post exposure) is the first exposure to the pool and therefore rats finding the escape platform on this trial is by chance, or if they reach the 60-minute time limit, they are lead to the platform. This is reflected by the flatness in the histogram for Trial 1. During this first trial, rats tended to swim mostly at the pool perimeter, trying to find purchase on the pool walls. Subsequent trials show increasing improvement. On Stage Day 1, Escape Latency decreases progressively from Trial 2 to Trial 4 showing that rats are acquiring the set of behaviors to escape more quickly – understanding that

there is an escape platform, and remembering its location. Stage Day 3 & Day 6 show a stark difference from Stage Day 1. Stage Day 4 show a slight regression in Escape Latency as the platform location is no longer in a quadrant, but was moved to the center of the pool.



**Figure 19 – Histograms of Unexposed, Exposed + Saline and Exposed + B12r rats as a single population with Escape Latency times represented in histogram. Left histograms of trials are for all stages and show increasing proficiency from Trial 1 to Trial 4. Right histogram of stages are for all trials and show MWM rule learning improvement from Stage Day to Stage Day 8.**

Analysis of the *Escape Latency* factor showed no significant escape probability differences between Unexposed, Exposed + Saline, and Exposed + B12r groups. The probability of an Unexposed rat escaping the MWM versus an Exposed + Saline rat was 1.04 to 1 with a 95% Confidence Interval (C.I.) of [0.85, 1.27]. The probability of an Exposed+B12r rat escaping the MWM versus an Exposed + Saline rat was 1.05 to 1 (95% C.I. of [0.89, 1.25]). The probability

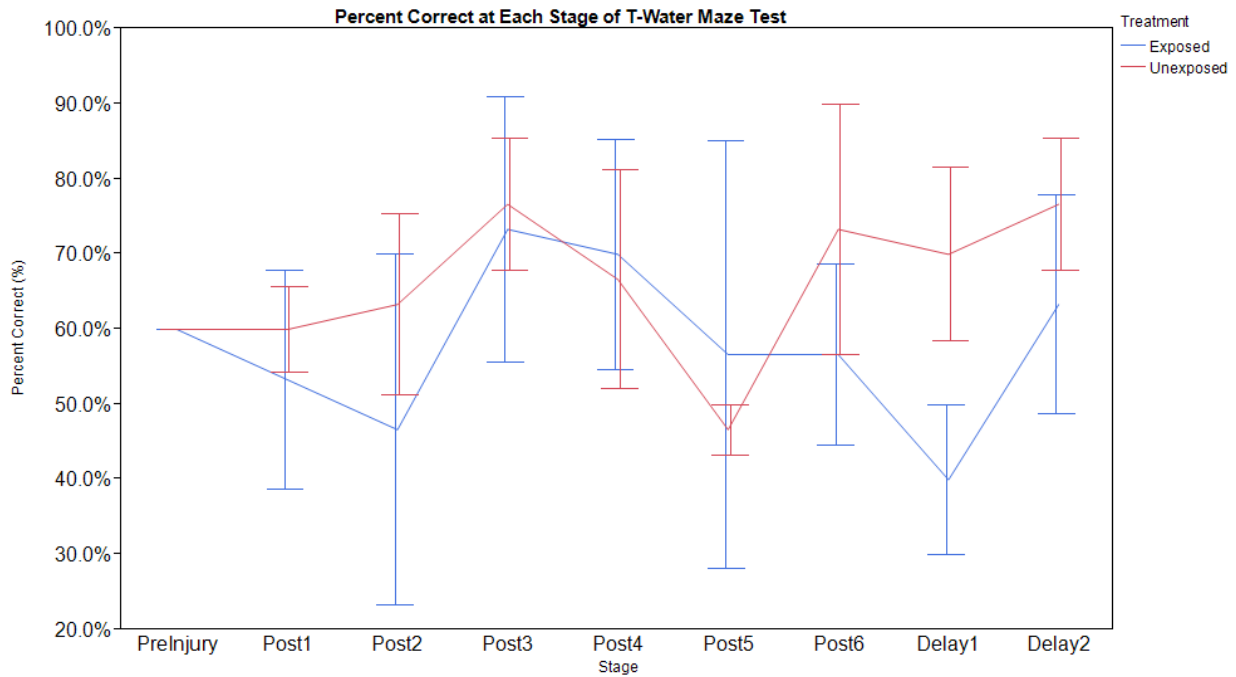
of an Exposed+B12r rat escaping the MWM over an Unexposed rat was 1.05 to 1 (95% C.I. of [0.84, 1.23]).

The analysis of the *mean speed* (p-value = 0.1162, DF=5, F-Ratio=1.7692) factor showed no significant differences between Unexposed, Exposed+Saline and Exposed+B12r.

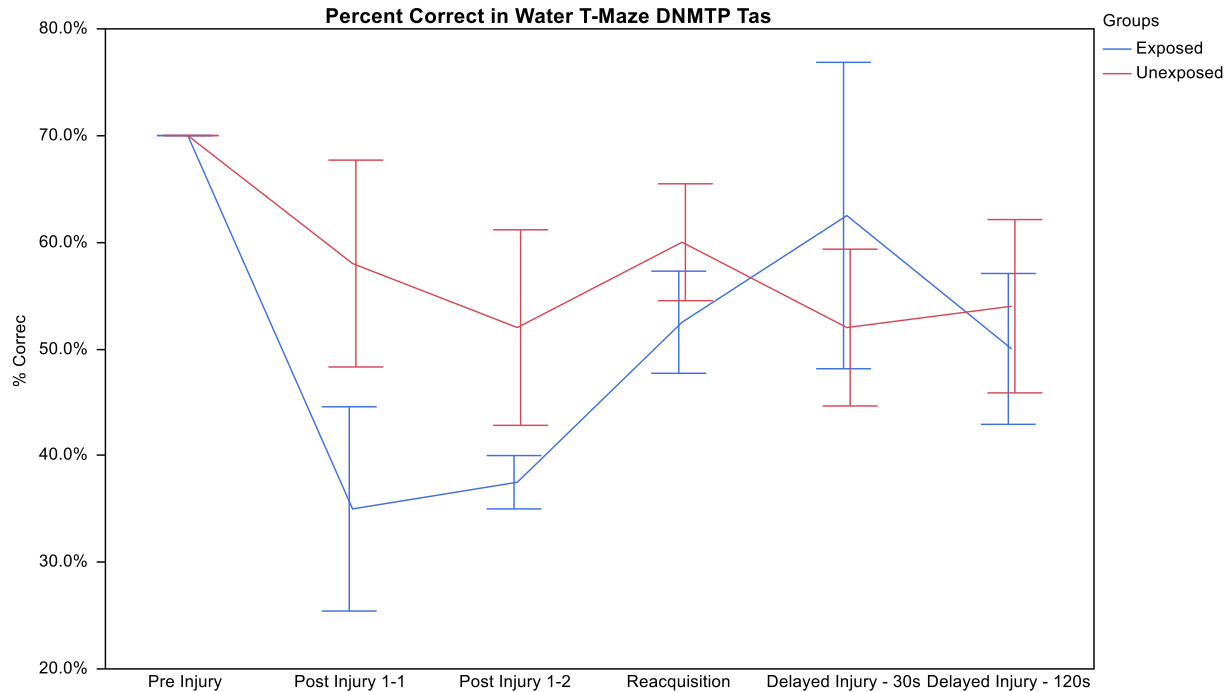
#### *Water T-Maze Pilots*

The Water T-Maze Pilots showed no statistically significant differences between Exposed and Unexposed rats. Figure 20 and Figure 21 show a separation of means at certain post-injury events. However, the separations were not significant. The data showed high variability between subjects. Figure 20 shows a potential to differentiate between Unexposed and Exposed CO-Injured rats for next day neurological sequelae but not for delayed neurological sequelae. Additionally, the difference needed to insert a treatment group, however, may not be enough. Statistical comparison by Repeated Measures ANOVA Student's t results in a non-significant difference between Expose and Unexposed of: Difference = 0.064%, p-value = 0.1588, 95% C.I. of [-0.026, 0.154]. Figure 21 also shows a separation in the first two days post injury, and also 20 days later, but neither were statistically significant.





**Figure 20 - Percent Correct Mean Plot +/- S.E. of the First Pilot for the Water T-Maze. N=3 for each group. Normalized at Pre-Injury by subtracting scores to result in 60% correct for all rats. Post1 – Post6 are post injury tests, and Delay 1 & 2 are tests delayed to Days 20 and 21.**



**Figure 21 - Percent Correct Mean Plot +/- S.E. of the Second Pilot for the Water T-Maze. Normalized at Pre-Injury by subtracting scores to result in 70% correct for all rats. Reacquisition occurred at 20 days post-injury.**

## Discussion

### *Raman Analysis of COHb Changes with Antidote*

Our *in vitro* experiments showed COHb levels reduced by 77 percentage points in 55 minutes with B12r + O<sub>2</sub>. This shows a large improvement over the O<sub>2</sub>-only trial that reduced COHb levels by 62 percentage points in 112 minutes. The two trials used the same blood donor. During the O<sub>2</sub>-only trial, blood samples were taken, but only an initial blood sample was taken in the B12r + O<sub>2</sub> trial. Collection of the Raman spectrum was therefore easier which is why there are more data points in the B12r + O<sub>2</sub> trial. We don't believe this difference in collection frequency affects the results. This experiment gives evidence that answer our objectives in using Raman to detect Hb changes with B12r, and that B12r may indeed provide a faster clearance of CO from poisoned blood. More paired trials are needed between O<sub>2</sub>-only and B12r + O<sub>2</sub> treatments. The Maquet™ system proved to be an extremely efficient gas-exchange device. Administration of 100% O<sub>2</sub> via the Maquet oxygenator may be too strong of an influence in this *in vitro* set up and may wash out the effects of B12r. In humans administration of 100% O<sub>2</sub> has clearance expectations of up to 6 hours for non-fatal CO exposures (20-30% COHb).<sup>5</sup> This is a wide range due to human variability, but at the slow end, a 20% point reduction in six hours seems to be a plausible expectation *in vivo*. Repeating this experiment with air instead of 100% O<sub>2</sub> may better demonstrate the efficacy of B12r.

The non-gas exchange trials of COHb mixed with B12r allowed us to observe relationships between the B12r interference on the Raman spectra of Hb. As observed in Figure 15, *v*<sub>4</sub> band's relative intensity drops with addition of B12r in the blood. This may be attributed to excitation energy absorption by the B12r as well as the absorption of the emitted energies of the Raman shifted light. The absorption would be in line with the Beer-Lambert law, that absorption of

specific energies of light has a predictable absorption profile proportional to concentration. An exploitable relationship exists with *dual* absorption, and more trials are needed to narrow down the model. The 407.6nm excitation energy is being absorbed and thus does not excite the molecular bonds as much. The second absorption event is on the light emitted from the vibrational bonds, and each peak has a different absorption profile since it is a different wavelength than the excitation source. For example, the 505 peak is due to emission of a 415nm light, and the 1950 peak is a 441nm wavelength emission. In this thesis, we have not completely satisfied our objective to quantify B12r concentration in blood; though a relationship is evident. Absorption profiles for B12r and the excitation laser could potentially be used in a mathematical model to measure B12r concentration.

The non-gas exchange experiment also produced some results that were unanticipated. Figure 17 shows the complete switch from an oxygenated blood spectrum to a deoxygenated blood spectrum by introduction of B12r into oxygenated blood. Some preliminary investigation has already been done that is not included in this thesis. B12r appears to be making sufficient changes to pH causing a lower affinity of Hb to O<sub>2</sub>. There is also a compound effect from CO and CO<sub>2</sub>. The presence of CO causes a higher affinity of Hb to O<sub>2</sub>, and CO<sub>2</sub> causes a lower affinity of Hb to O<sub>2</sub>. Therefore, when the B12r causes CO to catalyze to CO<sub>2</sub>, and reversal in O<sub>2</sub> affinity may be the cause of this drastic conversion to DexoyHb. Additionally, O<sub>2</sub> might be affected by B12r directly as we are using B12r to mimic hemoglobin's affinity for CO. It is plausible that B12r may have an affinity for O<sub>2</sub>. Future biochemical studies will be beneficial to measure affinities of B12r to both CO and O<sub>2</sub>.

### *Behavioral Trials*

Although modeled after previous work in which differences were found between CO-injured rats and uninjured rats in a MWM test, our modifications produced no significant differences that can be interpreted from the odds of escape between Unexposed, Exposed+Saline and Exposed+B12r groups.<sup>21</sup> The Time-to-Event statistical method chosen in this study is different from the repeated measures analysis of variance typically chosen; however, performing repeated measures ANOVA ( $F_{2, 830} = 0.6546$ ,  $p\text{-value} = 0.5199$ ) also show no statistical significance. The exposure protocol used by Sun et al exposed Sprague Dawley rats to 1000 ppm of CO for 40 min followed by up to 20 min of 3000 ppm CO or until the animal fainted.<sup>21</sup> There are various other injury protocols as well. One showed that 2500 ppm CO exposure for 60 min may not produce syncope, but resulted in some syncope and deaths after 75 min in one study; and in another study, 3000 ppm for a little greater than 60 min produced some deaths.<sup>17,18</sup> The decision to increase the concentrations used to 2500 ppm for 60 min, followed by 6000 ppm for up to 10 min or until syncope stemmed from the need to ensure exposure while keeping mortality rate low. The CO-injury appeared sufficient since we observed syncope in most subjects as well as observed a 14.7% mortality. The Long-Evans strain used is not novel in carbon monoxide studies with neurocognitive tests, and some have even used Wistar and Fisher Inbred strains.<sup>3,17,20</sup> But the only studies that have used the MWM with carbon monoxide poisoning used the Sprague-Dawley strain.<sup>21,22,24</sup> Selection of the Long-Evans strain may be a minor factor for the differences in outcome.

The MWM can be conducted with differing protocols, and here is where the main difference lies between this study and some of the literature guides for MWM on CO Injury. The place-navigation protocol used in literature was pilot tested prior to our study, and we found no

significant difference between exposed and unexposed rats. The place-navigation protocol involved finding the hidden escape platform from varying starting compass points (north, south, east or west) in the pool; and the platform location was fixed—never relocated between trials or between stages.<sup>21,25</sup> This task of remembering a fixed though hidden location seemed too simplistic. We modified the protocol to stress working memory using a delayed matching to place protocol as described in the methods section, and we were encourage with early trends, but in the end, no differences could be found.

After the results of the MWM, we sought another cognitive behavioral test for CO Injury. Experts at VCU indicated that finding an appropriate test can be a trial-and-error experience. The WTM to our knowledge has not been used with a CO injury model, but was successfully used in traumatic brain injury experiments with rats. It was also an attractive option due to the availability of a T-Maze that can be borrowed to place into the MWM pool, and therefore, a quick set-up. Our pilot results were more promising than the MWM results to differentiate between Exposed and Unexposed SD Rats. There were trends of separation between the groups despite no significance. However, the variability of scores from the rats was high, and the numbers, time, and resources for a sufficiently powered study would have been too great.

We harvested and stored brain samples of the rats from both studies. Per literature, the cortex, hippocampus and basal ganglia regions of rats injured with CO exposure show increased neural cell damage and/or death as well as increased microglial activation.<sup>18,19,21,26</sup> Our future histology studies will verify brain injury by our CO injury model. We may also be able to identify rats that were exposed to CO but due to susceptibility, may not have been injured sufficiently and re-analyze the MWM and WTM data. Preliminary H&E stains show new vascular growth in the brains of our CO exposed subjects versus normal vasculature in unexposed rats which indicates

that injury was occurring. The injury protocol was the same for the Long Evans rats used in the MWM and the Sprague Dawley rats used in the WTM. The future pathohistology results we obtain may shed light on strain differences of CO injury.

For this second part of the study, we could not meet the objectives due to the inability to differentiate between control subjects and injured subjects. For future studies, a radial arm maze might be another option to explore since this has been used in CO injury experiments using rats.

## Summary

Our aim to develop a novel treatment for victims of CO poisoning is highly applicable to public health concerns. In addition to the 15,000 non-fire CO exposures reported annually, there were over 500,000 structure fires in 2000.<sup>27</sup> A field administered, quick-acting treatment would be of tremendous value. Our previous work in the lab gave us an indication that this can be done with human blood by measuring CO<sub>2</sub> generation in an *in vitro* bench top study of B12r treated blood.<sup>8</sup> Our present work has given us further insight into B12r's action with human blood and has raised further questions to answer. B12r treatment coupled with the current treatment of 100% O<sub>2</sub> can greatly enhance removal of CO. We find that the O<sub>2</sub> treatment is also critical as the B12r affects HbO<sub>2</sub> if not O<sub>2</sub> directly. We show that Raman spectroscopy is a potential method to study Hb forms even with the interference of B12r. This technology to measure B12r concentration alongside concentrations of Hb forms in human blood with a B12 presence can already be valuable for victims of cyanide poisoning since Cyanokit™, a B12 intravenous treatment, muddles clinical diagnostics machines using absorbance spectroscopy. Future work here is the natural progression to *in vivo* experimentation with a micro-circulation Raman spectroscopy system with small animal models for CO injury and treatment with B12r, and further studies with robust computations to measure B12r in blood using combine Raman and absorbance spectroscopy. Concurrently, studies to find a reliable model for testing neurological sequelae in CO injury is necessary. The rat species may not be the most appropriate since the cognitive impairment in humans is often at higher levels of reasoning and operation.

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## **Vita**

Leonardo G. Somera, III was born in the Philippines. As the son of a U.S. Navy Sailor, he moved to the United States of America when he was three years old. After graduating from Salem High School, Virginia Beach, VA, he attended Tidewater Community College earning an Associates in Science in Engineering. Afterwards he attended the University of Virginia where he obtained a Bachelor of Science in Engineering Science with a focus in Biomedical Engineering. After graduation, Leonardo worked as a Lab Technician at the Anesthesiology Core Laboratories, Medical College of Virginia (presently Virginia Commonwealth University Health System) doing work in decompression sickness, and hemoglobin saturation studies with Raman spectroscopy. He later commissioned into the United States Air Force as a Bioenvironmental Engineer. In 2012, he became a Graduate student at the Department of Biology, Virginia Commonwealth University, via the Air Force Institute of Technology's Civilian Institute program. He is scheduled to receive his Master of Science in Biology in May of 2014.