STUDIES ON THE REACTION OF HIGH-DOSE HYDROXOCOBALAMIN AND ASCORBIC ACID WITH CARBON MONOXIDE: IMPLICATIONS FOR TREATMENT OF CARBON MONOXIDE POISONING

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STUDIES ON THE REACTION OF HIGH-DOSE HYDROXOCOBALAMIN AND ASCORBIC ACID WITH CARBON MONOXIDE: IMPLICATIONS FOR TREATMENT OF CARBON MONOXIDE POISONING.

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

by

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March, 2013
First and foremost I would like to thank Dr. Bruce Spiess for his constant support and encouragement, and for allowing me use of his time, resources, and funds. Without his generosity none of this would have been possible. Thanks to Dr. Michael J Feldman, Dr. Andrea Pozez, and Dr. Abel Gebre-Giorgis. They were there from the very beginning and have been there for me throughout this entire project. Thanks to Capt. Leonardo Somera for his help in the lab, statistical analysis and help with tables and figures, and for his friendship. Thanks to Dr. Jim Terner for the use of his lab and his tireless effort and support with raman analysis. Thanks also to Mr. Brian Berger for sharing his knowledge and experience and all of his logistical support and problem solving. Thanks to the entire VCURES team, especially Dr. Penny Reynolds for her assistance with statistics and manuscript editing. Finally, thanks to my wife and kids who have dealt with the long days and sleepless nights and are both my support and my inspiration for all that I do.
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LIST OF ABBREVIATIONS

**AUC.** Area Under the Curve. The integral of the difference between two points along the x-axis of a graph and the y-value between those same points where \( y = f(x) \). In this case it used as a measure of the total magnitude of CO\(_2\) produced by the system in a given time period, expressed as ppm-min.

**B\(_{12}\).** An essential and water-soluble vitamin. It exists in at least two main forms in the body. Distinguished based upon the upper-axial ligand (typically either –CN or –OH). The coenzyme form has either a methyl group or an adenosine group as the upper axial ligand.

**cGMP.** Cyclic Guanosine Monophosphate. A cyclic nucleotide derived from guanosine triphosphate, it is a key component in many second messenger systems throughout the body, typically via activation of intracellular protein kinases.

**Ca\(^{2+}\).** Calcium Ion.

**CO.** Carbon monoxide. A carbon triple-bonded to monatomic oxygen.
CO$_2$. Carbon Dioxide. A carbon with two double-bonded monatomic oxygens.

COHgB. Carboxyhemoglobin. Hemoglobin that is bound to carbon monoxide as well as, or instead of, diatomic oxygen.

COHgb%. Percentage of Carboxyhemoglobin. A value that is determined typically by absorbance spectroscopy and is expressed as the amount of carbon monoxide bound hemoglobin, divided by the total hemoglobin multiplied by 100.

CORM's. Carbon Monoxide Releasing Molecules. A class of metal containing organic compounds that are capable of reversibly binding carbon monoxide and releasing it at a controlled rate under specific conditions. They are being used experimentally to test the effects of carbon monoxide in targeted sites, and potentially as therapeutic compounds.

**ctHb.** Concentration of Total Hemoglobin. An abbreviation used by some monitoring devices to report the total concentration of hemoglobin in a sample. This is typically reported in either g/dL or g%.

**DNA.** Deoxyribonucleic Acid. The molecule that encodes the genetic or cellular instructions used for the development and functioning of nearly all known living organisms.

**DNS.** Delayed Neurologic Sequelae. A descriptive term used in reference to the syndrome of neurologic dysfunction that often occurs in patients exposed to toxic levels of carbon monoxide. Since signs and symptoms often occur after apparent resolution of acute carbon monoxide toxicity it is referred to as “delayed”. This term is roughly equivalent to the term “prolonged neurologic sequelae”.

**ECG.** Electrocardiogram. A device (or the report that it produces) that uses sensitive electrodes, connected at specific points to the human body, in order to monitor the electrical activity of the heart and detect abnormalities. It is often referred to as EKG as well.

**ED.** Emergency Department. The area of a hospital that assesses and treats patients who have an urgent need for medical attention.
**FCOHb.** Fraction of Carboxyhemoglobin. An alternate abbreviation for carboxyhemoglobin concentration used by some monitoring devices. This is a percentage value.

**GTP.** Guanosine Triphosphate. A purine nucleoside triphosphate that is used as a substrate for both RNA and DNA synthesis, and as an energy source in numerous metabolic reactions.

**HbCO.** Carboxyhemoglobin. An alternate abbreviation for carboxyhemoglobin concentration used by some monitoring devices. This is a percentage value.

**HBO.** Hyperbaric Oxygen Therapy. Refers to the process of placing a patient into a sealed chamber and increasing the % of oxygen in the chamber while at the same time increasing the pressure to 2-3 times normal atmospheric pressure. This increases the solubility of oxygen and other gases in bodily fluids.

**Hctc.** Hematocrit Concentration. Also known as packed cell volume or erythrocyte volume fraction, it is a measure of the volume% of red blood cells in blood. It is typically measured by multiplying the red cell count by the mean cell volume.
**HgB.** Hemoglobin. A globular protein found in human red blood cells that is capable of carrying diatomic oxygen. It is composed of four subunits, each of which is bound to a heme moiety (a porphyrin ring with ferrous iron at its core).

**HPLC.** High-Pressure Liquid Chromatography. A chemical separation and analysis technique that allows for separation and identification of an analyte of interest from a complex matrix.

**HO.** Heme Oxygenase. The intracellular enzyme responsible for heme degradation via conversion of heme into protoporphyrin IX, and carbon monoxide. There are three types so far identified (HO-1, HO-2, and HO-3).

**HVAC.** Heating, Ventilation, and Air Conditioning. The technology of indoor air supply, air quality, and temperature control. The system or systems that maintain a constant temperature within indoor environments and ensure adequate air supply to enclosed spaces.

**IL-1β.** Interleukin 1 Beta. An immune system cytokine that is involved in systemic inflammation and multiple other immune system processes including apoptosis and cellular proliferation.
**IRB.** Institutional Review Board. The department of a university or research organization that specifically reviews all projects that fall under the dual-headings of “research” and “human subjects”. All projects involving human subject research must be reviewed and approved by this board prior to commencing.

**LPS.** Lipopolysaccharide. A family of large molecules consisting of a lipid and a polysaccharide that are found on the cell walls of gram negative bacteria. They act as endotoxins, and illicit powerful immune system and inflammatory reactions when administered to animals.

**MAPK.** Mitogen Activated Protein Kinases. Serine/Threonine specific protein kinases that direct cellular responses to a variety of stimuli including mitogens and pro-inflammatory cytokines such as TNF-alpha.

**MMP’s.** Matrix Metalloproteinases. Zinc dependent endopeptidases capable of degrading extracellular matrix proteins.

**MS.** Mass Spectrometry. A chemical analysis tool that creates gas-phase ions from a liquid or gas solution of interest and then identifies the molecular species in the solution based upon their mass/charge ratio.
**NADPH.** Nicotinamide Adenine Dinucleotide Phosphate. NADPH is the reduced form of NADP⁺ and is a common physiologic reducing agent used in a number of reactions.

**NaHCO₃.** Sodium Bicarbonate. It dissociates in water to yield a sodium ion and the conjugate base of carbonic acid. Degradation by the enzyme carbonic anhydrase results in the formation of carbon dioxide.

**NaNO₂.** Sodium Nitrite. A powerful oxidizing agent with minimal interference on absorbance and resonance raman spectroscopy.

**NBO.** Normobaric Oxygen Therapy. Refers to the process of having a patient breathe in an increased % of oxygen at normal atmospheric pressure.

**NS.** Normal Saline. A solution of water and 0.9% sodium chloride. This is isotonic with blood, and used to prevent red cell lysis upon injection into red blood cell containing solutions like whole blood.

**O₂.** Diatomic Oxygen.

**OHCbl.** Hydroxocobalamin. One of at least two forms of vitamin B12, which contains an –OH group in the upper axial ligand binding site.
This term may be used interchangeably to refer generically to all forms of hydroxocobalamin or specifically to the non-reduced form.

**OHCbl\(^{1+}\)** Reduced hydroxocobalamin. In this case the cobalt moiety of hydroxocobalamin is in the 1+ oxidation state.

**OHCbl\(^{2+}\)** Reduced hydroxocobalamin. In this case the cobalt moiety of hydroxocobalamin is in the 2+ oxidation state.

**OHCbl\(^{3+}\)** Non-reduced (i.e., oxidized) hydroxocobalamin. In this case the cobalt moiety of hydroxocobalamin is in the 3+ oxidation state.

**p38 MAPK.** p38 Mitogen Activated Protein Kinases. A subfamily of protein kinases that are involved in the cellular response to stressful stimuli.

**pCO\(_2\).** Partial Pressure of Carbon Dioxide. A measure of the pressure of carbon dioxide in solution, relative to the pressure of other gases in the same solution.

**pH.** A measure of the activity of hydrogen ions in solution on a logarithmic scale.

**PNS.** Prolonged Neurologic Sequelae. A descriptive term used in reference to the syndrome of neurologic dysfunction that often occurs
in patients exposed to toxic levels of carbon monoxide. Since signs and symptoms often continue well after apparent resolution of acute carbon monoxide toxicity it is referred to as “prolonged”. This term is roughly equivalent to the term “delayed neurologic sequelae”.

**PPM.** Parts Per Million. A unitless notation for describing the volume fraction of a gas or liquid within a gas or liquid mixture.

**RBC.** Red Blood Cell. The cellular component of blood that contains hemoglobin and other enzymes.

**RR.** Resonance Raman Spectroscopy. An analytical technique that uses monochromatic laser light directed at a sample to identify compounds and/or monitor chemical reactions and their products.

**SAT.** Oxygen Saturation. An abbreviation used by some monitoring devices to indicate the %saturation of hemoglobin with oxygen.

**t_{1/2}**. Half-life. The time required for a thing to be reduced to half of its original concentration. It is a descriptive term of the decay-rate of an item of interest. In this article it is typically used to refer to the decay-rate of carboxyhemoglobin.
**TNF-α.** Tumor Necrosis Factor Alpha. An immune system cytokine that is involved in systemic inflammation and the acute phase reaction of immune system response.

**tHb.** Concentration of Total Hemoglobin. An abbreviation used by some monitoring devices to report the total concentration of hemoglobin in a sample. This is typically reported in either g/dL or g%. 
Abstract

STUDIES ON THE REACTION OF HIGH-DOSE HYDROXOCOBALAMIN AND ASCORBIC ACID WITH CARBON MONOXIDE: IMPLICATIONS FOR TREATMENT OF CARBON MONOXIDE POISONING.

By Joseph D. Roderique, MA, MS

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

Virginia Commonwealth University, 2013

Major Director: Dr. Bruce D. Spiess
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Based upon experimental evidence from the 1970’s we proposed that a reduced form of hydroxocobalamin should be capable of producing carbon dioxide (CO2) from carbon monoxide (CO) in blood, and that this conversion should be detectable. Using resonance raman spectroscopy we demonstrated that a mixture of hydroxocobalamin and ascorbic acid could create the reduced form of hydroxocobalamin. We used a closed-loop circulation system with a hollow-fiber membrane oxygenator to produce carboxyhemoglobin. Using sensitive gas monitoring equipment to the gas-out port of the
oxygenator we analyzed the CO and CO₂ concentrations coming from the oxygenator. The mixture of hydroxocobalamin and ascorbic acid caused a 5-fold increase in the CO₂ concentration of the gas-out flow, in comparison to baseline and negative controls. These findings offer initial support for the potential use of a mixture of hydroxocobalamin and ascorbic acid as an injectable antidote for carbon monoxide poisoning.
Introduction and Background:

1.1 Hypothesis:

Hydroxocobalamin (OHCbl) has been used in Europe as an antidote for cyanide poisoning and smoke inhalation for over 40 years.\(^1\) Research from the 1970’s indicated that a reduced form of OHCbl was capable of converting carbon monoxide (CO) into carbon dioxide (CO\(_2\)).\(^2\) The authors recognized the potential clinical implications of this finding but stated that they were unable to detect decarbonylation of carboxyhemoglobin (COHgb) when reduced OHCbl (OHCbl\(^{2+}\)) was added. Surprisingly, we have been unable to find any additional publications on the interaction of OHCbl\(^{2+}\) with COHgb and concluded that further investigations were warranted.

The formation of OHCbl\(^{2+}\) in solution may be accomplished with a multitude of different reducing agents, however not all reducing agents are suitable for injection into a living organism or approved for use in humans. Several potential reducing agents were considered and evaluated during pilot studies and, ultimately, L-ascorbic acid was chosen for this study. Ascorbic acid is a powerful reducing agent that is also non-toxic even at relatively high-doses.\(^3\,^4\)

We found that a mixture of OHCbl and ascorbic acid in deoxygenated normal saline (herein referred to as “the antidote”) could produce the reduced form of OHCbl, as demonstrated by characteristic changes on Resonance Raman spectroscopy. Therefore we hypothesized that this mixture should be capable of producing CO\(_2\) from CO in blood. CO is typically removed from the body by the use of either high-flow atmospheric or hyperbaric O\(_2\). The conversion of CO to CO\(_2\) would be a novel approach
for CO detoxification. We determined that if this conversion were large enough to produce a potentially clinically relevant effect then it should be detectable by monitoring CO₂ generation from the blood using a closed-loop artificial circulation system.

1.2 CO Poisoning:

1.2.1 Magnitude of the Problem:

Carbon monoxide (CO) is a colorless, odorless, and tasteless gas that is formed from incomplete combustion of hydrocarbons. CO poisoning is a major public health concern, both in the United States and worldwide. CO may be encountered as a sole toxicant such as in a suicide attempt using automobile exhaust, or as one of several toxicants such as in smoke exposure during a fire. CO alone is responsible for 40,000 ED visits annually in the United States and at least 5000-6000 deaths each year.⁵⁻⁷ In the “World Health Organization European Member States” there were 140,490 CO related deaths reported between 1980-2008.⁸ These numbers only reflect those cases where CO was the primary or only exposure. It is suspected that the actual incidence is much greater since COHgb levels are not routinely tested and CO poisoning is often mistaken for other illnesses.⁶ If one adds smoke inhalation to this (a major component of which is CO), then the numbers increase significantly. In the United States, it is estimated that 310,000-670,000 people are exposed to the toxic effects of smoke inhalation in residential fires each year, and that 20,000-30,000 are reported as injured or killed.⁹

While smoke inhalation represents the majority of most unintentional cases of CO exposure, there are many others and the magnitude of these varies by both season
and location. Other sources include malfunctioning heaters or hot-water heaters, improperly vented heating or cooking appliances (kerosene heaters, propane stoves, charcoal grills, generators and automotive exhaust).\textsuperscript{6,10–12} Large-scale exposures often occur in the setting of office buildings or gathering places where a heater or oil-burning furnace malfunctions or leaks exhaust fumes into the HVAC system. Electrical wire insulation has also been shown to produce significant amounts of CO when wires overheat and begin to burn through the insulation. Natural disasters such as hurricanes, tornadoes, and earthquakes have been shown to cause a dramatic rise in CO poisonings immediately following the disaster, mainly due to fires, damaged electrical wiring and improper use of gas-powered generators.\textsuperscript{11} Exposures can also occur in the workplace and healthcare settings from improperly connected anesthesia circuits and from exposure to compounds such as methylene chloride.\textsuperscript{13}

1.2.2 Pathophysiology:

No matter the source, the effect of CO is the same. It diffuses rapidly across the pulmonary epithelium and binds to the iron moiety of heme and other hemoproteins with 200-300 times the affinity of $O_2$. The amount of carboxyhemoglobin (COHgb) that develops is highly variable and depends upon the concentration of CO in the inhaled air, the duration of exposure, heart rate, and the minute respiration. CO is endogenously produced and is ubiquitous in nature. All humans have at least some amount of COHgb in their blood, typically ranging from 1-3%. Smokers and those who live in large or polluted urban environments can have concentrations from 5-15% at baseline.\textsuperscript{14} It is believed that other sources of oxidative stress such as diabetes, chronic ulcers, autoimmune disorders and critical illness may also lead to elevated levels of COHgb
that are above that of the normal population and several centers are actively studying this phenomenon.\textsuperscript{15,16}

Due to the binding chemistry of CO to hemoglobin (Hgb), it induces a conformational change in the shape of Hgb that reduces the ability of the other three \( O_2 \) binding sites to off-load \( O_2 \) to the tissues. This is represented as a left-shift of the oxy-hemoglobin dissociation curve. The combination of the left-shift of the oxy-hemoglobin dissociation curve, as well as the presence of CO on sites that are normally occupied by \( O_2 \) are believed to cause a decrease in tissue \( O_2 \) delivery resulting in a hypoxic/ischemic form of injury, especially in \( O_2 \) sensitive tissues and watershed vascular beds.

Beyond its impairment of \( O_2 \) delivery, it has been shown that CO is also capable of binding numerous other hemoproteins in the body including myoglobin, the cytochromes, and NADPH reductase among others. The exact extent to which binding of these proteins plays a role in the toxic effects of CO remains controversial, but it believed that at least 10-15\% of the CO which is inhaled winds up in the extravascular space bound to proteins like those mentioned above.\textsuperscript{17,18} Ultimately, it is felt that this results in decreased \( O_2 \) utilization and mitochondrial dysfunction particularly in tissues like the heart and brain, where dysfunction continues despite adequate \( O_2 \) delivery.\textsuperscript{19–21} The effects on the brain specifically, are referred to as delayed neurologic sequelae (DNS) and/or prolonged neurologic sequelae (PNS). The exact mechanism of DNS/PNS remains uncertain, but several recent studies have indicated that it may be a result of brain lipid peroxidation that leads to an exaggerated immune response.\textsuperscript{22} These studies have implicated xanthine oxidoreductase as a potential mediator of this
lipid peroxidation but more data are needed.\textsuperscript{21} What has become clear in recent years is that the neurologic injury subsequent to CO poisoning is distinct from a simple hypoxic form of injury and cannot be attributed solely to the effects of CO upon hemoglobin or oxidative phosphorylation.\textsuperscript{23–25}

\textbf{1.2.3 Clinical Presentation and Diagnosis:}

Diagnosis of CO poisoning, particularly when it is unintentional, is often very difficult.\textsuperscript{18,26} CO poisoning is not a condition that any one clinician is likely to encounter on a routine basis except for those who specifically work in the fields of trauma, burn, and emergency medicine. Unless a history of CO exposure is provided, CO poisoning presents much like an acute viral illness. Headache (most common), malaise, nausea, dizziness and (if the exposure is large enough) loss of consciousness are the most frequently encountered signs and symptoms. The classic description of cherry-red blood, or bright red skin or lips is rarely encountered except when the COHgb is extremely high and is, therefore, not useful as a marker of CO toxicity.\textsuperscript{10,17,18} Unfortunately, due to the nonspecific nature of the signs and symptoms, CO poisoning is often misdiagnosed. Since testing for COHgb is not routine, it must be requested by the ordering clinician or it will be overlooked. The recent development of a rapid and reliable device for noninvasive assessment of COHgb concentration may provide a much needed breakthrough in the diagnosis of CO poisoning.\textsuperscript{27–29} Unfortunately, the accuracy of these devices has been called into question repeatedly, with several studies suggesting that while they may be beneficial as a routine screening tool in patients at risk they are not a substitute for laboratory COHgb measurement.\textsuperscript{30,31} Any time a patient is suspected of having CO toxicity it is recommended that a blood sample be sent for
oximetry analysis immediately and the patient should be placed on 100% O₂ by a non-
rebreather mask. If a case of suspected CO poisoning is confirmed, then additional
tests should be ordered, including arterial blood gas (to check for acidosis), ECG (to
check for dysrhythmias) and cardiac biomarkers (to check for myocardial injury).

1.2.4 Complications:

As many as one-third of patients with CO poisoning will develop evidence of acute myocardial injury.³² This has been shown to lead to a significant increase in mortality, with these patients having three times the risk of death as their non-poisoned counterparts.³³ Again, the mechanism of this injury remains a subject of controversy and intense research. The classic paradigm of hypoxic/ischemic injury is insufficient to explain the disturbances that are seen. While it is likely that inhibition of cytochromes and/or myocardial myoglobin play a role in this, several studies have shown that CO may be capable of directly interacting with ion channels leading to arrhythmias and contractile dysfunction of cardiac muscle.³⁴–³⁸

As previously mentioned, a second major complication of CO poisoning is the development of DNS/PNS. Studies have shown that this can occur in up to 40% of patients and can manifest up to 240 days following discharge from the hospital.³⁹–⁴³ Most often however, the symptoms of DNS/PNS arise within the first 30 days following exposure, and the deficits typically persist for at least a year or more. Symptoms of DNS/PNS include cognitive deficits, personality changes, focal neurologic deficits, movement disorders and balance disorders. Surprisingly, the development of DNS/PNS is only poorly associated with COHgb concentration.⁴⁰ Patients who have lost
consciousness at some point during or immediately following exposure have been shown to be at the highest risk. Additional complications include acute lung injury, end-organ failure, metabolic acidosis, and (in pregnant women) fetal distress with a high potential for fetal death.

1.2.5 Management

Presently, the only therapy for CO poisoning is the delivery of oxygen (O\textsubscript{2}). This can be subdivided into atmospheric pressure O\textsubscript{2} (i.e., normobaric or NBO) and high-pressure O\textsubscript{2} (i.e., hyperbaric or HBO). NBO has the advantage that all hospitals and many EMS providers are able to deliver NBO as soon as the diagnosis is made or suspected. NBO can reduce the half-life of COHgb from 5 hours, down to about 60-90 minutes. HBO on the other hand can reduce the half-life of COHgb down to about 15-30 minutes. HBO has the advantage that (due to the high pressure) it can substantially increase the solubility of O\textsubscript{2} in plasma, and thus increases the partial pressure of O\textsubscript{2} in the blood from 0.3 mL/dL to around 6.0 mL/dL (depending upon the protocol used).

The primary indication for the use of HBO is in patients whose COHgb\% is greater than 25-40\% and/or who show evidence of end-organ damage (i.e., acidosis, renal dysfunction) ECG changes, or elevated cardiac biomarkers.\textsuperscript{18,39,44} The main goal of HBO is to increase O\textsubscript{2} delivery to tissues via unbound O\textsubscript{2} dissolved in plasma, restore Hgb to normal functioning as quickly as possible, and to prevent acute myocardial injury and DNS/PNS. Unfortunately, despite a multitude of studies on the efficacy of HBO over the past 30 years, the use of this therapy remains highly controversial.\textsuperscript{45,46} This is in large part due to the lack of available facilities, difficulty of
transferring patients, and the inability to access a patient quickly if they begin doing poorly once they are in the chamber. The inadequacies of both NBO and HBO for treating CO poisoning and preventing the sequelae of CO poisoning are the driving forces behind trying to find a better way to treat this significant public health problem.47

1.3 Chemistry and History of CO:

The CO molecule is a carbon triple-bonded to monatomic oxygen (MW=28.01 g/mol) and is characterized by a bond length of 112.8 pm.48 There is a lone pair on both the carbon and the oxygen, and very little charge separation between them. Thus, CO is a relatively unreactive gas, with a b.p. of -191.5 °C. It has ten valence electrons, four from the carbon and six from the oxygen (dipole moment 0.122 D). CO coordinates only to main group elements that are strong electron acceptors. CO also forms stable complexes with many transition metals in low oxidation states, especially those from groups 6 to 11. This explains its reactivity with Heme (Iron, group 8), Cytochrome c (Copper, Group 11), and potentially Vitamin B₁₂ (Cobalt, Group 9).

The French physiologist Claude Bernard presented one of the first documented studies of CO, and its toxic effects on humans, in 1857 in a series of lectures that have become one of the seminal works of the field of Toxicology.49 By the 1940’s it had been demonstrated that the binding of CO to Hgb changes the shape of the oxyhemoglobin dissociation curve to hyperbolic instead of sigmoid (i.e. a flattened and downward shift) and shifts the curve to the left.50 This means that not only is the O₂ saturation of Hgb lower in the presence of COHgb, but the unloading of O₂ from Hgb in the tissues is also diminished.51 We now know that besides its interactions with heme, CO also binds to
the mitochondrial heme protein cytochrome c oxidase, as well as microsomal cytochrome P450 enzymes.\textsuperscript{52,53} Then in the 1950’s and 60’s CO was shown to be formed endogenously by the degradation of Hgb \textit{via} the enzyme heme oxygenase (HO).\textsuperscript{54–57} Initially, it was felt that this was simply a byproduct of Hgb degradation without a direct purpose.

### 1.4 Gaps in Current Understanding:

Douglas and Haldane are the most famous for their work on CO and its effects on Hgb, a fact they came across while trying to better understand the reversible nature of the binding of $O_2$ to Hgb.\textsuperscript{50,58} Their work in this area is still taught in medical schools around the world and we shall herein refer to it as the “carboxyhemoglobin theory of CO toxicity.” While the efforts of Douglas and Haldane were, and continue to be, outstanding science, the question remains: is the primary source of toxicity for CO due to the formation of COHgb and subsequent loss of adequate $O_2$ delivery to vital tissues? The modern answer to this important question, based upon the work by a number of experts in this field is a resounding, “No!”\textsuperscript{18,47,59}

There is no question that CO binds to Hgb with an affinity somewhere between 200-300 times greater than that of $O_2$.\textsuperscript{50} There is also no question that the binding of CO to Hgb causes a leftward and downward shift of the COHgb dissociation curve, lowering the $O_2$ carrying capacity of Hgb and causing Hgb to bind more tightly to $O_2$.\textsuperscript{50,58,60} There is however a very big question surrounding whether or not these changes result in meaningfully diminished $O_2$ delivery to the tissues that need them for aerobic respiration. Multiple studies have shown quite convincingly that except when the
COHgb concentration exceeds 70-80%, or in situations where the poisoned individual is in an environment deprived of O₂ or engaged in metabolically demanding activities, the poisoned Hgb is still able to deliver an adequate supply of O₂ to tissues that need it most. This is in part due to the fact that Hgb has a very large functional reserve that we rarely ever tap into, and because the body is extremely efficient at altering the dissociation curve in order to maximize O₂ delivery and extraction efficiency.

These problems with the carboxyhemoglobin theory were noticed early on and, indeed, Haldane’s own son sought to disprove the theory. One major problem which was immediately apparent and remains to this day, is that the COHgb concentration of a patient bears little or no relevance to the patients outcome or the severity of their injury. This is particularly true in pregnant women who are exposed to CO. In these cases, the effects on the fetus are even more variable, despite the fact that the fetus is not inhaling CO directly. If the injury were due primarily to ischemia, as the carboxyhemoglobin theory suggests, then a patient’s outcome and injury severity should be a function of their COHgb%. In other words, as a patient’s COHgb% increases, so too should the severity of their injury. Instead what we see is a wide variance in patient presentation and outcomes across a wide range of COHgb values, even when other confounding variables have been accounted for. Patients with “fatal” levels sometimes survive and do fairly well, while others with only mild to moderate elevations of COHgb may die or do very poorly, often suffering for years from neurologic and cardiac complications. This points to the idea that some other process must be involved, but it is one that scientists have had difficulty locating until recently.
1.5 Therapeutic applications of CO and what that teaches us:

It was demonstrated in the late 1980s that both NO and CO, applied exogenously, stimulated vessel relaxation.\textsuperscript{68} It was then shown that the mechanism for this was through the second messenger guanylate cyclase system (a heme-containing protein), which converts guanosine triphosphate (GTP) to cyclic-guanosine monophosphate (cGMP).\textsuperscript{68,69} More than a mere curiosity, both of these gaseous compounds are produced endogenously and on purpose, for it now appears that there are vital functions carried out by these simple gases. Several very recent studies have indicated that an impaired HO/CO system may be an underlying cause of vascular resistance in diseases such as systemic and pulmonary hypertension among others.\textsuperscript{70–72} These studies have indicated that while Heme Oxygenase-1 (HO-1) is present in all mammalian cells, its expression is increased in cells subjected to conditions of increased oxidative stress, which is the common denominator in the progression of vascular dysfunction, cardiovascular disorders and inflammatory diseases.\textsuperscript{73–75} Rather than being a cause of the damage, however, the HO-1/CO upregulation appears to be a defensive and protective response by the cell.\textsuperscript{74,76–78}

At physiologically relevant concentrations CO exhibits potent vasorelaxant effects which are independent of NO and this allows for greater blood flow to tissues undergoing stress.\textsuperscript{70,79,80} However, the effects of CO are not restricted to guanylate cyclase activation. Other studies have demonstrated that CO directly effects the opening of big-conductance calcium-activated potassium channels (BK\textsubscript{Ca}) which leads to membrane hyperpolarization, thereby closing voltage-dependent Ca\textsuperscript{2+} channels, and
reducing resting Ca^{2+} concentration of smooth muscle cells.\textsuperscript{38,81,82} The end result of this process is vasodilation by smooth muscle relaxation.\textsuperscript{82}

The effects of CO go far beyond vasorelaxation. A study in 2000 demonstrated that low concentrations of CO gas (250 ppm) applied to murine macrophages or administered directly to rodents, significantly reduced the inflammatory response to lipopolysaccharide (LPS). The authors demonstrated that CO gas inhibited the expression of pro-inflammatory cytokines (TNF-\(\alpha\), IL-1\(\beta\)) and increased the expression of anti-inflammatory mediators such as IL-10 in response to LPS.\textsuperscript{83} The authors also showed that CO mediated these anti-inflammatory effects through a pathway involving mitogen-activated protein kinases (MAPK). Several other studies involving the use of Carbon Monoxide Releasing Molecules (CORM’s), showed that these compounds were able to down-regulate Matrix Metalloproteinase (MMP) expression and inhibit cartilage degradation in arthritis models and alveolar macrophages.\textsuperscript{84–86} This makes sense since MMP’s normally contain zinc which, although not technically a transition metal, shares many properties in common with them and is a common moiety in many proteins.

CO also appears to have anti-apoptotic properties. CO has been shown in several studies to prevent apoptosis in endothelial cells, vascular smooth muscle cells, hepatocytes, and neurons.\textsuperscript{87–90} This effect appears to involve phosphorylation and activation of p38 MAPK, although the precise mechanism has yet to be worked out. There are several other biological substrates that have been discovered in recent years and the list continues to grow.\textsuperscript{91,92} With so many endogenous functions of CO in the body, is it any wonder that scientists have had difficulty determining the primary cause of CO toxicity?
Most of the research into treating this significant issue has focused on methods of increasing O₂ delivery, either via high-pressure O₂ (hyperbaric oxygen therapy, HBO) or through O₂ carrier compounds such as perfluorocarbons and other blood substitutes, even including blood donation.\textsuperscript{18,93,94} Yet all of these therapies are based upon the belief that CO toxicity is due to poisoned Hgb and insufficient O₂ delivery to vital organs. We say belief because this is a point of view which has never in fact been definitively proven, and recently the bulk of scientific evidence now suggests that this view is at the very least incomplete.\textsuperscript{18,47} Furthermore, even if ischemia were a significant component of CO poisoning there are many other, now well proven, physiologic processes that are disrupted through CO overdose and which could (and should) be therapeutic targets.\textsuperscript{18,47}

1.6 Recent Developments and Their Implications:

There is a saying in pharmaceutical circles that says “the poison is in the dose” (from Paracelsus, “the dose makes the poison”). Following the discovery of Nitric Oxide as an endogenously produced gas with regulatory and cellular transmitter-like properties, scientists began to look for other “gasotransmitters”.\textsuperscript{74,95,96} It turns out that CO, is more than just a poison, it can actually be a medicine when given at extremely low doses.\textsuperscript{78,83–86} Moreover, CO has been shown to be produced endogenously as a cellular protectant by nearly every cell in our bodies when they are subjected to situations of oxidative stress or injury.\textsuperscript{57,75–77,97,98} This discovery led scientists to question if there were other natural functions of CO in the body beyond cellular protection, in much the same way as there were for nitric oxide. What they have found is that CO plays a major role in multiple cellular processes in almost every organ
system, and in system-specific ways. These include interactions with soluble guanylate cyclase, big-conductance potassium-gated calcium channels (BkCa), sodium channels, nitric oxide and nitric oxide synthase, mitochondria, cytochromes, NADPH oxidase, and xanthine oxidase to name just a few, and the list is growing every day. Of course, if CO plays an important regulatory role in all of these pathways in a normal healthy individual, then it stands to reason that an overdose of CO would result in a dysregulation of all of these pathways. Multiple studies have now shown that this is exactly what happens and that this finally provides a logical explanation for the extreme variability that is encountered in patients with this form of poisoning. This also explains why O2 (whether at high pressure or atmospheric pressure) is such a poor solution for this injury. If CO were, indeed, a simple case of ischemia, then high-flow, high-pressure O2 would clearly be the best choice.

The mechanism of CO’s toxicity has presumably been known for over 100 years now, yet in all that time the treatment for this major public health concern has remained relatively unchanged, except for the substitution of high-flow atmospheric O2 with hyperbaric O2 as the standard of care. While patients and studies vary significantly, it has been shown that normobaric 100% O2 (NBO) can reduce the half-life \( t_{1/2} \) of COHgb from about 5 hours (on room air) down to 60-90 minutes although the exact time is still controversial. Hyperbaric O2 (HBO) can reduce the \( t_{1/2} \) even further to approximately 15-30 minutes. Despite this, the use of HBO remains controversial. HBO is expensive, has limited availability (typically requiring transfer of critically ill patients), and has even been shown in some studies to increase morbidity.
and the severity of neurologic sequelae.\textsuperscript{39,46,106,108,109} NBO is readily available and is typically employed by first responders. However, even the reduction of the $t_{1/2}$ to 30 minutes is insufficient for the most severely poisoned patients (i.e., those in cardiopulmonary collapse), nor does it always prevent neurologic sequelae.\textsuperscript{18,46,47,63,64} In recent years, the use of both NBO and HBO is being increasingly called into question.\textsuperscript{18,108}

Under conditions of hypoxia or anoxia, neurons begin to suffer irreparable damage in less than 10 minutes.\textsuperscript{110} It is, therefore, widely accepted that in the case of CO poisoning, the time to treatment is far more important than the mode of treatment. Even small delays in therapy administration can lead to significant increases in morbidity and mortality.\textsuperscript{18,46,63} Yet the questions remain concerning which therapy is best. Despite the seemingly innocuous nature of O$_2$ therapy, awareness is increasing of the potential dangers of too much O$_2$. Oxygen therapy has the potential for significant free radical generation that can damage DNA, cell membranes, and even effect cellular respiration.\textsuperscript{22} It has already been shown that CO also produces free radicals, leading to cellular damage and that this may play a critical role in the mechanism of CO toxicity.\textsuperscript{21,22,99,111–115} It is possible and even likely, that the addition of high concentrations of O$_2$ to this scenario may further exacerbate the free radical damage that is occurring in a patient suffering from CO poisoning.\textsuperscript{22} A second negative effect of O$_2$ therapy is due to its action as a ventilatory stimulant. Hyperoxia can leads to hyperventilation causing a reduction in pCO$_2$ (hypocapnia).\textsuperscript{116} Both the cerebral and coronary vascular beds are CO$_2$ sensitive, and a reduction in pCO$_2$ leads to a reduction in blood flow to these organs. It is unlikely that the small increase in pO$_2$ that occurs
with NBO is large enough to compensate for the reduction in blood flow that is caused by the resultant hypocapnia. Thus it is likely that O₂ therapy (the standard of care for >100 years) may in fact exacerbate the ischemic injury caused by CO to some organ systems. This may help finally to explain some of the variability seen in clinical trials of NBO and HBO for CO poisoning.

In the past twenty years, additional mechanisms of CO toxicity that extend beyond its interaction with hemoglobin (Hgb) have been well elucidated. These extra-hemoglobin effects may not be treatable by O₂ therapy and may further explain why HBO may be detrimental in some cases. The controversial nature of HBO, and the inadequacy of NBO obviate the need for a new therapy for CO poisoning that is deployable by first responders, and which is capable of mitigating the extra-hemoglobin effects of CO. We will herein present initial examinations into the interactions of high-dose OHCbl and ascorbic acid with CO and COHgb and the implications for the first ever injectable antidote for CO poisoning.

1.7 Hydroxocobalamin:

Hydroxocobalamin is one of four compounds commonly referred to collectively as Vitamin B₁₂. These are hydroxocobalamin, cyanocobalamin, adenosylcobalamin, and methylcobalamin (See Figure 1). What is fascinating about the B₁₂ group of compounds (known to chemists as “corrinoids”) is that the central ring structure is nearly identical to the heme moiety of Hgb. The side chains notwithstanding, the central “corrin” ring is identical to the porphyrin ring of heme except that it is missing one carbon and the central metal atom is cobalt instead of iron (See Figure 2).
Figure 1: B$_{12}$ "Corrinoid" family of molecules depicting functional group differences
Figure 2: Structural similarities between the porphyrin ring of heme and the corrin ring of B$_{12}$. Note the lack of a carbon between positions 5 and 6 on the corrin ring.
Indeed, this similarity was exploited in early attempts to produce synthetic O₂ carriers to replace the need for blood products when patients lost large amounts of blood. What is even more fascinating is that OHCbl has been used in Europe for almost 40 years as a very effective antidote against cyanide poisoning, which often occurs concomitantly with CO poisoning in structural fires. It is given in massive doses of 5-15 grams per patient with few or no side effects, and no serious adverse events yet reported. It is safe for use in patients taking antidepressants, as well as in pregnant and nursing women. Furthermore, some basic lab research from the late 60’s and early 70’s seemed to indicate that it exhibited limited reactivity with CO under the right conditions. Unfortunately, those results were apparently never followed up on in a translational way. Those early results indicated that under the right conditions OHCbl was capable of converting CO into CO₂, thus rendering it harmless.

Beyond its potential effects directly upon CO (which we will investigate in the remainder of this paper), OHCbl has been shown to interact with multiple bodily systems that are involved in CO poisoning. OHCbl has been shown to inhibit certain forms of nitric oxide synthase, as well as to bind nitric oxide directly. OHCbl has also been shown to neutralize peroxide, superoxide, and peroxynitrite, along with other free radicals in a glutathione sparing fashion and thereby inhibit the damaging effects of free radical damage (a major component of CO toxicity). It has also been shown to regulate Nfkβ and TNFα expression, as well as promote an anti-inflammatory state in leukocytes (ie macrophages and neutrophils).
1.8 Hydroxocobalamin and CO:

The first to prove that OHCbl was capable of reacting with CO was Gerhard N. Schrauzer in 1970.\textsuperscript{2} He demonstrated in-vitro that under the right conditions OHCbl was capable of converting CO into carbon dioxide (CO\textsubscript{2}). He performed the first studies on the kinetics of the reaction and was the first to propose a mechanism. He also tested the reaction in the presence of COHgb but was unable to demonstrate decarbonylation and never published any additional findings on the subject. According to Schrauzer’s work, he proposed that OHCbl was only capable of reacting with free CO, not bound CO (as in COHgb). He further proposed that this reaction required two molecules of OHCbl, one in the III+ oxidation state (OHCbl\textsuperscript{3+}), and one in the reduced II+ oxidation state (OHCbl\textsuperscript{2+}).

He suggested that the CO bound the OHCbl\textsuperscript{2+}, and then formed a transient bis-complex with the non-reduced OHCbl and via a series of electron transfer steps the CO was converted to CO\textsubscript{2} in the presence of water while the OHCbl\textsuperscript{3+} became OHCbl\textsuperscript{2+} and the OHCbl\textsuperscript{2+} became OHCbl\textsuperscript{1+} (See Figure 3). Unfortunately, this mechanism has not yet been proven, and indeed our work seems to indicate that a different mechanism may, in fact, be at work.
Figure 3: Demonstrates two proposed mechanisms for the reaction of carbon monoxide (CO) with reduced hydroxocobalamin (OHCbl$^{2+}$). CO is known to form semi-stable complexes with transition metals (groups 6-10) in low oxidation states. $\pi$-back-bonding between the filled d-orbitals on the cobalt and the empty anti-bonding $\pi^*$-orbitals of CO result in a linear Co-CO bond. This results in a buildup of negative charge on the oxygen which makes it vulnerable to attack by hydroxide, and leads to formation of a metallo-carboxylic acid. This can then be deprotonated ultimately leading to the release of CO$_2$ and leaving the two electrons on the metal, a condition which is thought to be stabilized by the corrin ring. In one theory, one of the two electrons can then be passed on to an electron acceptor, such as oxidized hydroxocobalamin (OHCbl$^{3+}$). In another theory, the two electrons can be neutralized with two protons to give a metal di-hydride which can then lose H$_2$. 
Numerous studies have shown that CO can form stable complexes with transition metals (M) in low oxidation states.\textsuperscript{73,138–140} The lone pair of electrons on the carbon can form an $\sigma$-bond with the metal. In addition $\pi$-back-bonding from the filled $d$-orbitals on the metal to the empty anti-bonding $\pi^*$-orbitals on the CO may occur. This $\pi$-back-bonding causes a build-up of negative charge on the O$_2$, making it more reactive and allowing interactions with electrophiles. In the case of OHCbl, it is likely that the metal-CO complex (M-CO) is formed and then attacked by hydroxide [OH]$^-$ to produce [M-COO-H]$^-$ which is then deprotonated to yield [M-CO$_2$]$^{2-}$. The reaction then continues with the dissociation of CO$_2$, which leaves two electrons on the metal. These electrons are then passed on to an electron receptor (or receptors).

During the early phases of our work we tested varying amounts of different reducing agents in an effort to establish what combination would prove most effective. What we found was that the combination with ascorbic acid appeared to have a uniquely favorable profile compared with other reducing agents. Even more interesting was that the reaction proceeded even in the presence of excess amounts of ascorbic acid. If the mechanism proposed by Schrauzer was correct then the reaction should not have occurred since all of the available OHCbl would have been reduced already by the presence of excess ascorbic acid. What we found was that excess concentrations of ascorbic acid not only allowed the reaction to go forward but apparently enhanced it. Indeed our chosen concentration of ascorbic acid was nearly eight times the molar concentration of OHCbl. This finding has led us to propose a second mechanism.
Ascorbic acid is rather unique in that it behaves much like a vinylogous carboxylic acid and can therefore donate multiple protons per molecule of ascorbic acid. OHCbl normally contains a 5,6-dimethylbenzimidazole ribonucleotide tail that is coordinated to the central cobalt atom in the lower axial position (See Figure 4).
L-Ascorbic Acid.
This behaves as a vinylogous carboxylic acid. The resonance structures stabilize the conjugate base, and it can also exist in a fully oxidized form wherein both of the ring hydroxyl groups are double bonded oxygens.

Figure 4: Hydroxocobalamin (above).
Note the –OH group in the upper axial ligand binding site.
This large and bulky structure means that typically only the upper axial ligand binding site of OHCbl is available. However, the coordinate covalent bond between the imino nitrogen of the imidazole ring and the central cobalt can be hydrolytically cleaved in the presence of an acid or base. It has been previously demonstrated that the reactivity of OHCbl can be greatly improved in some reactions by switching into what is known as the base-off form (See Figure 5). Protonation of the imino nitrogen and cleavage of the coordinate covalent bond between it and the cobalt causes the benzimidazole base to "swing" out away from the central corrin ring, thus exposing the lower axial ligand binding site of OHCbl. In this form, there is considerably less steric hindrance around the active site which may make coordination with CO more favorable. We propose that once in the base-off form, CO coordinates with the central cobalt [M-CO] (See Figure 6).
Figure 5: Base-Off form of Hydroxocobalamin

Figure 6: Progression from the base-on to the base-off form of hydroxocobalamin and coordination with CO.
This allows nucleophilic attack on the carbonyl by the hydroxyl group attached to the cobalt in the upper axial position, resulting in the formation of a carboxylic acid [M-COO-H]$^-$.

The carboxylic acid deprotonates to form [M-COO]$^{2-}$ which then dissociates to form [M]$^{2-} + [CO_2]$. From here, the mechanism is less clear. The initial bond with CO and the dissociation of CO$_2$ would be expected to transfer two electrons to the cobalt such that it would wind up in the I$^+$ oxidation state (OHCbl$^{1+}$). This is an extremely unstable complex and it is likely that the excess electrons would be transferred to protons (from water or from ascorbic acid) to form a stable metal dihydride. Alternatively the metal dihydride may further dissociate with the formation of H$_2$ gas. There is some precedent for this reaction mechanism based upon the reaction of CO with other transition metal complexes, and based upon the reaction of NO with OHCbl and similar compounds.$^{73,138-140}$ In a Hgb containing system such as ours, it may also be possible that the excess electrons are transferred either directly or via a radical to the iron of the heme moiety on Hgb resulting in the formation of methemoglobin.$^{145}$ Nevertheless, the exact mechanism in this specific case remains to be proven.

Based upon Dr. Schrauzer’s work, and based upon the work of others with similar compounds (such as NO, and CO with other catalysts) we generated our first hypothesis. It is that, under the right conditions, OHCbl is capable of being used as an antidote for CO and that, if the reaction occurred as expected, then it should be possible to monitor it using an artificial lung with very careful monitoring of the CO$_2$ being “exhaled” or “out-gassed” by the artificial lung.
1.9 Basic Approach:

1.9.1 Circulation System:

We used a closed-loop circulation system containing fresh whole human blood and connected to a hollow-fiber membrane oxygenator to produce COHgb and monitor the concentration of CO and CO₂ coming from the oxygenator. We verified that we could change the CO₂ concentration of the blood and detect this as a change in the gas-out concentration of CO₂ (± 0.05% v/v within 2.4 sec) using sensitive gas monitoring equipment connected to the gas-out port of the oxygenator. Using this system, we then injected high-dose combinations of OHCbl and ascorbic acid in NS (0.9% NaCl solution), at three different doses in order to detect any changes in CO₂ concentration in the gas-out flow from the oxygenator.

1.9.2 Raman Analysis:

Due to the complex chemical nature of the compounds involved in this study, it was critical to be able to demonstrate that we were able to generate the reduced form of OHCbl and to find a more accurate method of measuring the reactions between OHCbl, ascorbic acid, Hgb, and CO. We chose to use Resonance Raman (RR) spectroscopy in order to analyze the compounds we were testing and to monitor the reactions.

RR spectroscopy was first developed in the late 1920’s, and since that time it has improved dramatically. RR spectroscopy analyzes scattered light that has been passed through, or reflected from, a sample. By using a highly uniform (monochromatic) light source of a known wavelength (typically a high-powered tunable laser) the light is directed towards an analyte that may be solid, liquid, or gas. Although the majority of
the excited photons that enter the sample will pass through unaltered, some of those photons will strike molecules and be scattered in all directions. Of those photons that are scattered, most will have the same wavelength as the incident light (i.e., the wavelength of the laser that generated them). However, some of those scattered photons will be slightly shifted in wavelength, and it is this shift that RR spectroscopy detects and uses for analysis.

The shift in wavelength occurs because the photons are temporarily absorbed by the molecule which changes the vibrational energy of that molecule (typically by changing the molecular orbital of its electrons). When the photon is reemitted from the molecule, it may transition to either a higher or lower vibrational energy state than it was in before it absorbed the photon. The energy that is either absorbed by the photon or donated from the photon causes a slight shift in the wavelength of that photon that is then detected by the spectral camera.

Since these spectral shifts are very small and the signals are extremely faint, they can be very difficult to detect. However, if one knows something about the chemical structure of the analyte of interest, then it is possible to tune the incident laser light to match the vibrational frequency of the chemical bonds of interest. By tuning the laser to the appropriate frequency specific bonds will resonate leading to a million-fold increase in signal intensity. Likewise, if the sample is an unknown structure then it is possible to tune the laser over a range of frequencies and note where resonance occurs. Since specific bond types have specific resonant frequencies it is possible to then use RR spectroscopy to gain basic structural analysis information, which, when
coupled with a secondary technology such as mass spec, allows for definitive structural identification.

Unlike other analytical techniques, RR spectroscopy has the advantage of being extremely fast, it is non-destructive, and requires only incredibly small sample volumes for analysis. Compared to other spectroscopic techniques such as infrared (IR) and absorbance (Abs) spectroscopy, the bands from RR are higher resolution. This is critical in dealing with combinations of B_{12} species and Hgb, since these compounds share several wavelengths on Abs spec that makes them difficult or impossible to distinguish. Studies have shown that there are highly characteristic changes that occur in the RR spectra of carboxylic acids and amines, due to the addition or removal of a proton. Other studies have also shown that it is possible to monitor changes in the oxidation state of specific bonds within a molecule due to characteristic changes in the RR spectrum. Thus, it is possible to monitor ascorbic acid for evidence of both deprotonation and oxidation, and to monitor OHCbl for evidence of reduction or oxidation, as well as the binding of CO.

Due to the specificity of RR spectra, all of these reactions can be monitored in blood since the spectral lines for various Hgb species are distinct from those of OHCbl and ascorbic acid. The intensity of the peak on RR spectra is proportional to concentration and thus RR spectra can also be used for quantitation. Although not yet complete, we are using this technology to quantify the amount of COHgb present in a sample of blood, and to monitor the rate of change (in real time) both in the presence and absence of the antidote in order to assess if there is any notable effect. This
technology is capable of in-vivo use by directing the RR laser to the sublingual capillary bed, and we are actively working to develop this technology commercially.

In the present study we used RR spectroscopy to identify the formation and quantity of OHCbl^{2+} in our antidote mixture. We also used it to create a reference standard for quantification of COHgb\% in whole blood (for later development of an in-vivo diagnostic device) and to monitor specific changes in OHCbl, ascorbic acid, and Hgb.

1.10 Summary of Findings:

Sodium bicarbonate (NaHCO₃) reacts with carbonic anhydrase in blood to produce CO₂. Therefore, we used an injection of NaHCO₃ as a systems check to verify that a change in the CO₂ concentration of the blood would be detectable in the gas-out concentration of CO₂ using our equipment. The specified resolution of our CO₂ detector was listed as 0.1\% (1000ppm) at 1 sample per second. By taking 10 samples per second, the effective resolution was increased to 0.035\% (350ppm). We found that the average signal variance was around 250ppm during sampling, but that we could reliably detect a change of >500ppm and thus we set this as our minimum level for significance. We injected 5mL of a standard 2.5\% (25g/L) NaHCO₃ solution and observed the predicted rapid increase in CO₂ concentration (>2,000ppm increase over baseline) in the gas-out flow. Two negative controls in blood containing COHgb: normal saline (NS), and OHCbl alone (without ascorbic acid), produced an increase of less than 250ppm CO₂. The third negative control in blood containing COHgb: ascorbic acid (reducing
agent) produced a median increase in the CO₂ concentration of the gas-out flow of 400ppm over 5 trials.

A fourth negative control: OHCbl and ascorbic acid injected into normal blood that contained less than 1% carboxyhemoglobin (COHgb), produced a highly variable increase in the CO₂ concentration of the gas-out flow with a mean value of 1500ppm over baseline. The reason for this is not yet clear.

The mixture of OHCbl and ascorbic acid, when injected into blood containing COHgb, caused a significant increase in the CO₂ concentration of the gas-out flow, in comparison to baseline and negative controls with a median of 1200ppm over baseline.
Methods

2.1 Materials:

All chemicals and reagents were supplied by Sigma-Aldrich unless otherwise specified and were of analytical/reagent grade or better. We used the Maquet Pediatric Quadrox-iD® hollow-fiber membrane oxygenators. For consistency, all tubing and supplies were also from Maquet Getinge Corp. Roller pump was from Stöckert/Shiley®. Continuous gas monitoring data were amplified and acquired by an MP150® system from BIOPAC corp. using AcqKnowledge 4.0 software®. Carbon Dioxide (CO₂) concentration was captured by a CO₂100c module from BIOPAC Corp. Carbon Monoxide (CO) concentration was captured by a Horiba VIA-510® model CO monitor. Heat exchanger was a Thermo-Haake model DC 10®. All gases used were supplied by Airgas International. Gas flow was monitored and maintained by universal flow meters from Aalborg Instruments Inc. Blood-gas analyses were performed using an OSM3® and an ABL 725® from Radiometer Copenhagen®.

2.2 Resonance Raman Analysis:

We employed the use of resonance raman (RR) spectroscopy in order to determine whether we had achieved adequate formation of the reduced form of OHCbl, as well to monitor the kinetics of the reaction and monitor certain characteristic changes in Hgb. RR spectra were obtained with 5 mw or less of 406.7 nm laser excitation from a Coherent Sabre DBW krypton ion laser system. The laser excitation was focused to a 100 micron beam waist onto samples that were contained in melting point capillaries. The capillaries were subjected to lateral motion to lessen possible photolytic
effects by the laser excitation. Raman scattering was collected by a Canon 50 mm f/0.95 camera lens and focused onto a 70 micron entrance slit of a Spex (Horiba) model 1870 0.5 meter spectrograph fitted with interchangeable 1200 and 1800 lines/mm holographic gratings (Jobin-Yvon/Horiba). The spectrograph was coupled to a Pylon (Princeton Instruments) liquid nitrogen cooled CCD detector with 400 x 1340 pixel scanning array. Scans were generally completed in 120 seconds. The scanning and data acquisition software was Winspec from Princeton Instruments. Data were further processed by GRAMS/AI version 8 (Thermo Fisher Scientific) and Origin-Pro version 7.5 (OriginLab Corp.).

2.3 Blood Collection:

For each experiment we obtained 600 mL of fresh whole human blood, with standard consent, from patients seen in our Apheresis clinic and anti-coagulated with 70 mL of CPD-A1 in standard fashion. All patients signed a standard informed-consent form that allows and acknowledges that blood and tissue taken in our institution may be used for research/scientific purposes. The head of our institutional review board (IRB) determined that this study was not “human subject research” and as such not subject to IRB review since blood was being collected as part of a routine procedure (and not for this study directly), was permanently de-identified and completely anonymous (no one involved in this study had contact with patients or patient information). Blood donated from the apheresis clinic was limited to donors with hemochromatosis, porphyria, and polycythemia. We used the blood within 72 hours of collection, most often within 24-48 hours following collection.
2.4 Circulation System:

Figure 7 is an illustration of the closed-loop artificial circulation system that we used for this study. With each experiment, approximately 150 mL of blood was injected into the system until it was full, and there was no longer any air visible in any of the tubing or in the oxygenator. After priming the system with blood the roller-pump was set to a rate of 250 mL/minute which circulated roughly the entire blood volume about 1.5 times per minute. We incorporated fluid sampling ports with luer-lock adapters and three-way stopcocks into the circulation pathway to allow for blood sampling and antidote injection. The CO and CO₂ monitors were set to a sampling rate of 10 samples/second.
Figure 7: Illustration depicting the closed-loop circulation system we used for these experiments.
2.5 Equilibration:

The blood we obtained was venous blood and thus contained a significant amount of residual CO₂. Therefore, we provided an initial equilibration period for the blood to off-load CO₂ under a constant flow of medical grade air (20-22% v/v O₂; <400 ppm CO₂; 78-80% v/v N₂) until a flat and stable baseline of CO₂ was achieved as indicated by a near-zero slope. Following this equilibration period, a 0.6mL sample was taken and analyzed for standard electrolyte, blood gas, and oximetry values.

2.6 CO Poisoning:

After baseline data were obtained, the in-flow gas mixture was switched to 6,000 ppm CO in research grade air (0.5838% v/v CO, remainder air) for twenty minutes at a flow rate of approximately 178 mL/min. A second 0.6 mL blood sample was taken and analyzed as before. If a value of 50% (+/-5%) carboxyhemoglobin (COHgb) was established then the in-flow gas mixture was returned to medical grade air at a flow rate of approximately 178 mL/min. If the COHgb level was too low, then the flow of CO was maintained at 5-10 minute intervals until the desired level was achieved.

Since the amount of CO₂ produced presumably depended upon the relative concentration of reduced OHCbl to CO (i.e., COHgb) every attempt was made to stay between 45-55% COHgb. This was intended to prevent an abnormally high or low COHgb concentration from independently increasing or decreasing the amount of CO₂ produced. A table of the range of blood gas values for each experiment is provided (Table 1).
### Summary Statistics: Post CO Exposure & Pre-Antidote Injection

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1 - Measurement from OSM3; 2 - Measurement from ABL700

### Table 1: Range of captured blood-gas values for the blood used during testing.

One blood sample was taken after the blood was warmed and oxygenated in the closed-loop circulation system. Following 20 minutes of CO exposure, a second sample was obtained to ensure that between 45-55% COHgb formation had been achieved prior to injection of the antidote, and additional time was allowed if necessary. All blood samples were run on both an OSM3 and ABL 725, from Radiometer Copenhagen. If we encountered a variance between the values provided by the two instruments of >3% we automatically tested a second sample. If the variance continued, then the problem was assumed to be in the blood and the experiment was stopped before antidote injection.

This table represents the final values obtained just prior to injection of the antidote. No post-injection blood-gas data are available since hydroxocobalamin causes significant interference with absorbance spectrometry and invalidates the readings.
2.7 Antidote Creation:

The antidote consists of a mixture of OHCbl and ascorbic acid in NS. Ascorbic acid is thought to reduce the oxidation state of OHCbl. The reduced form of OHCbl (OHCbl$^{2+}$) is extremely reactive toward any potential oxidant, particularly O$_2$ itself. Thus, the addition of ascorbic acid to OHCbl had to be carried out in a glove box under nitrogen environment. To prevent red cell lysis, deoxygenated NS (0.9% NaCl in water, Baxter, Inc.) was used as the solvent. The NS was deoxygenated by placing it in a cleaned, sterilized flat-bottomed glass vacuum flask and placed under vacuum for 30 minutes, and then flushed with nitrogen gas while still under vacuum for an additional 60 minutes. Approximately 1 hour prior to injection, the selected amounts of OHCbl and ascorbic acid were measured and placed into a sterile container with 5 mL of deoxygenated NS and mixed until fully dissolved. The antidote solution was then transferred from the sterile mixing container into a 5 mL syringe and placed into the glove-box airlock under continuous nitrogen flow until it was needed.

2.8 Antidote Injection:

Following adequate formation of COHgb and transition back to medical grade air the gas-out concentration of CO was monitored until it achieved steady state typically around 400 ppm. The CO monitoring line was then clamped so that all out-flow gas would go through the CO$_2$ monitor in order to allow for accurate measurement of total CO$_2$ gas-out concentration. The CO$_2$ concentration was monitored for an additional 5 minutes following clamping of the CO monitoring line to ensure an accurate baseline prior to antidote administration. At that steady state a 5 mL solution of the antidote was infused
into the flowing blood. Gas-out concentration of CO₂ was continuously measured at a sample rate of 10 samples/sec after infusion of the antidote for 30 minutes.

According to the experiments performed by Schrauzer et al. in the 1970’s, two molecules of OHCbl are required to reduce one molecule of CO. In addition, their work suggested that the rate of the reaction was partially dependent upon the ratio of reduced to non-reduced OHCbl. We used to the following calculations to estimate the concentration needed to achieve 1:1 concentration of CO:OHCbl in our system. Hgb has an O₂ binding capacity of 1.36-1.37mL O₂ per gram Hgb. The density of O₂ is .001331 g/mL, and the molecular weight of O₂ is 15.9994 g/mol. Thus, the O₂ carrying capacity of Hgb is 1.114x10⁻⁴ moles of O₂ per gram Hgb if the Hgb is 100% saturated with O₂. Since CO and O₂ share the same binding sites, we use the same value. Therefore, at 50% saturation, the value would be 5.57x10⁻⁵ moles of CO per gram of Hgb. Our system, contained 150 mL of blood, with an average Hgb value of 130 g/L. Thus the total grams of Hgb in our system are calculated as approximately 19.5 grams. The total moles of CO at 50% saturation in our system were calculated to be approximately 1.0862x10⁻³ moles CO. Multiplying this value by the molecular weight of OHCbl (1382.82 g/mol) provides us with 1.5 grams of OHCbl needed to provide molar equivalency for the amount of CO bound to Hgb in blood. Due to the expense and limited availability of OHCbl, we chose a value of half of this amount as our initial starting dose.

In total, three doses were chosen: 700 mg of OHCbl with 700 mg ascorbic acid (1/2 molar equivalency), 300 mg of OHCbl with 300 mg ascorbic acid (1/2 initial), and 300 mg OHCbl with 150 mg ascorbic acid (to evaluate the mechanism). Although the
precise mechanism of the reaction has not yet been definitively proven, the proposed mechanism suggests that the rate of reaction is partly dependent upon the ratio of reduced to non-reduced OHCbl since both may be necessary for the reaction to occur. Therefore, we chose to evaluate whether or not a change would be observable when we altered the ratio of ascorbic acid to OHCbl, in addition to any change that might be observable from altering the dose of OHCbl overall. Thus, the 3 doses chosen were: 700 mg:700 mg, 300 mg:300 mg, and 300 mg:150 mg, as a ratio of OHCbl:ascorbic acid respectively.

2.9 Controls:

Both positive and negative controls were used in order to minimize the risk of a type I error. For the positive control, a standard 2.5% solution of sodium bicarbonate (NaHCO₃) in water was used. Following the identical set of experimental procedures listed above, 5 mL of 2.5% NaHCO₃ solution was injected after formation of COHgb instead of the actual antidote. The gas-out CO₂ concentration was monitored for 30 minutes following injection of the positive control solution.

There were three negative controls: deoxygenated NS, ascorbic acid alone, and OHCbl alone. Following the procedures listed above, either 5 mL of deoxygenated NS (0.9% NaCl), 350 mg of ascorbic acid alone in 5 mL of deoxygenated NS, or 350 mg of OHCbl alone in 5 mL of deoxygenated NS were injected into the oxygenator. To demonstrate the synergy of the combination of ascorbic acid and OHCbl, the 350 mg OHCbl control injections were followed by injection of 350 mg of ascorbic acid. The gas-
out CO$_2$ concentration was monitored for 30 minutes following injection of the negative control solutions.

2.10 Analysis:

Data from the CO and CO$_2$ analyzers were routed through the MP150 system and captured by AcqKnowledge™ 4.0 software. Values were in ppm/minute, plotted over 30 minutes from the time of antidote injection. These values were imported into an Excel™ spreadsheet and analyzed using JMP™ 10 software for statistical analysis and cleanup. The graphs in Figure 17 were created using JMP 10, and represent the mean values of the 5 sample runs at each dose in ppm per unit time. Four of the five sample runs at each dose were paired, using the same unit of blood for each of the three doses tested. For these four samples the median and interquartile range values for the area under the curve of each paired data set were analyzed using the Wilcoxon signed-rank test.
Results

3.1 Resonance Raman Analysis of OHCbl and ascorbic acid:

The RR spectrum of $\text{B}_{12a}$ is dominated by a very large band at 1499 cm$^{-1}$ that is due to $\text{-C=C-}$ bond stretching in the macrocycle (Fig 8). This indicates that the OHCbl used for our studies was very pure, and matches published spectra.$^{147}$ The purity of the OHCbl was further verified with HPLC-MS (high pressure liquid chromatography and mass spectrometry) analysis, which also indicated a purity of >99%. Raman analysis of the L-ascorbic acid used, also indicated high-purity, and provided a strong peak in the region of 1690 cm$^{-1}$ which matches published spectra.$^{148,149}$ The broad band in this region is due to the $\nu(\text{C=O})$ stretch of the lacton and disappears upon deprotonation with the appearance of a new band near 1595 cm$^{-1}$. The combination of OHCbl and ascorbic acid showed a loss of the ascorbic acid peak at 1690 cm$^{-1}$, a loss of intensity of the 1496 cm$^{-1}$ peak and formation of strong peaks at 1602 cm$^{-1}$, 1537 cm$^{-1}$, and 1352 cm$^{-1}$. These changes are indicative of the deprotonation of ascorbic acid and the formation of OHCbl$^{2+}$. 
Figure 8: RR spectra of OHCbl in NS (a), OHCbl+ascorbic acid in NS (b), and ascorbic acid in NS (c). Key peaks for OHCbl (a) are 1499 cm$^{-1}$, 1199 cm$^{-1}$, and 1163 cm$^{-1}$. Key peaks for ascorbic acid (c) are 1693 cm$^{-1}$, 1148 cm$^{-1}$, and 828 cm$^{-1}$. Key peaks for the mixture of OHCbl and ascorbic acid (b) are 1602 cm$^{-1}$, 1537 cm$^{-1}$, 1496 cm$^{-1}$ and 1352 cm$^{-1}$. The decrease in intensity at 1499 cm$^{-1}$ with the appearance or strengthening of the peaks at 1602 cm$^{-1}$ and 1352 cm$^{-1}$ are strong indicators of the formation of OHCbl$^{2+}$. 
Sodium nitrite (a powerful oxidizing agent that does not interfere with RR analysis) was added to the mixture of OHCbl and ascorbic acid, in order to oxidize the OHCbl$^{2+}$ to OHCbl$^{3+}$ (Fig. 9). RR analysis shows only a single intense peak at 1500 cm$^{-1}$ with loss of the peaks at 1602 cm$^{-1}$, 1537 cm$^{-1}$, and 1352 cm$^{-1}$, consistent with oxidation of OHCbl$^{2+}$ to OHCbl$^{3+}$. A solution of methylcobalamin was analyzed in order to look for the types of spectral shifts that might occur with substitutions in the axial ligands of the cobalamin species. The spectrum of methylcobalamin is remarkably different from that of OHCbl, with three bands predominating at 1487 cm$^{-1}$, 1538 cm$^{-1}$, and 1591 cm$^{-1}$. Attempts to alter the spectrum of methylcobalamin by oxidation with sodium nitrite yielded no detectable spectral changes.
Figure 9: RR spectra of OHCbl with ascorbic acid and sodium nitrite (a), methylcobalamin (b), and methylcobalamin with sodium nitrite (c). Key peaks for OHCbl with ascorbic acid and sodium nitrite (a) are 1500 cm\(^{-1}\), 1202 cm\(^{-1}\), and 1164 cm\(^{-1}\). Key peaks for methylcobalamin (b) are 1487 cm\(^{-1}\), 1538 cm\(^{-1}\), and 1591 cm\(^{-1}\). Key peaks for methylcobalamin with sodium nitrite (c) are the same as those for methylcobalamin alone: 1487 cm\(^{-1}\), 1538 cm\(^{-1}\), and 1591 cm\(^{-1}\).
3.2 Resonance Raman Analysis of Blood:

Internal standards of fresh whole blood were prepared and analyzed in order to establish parameters for accurate quantitation and identification of the various states of Hgb in our samples (Fig. 10). The deoxyhemoglobin (O$_2$Hgb% < 6.9%) sample showed two intense peaks at 1356 cm$^{-1}$ and 1472 cm$^{-1}$. The methemoglobin samples had two intense peaks at 1371 cm$^{-1}$ and 1563 cm$^{-1}$. The oxyhemoglobin (O$_2$Hgb% = 100%) samples had two intense peaks at 1377 cm$^{-1}$ and 1639 cm$^{-1}$. These peaks were all in agreement with published spectra.$^{150,151}$ The COHgb (COHgb% > 87%) sample showed two intense peaks at 1375 cm$^{-1}$ and 675 cm$^{-1}$. 

Figure 10: RR spectra of deoxyhemoglobin (a), methemoglobin (b), oxyhemoglobin (c), and COHgb (d). Key peaks for deoxyhemoglobin (a) are 1356 cm\(^{-1}\) and 1472 cm\(^{-1}\). Key peaks for methemoglobin (b) are 1371 cm\(^{-1}\) and 1563 cm\(^{-1}\). Key peaks for oxyhemoglobin (c) are 1377 cm\(^{-1}\) and 1639 cm\(^{-1}\). Key peaks for COHgb (d) are 1375 cm\(^{-1}\) and 675 cm\(^{-1}\) (not shown). These were internal standards created in our lab, and verified using a standard laboratory co-oximeter. This allows for definitive identification of these species when analyzing a complex matrix or mixed sample.
Initial scans of a blood sample containing 49.2% COHgb showed a double-peak at 1359 cm\(^{-1}\) and 1377 cm\(^{-1}\) as well as an intense peak at 1473 cm\(^{-1}\) (Fig. 11). These peaks are consistent with a mix of deoxyhemoglobin and oxyhemoglobin. It is well known that the high energy of the RR laser can induce photolysis of CO from Hgb and this feature has been often utilized to study the kinetics and electronic intermediates of Hgb and other hemoproteins (such as the cytochromes).\(^{53,151,152}\) Thus, it is likely that this represents photolyzed COHgb rather than true deoxyhemoglobin. Oscillating the sample during the scan by the laser, can reduce the photolytic effects and allow for a true scan of COHgb. This is seen in line (b) with a reduction of the 1359 cm\(^{-1}\) peak and a new peak at 1375 cm\(^{-1}\). We allowed the sample to settle overnight and then differentially focused the laser on the red blood cells (RBC’s) and then the plasma. This indicated the presence of some Hgb, (presumably free Hgb) in the plasma. This also indicated that the plasma fraction of Hgb was almost entirely oxyhemoglobin, an unexpected finding considering that this blood contained a significant amount of CO.
Figure 11: RR spectra of a blood sample containing 49.2% COHgb. The capillary was placed on its side and the blood was allowed to settle in the capillary overnight to separate the plasma from the RBC’s. The laser was differentially focused on the RBC’s and then the plasma. (a) RBC scan without oscillating the sample, (b) RBC scan with sample oscillation, (c) plasma scan with oscillation. The internal standard of oxyhemoglobin (d), is placed here for reference comparison.
3.3 Resonance Raman Analysis of the antidote in poisoned blood:

RR analysis of poisoned blood was taken at two time points following antidote administration, one at 20 minutes and the second at 70 minutes (Fig. 12). Again, the blood was allowed to settle and the laser was differentially focused on the RBC’s and the plasma. The predominant peaks at 1359 cm\(^{-1}\) and 1473 cm\(^{-1}\) in the RBC’s are indicative of deoxyhemoglobin. This may be true deoxyhemoglobin, or it may be due to laser photolysis of COHgb. Interestingly, there is no evidence of oxyhemoglobin in the RBC fraction, as was seen in the same blood prior to antidote administration. Conspicuously absent from this spectrum are peaks for OHCbl and ascorbic acid (expected peaks at 1499 cm\(^{-1}\), 1602 cm\(^{-1}\), 1537 cm\(^{-1}\), and 1352 cm\(^{-1}\)). The plasma fraction again appears to contain free Hgb. In contrast to the same blood prior to antidote administration, the free Hgb is now entirely methemoglobin. In this closed system, therefore, the Hgb species appear to exist only as either deoxy/carboxyhemoglobin or methemoglobin when exposed to the antidote mixture dependent on whether the Hgb is free or cellularly bound. These results were entirely unexpected and at present we cannot explain these findings.
Figure 12: RR spectra using the same blood shown in figure 11, containing 49.2% COHgb following antidote administration. After a baseline sample was obtained, the 300mg:300mg antidote mixture was injected and two additional samples were taken at 20 min and 70 min post antidote injection. The capillary was placed on its side and the blood was allowed to settle in the capillary overnight to separate the plasma from the RBC’s. The laser was differentially focused on the RBC’s and then the plasma. (a) RBC scan at 20 min post antidote injection, (b) RBC scan at 70 min, (c) plasma scan at 20 min post antidote injection, (d) plasma scan at 70 min post antidote injection.
3.4 Control Runs:

We checked the viability of the closed-loop circulation system for detecting changes in the gas-out CO₂ concentration through the use of a 2.5% NaHCO₃ injection (Fig. 13). NaHCO₃ reacts vigorously with carbonic anhydrase in blood to produce CO₂. We predicted that this rise in CO₂ concentration of the blood would be detectable as a subsequent rise (>0.1% v/v within 10 sec) in the gas-out concentration of CO₂. The 2.5% sodium bicarbonate injection resulted in a nearly 3-fold increase over baseline (from 100 ppm to 2,500 ppm) in 15 seconds in the gas-out CO₂ concentration.
Figure 13: Following a warming and equilibration period, 10mL of freshly prepared 2.5% NaHCO$_3$ solution in NS were injected into the system. The line shows the mean response of the CO$_2$ generated (in ppm) over time. This was performed to demonstrate the ability of the system to detect changes in the CO$_2$ concentration when a substance was injected into it that caused a rise in pCO$_2$. This was also done to demonstrate the graphical appearance of CO$_2$ generation due to an acid-base type reaction.
As shown in figure 14 we tested each component of the antidote individually (deoxygenated NS, OHCbl, and ascorbic acid) in order to determine whether any rise in the gas-out CO$_2$ concentration was due to an individual component or due to the combination only. We also evaluated the combination of OHCbl and ascorbic acid in blood that was essentially free of COHgb to test whether a subsequent rise in the gas-out CO$_2$ concentration was due to a reaction of the antidote with some component of the blood other than either CO or COHgb.

We used NS as our first negative control since it is non-reactive. The injection of 5mL of deoxygenated NS showed a slight increase in the gas-out CO$_2$ concentration in the presence of COHgb with a median value of 250 ppm over a total period of 30 minutes. Injection of 350mg of OHCbl in 5mL deoxygenated NS showed a median increase of 250 ppm over a total period of 30 minutes. Injection of 350mg ascorbic acid in 5mL NS showed a median increase in the gas-out CO$_2$ concentration of 400 ppm over a total period of 30 minutes in the presence of COHgb. Surprisingly, injection into normal blood that contained less than 1% COHgb, of 300mg of OHCbl and 300mg ascorbic acid in 5mL NS also showed a median increase of 1500 ppm over a total period of 30 minutes in the gas-out CO$_2$ concentration.
Control Experiments: CO2 Generation vs Time

\( (mg \text{ hydroxocobalamin}:mg \text{ ascorbic acid}) \)

Figure 14: Median response by the four control experiments. The injection of NS alone and OHCbl alone each showed no response. As expected, the injection of 300mg of ascorbic acid showed a moderate response that was similar (although less pronounced) to that of the bicarbonate injection. Surprisingly, the injection of the combination of OHCbl and ascorbic acid into blood that contained <1% COHgb resulted in a significant increase in CO\textsubscript{2} concentration.
3.5 Serial Injections:

We performed the NS and the OHCbl negative controls in a serial fashion, in order to assess and demonstrate the unique nature of the combination of ascorbic acid and OHCbl. After forming COHgb using the closed-loop circulation system we injected the NS, followed by injection of the OHCbl, and then by injection of ascorbic acid at 30-minute intervals. Figure 15 demonstrates the difference in the activity of the mixture of ascorbic acid and OHCbl (reduced versus non-reduced OHCbl) for reacting with CO in-vitro. Neither the injection of NS, nor the injection of OHCbl resulted in a significant (i.e., <500ppm) change in the gas-out concentration of CO₂. The subsequent administration of ascorbic acid, resulted in a 3-fold increase in the gas-out concentration of CO₂ with a median increase of 1100ppm over baseline. Injection of the solution of pre-mixed OHCbl and ascorbic acid into blood containing 45-55% COHgb resulted in a 3-4 fold increase in the gas-out concentration of CO₂.
Figure 15: 5 samples were performed by injecting each control solution in a serial or sequential fashion. Sequential injection of 5mL deoxygenated NS, followed by 350mg OHChbl in 5mL deoxygenated NS, followed by 350mg ascorbic acid in 5mL deoxygenated NS. The line shows the mean response of the 5 samples in CO$_2$ generated (in ppm) over time. This was performed to demonstrate the unique synergy of the combination of ascorbic acid with OHChbl as opposed to the lack of reaction of the individual components.
We found with some samples that injection of the antidote mixture into blood that was not poisoned with CO nevertheless resulted in a significant increase in CO₂ concentration. Therefore, we attempted to determine where this CO₂ might be coming from. In order to assess whether the CO₂ being produced was due to a breakdown product from the combination of OHCbl and ascorbic acid, we filled our circulating system with NS and injected the antidote mixture. As shown in figure 16, there was no detectable increase in the gas-out concentration of CO₂. To determine whether the CO₂ produced was due to interaction of the antidote with some component of blood we then performed two serial injections, the first with plasma and the second with packed red blood cells. Both injections resulted in a rapid and transient rise in the gas-out concentration of CO₂. However, in both cases the CO₂ returned to baseline within five minutes.
Sequential Events with Circulating Saline:
1) 300:300, 2) 10ccs Plasma, 3) 10ccs RBCs

Figure 16: Injection of a mixture of 300 mg OHCbl plus 300 mg ascorbic acid in 5mL of deoxygenated NS into the circulation system which contained only NS instead of whole blood. Sequential injection of 10 mL plasma followed by 10 mL packed red blood cells. The line shows the mean response of the 5 samples in CO₂ generated (in ppm) over time. This was performed to demonstrate that the CO₂ produced was not due to a breakdown product of the mixture of OHCbl and ascorbic acid, and to ascertain whether it might be due to a reaction with some blood component other than CO₂.
3.6 Mean Dose Response:

Figure 17 shows the mean values of CO$_2$ produced over time according to the amount of antidote that was injected into the blood. As seen from the graphs, all three doses resulted in a five to eight-fold increase in the gas-out concentration of CO$_2$ compared with controls. Also visible in the graph is the difference between the high-dose (700 mg:700 mg) and the low-dose (300 mg:300 mg).
Fig 17: N=5 trials of each dose: 700mg hydroxocobalamin with 700 mg ascorbic acid; 300mg OHCbl with 300 mg ascorbic acid; 300mg OHCbl with 150 mg ascorbic acid. Each line represents the mean response over time and is plotted to show the central tendency of CO₂ generation (in ppm) for each dose mixture over a 30 minute period. The controls (N=5 for each) were: deoxygenated NS (0.9% NaCl solution), 350 mg OHCbl in 5mL deoxygenated NS, and 350mg ascorbic acid in 5mL deoxygenated NS. The control line above represents the aggregate central tendency for all three.
3.7 Dependent (Paired) Experiments:

Performing three dose-experiments per unit of blood allowed us to collect and compare paired (dependent) data on the dose-response relationship within the same unit of blood for each dose for four out of the five samples. We looked at two dependent mean comparisons: Proportionate Dose and Non-Proportionate Dose. The doses are displayed in \textit{mg of OHCbl: mg of ascorbic acid}. In the Proportionate Dose case, we tested a low dose, 300mg:300mg, to a high dose, 700mg:700mg, from the same blood donor, and repeated this five times with five unique donors. In the Non-Proportionate Dose case, we compared 300mg:300mg dose with a 300mg:150mg dose on blood from the same donor, and repeated this four times with four unique donors. The area under the curve (AUC) from the time response curves from these samples was measured, and represents the magnitude of CO$_2$ generated from the system in units of ppm-min. The AUC was normalized by extrapolating out 30 min of data from 4 min of actual baseline measurements, and subtracting the 30 min baseline AUC from the 30 min AUC of the post-antidote curve. Analysis of the differences in the AUC was performed by Wilcoxon signed-rank test due to the small sample sizes, and compared against a significance of $\alpha = 0.05$. The one-sided signed t-test = 6.25, DF = 3 and the p-value = 0.0041 for the Proportionate Dose Comparison. The Non-Proportionate Dose Comparison produced a signed t-test = 0.27, DF = 3 and p-value = 0.3125. These results are displayed in table 2.
### Table 2: Paired (dependent) data using blood from the same donor (N = 4). Two types of dose comparisons:

1) *Proportionate* dose increase where both the OHCbl and ascorbic acid doses were increased proportionately.

2) *Non-proportionate* increases with an increase in the ascorbic acid while keeping the OHCbl constant.

The area under the curve (AUC) from the dose response over time was measured, and represents the magnitude of CO$_2$ generated from the system in units of ppm-min.

The proportionate dose comparison produced a signed t-test = 6.25, DF = 3 and the *p*-value = 0.0041.

The non-proportionate dose comparison produced a signed t-test = 0.27, DF = 3 and *p*-value = 0.3125.

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4.1 Summary:

The results of this study indicate that the mixture of OHCbl and ascorbic acid in blood containing COHgb may be capable of converting CO to CO₂. The magnitude of this conversion is uncertain, however, and will require further investigation. This is an important finding with significant clinical implications since both of these compounds are safe and approved for use in humans even at extremely high doses. ¹ ³ Although both ascorbic acid and OHCbl are being used increasingly in the settings of trauma, burn, and smoke inhalation, this is the first study that suggests a novel set of capabilities when the two are combined at high dose. This study also indicates that these compounds may be effective whether they are given together or separately but spaced closely together. The potential to extract CO from the body, by its conversion to CO₂, independently of high-flow or high-pressure O₂ is a novel finding.

4.2 Resonance Raman Analysis:

All corrinoid compounds contain very strong absorption bands in the visible and near-ultraviolet wavelengths, due to the π-π* electronic transitions.¹⁴⁷ Therefore, tuning the laser to these ranges (i.e. 400-500 nm) produces strong resonance enhancement of raman bands associated with vibrations of the corrin ring which contains –C=C- bonds (that contain π-bonding electrons). Cobalt contains valence electrons within the d-orbital system. Thus changes in the electronic state of the cobalt molecular orbitals (d-d transitions) may also be detectable by using laser wavelengths in the 476-606 nm range.¹⁵³ This range appears to be particularly sensitive to alterations in the lower axial
ligand (i.e. base-off versus base-on form of OHCbl). Although we have not yet been able to investigate this ourselves, it would be beneficial in determining the mechanism of the reaction and demonstrating formation of the base-off form of OHCbl. Several studies have shown that the spectra of OHCbl$^{3+}$, OHCbl$^{2+}$, and OHCbl$^{1+}$ are distinct, and that detection of the base-off form of OHCbl is also possible.$^{147,148,153–156}$

In our studies so far the loss of the 1690 cm$^{-1}$ peak along with the appearance of a new peak at 1600 cm$^{-1}$, (when hydroxocobalamin and ascorbic acid were mixed) were indicative of deprotonation of the hydroxyl group on the ring structure of ascorbic acid.$^{149}$ In the same mixture the decrease in intensity at 1496 cm$^{-1}$ along with the appearance of new bands at 1600 cm$^{-1}$ and 1352 cm$^{-1}$ indicate successful formation of OHCbl$^{2+}$ species in solution.$^{147,148}$ This also suggests that the 406.7 nm wavelength is quite sensitive at detecting the $\pi-\pi^*$ electronic transitions occurring in the corrin macrocycle due to changes in the oxidation state of the coordinated cobalt and subsequent delocalization of the additional electrons. Unfortunately, this wavelength appears to be less sensitive at detecting the Cobalt d-d transitions that have been shown to accompany alterations in the lower axial ligand, although we are continuing to study this.

The addition of sodium nitrite (a powerful oxidizing agent that does not interfere with RR analysis) to the mixture of OHCbl and ascorbic acid, was expected to re-oxidize the OHCbl$^{2+}$ to OHCbl$^{3+}$. RR analysis shows only a single intense peak at 1500 cm$^{-1}$ with loss of the peaks at 1602 cm$^{-1}$, 1537 cm$^{-1}$, and 1352 cm$^{-1}$, consistent with reoxidation of OHCbl$^{2+}$ to OHCbl$^{3+}$. This offers further evidence that we successfully...
formed reduced OHCbl via the addition of ascorbic acid, since we were able to reverse the spectral changes by addition of an oxidizing agent.

Changes in specific bonds or bond types often result in characteristic changes of RR spectra. For example, attachment of different ligands at the same site will often shift the RR spectrum in a characteristic fashion. A shift towards a longer wavelength (compared with the incident beam) is called a red-shift, and a shift towards a shorter wavelength is called a blue-shift. In the case of OHCbl, it is possible to study the effects of differential ligand binding to the central cobalt in the upper axial position (six-coordinated cobalt) using known compounds such as OHCbl, methylcobalamin, and adenosylcobalamin. By knowing the properties of these bonds, and whether they are likely to be electron donating or electron accepting, it becomes possible to look at an unknown ligand bound to the upper axial position and make some conclusions about its nature.

We analyzed a solution of methylcobalamin in order to look for the types of spectral shifts that might occur with substitutions in the upper axial ligand of the cobalamin species. The binding of the methyl group is much stronger than that of hydroxide, water, or CO and would therefore be expected to provide quite a different spectrum. We found that the spectrum of methylcobalamin was, indeed, quite different from that of OHCbl, with three bands predominating at 1487 cm⁻¹, 1538 cm⁻¹, and 1591 cm⁻¹. The attempts to alter the spectrum of methylcobalamin by reduction with sodium thiosulfate (a powerful reductant with minimal interference with RR) or oxidation with sodium nitrite yielded no detectable spectral changes. This provided us with some valuable information when identifying peaks in the mixture of poisoned blood and the
antidote since it would allow us to determine not only whether the OHCbl was in the reduced or oxidized state, but also potentially to determine if there was perhaps a different ligand bound in the upper axial site.

Most studies involving Hgb and other heme proteins have relied upon lasers with longer wavelengths closer to the visible spectrum, and near the absorbance maxima of Hgb around 525 nm. The use of near-UV or deep violet lasers such as the 406.7 nm Krypton ion laser used in this study is far less common and indeed some species have never been analyzed using this specific wavelength. Our results show that Hgb is quite amenable to RR analysis at this wavelength, with clear identification of each of the primary Hgb species (oxy, deoxy, and met).

The analysis of blood containing 49.2% COHgb was complicated by the phenomenon of photolysis. COHgb is quite stable under anaerobic or sealed conditions, allowing us to analyze it both immediately after sampling and several hours to days later. Since the blood was exposed to a normal partial pressure of O₂ during CO exposure and prior to sampling, there should be no deoxyhemoglobin present in the sample (or very little). Therefore, the initial spectra obtained indicates probable photolysis of COHgb, thereby yielding the spectrum for R-state deoxyhemoglobin. This problem can be solved by keeping the sample flowing, or by oscillating the sample rapidly in addition to making the laser focus more diffuse and/or reducing the laser power. This effect is partly seen with the second scan of the same sample in which the sample was oscillated during the laser exposure. The decrease in intensity of the 1356 cm⁻¹ peak and the emergence of the 1371 cm⁻¹ peak are indicative of the presence of COHgb and a reduction in the amount of deoxyhemoglobin being detected. Further
reducing the laser power, more rapid sample oscillation, and/or use of a pulsatile laser have been shown to reduce photolysis and should result in a more accurate analysis of COHgb concentration.

Another fascinating finding with the COHgb sample was the presence of only oxyhemoglobin in the plasma. While the finding of some amount of free Hgb is quite common, particularly in blood that has been stored and manipulated, we expected that it would be a mixture of oxyhemoglobin and deoxy/carboxyhemoglobin. It may be that the free Hgb is able to dissociate CO more rapidly than cellularly bound Hgb, possibly due to changes in pH or the microenvironment.

The analysis of the blood containing both COHgb and the antidote mixture likewise presented some unexpected results. Previous scans using a combination of OHCbl in unpoisoned blood, and OHCbl + ascorbic acid in unpoisoned blood had shown that we could clearly detect and distinguish each species in the mixture using our RR system. Yet when we injected the antidote mixture into poisoned blood (i.e., containing COHgb) we lose the RR signal for both OHCbl and ascorbic acid entirely. Furthermore, in this system we find that the RBC’s contain only deoxy/carboxyhemoglobin and that the free Hgb is completely converted into methemoglobin. A recent study from 2008 (and the only one of its kind) indicated that extremely high doses of OHCbl, like those we are using, seemed to convert deoxyhemoglobin into methemoglobin. However, they only tested free porcine Hgb and not cellular bound or human Hgb. Our results would appear to corroborate this finding, although we need to study this further.
The finding of increased levels of methemoglobin is perplexing and needs further study, in part because it runs counter to the currently understood mechanisms of methemoglobin formation. Methemoglobin is formed when the iron moiety of heme is converted from $\text{Fe}^{2+}$ to $\text{Fe}^{3+}$ due to loss of an electron. The finding of increased levels of methemoglobin in the presence of OHCbl would seem to indicate that OHCbl is somehow removing electrons from Hgb. If this were true, it would be a novel finding since it is likely proceeding through the action of a radical intermediary.

It had occurred to us that the formation of methemoglobin might be an explanation for the additional CO$_2$ concentration we see in the gas-out flow after injection of the antidote mixture. Since methemoglobin loses its ability to carry O$_2$ as well as CO and CO$_2$. However, if this were the case then we would have expected that OHCbl alone would have caused an increase in CO$_2$ concentration. Yet, as we have shown, the addition of high-dose OHCbl alone caused almost no change in CO$_2$ concentration. It was only after the addition of ascorbic acid to the mixture of OHCbl and blood that we saw the significant increase in CO$_2$ concentration. This might seem to suggest that the ascorbic acid is somehow contributing to the formation of methemoglobin by OHCbl. However, ascorbic acid is a reducing agent not an oxidizing agent and it has in fact been used as an antidote for methemoglobin.$^{157-159}$ Therefore, it seems unlikely that the ascorbic acid is contributing to methemoglobin formation; but it also begs the question of what is causing methemoglobin formation? Regardless of the cause, this study also indicates that cellular bound Hgb is relatively protected since there is no evidence of methemoglobin in the RBC’s. However, there is also no evidence of oxyhemoglobin in the RBC’s. This may indicate that the conversion of CO
to CO₂ proceeds through an O₂ radical or some other process that results in stripping of O₂ from Hgb, leaving only deoxyhemoglobin behind. The formation of the O₂ radical could also explain the formation of methemoglobin via loss of the heme electron to the O₂. All of these questions remain to be explored in more detail.

Since RR analysis is capable of quantitation, we had intended to utilize this for the purposes of quantifying COHgb and monitoring its decay rate in solution both with and without the presence of the antidote mixture. This would provide an important piece of additional information regarding the ability of the antidote to speed up elimination of CO from Hgb via conversion to CO₂. The various states of Hgb, particularly oxy, met and COHgb have some overlap on the RR spectrum. However, by identifying specific peaks representative of particular Hgb species, it is possible to perform a Gaussian deconvolution of the relative contribution of each peak to the area under the curve. This allows for definitive quantitation when compared against reference standards for the particular RR system being used. The identification of a unique COHgb peak using the 406.7 nm laser is a new and unpublished finding. The photolytic effects of the laser present challenges in ensuring accuracy of quantification that must be overcome reliably. Therefore, we are not yet ready to definitively quantify COHgb in these samples but that work is ongoing.

4.3 Controls:

In the course of these experiments we found that we at times achieved significant levels of CO₂ when injecting the antidote into blood that we had not poisoned. In some cases this may have been due to the presence of high baseline levels of COHgb (one
patient had a baseline of >10% COHgb and thus may have been a smoker). We tried several different units of blood from different patients and found extreme variability in the amount of CO₂ that was produced upon addition of the antidote even though the blood was “unpoisoned.” However, even after flushing the blood under pure O₂ for two hours, in several cases we still had apparently high levels of CO₂ being produced upon injection of the antidote. There is some evidence that suggests that CO₂ may be produced as a degradation product of ascorbic acid and/or Hgb. However, when we injected the antidote mixture into NS we were unable to detect any change in CO₂ concentration, indicating that no CO₂ was being produced or, at least, not enough to explain our results. We followed this with injections of plasma and then packed RBC’s into the antidote mixture. There was a rapid initial spike of CO₂ that quickly diminished in both cases and was consistent with off-gasing of CO₂ that was already present in the blood/plasma. In both cases, the CO₂ fell rapidly to zero, instead of holding the sustained high concentration that we had seen when the antidote was injected into poisoned blood. This is indicative of a unique reaction that is occurring when the antidote is injected into whole blood. The CO₂ produced by this reaction is greater than the sum of the CO₂ produced when any of the components are injected individually, and is not due to a reaction between the components themselves but clearly relies upon some factor that is present in the blood. This may be the expected conversion of CO to CO₂, but we cannot rule out the possibility that there is another source.

4.4 Antidote Trials:

Our evidence indicates that the combination of OHCbl and ascorbic acid results in the production of a significant quantity of CO₂ when injected into whole blood, and
that this reaction goes well beyond a simple acid-base reaction. This suggests that the conversion of CO to CO₂ by this mixture may be possible. However, the interaction of the antidote with some other component of blood cannot be ruled out. We found that OHCbl alone results in little or no CO₂ production that we could detect when injected into blood containing CO, suggesting limited reactivity although these results were not conclusive. Its efficacy in terms of other physiologic processes in the face of CO poisoning has yet to be demonstrated.

4.5 Mechanism:

The mechanism suggested by Schrauzer et al. was that a combination of the reduced and non-reduced forms of OHCbl allowed this reaction to proceed, but our evidence does not directly support this theory. In our study the concentration of ascorbic acid was nearly eight times the molar concentration of OHCbl, which should have been more than sufficient to fully reduce the OHCbl, yet the reaction still went forward. If, as was suggested, the reaction required both reduced and non-reduced OHCbl in order to work, then a reduction in the concentration of ascorbic acid should have resulted in an increase in the area under the curve (AUC). Instead, lowering the ascorbic acid concentration appeared to decrease the AUC. If the mechanism proposed by Schrauzer et al. were correct, it might also be reasonable to expect that any reducing agent would allow for this reaction to proceed, but we have not yet tested this theory.

4.6 Limitations:

This study is limited in that it does not study the kinetics or mechanism of this reaction directly. While there is strong evidence that the production of CO₂ is from CO,
these experiments were only designed to study this indirectly. Whether the additional CO₂ produced when the antidote mixture is injected is due in whole or in part to the conversion of CO to CO₂ cannot be conclusively determined from the present study.

In addition, while the delayed mixture of ascorbic acid and OHCbl works in this artificial system, it is unknown whether this would work in a living system where both OHCbl and ascorbic acid may be taken up by cells or move into the intracellular space. CO has many effects on physiologic systems throughout the body. The interaction of the ascorbic acid/OHCbl mixture with these systems was also not studied here. While the conversion of CO to CO₂ is promising, it does not directly translate into a clinical benefit. Therefore, further studies in living mammalian systems are required.

4.7 Future Directions:

4.7.1 The Big Picture:

CO poisoning whether alone or as a component of the broader injury of smoke inhalation injury, remains a significant public health problem the world over. The focus of the present study was to demonstrate whether or not OHCbl could react with CO in blood and convert it to CO₂ according to the reaction first worked out by Schrauzer et al. We recognize that there is more at stake here and that the real value of this antidote lies not solely in its reactivity with CO directly but, instead, in its other effects. CO overdose has a myriad of effects in the body. Two specific effects have been well researched and there is evidence to suggest that these may be potential secondary targets for the action of this high-dose mixture of OHCbl and ascorbic acid.
4.7.2 Nitric Oxide:

The production and release of nitric oxide has recently been shown in numerous studies to be a significant effect of CO and a likely culprit in the wide-ranging effects of CO toxicity.\textsuperscript{23,25,96,99,100,160,161} CO causes the release of nitric oxide from the hemoproteins to which it is normally bound, thus causing a rapid rise in free nitric oxide levels both intracellularly and extracellularly. At the same time, CO has also been shown to be capable of activating certain forms of nitric oxide synthase, resulting in an overall increased production of nitric oxide.\textsuperscript{79,99} Besides the well-known vasodilatory effects of nitric oxide, it is also a significant regulatory molecule with multiple effects on various cellular processes.\textsuperscript{80} Nitric oxide is also a powerful oxidizing agent and high levels of it can lead to cellular and DNA damage, thereby causing cell death and immune system activation.\textsuperscript{161} The reduced form of OHCbl is significant in this situation because it has been shown in several studies to be capable of not only scavenging free nitric oxide (potentially via formation of a nitrosyl derivative), but also of downregulating several forms of nitric oxide synthase.\textsuperscript{130,132,162,163} Furthermore, there is both direct and indirect evidence that properly administered OHCbl can have immunomodulatory effects on neutrophils and macrophages encouraging an anti-inflammatory state as opposed to the pro-inflammatory state induced by the presence of high concentrations of nitric oxide and oxidized lipids.\textsuperscript{128,129,133–137}

4.7.3 Reactive Oxygen Species:

The production of reactive oxygen species is a second major component of CO poisoning that has recently been demonstrated. Studies have shown that CO is capable
of inducing the formation of reactive oxygen species intracellularly.\textsuperscript{22,25,100,111,115,120,161,164,165} It appears to do this primarily through interaction with the mitochondria. There are likely several mechanisms at work here. At least one mechanism involves the conversion of xanthine dehydrogenase into xanthine oxidase causing the formation of peroxide. Another mechanism is the conversion of elevated levels of nitric oxide into peroxynitrite (ONOO\(^-\)) which is a longer lasting and more potent free radical than nitric oxide itself. However, peroxide and superoxide have also been shown to be produced as a direct result of excess CO exposure.\textsuperscript{25,99,100} The effects of excess levels of free radicals are widespread and include DNA/RNA damage, lipid peroxidation, cell death and immune system activation, among others. Again, the reduced form of OHCbl has been shown to be capable of neutralizing these free radicals and modulating the immune system towards an anti-inflammatory state.\textsuperscript{129,162,163,166,167} OHCbl is also essential for DNA formation and repair as well as for maintaining the ability of the cell to utilize O\(_2\) for aerobic metabolism.

4.8 Conclusions:

This study indicates that the production of CO\(_2\) in whole blood by OHCbl and ascorbic acid is synergistic. This study also demonstrates that OHCbl\(^{2+}\) can be effectively produced with the addition of ascorbic acid. Although the non-reduced form of OHCbl is effective at binding and neutralizing cyanide, the literature suggests that this form is ineffective at interacting with CO, nitric oxide, or reactive oxygen species. Our research provides some evidence in support of the improved efficacy and functionality of the reduced form of OHCbl for use in CO poisoning and/or smoke inhalation injury.
4.9 More is Needed:

There is still much work to be done to prove the efficacy of the reduced form of OHCbl as an antidote for CO poisoning as mentioned herein. One method for determining the precise source of CO₂ production in this case, would be to use labeled CO, as well as labeled ascorbic acid. The CO₂ produced could then be captured and analyzed to see whether it was coming from CO or from a breakdown product of ascorbic acid or from something else. Demonstrating the evolution of labeled CO₂ following addition of labeled CO would be more precise and definitive. Ultimately, however, the real question is whether or not OHCbl (reduced or non-reduced) offers neuroprotective and/or cardioprotective benefits resulting in an improvement in clinical outcomes. This can only be demonstrated through a clinical trial in animals and then humans. We are actively engaged in performing animal studies as well as pursuing approval for human trials to determine the ultimate potential for this antidote as an injectable therapy for CO poisoning, although the results of these trials are still at least a year away. Even in the event that this antidote should ultimately prove ineffective or unusable, we sincerely hope that this work will inspire a new era in the treatment and understanding of CO poisoning leading ultimately to one or several injectable antidotes as one solution for this significant global health problem.
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