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Hyperbaric Oxygen in the Prevention of Carbon Monoxide Induced Delayed Neurological Sequelae in Male Sprague Dawley Rats (Rattus norvegicus)

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HYPERBARIC OXYGEN IN THE PREVENTION OF CARBON MONOXIDE INDUCED DELAYED NEUROLOGICAL SEQUELAE IN MALE SPRAGUE-DAWLEY RATS (*RATTUS NORVEGICUS)*

A Thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Chester P. Gut, Jr. ENTITLED Hyperbaric Oxygen in the Prevention of Carbon Monoxide Induced Delayed Neurological Sequelae In Male Sprague-Dawley Rats (*Rattus norvegicus)* BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Gut, Jr., Chester Preston., M.S. Department of Pharmacology and Toxicology, Boonschoft School of Medicine, Wright State University, 2010.
Hyperbaric Oxygen in the Prevention of Carbon Monoxide Induced Delayed Neurological Sequelae In Male Sprague-Dawley Rats (*Rattus norvegicus)*

In Carbon Monoxide (CO) induced Delayed Neurological Sequelae (DNS) clinical signs develop 1 to 6 weeks after CO has cleared the body. The aim of this experiment was to develop a model of CO induced DNS which closely mimics "real world" conditions both in exposure and treatment. The model was challenged with hyperbaric or normobaric oxygen, or room air. Basic behaviors were measured by Open Field test on days 1, 7, 14 post exposure and treatment. No significant difference in behavior was observed between exposed and control animals or between treatment groups. Histological analyses showed no DNA or necrotic damage to the basal ganglia, cortex, or hippocampus in CO exposed animals at time of euthanasia regardless of treatment. Data suggest variability in tolerance to CO and development of DNS. Data also indicates that the *in vivo* COHb half-life is not constant across species.

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BACKGROUND

Carbon monoxide (CO) is a simple, yet highly toxic diatomic gas. The toxicity of CO has been known since ancient times where it was used as a means of execution, and is suspected in the deaths of two Byzantine emperors (Lascaratos and Marketos 1998; Prockop and Chichkova 2007). Exogenously, CO is the product of incomplete combustion of hydrocarbons, thus, it is ubiquitous outside the body (Prockop and Chichkova 2007). Endogenously, CO is produced by the breakdown of heme into bile pigments. (Ryter, Alam et al. 2006) The resultant CO is then used as a vasodialator in conjunction with NO, and may modulate long-term potentiation and neurotransmitter release (Haldane 1895; Zhuo, Small et al. 1993; Piantadosi, Zhang et al. 1997). CO is also a signaling molecule involved with apoptosis and inflammation (Prockop and Chichkova 2007). Despite its ubiquity, CO is one of the most deadly toxins per capita on the planet. It has been estimated that more than half of the fatal poisonings around the world are caused by CO (Raub, Mathieu-Nolf et al. 2000).

Several physical characteristics of CO contribute to the deadly nature of the gas. It is completely undetectable by humans without mechanical means. It is odorless, tasteless, and invisible, at any concentration. The molecular weight of CO is 28 (the weighted average molecular weight of atmospheric air at sea level is approximately 29) (Penney 2008). CO is completely miscible in air, it will not pool within any particular area of a given space. Although these characteristics certainly contribute to the severe toxicity of CO, it is hemoglobin's affinity for it that makes the gas so deadly to humans.

Inside the mammalian body, hemoglobin has an affinity for CO between 210-240 times that of oxygen (O_2)(Haldane 1895; Allen and Root 1957; Garland and Pearce 1967; MacMillan 1977; Ginsberg 1985). Even though the resulting compound, carboxyhemoglobin (COHb) is readily made, the CO is not as rapidly released from it, severely reducing the oxygen carrying capacity of the blood. The half-life for COHb in humans is well established at 4 – 6 hours in normal, atmospheric air (Haldane 1895; Allen and Root 1957). This relatively long half-life results in a prolonged O_2 deficiency (hypoxia) in the higher metabolic tissues. Due to their high metabolic rates, the two organs most affected by the resultant lack of oxygen are the heart and brain. The tissue site of COHb formation, the lung, is relatively transparent to CO toxicity (Penney 2008). CO also binds to myoglobin further exacerbating hypoxia in the brain, heart, and other highly metabolic tissue (Prockop and Chichkova 2007; Wolf, Lavonas et al. 2008). CO also causes tighter binding of O_2 to oxyhemoglobin, shifting the oxygen dissociation curve to the left, further impeding the release of the available O_2 in arterial blood (Penney 2008).

The symptoms of CO intoxication vary widely ranging from mild febrile symptoms to coma and death (Raub, Mathieu-Nolf et al. 2000; Prockop and Chichkova 2007; Penney 2008). It is estimated that in approximately 50% of CO intoxication cases short-term symptoms include, but are not limited to, headache, weakness, nausea, confusion, and shortness of breath (Prockop and Chichkova 2007). Headache occurs in 84% of reported cases of CO intoxication (Handa and Tai 2005). It has been noted that because of the nonspecificity of these symptoms CO intoxication is often misdiagnosed, or missed completely upon presentation at the hospital (Raub, Mathieu-Nolf et al. 2000; Penney 2008). It is also noted that COHb measurements taken upon arrival at the hospital do not correlate with the symptoms or their severity (Garland and Pearce 1967; Choi, Kim et al. 1995; Thom, Taber et

al. 1995). It is interesting that the one symptom most commonly associate with CO intoxication, the cherry-red coloration of the skin and cyanosis are not frequently seen (Lapresle and Fardeau 1967).

Delayed neurological sequelae (DNS) are one of the more bizarre and insidious clinical presentations of acute CO intoxication. This symptom is as bizarre as it is tragic. It appears anywhere from 1 – 6 weeks after CO has cleared the body (Garland and Pearce 1967; Lee and Marsden 1994; Choi, Kim et al. 1995). The clinical signs of DNS include, but are not limited to, cognitive deficit, severe dementia, psychosis, Parkinsonism (shaking), and incontinence (Garland and Pearce 1967; Lee and Marsden 1994; Choi, Kim et al. 1995; Mimura, Harada et al. 1999). DNS occurs in 0.2-40% of those individuals who survive acute CO intoxication (Lee and Marsden 1994; White 2008). In two thirds of these individuals the DNS will be persistent (Raphael, Elkharrat et al. 1989; Thom, Taber et al. 1995; Weaver, Hopkins et al. 2002). Since DNS occurs after the blood COHb concentrations return to baseline levels, CO induced hypoxia can be ruled out as its cause (Piantadosi, Zhang et al. 1997). Piantandosi hypothesized that DNS was due to delayed neuronal damage incurred via mechanisms similar to those associated with ischemia (Piantadosi, Zhang et al. 1997).

Normobaric oxygen treatment (NBOT) is the standard treatment for CO intoxication, being available at all emergency medical facilities(White 2008). NBOT lowers the half-life of COHb from 4-6 hours to 1-1.5 hours, as Haldane first concluded in 1895 "the higher the oxygen tension the less dependent an animal is on its red corpuscles as oxygen carriers, since the oxygen simply dissolved in the blood becomes considerable when the oxygen tension is high" (Haldane 1895). This increases the dissolved oxygen in blood to 1.5 mL/dL, five time the normal amount (Prockop and Chichkova 2007) Although commonplace, NBOT

has not proven itself to be effective in the prevention and/or treatment for CO induced DNS (White 2008).

Hyperbaric oxygen treatment (HBOT) is 100% oxygen at increased pressure, usually 2 -3 atmospheres for 1 -1.5 hours (Simman 2010). Its main affect is to lower the half-life of COHb to approximately 20 minutes (Pace, Strajman et al. 1950; Sasaki 1975). Following Haldane's conclusion above, HBOT increases arterial oxygen tension, and therefore increases the dissolved oxygen to 6 mL/dL, 20 times the normal level (Prockop and Chichkova 2007). In addition to this benefit, HBOT has been demonstrated to prevent neutrophil adherrance to the brain microvascular endothelium, thus CO-mediated oxidative stress, which generates abnormalities in myelin basic proteins (Thom, Bhopale et al. 2004; Thom, Bhopale et al. 2006; Tomaszewski 2008). HBOT is the recommend treatment for CO intoxicated patients who are unconscious, and/or have a blood COHb level > 25%, (15% for a pregnant female) (Tibbles and Perrotta 1994; Hampson, Dunford et al. 1995; Ernst and Zibrak 1998; Thom 2002). Despite the seemingly inherent advantages to HBOT there is still great debate in the medical community on the effectiveness of HBOT as the sole treatment for CO intoxication (Gibson, Davis et al. 1991; Gilmer, Kilkenny et al. 2002; Isbister, McGettigan et al. 2003; Weaver, Valentine et al. 2007; Scheinkestel and Millar 2008).

This experiment was designed to support the theory that DNS is caused by delayed neuronal damage in the forms of either apoptosis or necrosis after acute CO intoxication. Exposure times and concentrations were determined based on a predictive curve COHb formation in rats by Benignus and Annau (Figure 1) coupled with Piantadosi's findings regarding behavior and neuronal damage 30 days post CO intoxication (Benignus and Annau 1994; Piantadosi, Zhang et al. 1997).

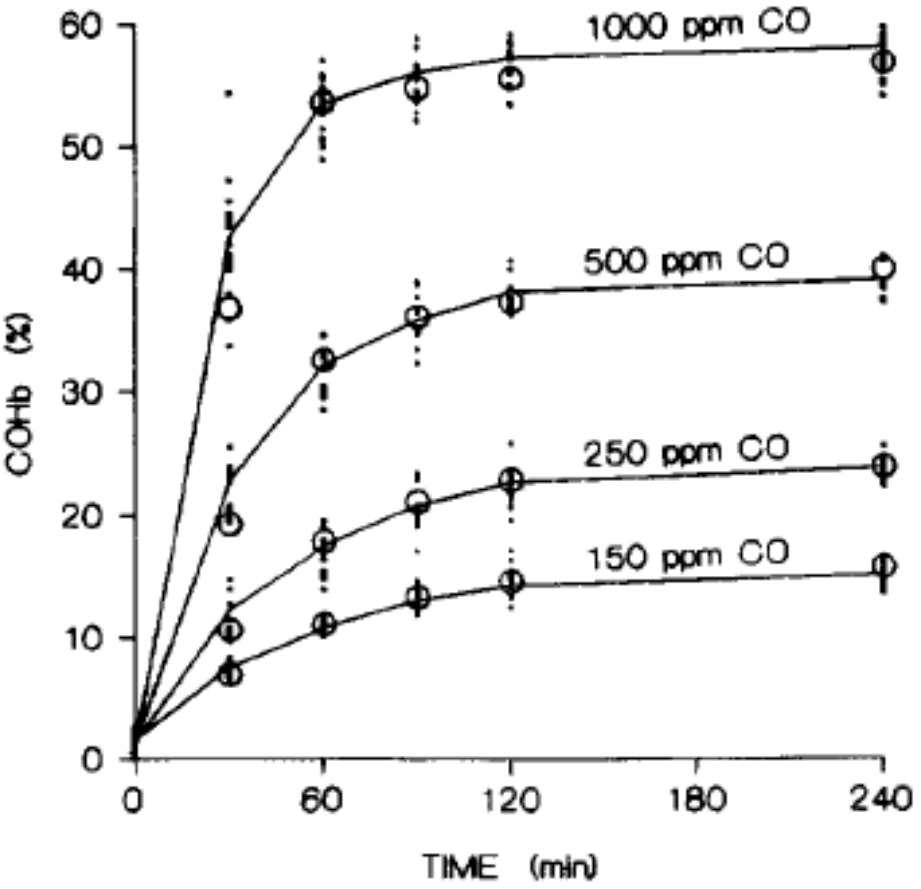


Figure 1: The **Benignus-Annau Curve**: Plots COHb% as function of time of exposure. Small points are observed individual values, circles are CFKE predicted values. Lines are drawn through means of observed data (Benignus and Annau 1994).

The experiment was designed in three phases. Phase I determined the exposure time and CO concentration. This was guided by the dosing curve generated by Benignus and Annau (Figure 1)(Benignus and Annau 1994). This curve predicts COHb levels for various concentrations of CO in air as a function of exposure time. Benignus and Annau developed this predictive curve using data obtained from experiments using inbred, Long Evans rats and we were using Sprague-Dawley rats as did Piantadosi (Benignus and Annau 1994; Piantadosi, Zhang et al. 1997). These two differences (different strain and outbred) require verification of the predictive model for this entire study.

The ultimate goal of this study was to provide evidence that HBOT is superior to NBOT in the prevention of CO induced DNS. In order to do this, it was necessary to induce DNS in a maximum number of CO exposed rats. To do this, we chose endpoints of 50-55% COHb blood level and syncope. These two endpoints are predictors for DNS development in humans, particularly syncope (Myers, Snyder et al. 1985; Lee and Marsden 1994; Choi, Kim et al. 1995; Thom, Taber et al. 1995; Mimura, Harada et al. 1999; Lee, Mak et al. 2001; Gilmer, Kilkenny et al. 2002; Weaver, Valentine et al. 2007). We postulated that these two endpoints would produce the desired effect in the maximum number of rats surviving the CO exposure. Phase II is the development of a viable rat behavioral model for DNS. A thorough review of the current literature showed a wide variety in CO exposure techniques, a lack of a standard rat model for DNS, and vague or nonexistent descriptions of visible clinical signs of CO induce DNS in aniumals (Piantadosi, Zhang et al. 1997; Gilmer, Kilkenny et al. 2002; Gu, Januszkiewicz et al. 2005). A measureable, repeatable, readily observable rat model of CO in would need to be developed for this and the following phase of this experiment. Phase II draws heavily on the work of Piantadosi. After Piantadosi, Sprague-Dawley rats were chosen for this experiment, and neurobehavioral tests were to be

performed on days 1, 7, 14, 21, and 28 post exposure (Piantadosi, Zhang et al. 1997). Unlike Piantadosi, our neurobehavioral test would be limited to Open Field tests as personnel and equipment constraints prevented the effective use of a Radial Arm Maze for testing learning and memory.

Phase III is designed to challenge the neurobehavioral model developed in Phase II with room air, normobaric oxygen, or hyperbaric oxygen. Phase III employs 100% O2 at 3 atmospheres of pressure for 1 hour as described (Tibbles and Perrotta 1994; Gilmer, Kilkenny et al. 2002; Weaver, Hopkins et al. 2002; Isbister, McGettigan et al. 2003; Scheinkestel and Millar 2008; Tomaszewski 2008; White 2008). Phase III occurs in two sub phases: immediate treatment or delayed treatment.

The histological regions of the brain selected for study are: the basal ganglia (exterior *globus pallidus*), hippocampus (*dentate gyrus*), and cortex per Piantadosi (Piantadosi, Zhang et al. 1997). These are the most metabolically active areas of the brain thus most likely to be affected by CO intoxication (Choi 1983; Ginsberg 1985; Ishimaru, Katoh et al. 1992).

HYPOTHESES AND OBJECTIVES

Hypothesis

The administration of Hyperbaric Oxygen after Carbon Monoxide intoxication will prove more effective in preventing delayed neurological sequelae than normobaric oxygen or room air.

Sub Hypotheses

1) Delayed Neurological Sequelae are produced by necrosis and/or increased levels of apoptosis in the basal ganglia, cortex, and/or hippocampus.
2) There is a therapeutic window outside of which hyperbaric oxygen is no more effective than normobaric oxygen or room air.

Objectives

1) Establish in rat a model of DNS following CO intoxication
2) Challenge model with hyperbaric or normobaric oxygen treatment
3) Establish treatment "window"

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) at 250-360g body weight were used in this study. The rats were kept in an AAALAC approved facility on a 12 hour light/dark cycle with food and water *ad libitum*. All rats were observed daily until euthanization (see below). All procedures involving live animals were approved by the WPAFB Institutional Animal Care and Use Committee (IACUC), Protocol # F-WA-2008-0107-A.

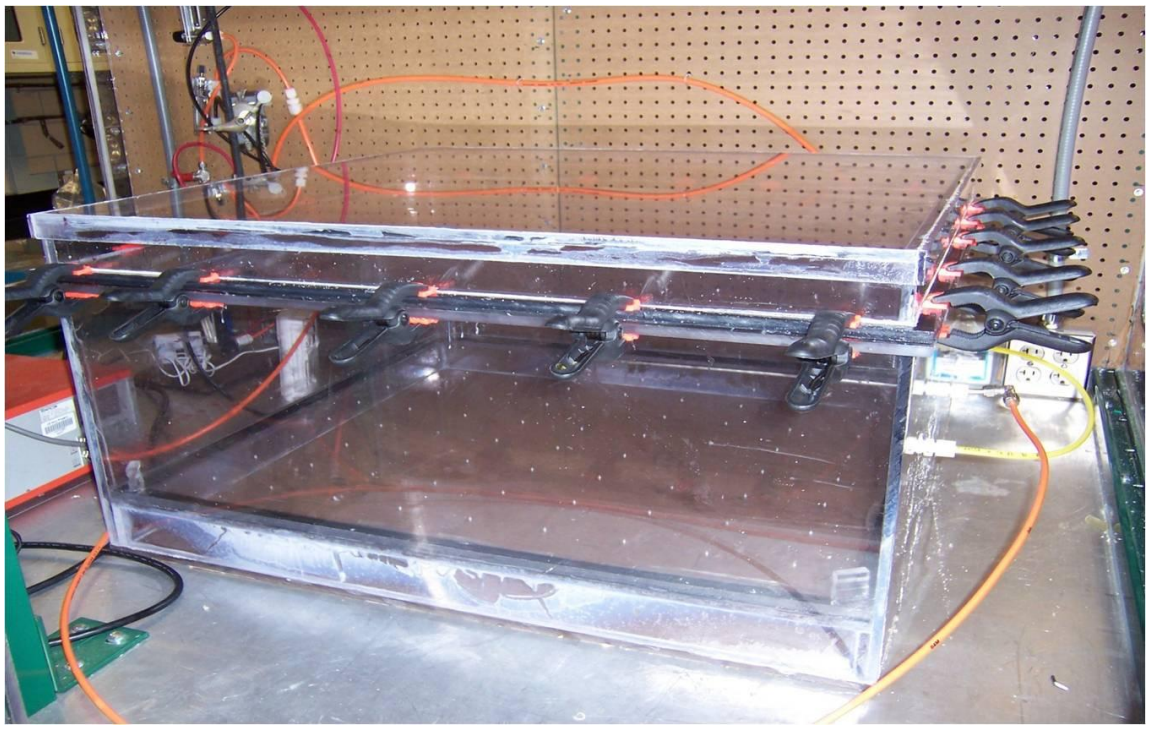
CO Exposure

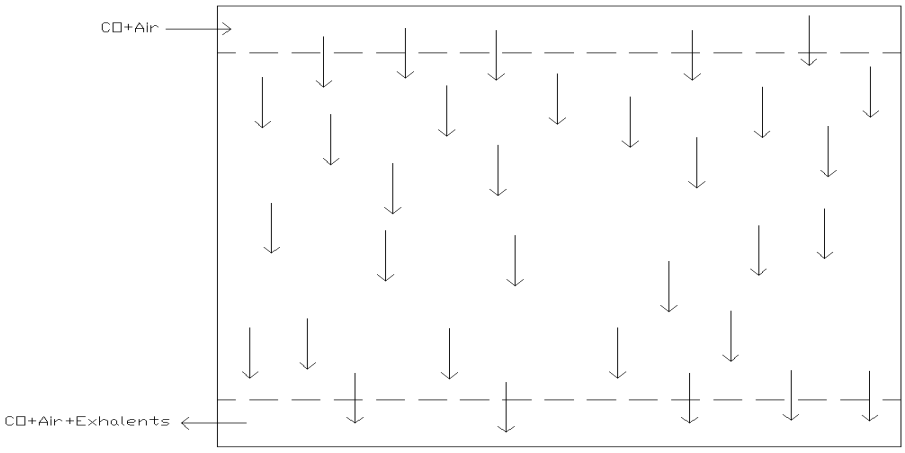
Determination of Exposure Time at 1000 ppm CO in Air

Individual groups of rats were exposed to one time 1000 ppm CO in air (Weiler Welding, Moraine, OH) in an exposure chamber of our own design (Reboulet, Lear et al. 2009) for a one time exposure at 75, 90, 105, 120, or 180 minutes until a carboxyhemoglobin level of least 50% and/or syncope (See Picture 1). CO concentrations were monitored in real time with a BINOS monitor (Infincon, East Syracuse, NY). Final exposure time repeated one time to confirm (See Figure 2).

Determination of Exposure Time at 3000 ppm CO in Air

Individual groups of rats were exposed to one time, 3000 ppm CO in air (Weiler Welding, Moraine, OH) in an exposure chamber of our own design (Reboulet, Lear et al. 2009) for a one time exposure at 135 minutes until a carboxyhemoglobin level of least 50% and/or



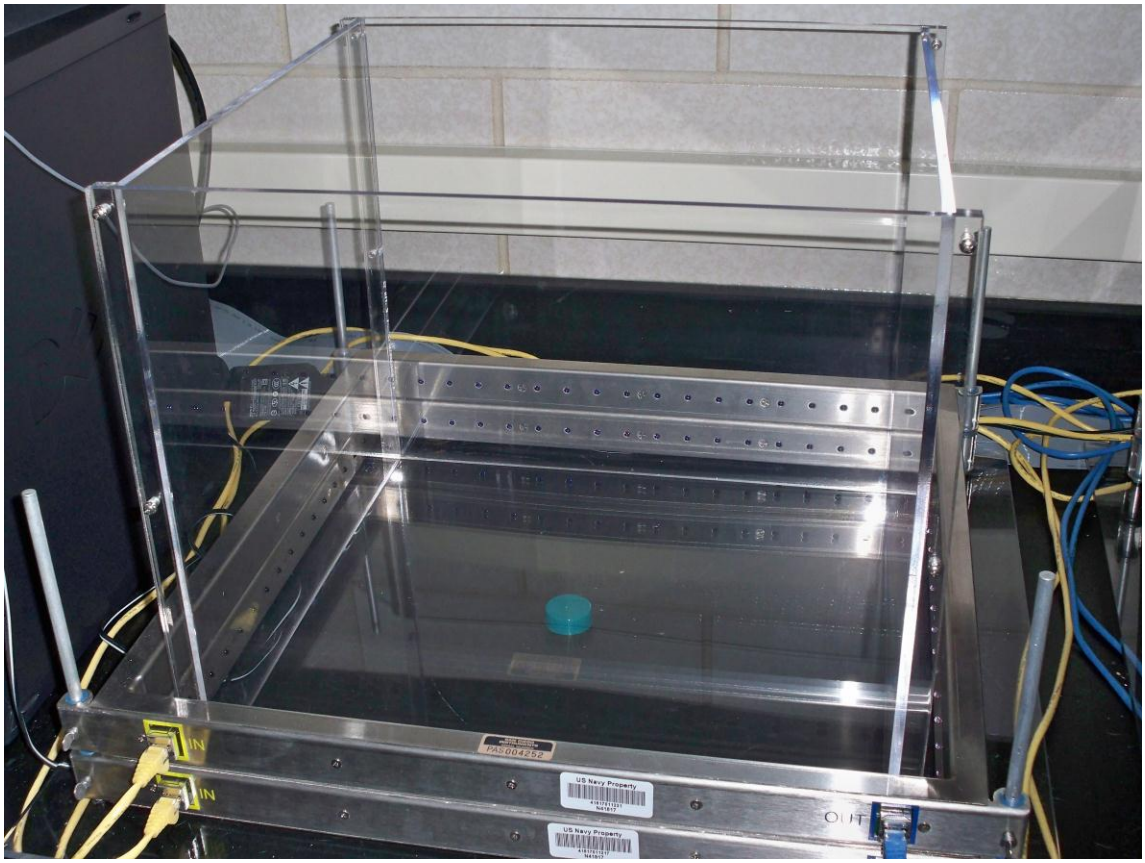


Picture 1: Photo and Schematic of the exposure chamber. Note how exhalents are drawn out through the bottom plenum. Chamber is under slight negative pressure. It provides 15 atmospheric exchanges per minute (Reboulet, Lear et al. 2009).

syncope (see Results). CO concentrations were monitored in real time with a Model 880A monitor (Rosemount Analytical, Solon, OH). Final exposure time was repeated to confirm (See Figure 2).

Development of CO Induced DNS Neurobehavioral Model

Rats were exposed to CO in air at either 1000 or 3000 ppm for times determined above. Rats were returned to their cages to recover in ambient room air. Control animals were placed in the exposure chamber for the same time period in only room air. All CO exposed rats were observed for mortality for 1 hour post exposure, and living animals were returned to the Vivarium. All rats had access to food and water *ad libitum* post time in the exposure chamber. At 24 hrs, 7, 14, 21, and 28 days post exposure, all rats (control and exposed) were individually studied in PAS-OF chambers (San Diego Instruments, San Diego, CA), where their movements (active time, number of rears, distance traveled) measured via light beam breaks (See Picture 2). The placement of the rats in the PAS chambers was designed so that no rat would be in the same cage twice or next to the same rat or rats twice. The duration of the testing was 30 minutes while their movements were recorded using PAS Recorder software (v.2.07.101 San Diego Instruments, San Diego, CA). The spacing of testing days was done to prevent habituation. A random selection of rats, both control and CO exposed, was video recorded with a Handycam HDR-SR11 digital camcorder (SONY, Japan) to capture any observable clinical signs of sequelae in CO exposed rats. All neurobehavioral testing was done in low light conditions with 65 decibels background, white noise was generated by a TX-903 tuner amplifier (Onkyo, Japan). This was done to control environmental conditions in the testing room as tightly as possible.



Picture 2: The PAS Open Field testing chambers. Movements are measure by the rats breaking light beams crisscrossing the chamber.

Figure 2: Master plan for optimization of CO exposure animal usage. Actual animal usage did not reflect what is pictured here. This general model was used for all CO exposure optimizations.

Exposure to CO Prior to Treatment in Hyperbaric Chamber

CO Exposed Rats

Rats were exposed to 3000 ppm CO in air for 75 minutes (not including a 20 minute ramp up time to 3000 ppm CO, and a 5 minute chamber evacuation time). After the CO was evacuated the rats were removed from the exposure chamber, and placed into the hyperbaric chamber. For the "immediate treatment" subgroup, the rats were placed in to the hyperbaric chamber immediately after the CO had been evacuated from the exposure chamber. For the "delayed treatment" subgroup, the rats were returned to their home cages for 4 hours. After four hours in their home cage the rats were returned to the dosing cages and placed into the hyperbaric chamber. During the 4 hours in their home cages the rats with food and water *ad libitum (*See Figure 3).

Control Rats

Control rats treated as described above without exposure to CO. These rats spent the same time in the exposure chamber, but were only exposed to room air.

One rat was kept completely naïve. It did not spend time in either the exposure chamber or the hyperbaric chamber. It was not exposed to CO or O_2. This rat's brain tissue served as the control tissue for the TUNEL and H&E analyses.

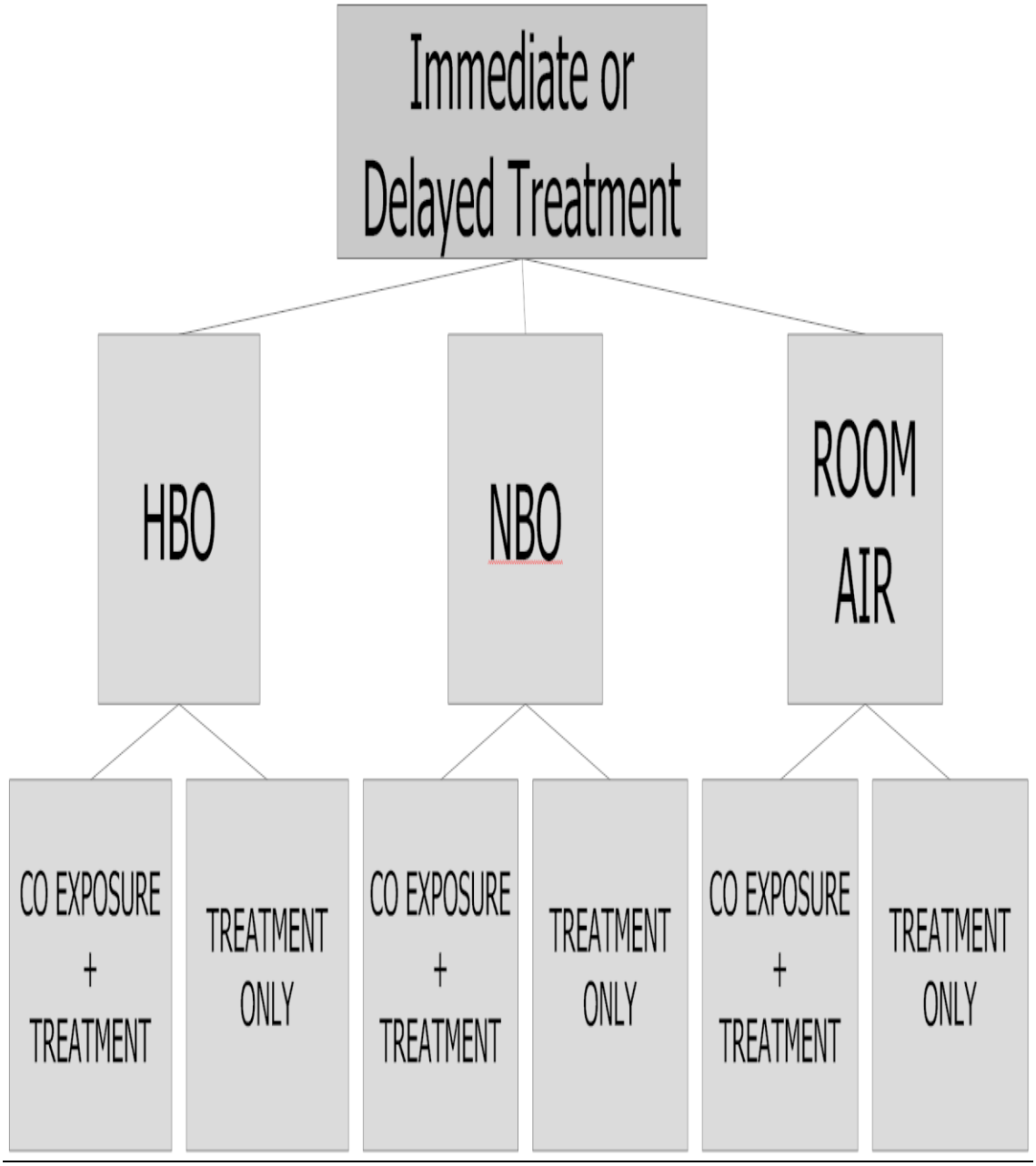


Figure 3: Master plan for treatment subgroups. This model was used for both immediate and delayed treatment procedures. This chart also shows the division of control and exposed rats.

Treatment of Rats in Hyperbaric Chamber

Room Air Treatment Sub Group

All rats(control or CO exposed) were placed in the hyperbaric chamber (Type II Animal Hyperbaric Chamber No. 33629 – Dixie Mfg. Co., Baltimore, MD) for 120 minutes and treated with room air at ambient temperature and pressure.

Hyperbaric Oxygen Treatment Sub Group

All rats (control and CO exposed) were placed into the hyperbaric chamber (Type II Animal Hyperbaric Chamber No. 33629 – Dixie Mfg. Co., Baltimore, MD) and treated with 3 atmospheres of 100% O_2 (See Picture 3) . The total time the rat spent in the hyperbaric chamber was 120 minutes. This time included 20 minutes pressurization and atmosphere change out to 100% O_2(99.999% Research Grade - Weiler Welding, Moraine, OH); 60 minutes at 3 atmospheres with 100% O_2; and 40 minutes decompression and venting of O_2 in chamber to room air. Oxygen levels were monitored using a Percent Oxygen Analyzer (Teledyne Analytical Instruments, City of Industry, CA).

Normobaric Oxygen Treatment Sub Group

All rats (control and CO exposed) were placed in the hyperbaric chamber and treated with 100% O_2 for a total of 120 minutes. In order to mimic the 100% O_2 exposure time of the hyperbaric treatment, the time included 5 minutes atmosphere change out to 100% O_2 and 5 minutes of venting out the 100% O_2 in the chamber to room air (20% O_2). Oxygen levels were monitored as above.

Picture 3: The hyperbaric chamber. It is design to provide up to 1500 psi. The experiment used 44.1 psi (3 ATA).

All rats, regardless of treatment sub group, were observed periodically for signs of stress or discomfort while in the hyperbaric chamber. After completion of treatment, the rats were removed from the chamber and returned to their home cages with food and water *ad libitum*. The rats were observed for 1 hour post treatment before being returned to the vivarium.

Post CO Exposure and Treatment Neurobehavioral Testing

All rats (control and CO exposed) were individually placed in PAS-OF boxes for 30 minutes as described earlier at: 24 hours, 7, and 14 days post exposure. Their movements were recorded as described. After neurobehavioral testing on day 14, the rats were anesthetized and euthanized. Their brains were harvested, processed and preserved as described.

Anesthesia, Blood Work, and Euthanasia

All rats were anesthetized prior to blood sampling and euthanasia with an intraperitoneal injection of 0.5 ml of sodium pentobarbital solution (Nembutol®, Ovation Pharmaceuticals, Deerfield, IL) using a LO-DOSE® U-100 insulin syringe with a 28 G, ½ inch needle (BD, Franklin Lakes, NJ). Invasive procedures were performed only after rats were no longer responsive to toe pinch.

Blood was drawn via heart stick with a 3 mL syringe with an 18G 1 ½ in needle (BD, Franklin Lakes, NJ). Blood was analyzed for HbCO% with an IL-682 COOximeter (Instrumentation Laboratories, Bedford, MA) Rats were euthanized immediately post blood draw via rapid decapitation.

Preparation and Preservation of Brain Tissue

Brains were removed from rats after decapitation. The brains were cut coronally at Bregma --6.00 mm and -2.04 mm to produce brain sections containing either the hippocampus or the basal ganglia. The posterior section containing the cerebellum was discarded. The desired section were then placed individually to cryomolds (Peel-A-Way Embedding Molds, Ted Pella, Redding, CA) with sufficient tissue freezing medium (TFM, Fisher Scientific, Pittsburgh, PA)to cover. Brain sections were placed in a freezer and frozen to -20°C. After 24 hours, frozen cubes were transferred to zip top freezer bags to prevent drying.

Histology

Tissue slices of the hippocampus and basal ganglia were made to a thickness of 7.5 μm on an OTF 5000 Crytostat (Bright Instrument Co. Ltd, Cambridgeshire, UK). Six slides of each brain section were prepared with three serial slices per slide.

Tissue slides were preserved in 4% paraformaldehyde in PBS for 20 minutes, rinsed in PBS, allowed to air dry, and returned to -20°C

Hemotoxylin and Eosin Staining

H&E staining was performed in an automatic DRS-601 Sakura Diversified Stainer (Sakura-FineTek, Torrence, CA) per established methods. Slides were cover slipped using a Sakura Tissue Tek Film (Sakura FineTek, Torrence, CA).

Terminal Deoxynucleotidyl Tranferase dUTP Nick End Labeling (TUNEL)

TUNEL assay was performed on 7.5 μm thick, cryopreserved brain tissue slices using the *In Situ* Cell Death Detection Kit, Fluorescein or TMR (Roche, Indianapolis, IN). The

manufacturer's assay protocol was followed with the following exceptions to optimize the procedure. All post fixation incubations were carried out at 37°C, permeablization was performed for 30 minutes, and the positive control incubation was performed for 15 minutes. The permeablization solution employed a 0.2% Triton X-100 solution in 1X PBS buffered with 0.1% sodium citrate. TUNEL labeled slides were treated with Prolong Gold Antifade (Invitrogen, Eugene, OR) and cover slipped with Fisherfinest Premium Cover Glass (Fisher Scientific, Pittsburgh, PA).

Image Capture

H&E

Tissue slices were viewed using a MI6000B microscope (Leica Microsystems (Switzerland) Limited CMS GmbH) with a 10X objective under phase contrast settings. Images were taken with a DFC300FX camera (Leica Microsystems (Switzerland) Limited CMS GmbH) and processed with Leica Application Suite software (v.2.4.0R1[Build 795] Leica Microsystems (Switzerland) Limited CMS GmbH).

TUNEL

Tissue slices were viewed using a BX51 microscope (Olympus America, Center Valley, PA) with a 20X objective under the fluoroscein thiocyanate (FITC) filter. TUNEL images were taken with a Qicam Fast 1394 camera (Quantitative Imaging Corp, San Francisco, CA) and processed with QCapture software (v.2.90.1, Quantitative Imaging Corp, San Francisco, CA).

STATISTICS

COHb %

All measured values of COHb % were input into Graphpad Prism v5.03 where mean values were calculated and compared using a two tailed, t-test with 99% confidence levels. P values less than 0.05 were accepted as significant.

Neurobehavioral Data

All data generated from the PAS-OF system was uploaded into Microsoft Excel 2007, were mean, standard deviation, and standard error of the mean were calculated. These values were uploaded into Graphpad Prism v5.0 for graphing.

Comparison and evaluation of the neurobehavioral data was performed by Maj David Kaziska, PhD of AFIT (Air Force Institute of Technology, WPAFB, OH). His explanation of these very complicated calculations is as follows:

Repeated Measures Analysis

The data was studied using a repeated measures analysis at a level of significance of $\alpha = .05$. (Footnote: general reference is Kutner, Nachtsheim, Neter, & Li, *Applied Linear Statistical Models*, 5th Edition). For each of the four response variables, a repeated-measures ANOVA model was fit in JMP 8 software with the factors treatment, timing of the treatment (immediate or delayed), day (the measurements were taken on three days, and the days were a week apart), and group (exposed or control), and two and three way interactions of these factors. Additionally, the factor of "Animal" was evaluated as a nested factor within each Group. Final models were fit for each response variable using a backward elimination in

which insignificant factors were removed. The hierarchical model convention under which main effects were retained for significant interactions was followed.

Results and Interpretation

The Fixed Effects Tests indicate factors which are significant, factors in that output whose p-values are less than $\alpha = .05$ are the significant factors. In addition, the nested factor "Animal" is significant in each of the models based on the large value of the displayed log likelihood (the p value is not displayed for this output but this p is zero to many decimal places).

Post Hoc Analysis

Post hoc analysis was performed using Tukey's multiple comparisons. The Tukey method was chosen because it is a robust analysis, not sensitive to violations of normality.

RESULTS

Determination Of CO Exposure Time

1000 ppm

Dosing time at 1000 ppm CO in air was determined to be 180 minutes, not including 20 minute "ramp up" and 5 minutes chamber evacuation. This was made after 8 separate exposures on 8 separate groups at 75, 90, 105, 120 and 180 minutes. Two exposures on two separate groups were done at 120 and 180 minutes (see Table 1).

3000 ppm

Dosing time at 3000 ppm CO in air was determined to be 135 minutes, not including 20 minute "ramp up" and 5 minutes chamber evacuation. This was made after two separate exposures at 90 and 135 minutes on two separate groups(see Table 2).

Lot #	N	CO Dose (ppm)	Duration (min)	Avg COHb%	Date
R12	2	1000	75	43.3	12/1/2008
R12	2	1000	90	47.6	12/2/2008
R12	2	1000	105	48.9	12/2/2008
R12	4	1000	120	46.8	12/5/2008
R13	2	1000	120	48.5	12/22/2008
R13	7	1000	120	51.6*	12/22/2008
R14	2	1000	180	51.5	1/27/2009
R14	4	1000	180	46.8	1/27/2009

* COHb measured individually over time

Table 1: Animal usage for1000 ppm CO exposure optimization

Lot #	N	CO Dose (ppm)	Duration (min)	Avg COHb %	Date
R20	4	3000 ppm	90	*	4/6/2009
R20	3	3000 ppm	135	55.4	4/7/2009

* COHb measured individually over time

Table 2: Animal usage for 3000 ppm CO exposure optimization

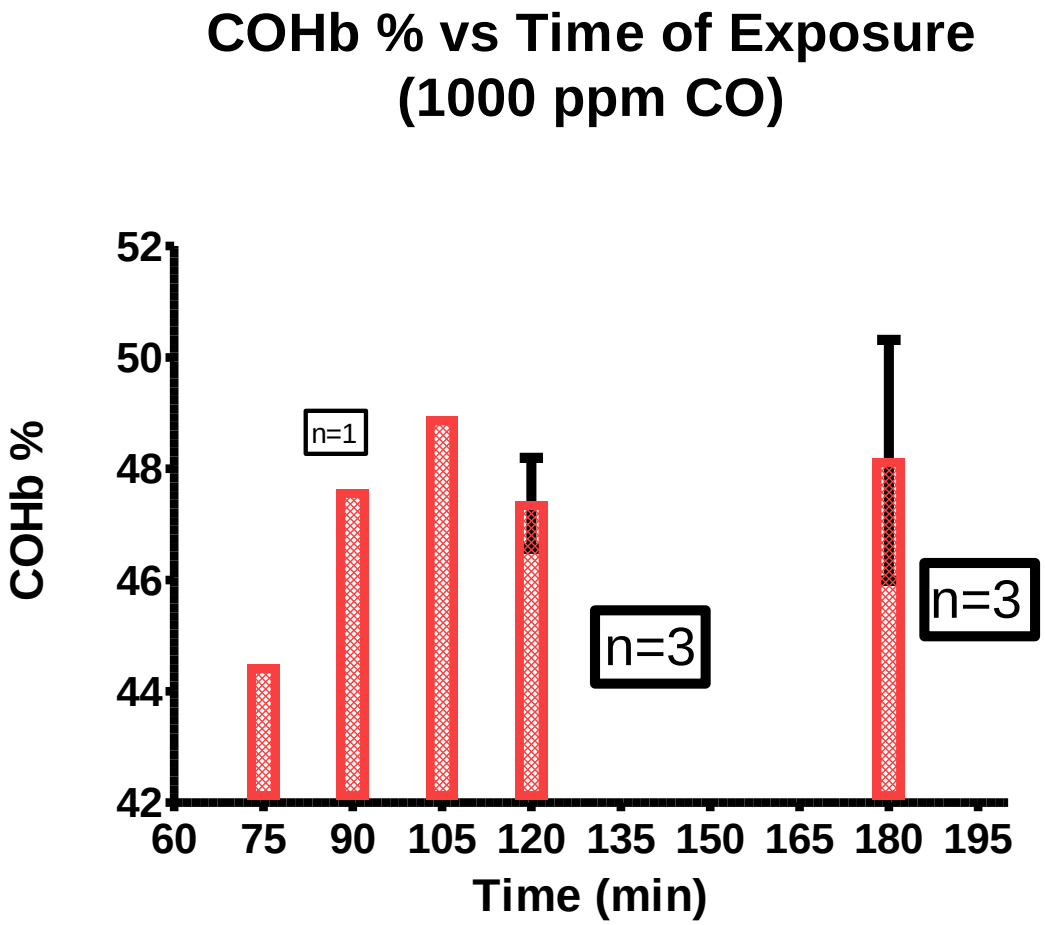


Figure 4: Graph showing plateau of COHb formation as a function of exposure time at 1000 ppm CO.

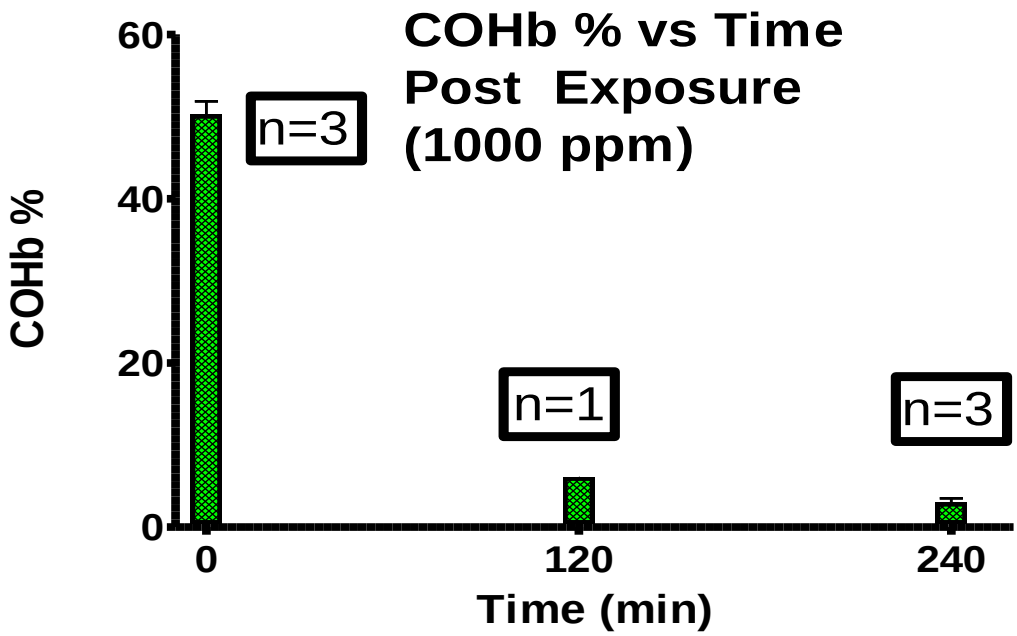
Determination of COHb Half-Life

1000 ppm

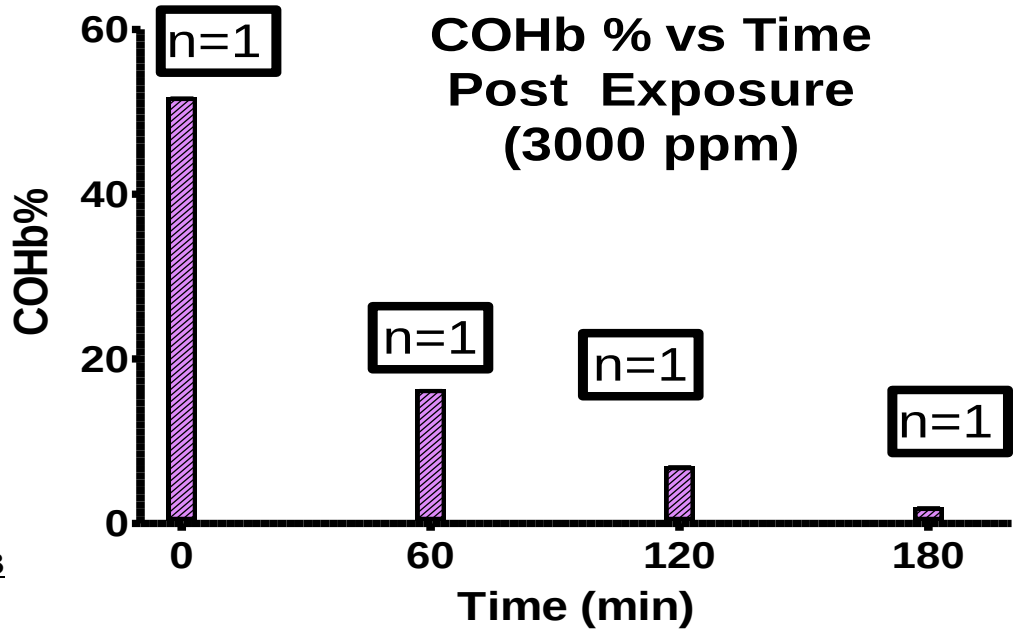
COHb half-life was determined by measuring the COHb percentages in individual rats at 0, 120, and 240 minutes post exposure to 1000 ppm CO for 120 minutes (exposure time does not include 20 minute "ramp up" and 5 minutes chamber evacuation)(Figure 4).

3000 ppm

COHb half-life was determined by measuring the COHb percentages in individual rats at 0, 60, 120, and 180 minutes post exposure to 3000 ppm CO for 90 minutes (exposure time does not include 20 minute "ramp up" and 5 minutes chamber evacuation)(See Figure 4).



A



B

Figure 5: Results of COHb measurement post CO exposure at 1000 ppm (A) and 3000 ppm CO (B). The 1000 ppm exposure was for 120 minutes. The 3000 ppm exposure was for 90 minutes. Note similarities in COHb half-life regardless of concentration. The number of rats used for each time point is indicated. One COHb reading was taken per rat.

DNS Neurobehavioral Model
1000 ppm

After exposing rats (n=16) to 1000 ppm CO in Air as described above, neurobehavioral testing yielded no statistically significant differences between control (n=8) rats and CO exposed rats at any time point. No fatalities resulted from this exposure (See Figure 5).

3000 ppm

After exposing rats (n=16) to 3000 ppm CO as described above, neurobehavioral testing yielded statistically significant differences between control (n=8) rats and CO exposed rats (n=12). The greatest difference was on day 14 post exposure (See Figure 6).

The protocol was modified to reflect this data. The neurobehavioral testing was shortened to 14 days post exposure.

Four rats (25%) died in the final 10 minutes of CO exposure. The COHb level was measured as described for the deceased rats (n=4) at 71.4 $\pm$ 0.7 %.

Dosing Modifications

It had been determined that CO exposure at 3000 ppm would last 135 minutes. When this was attempted, all the rats (n=16) died after only 120 minute exposure. All remaining rats (N=49) were resorted into three groups of 16 (control n=6, CO exposed n=10). The endpoint for the exposure was set at 25% mortality (the only endpoint we could use from the previous data (see above). Treatment options were kept as originally described. Both Vivarium staff and Charles River Laboratories, confirmed that this lot of animals were indeed outbred, Sprague-Dawley rats. Charles River Laboratories also confirmed these rats had come from the same breeding location as the rats used in the original 3000 ppm CO exposure time determination (see Methods and Materials). All equipment and gas was checked and found to be within specs.

Exposure duration was determined to be 75 minutes based on 25% mortality for all three treatment groups (room air, 100% hyperbaric oxygen, and 100% normobaric oxygen). All surviving rats were treated per the designated groups immediately prior to exposure as described.

This 75 minute CO exposure time would also be used for the "delayed treatment" phase of this experiment.

Treatment

No rats died during any treatment option in the hyperbaric chamber. If a rat survived the CO exposure then it survived through to the end of the experiment, without exception.

Neurobehavioral Testing Post Exposure and Treatment

No significant differences were found in the neurobehavioral data. This includes differences between control and CO exposed animals within treatment groups, and/or between treatment groups or sub groups (See Figures 7- 12).

Histology Post CO Exposure and/or Treatment

TUNEL

3000 ppm

No apoptosis was observed in the brain tissue sections of control or CO exposed rats at the time of sacrifice (15 days post exposure)(See Pictures 4-9).

H&E

3000 ppm Exposure

No eosinic neurons (necrosis) were observed in any of the brain tissue samples of the exposed and/or treated rats at the time of sacrifice (15 days post exposure) (See Pictures 10-15).

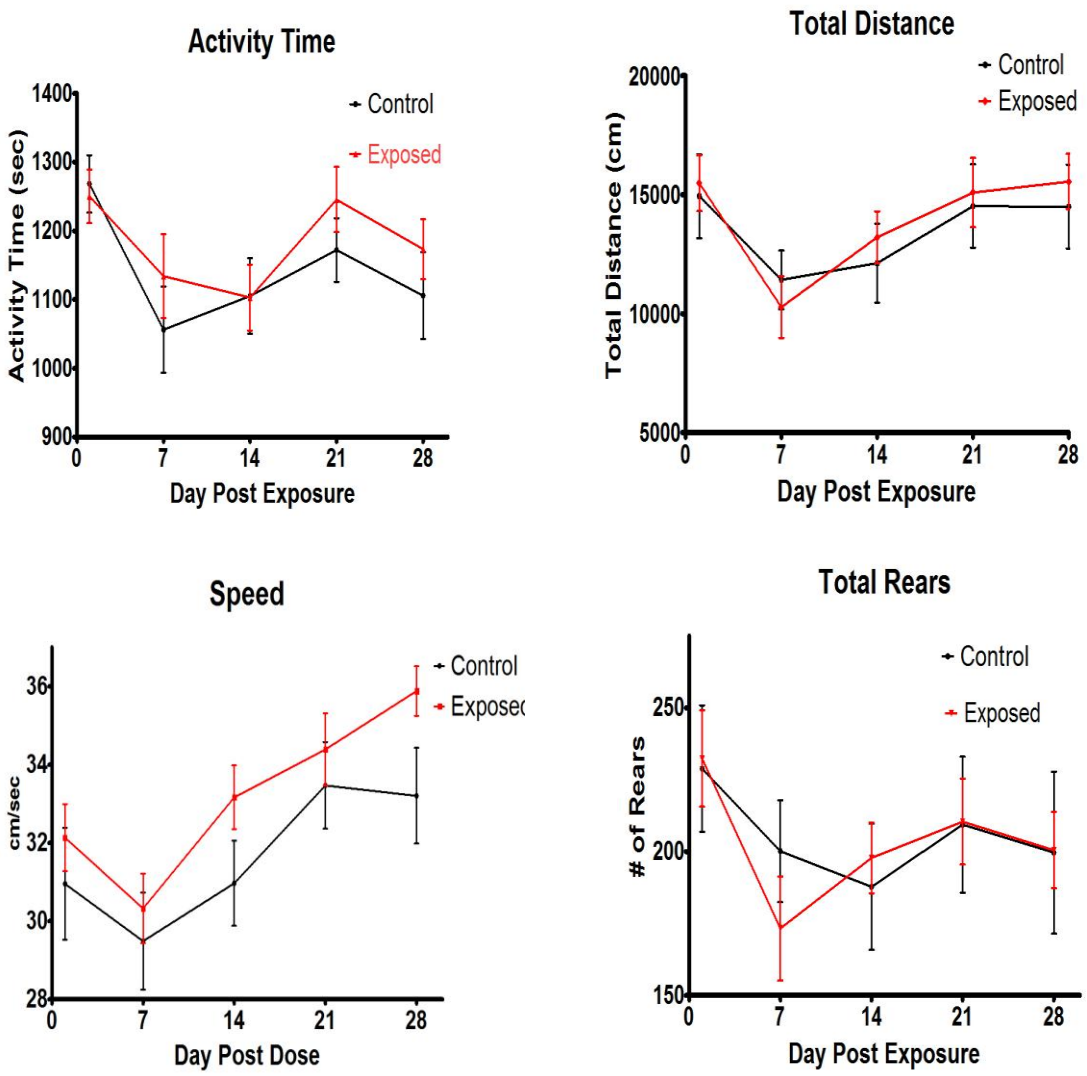


Figure 6: Neurobehavioral data from the 180 minute exposure at 1000 ppm CO. This exposure did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean $\pm$ SEM.

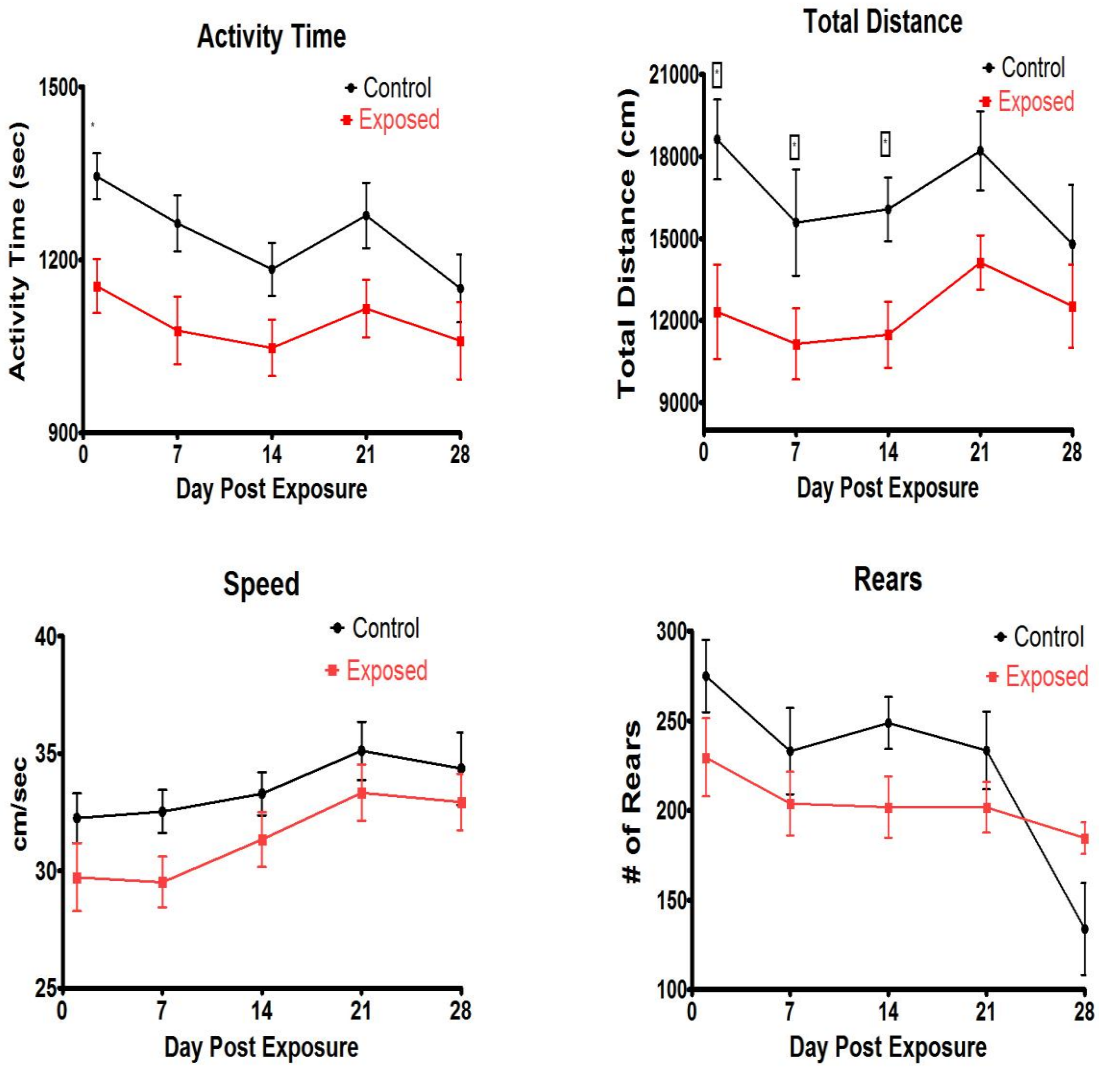


Figure 7: Neurobehavioral data from the 135 minute exposure at 3000 ppm CO. This exposure did produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean $\pm$ SEM. Differences peaked ($p=0.019$) at 14 days.

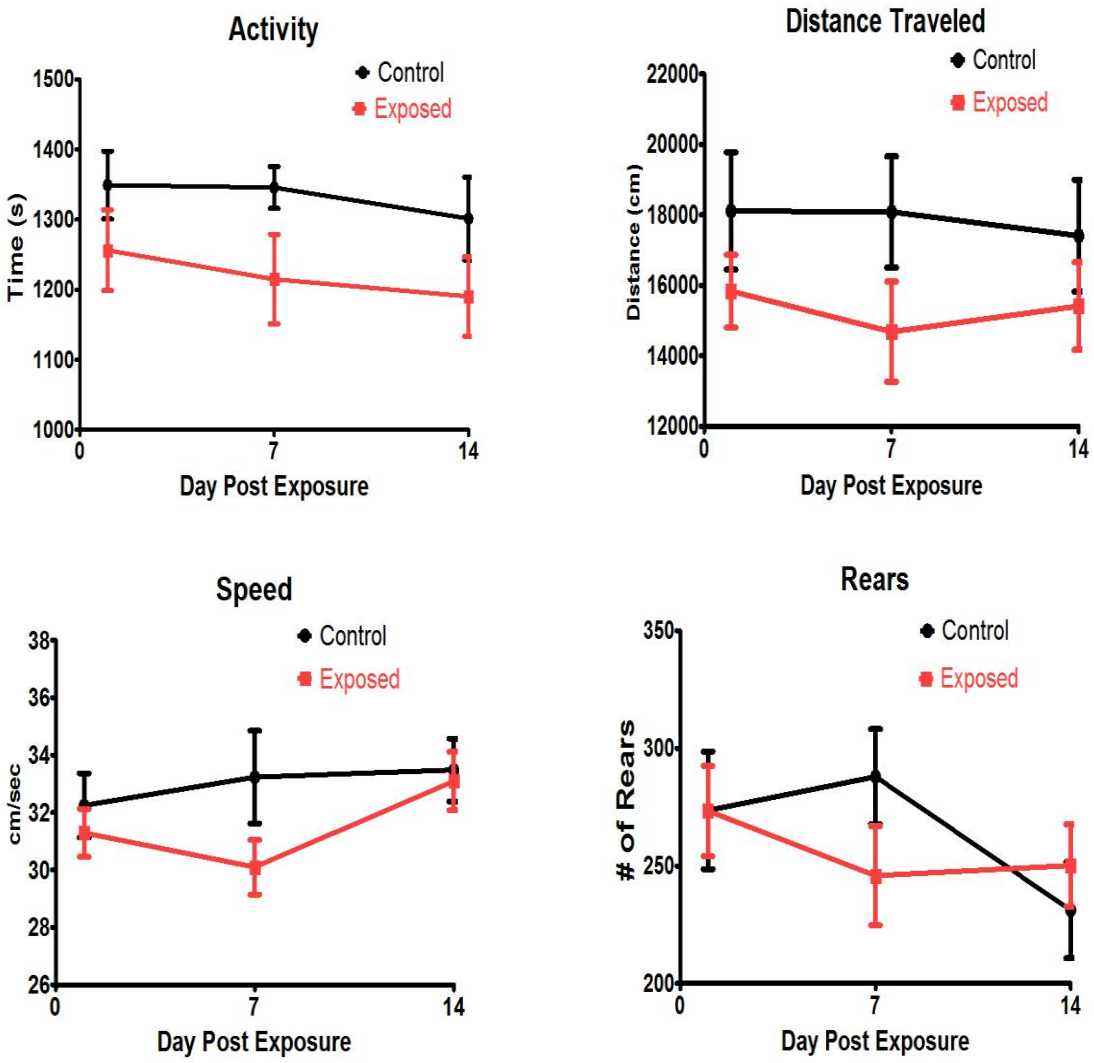


Figure 8: Neurobehavioral data from the 75 minute exposure at 3000 ppm CO with room air treatment immediately following. This exposure/treatment did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean $\pm$ SEM.

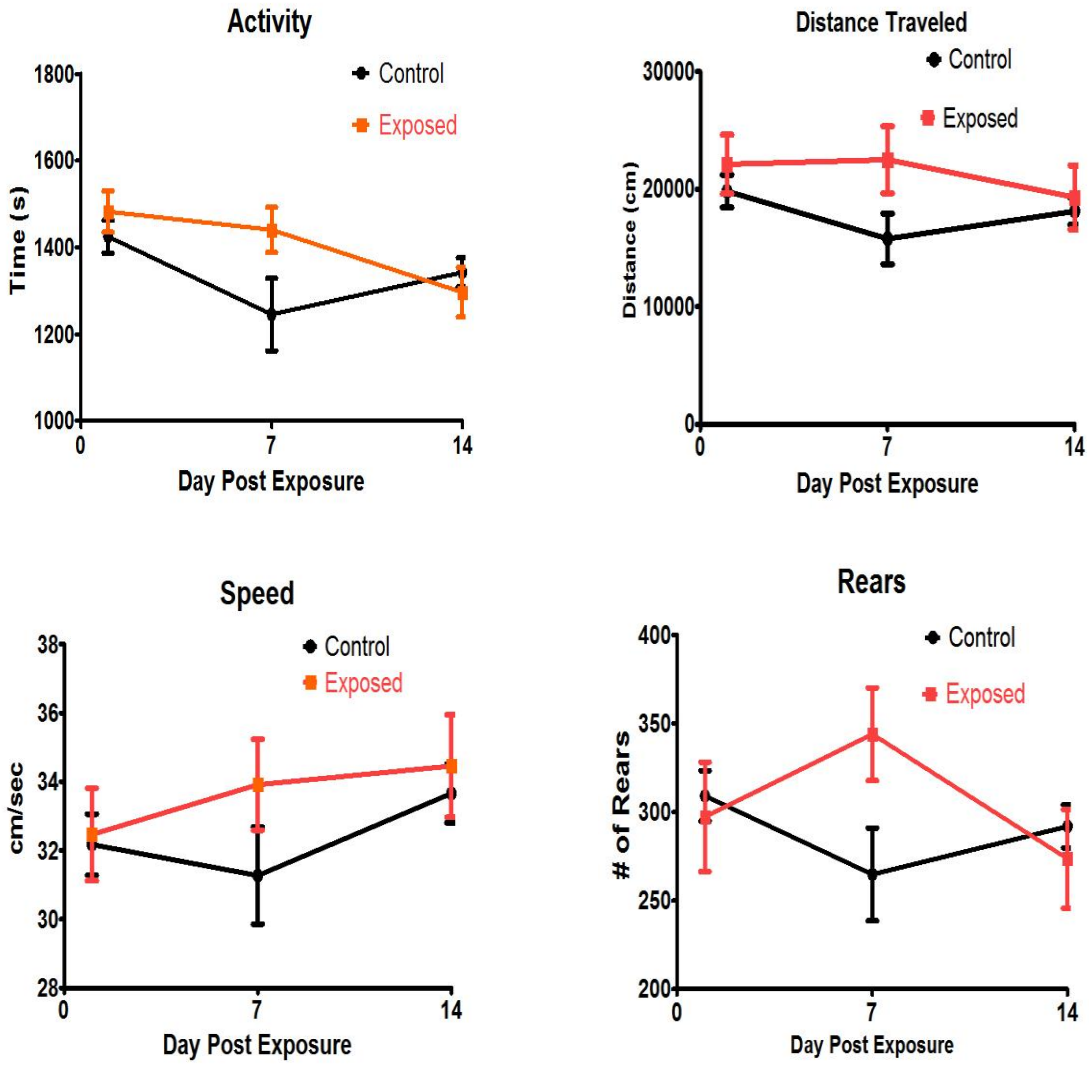


Figure 9: Neurobehavioral data from the 75 minute exposure at 3000 ppm CO with 60 minutes 100% HBOT immediately following. This exposure/treatment did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean ± SEM.

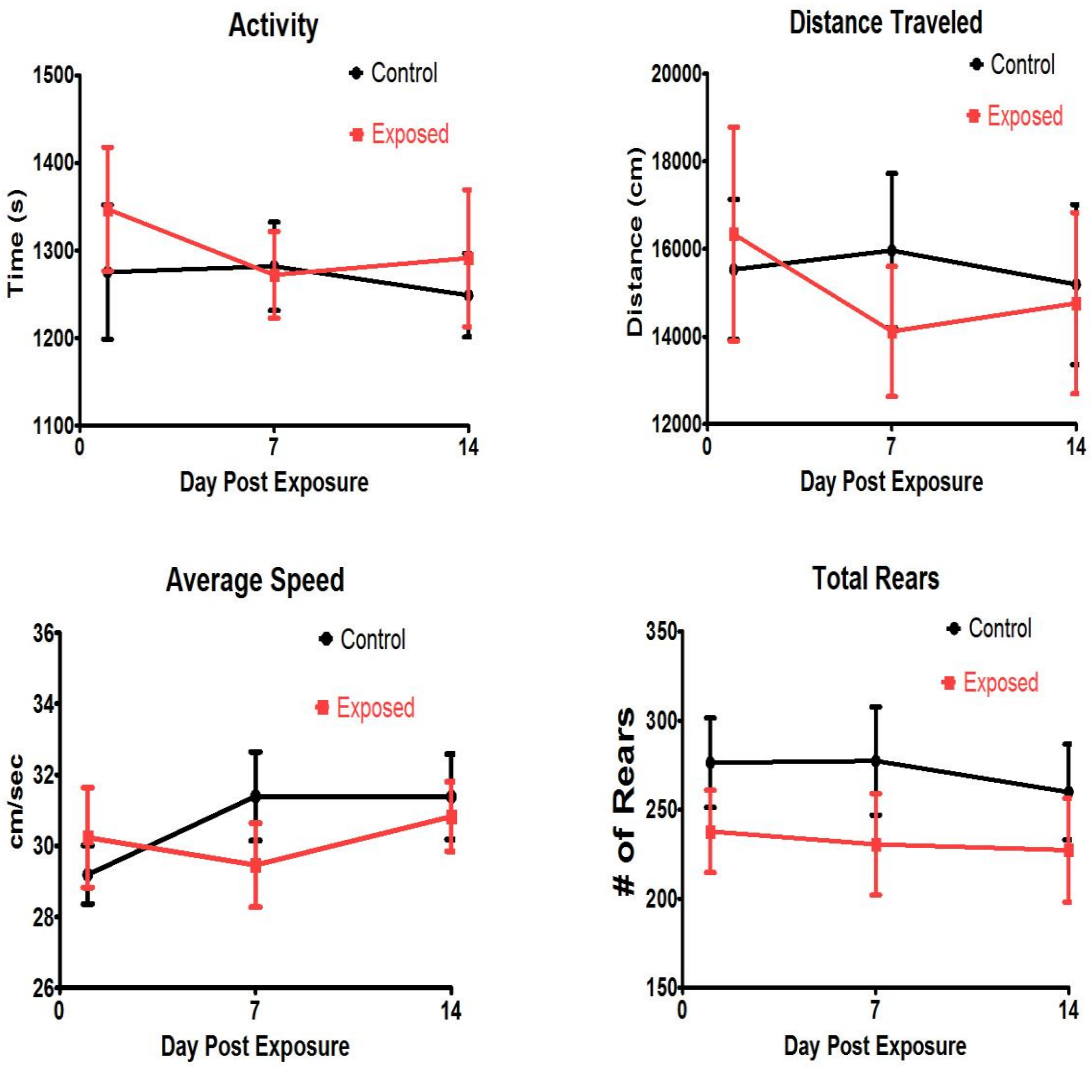


Figure 10: Neurobehavioral data from the 75 minute exposure at 3000 ppm CO with 120 minutes 100% NBOT immediately following. This exposure/treatment did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean ± SEM.

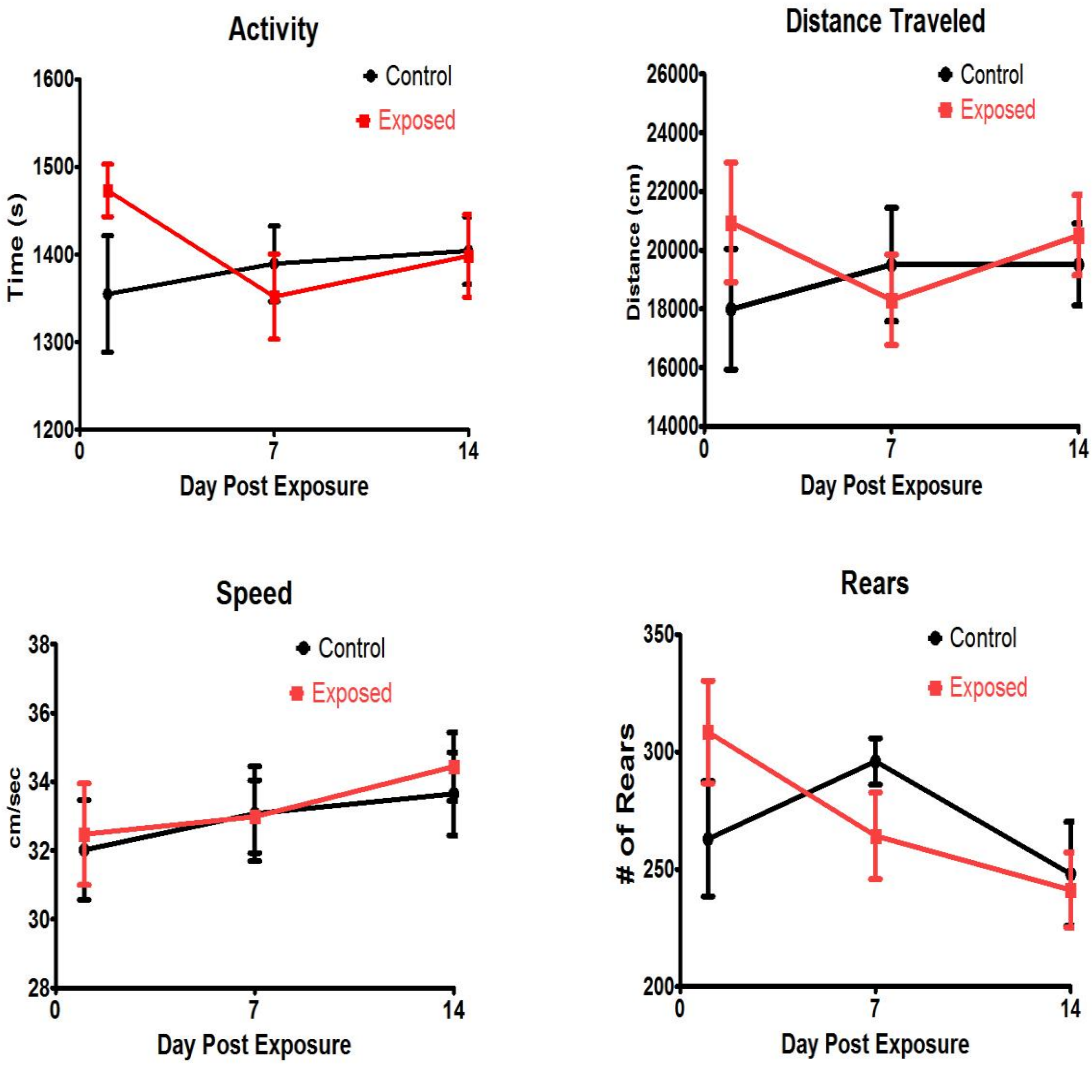


Figure 11: Neurobehavioral data from the 75 minute exposure at 3000 ppm CO with room air treatment after a 4 hour delay post exposure. This exposure/treatment did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean $\pm$ SEM.

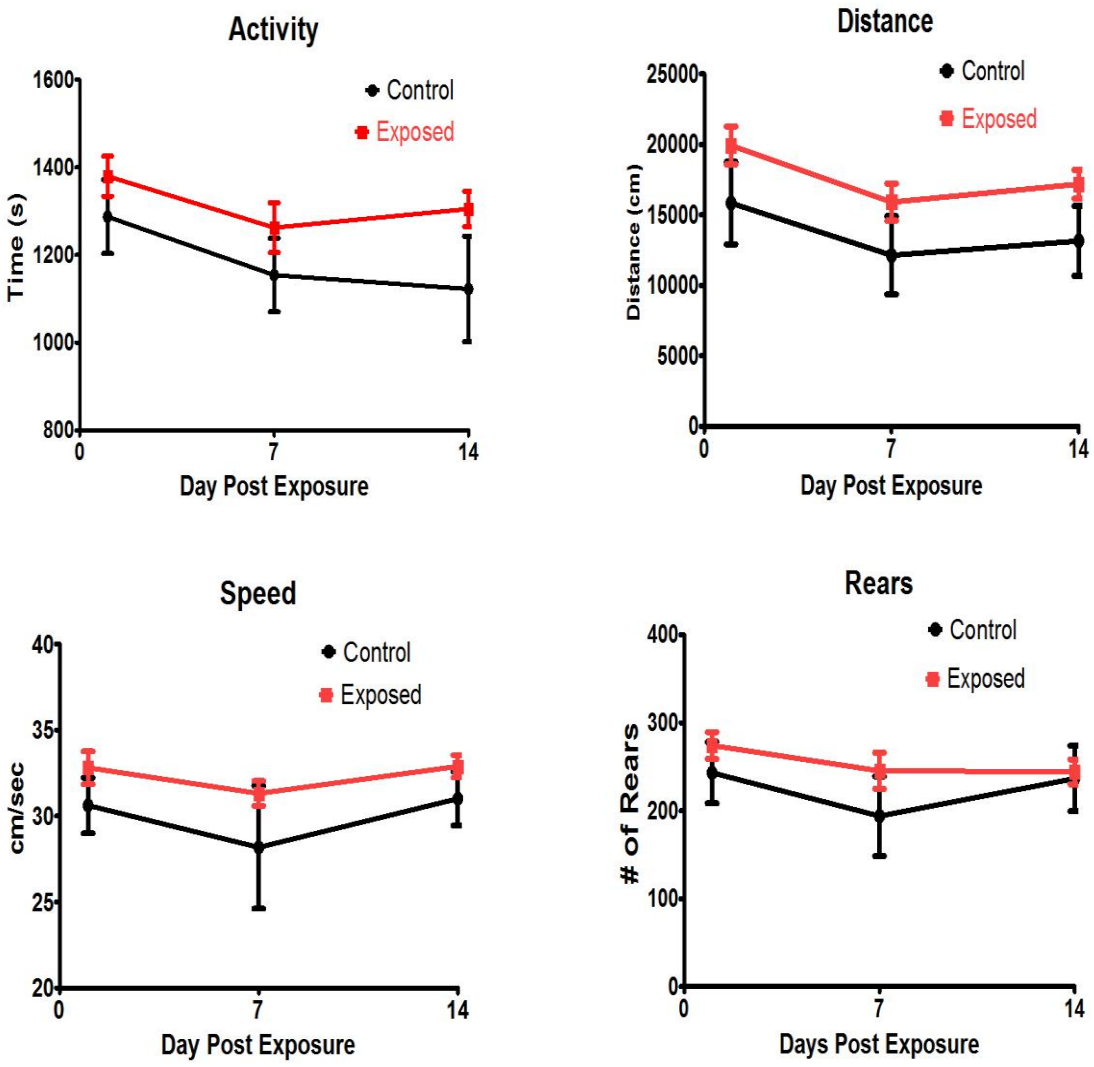


Figure 12: Neurobehavioral data from the 75 minute exposure at 3000 ppm CO with 60 minutes 100% HBOT after a 4 hour delay post exposure. This exposure/treatment did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean $\pm$ SEM.

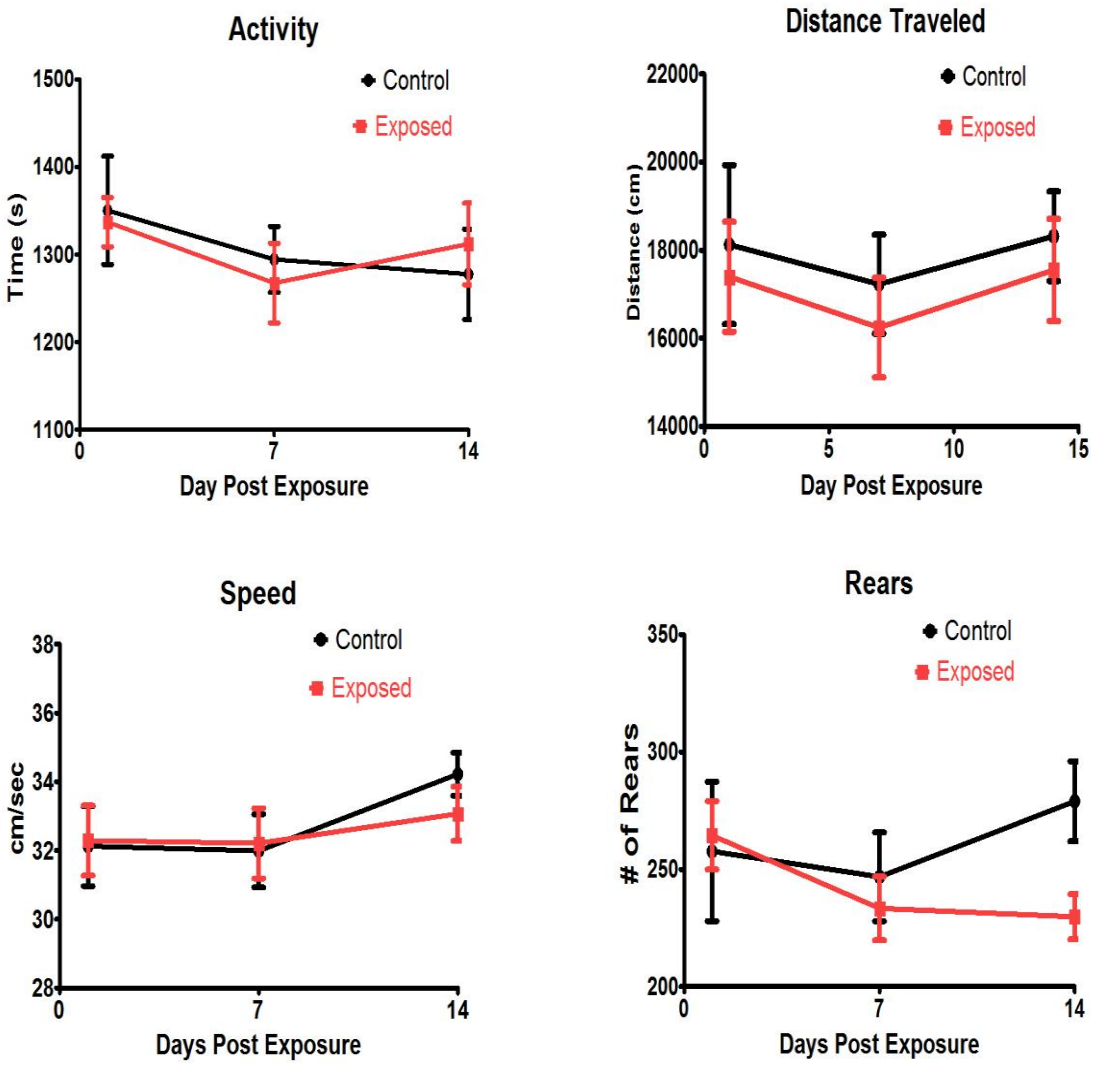
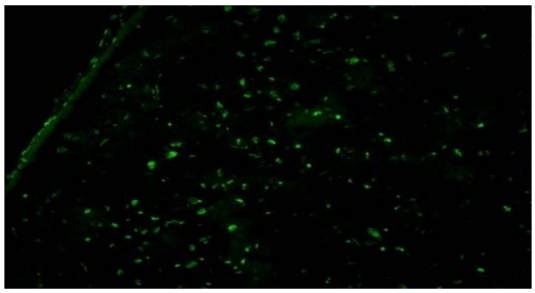
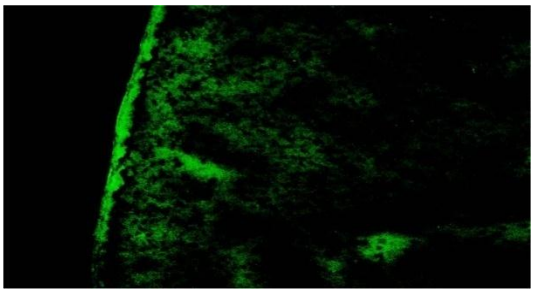


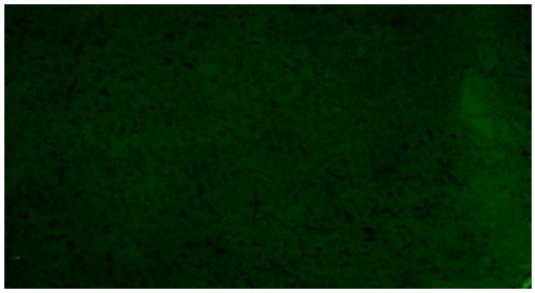
Figure 13: Neurobehavioral data from the 75 minute exposure at 3000 ppm CO with 120 minutes of 100% NBOT after a 4 hour delay post exposure. This exposure/treatment did not produce significant neurobehavioral differences ($p<.05$) between control and CO exposed rats. Values expressed as mean $\pm$ SEM.



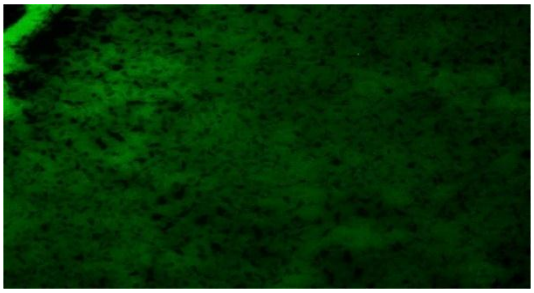
A – Positive Control (Naïve Rat)



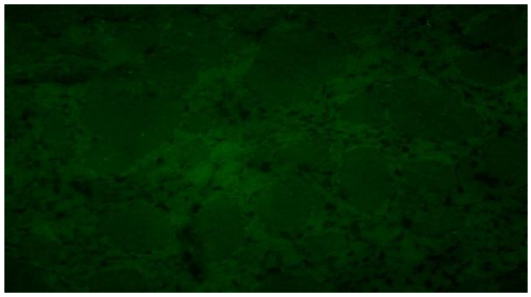
B – Negative Control (Naïve Rat)



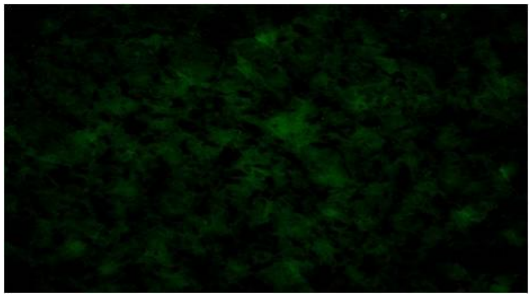
C – Control Cortex



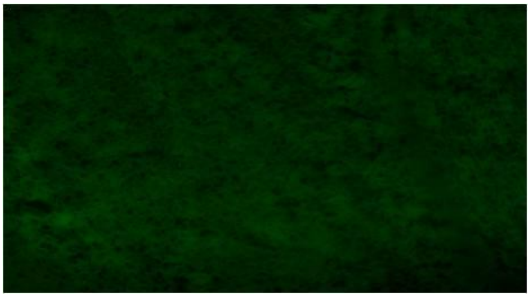
D – CO exposed Cortex



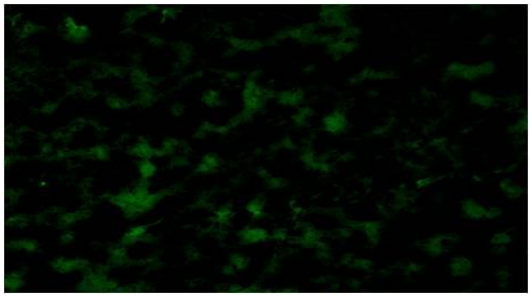
E – Control Basla Ganglia (EGP)



F – CO exposed Basal Ganglia (EGP)

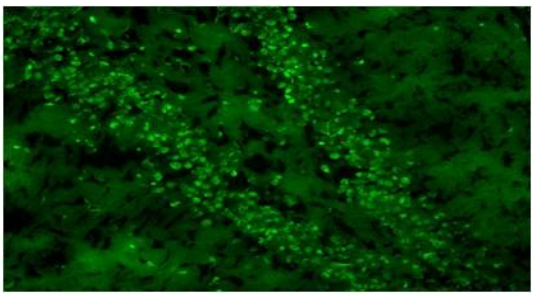


G – Control Hippocampus

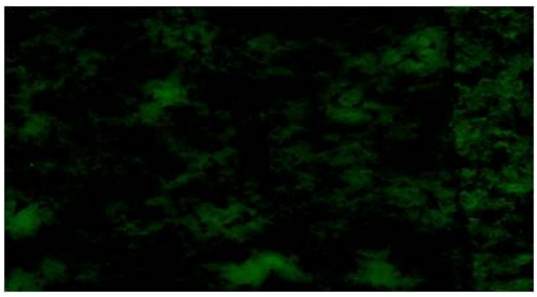


H- CO exposed Hippocampus

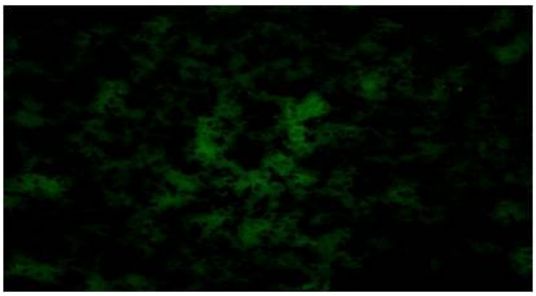
Picture 4: TUNEL Assay results on brain tissue for control and CO exposed rats treated immediately with room air. There are no apoptotic nuclei in either CO exposed or control rats. If damage due to CO exposure would have occurred it would have been in these rats. (20X Magnification)



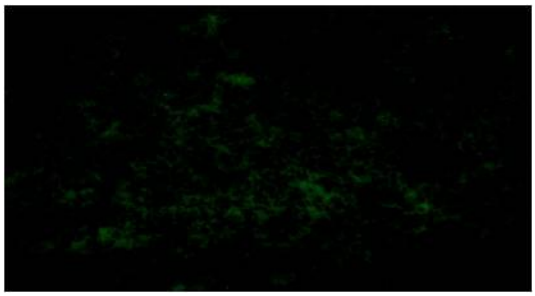
A – Positive Control (Naïve Rat)



B – Negative Control (Naïve Rat)

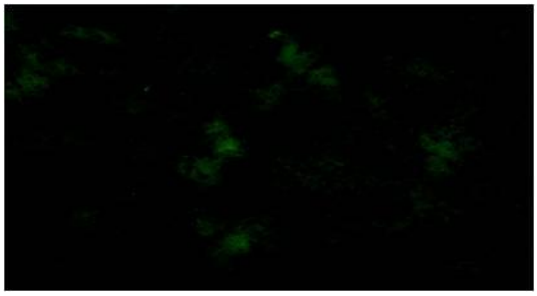


C – Control Cortex



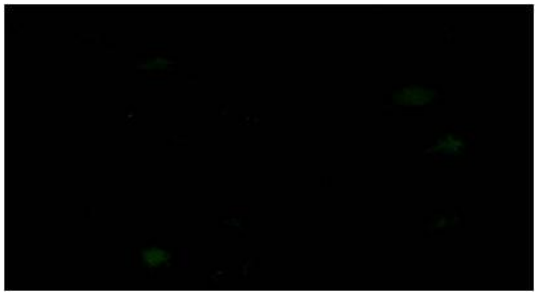
D – CO Exposed Cortex

E – Control Basal Ganglia (EGP)



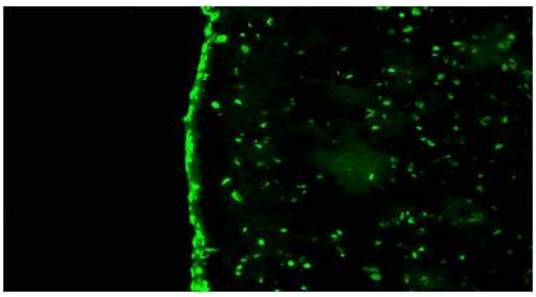
F – CO exposed Basal Ganglia (EGP)

G – Control Hippocampus

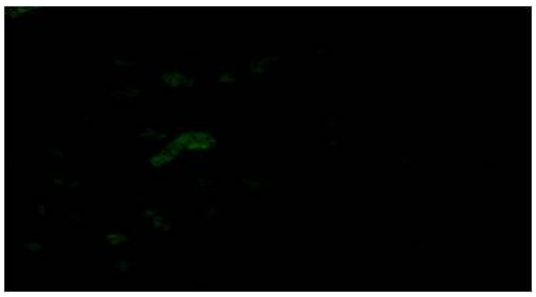


H – Co Exposed Hippocampus

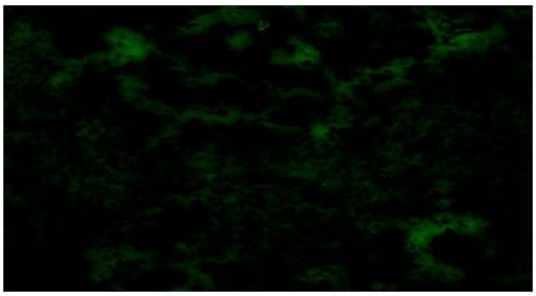
Picture 5 : TUNEL Assay results on brain tissue slices of control and CO exposed rats treated immediately with 100% hyperbaric oxygen treatment (HBOT). There are no apoptotic nuclei in either CO exposed or control rats. (20X Magnification)



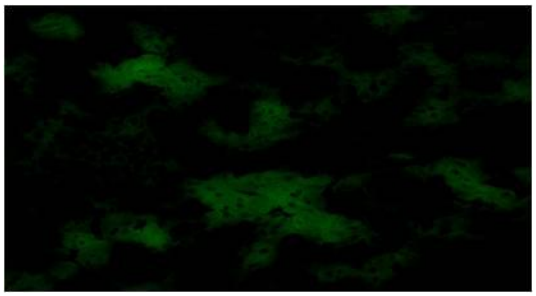
A – Positive Control (Naïve Rat)



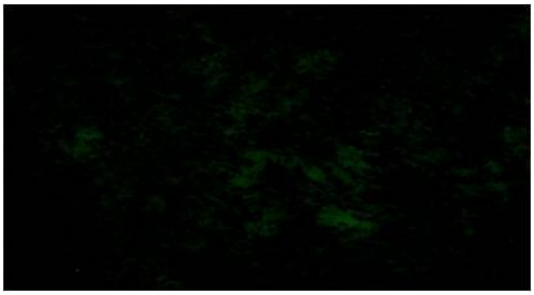
B – Negative Control (Naïve Rat)



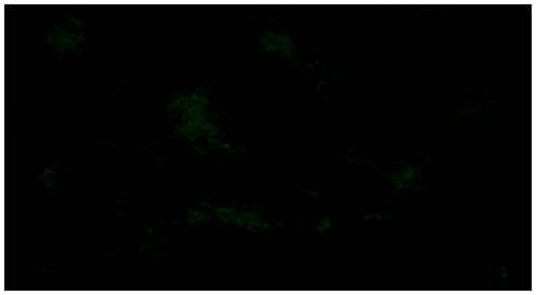
C – Control Cortex



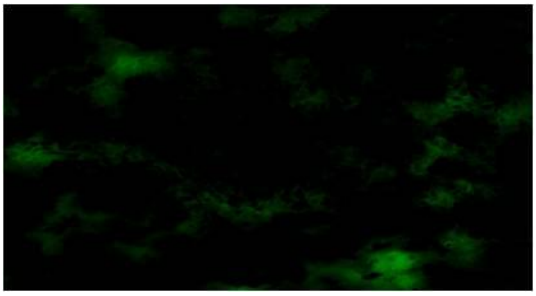
D – CO Exposed Cortex



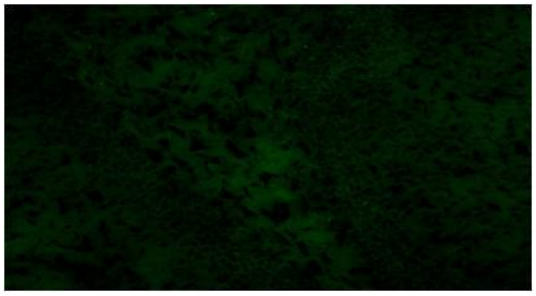
E – Control Basal Ganglia (EGP)



F – CO exposed Basal Ganglia (EGP)

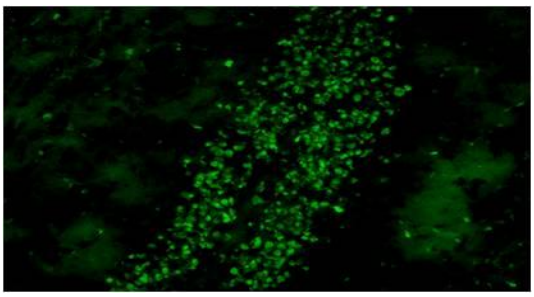


G – Control Hippocampus

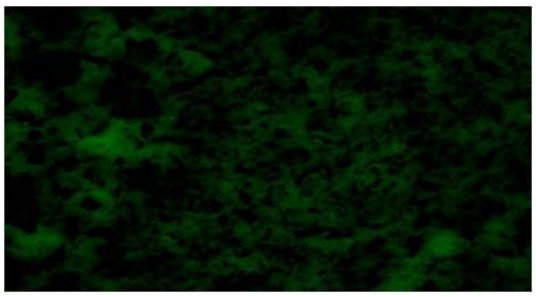


H – CO exposed Hippocampus

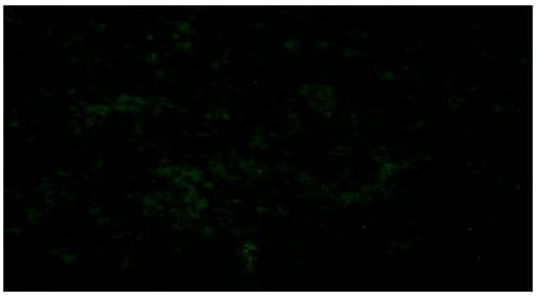
Picture 6: TUNEL Assay results on brain tissue slices of control and CO exposed rats receive 100% normobaric oxygen treatment (NBOT) immediately post exposure. No apoptotic nuclei are present regardless of exposure or treatment. (20X Magnification)



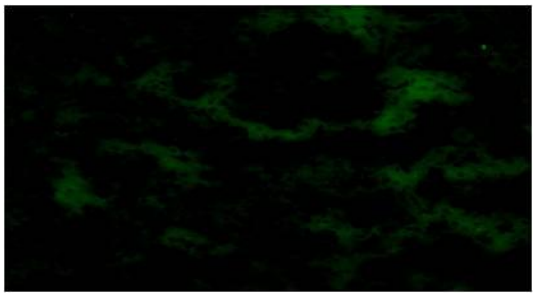
A – Positive Control (Naïve Rat)



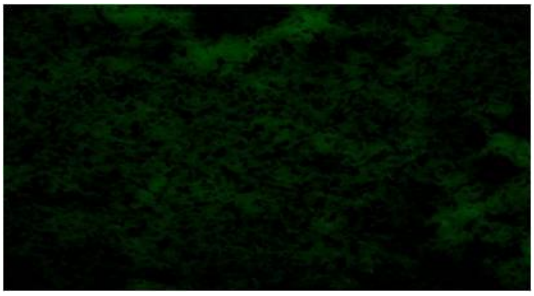
B – Negative Control (Naïve Rat)



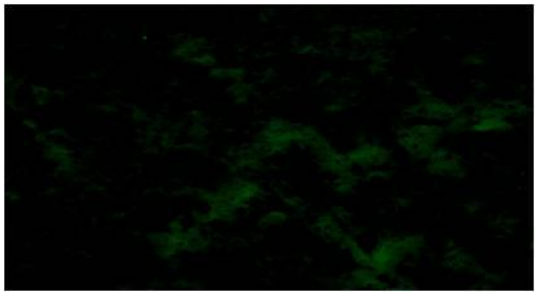
C – Control Cortex



D – CO exposed Cortex

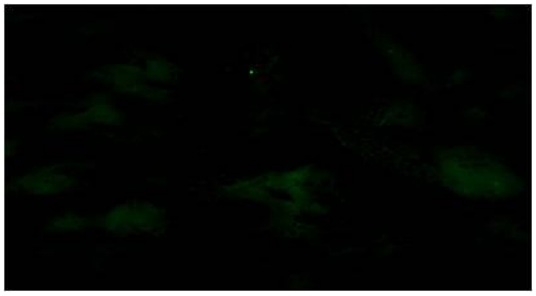


E – Control Basal Ganglia (EGP)



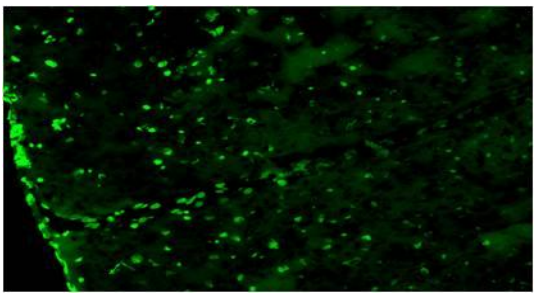
F – CO exposed Cortex (EGP)

G – Control Hippocampus

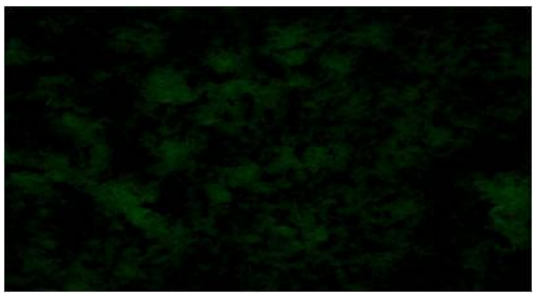


H – CO exposed Hippocampus

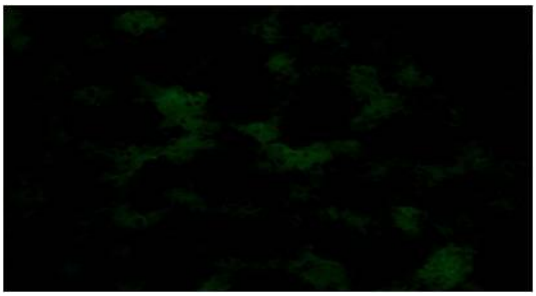
Picture 7: TUNEL Assay results on brain tissue slices for rats treated with room air 4 hours post exposure to room air or CO. The results are the same for the animals treated immediately with room air. No apoptotic nuclei are present regardless of CO exposure. (20X Magnification)



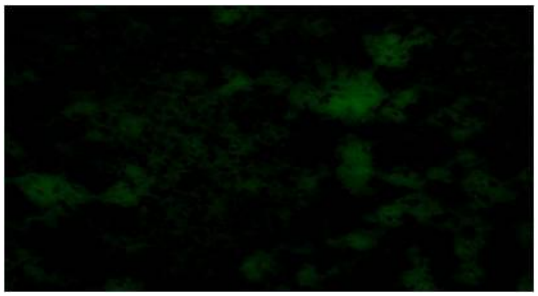
A – Positive Control (Naïve Rat)



B – Negative Control (Naïve Rat)

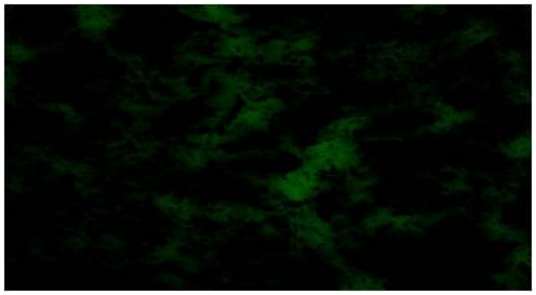


C – Control Cortex



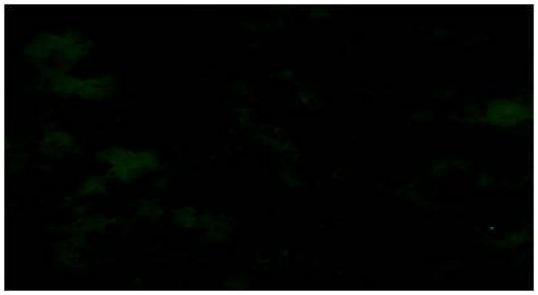
D – CO Exposed Cortex

E – Control Basal Ganglia (EGP)



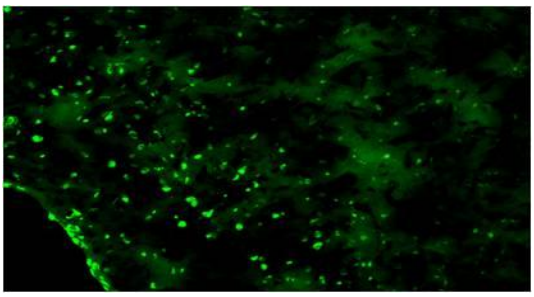
F – CO Exposed Basal Ganglia (EGP)

G – Control Hippocampus

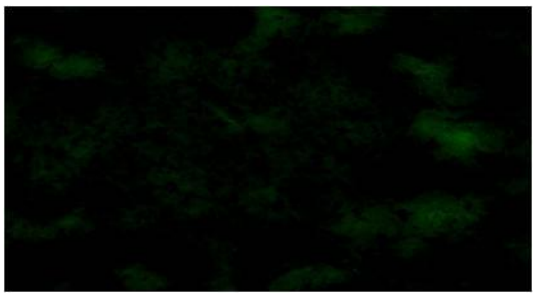


H – Co Exposed Hippocampus

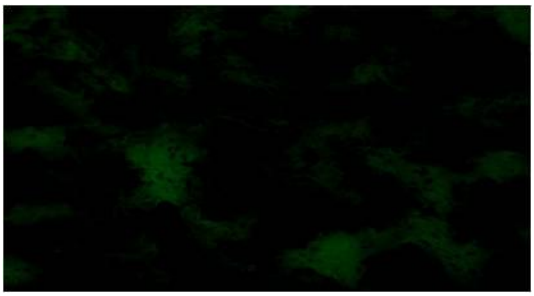
Picture 8: TUNEL Assay results on brain tissue slices for control and CO exposed rats receiving HBOT 4 hours post exposure to room air or CO. No apoptotic nuclei detected regardless of exposure or treatment. (20X Magnification)



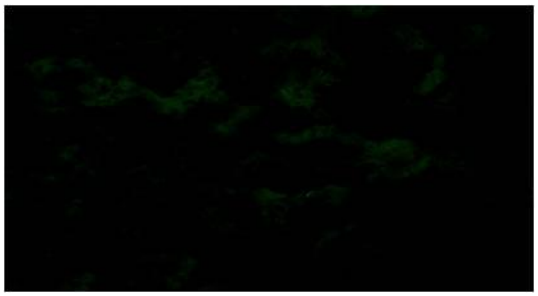
A – Positive Control (Naïve Rat)



B – Negative Control (Naïve Rat)

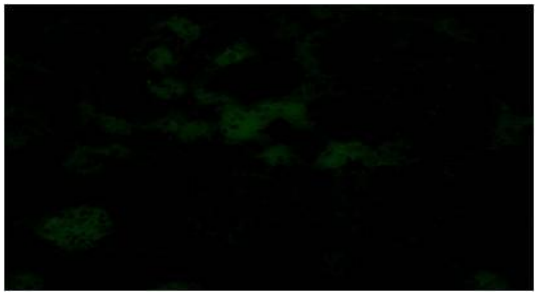


C – Control Cortex

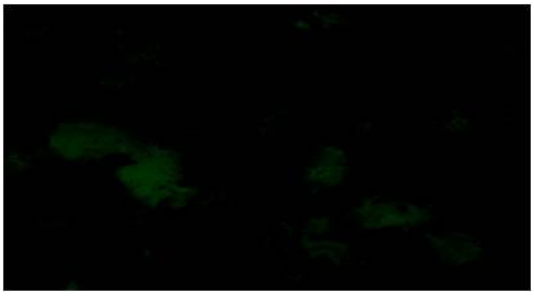


D – CO Exposed Cortex

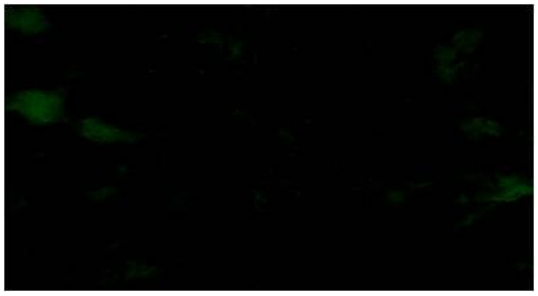
E – Control Basal Ganglia (EGP)



F – CO Exposed Basal Ganglia (EGP)

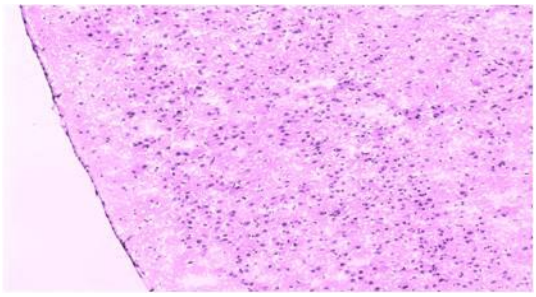


G – Control Hippocampus

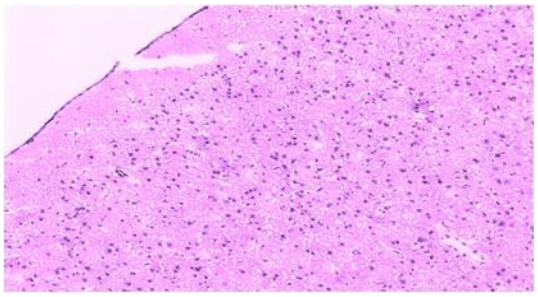


H – CO Exposed Hippocampus

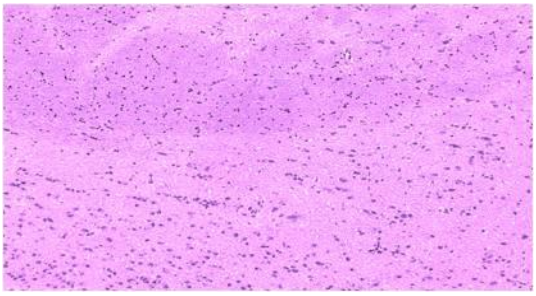
Picture 9: TUNEL Assay results on brain tissue slices for control and CO exposed rats receiving NBOT 4 hours post exposure to room air or CO. No apoptotic nuclei detected regardless of exposure or treatment. (20X Magnification)



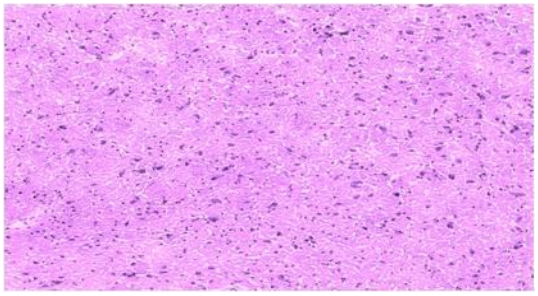
A – Control Cortex



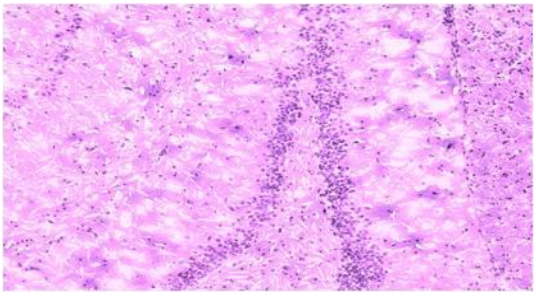
B – CO Exposed Cortex



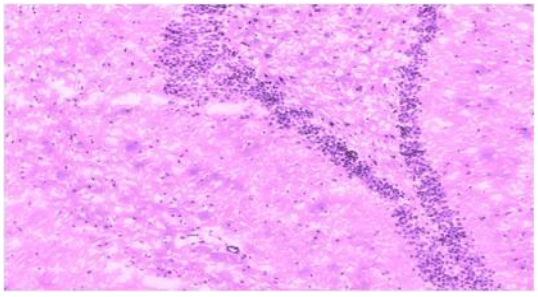
C – Control Basal Ganglia (EGP)



D – CO Exposed Basal Ganglia (EGP)

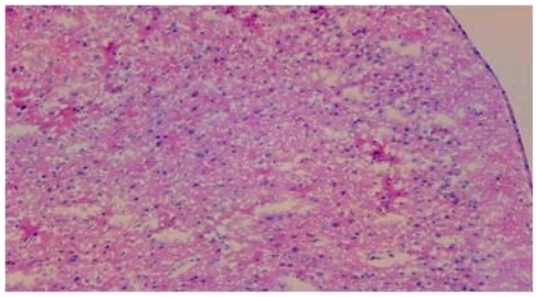


E – Control Hippocampus

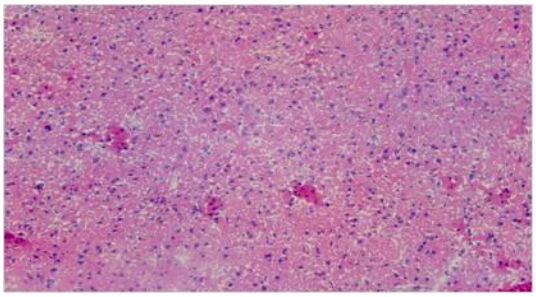


F – CO Exposed Hippocampus

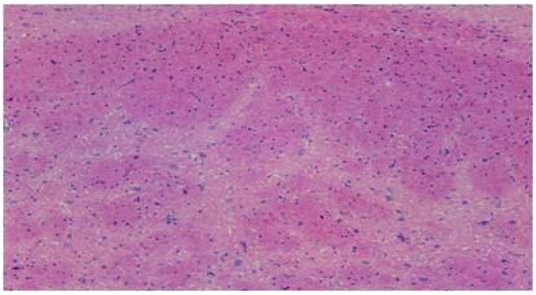
Picture 10: H&E stains on brain tissue of control and CO exposed rats. These rats were treated with room air immediately post exposure to room air or CO. No necrotic cells detected regardless of exposure. Theses picture were taken with an Olympus microscope (see Materials and Methods) at 10X Maginification.



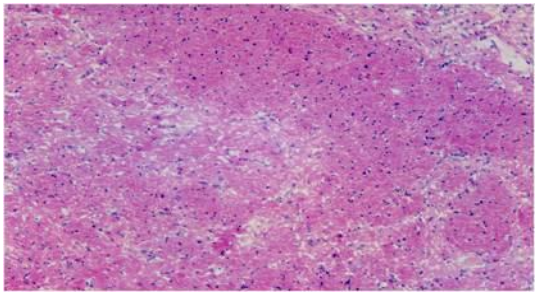
A – Control Cortex



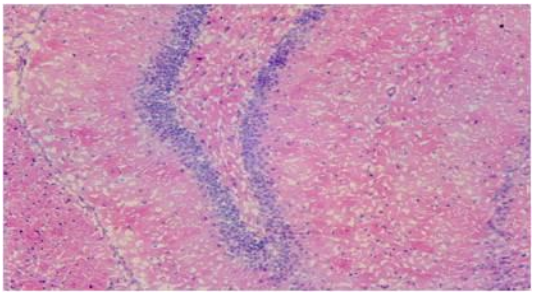
B- CO Exposed Cortex



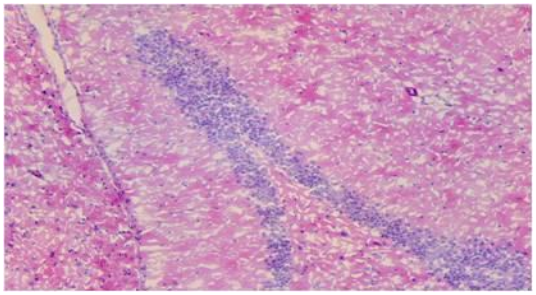
C – Control Basal Ganglia (EGP)



D – CO Exposed Basal Ganglia (EGP)

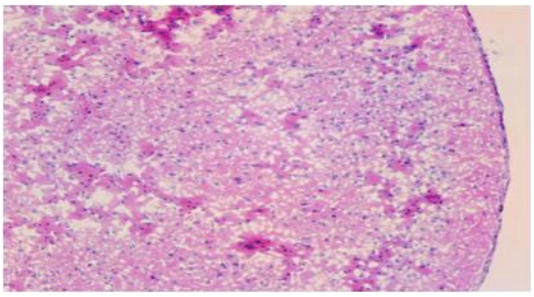


E – Control Hippocampus

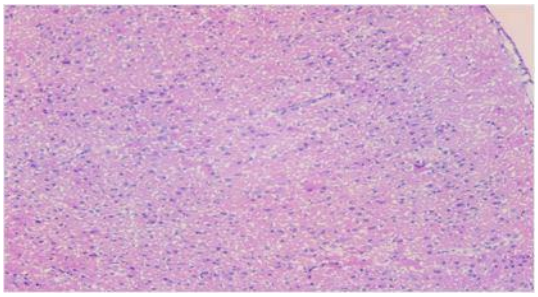


F – CO Exposed Hippocampus

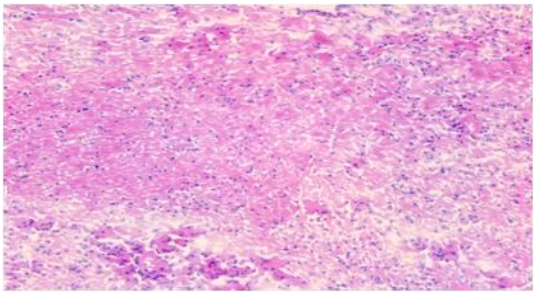
Picture 11: H&E stains on brain tissue of control and CO exposed rats. These rats were treated with HBOT immediately post exposure to room air or CO. No necrotic cells detected regardless of exposure or treatment. (10X Magnification)



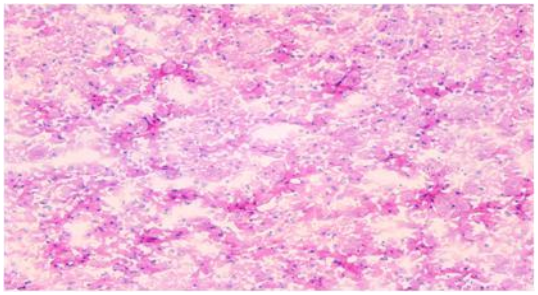
A – Control Cortex



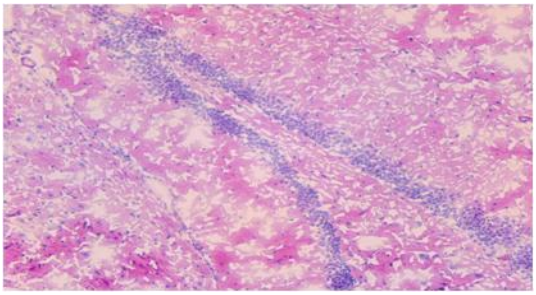
B - CO Exposed Cortex



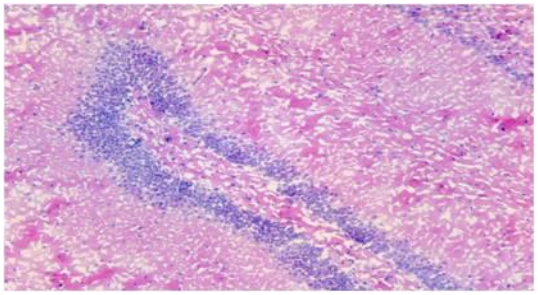
C – Control Basal Ganglia (EGP)



D – CO Exposed Basal Ganglia (EGP)

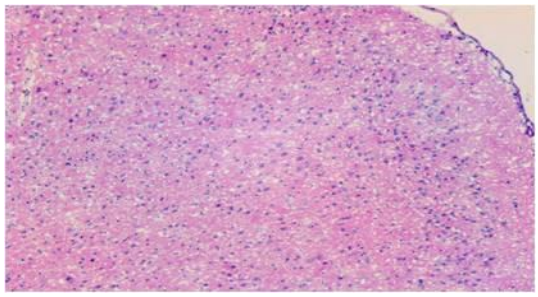


E – Control Hippocampus

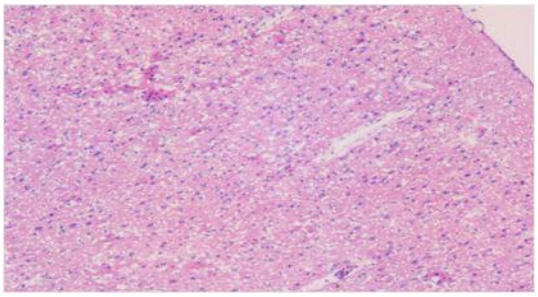


F – CO Exposed Hippocampus

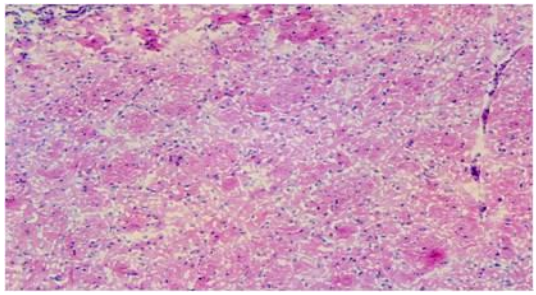
Picture 12: H&E stains on brain tissue of control and CO exposed rats. These rats were treated with room air immediately post exposure to room air or CO. No necrotic cells detected regardless of exposure or treatment. (10x Magnification)



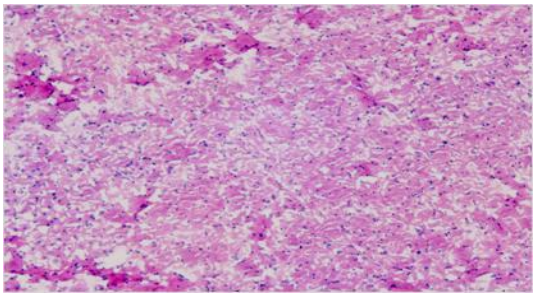
A – Control Cortex



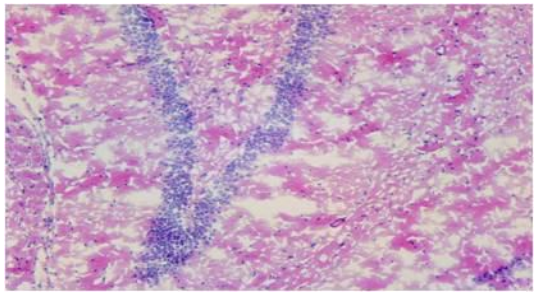
B – CO Exposed Cortex



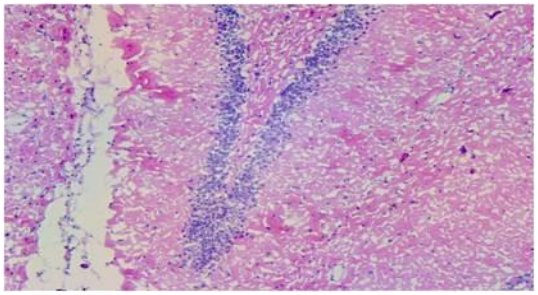
C – Control Basal Ganglia (EGP)



D – CO Exposed Basal Ganglia (EGP)

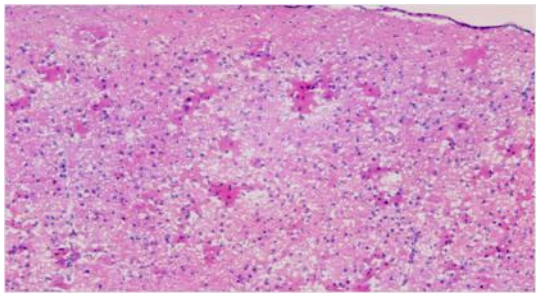


E – Control Hippocampus

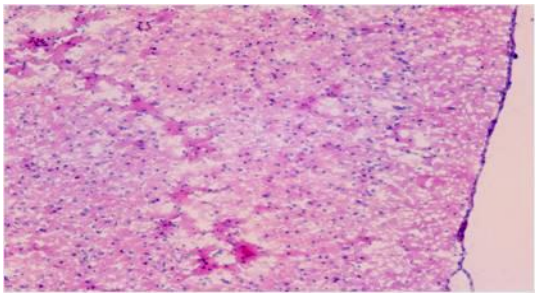


F – CO Exposed Hippocampus

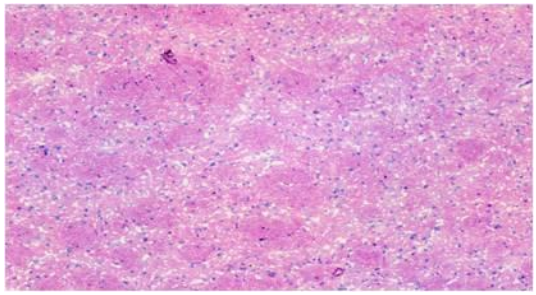
Picture 13: H&E stains on brain tissue of control and CO exposed rats. These rats were treated with room air i4 hours post exposure to room air or CO. No necrotic cells detected regardless of exposure or treatment. (10X Magnification)



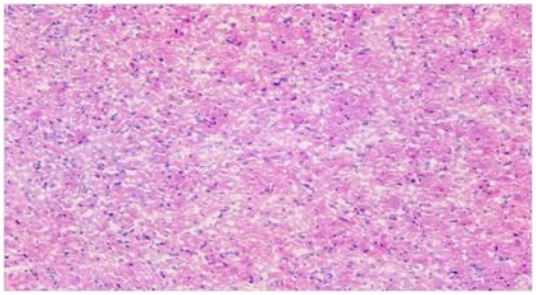
A – Control Cortex



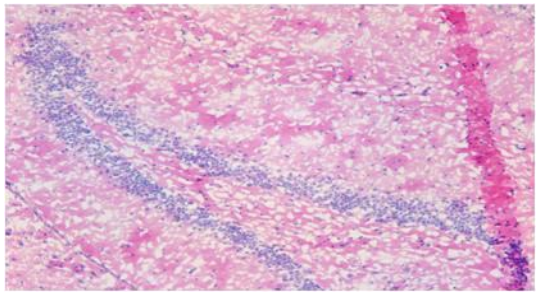
B – CO Exposed Cortex



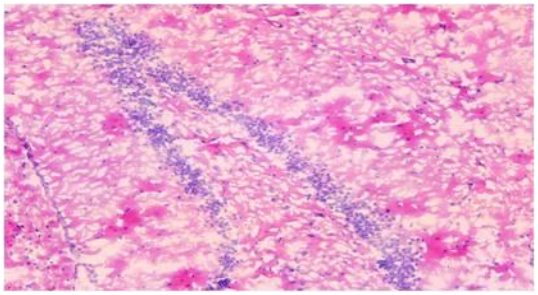
C – Control Basal Ganglia (EGP)



D – CO Exposed Basal Ganglia (EGP)

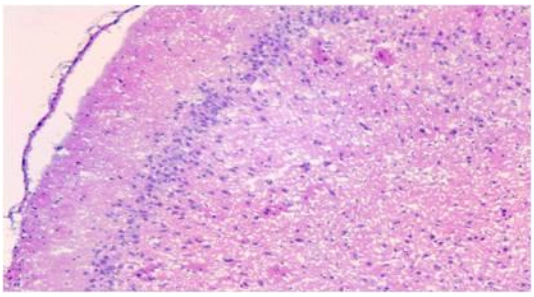


E – Control Hippocampus

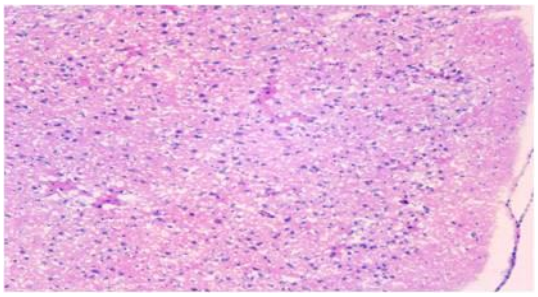


F – CO Exposed Hippocampus

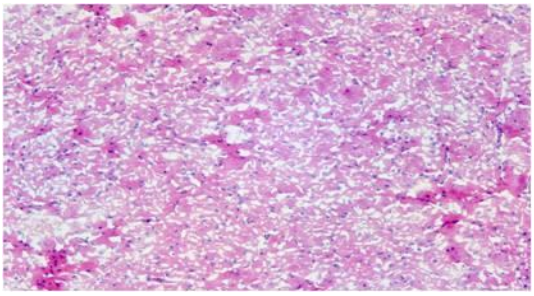
Picture 14: H&E stains on brain tissue of control and CO exposed rats. These rats were treated with HBOT 4 hours post exposure to room air or CO. No necrotic cells detected regardless of exposure or treatment. (10X Magnification)



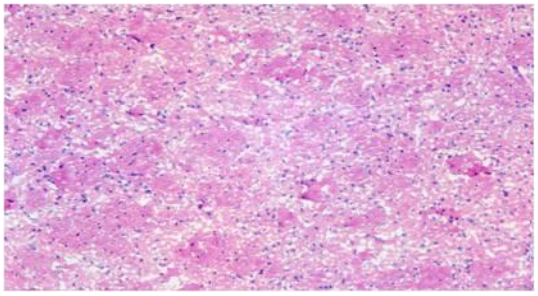
A – Control Cortex



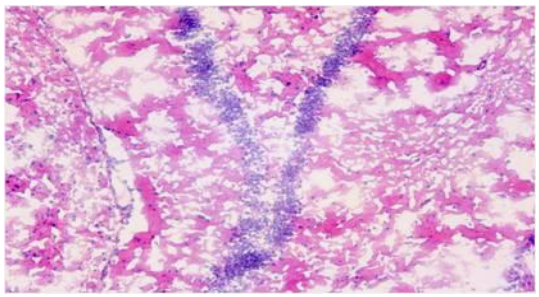
B – CO Exposed Cortex



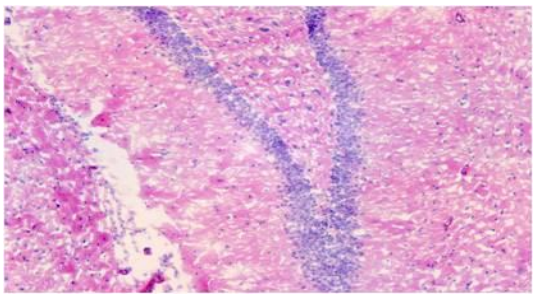
C – Control Basal Ganglia (EGP)



D – CO Exposed Basal Ganglia (EGP)



E – Control Hippocampus



F – CO Exposed Hippocampus

Picture 15: H&E stains on brain tissue of control and CO exposed rats. These rats were treated with NBOT 4 hours post exposure to room air or CO. No necrotic cells detected regardless of exposure or treatment. (10X Magnification)

DISCUSSION

The main objective of this study was to test the efficacy of hyperbaric oxygen treatment in preventing CO induced DNS. Specifically, we asked the following questions: 1) What are the observable clinical signs (both physical and neurobehavioral) of CO induced DNS in the Sprague-Dawley rat; 2) Is CO induced DNS due to apoptosis, necrosis, or a combination of the two; and 3) Is there a treatment "window" beyond which oxygen treatment of any kind is no longer an effective preventative measure for CO induced DNS? In order to answer these questions, the following methods were emploiyed: 1) Optimization of CO exposure to favor the development of CO induced DNS; 2) Development of a reproducible rat neurobehavioral model for CO induced DNS; 3) A challenge to the neurobehavioral model with room air; normobaric oxygen, and hyperbaric oxygen treatments both immediately after CO exposure, and 4 hours post CO exposure; 4) TUNEL analysis on brain tissue slices of the basal ganglia, cortex, and hippocampus to detect apoptosis; and 5) H & E staining on brain tissue slices of the basal ganglia, cortex, and hippocampus to detect necrosis.

Design of Exposure Chamber

This experiment was designed after the work done by Piantadosi (Piantadosi, Zhang et al. 1997). The final CO exposure level of 3000 ppm CO, the neurobehavioral testing time points, rat strain, and areas of the brain studied were all inspired by that work (Piantadosi, Zhang et al. 1997). The key differences were in the neurobehavioral tests performed, and the delivery of CO to the rats. Piantadosi used a "bolus" exposure method. This CO delivery method was essentially a large plastic bag where the atmosphere was forced out, and

then a dose of 40000 ppm CO was delivered to the rats causing them to lose consciousness (Piantadosi, Zhang et al. 1997). This experiment was designed from the beginning to closely resemble "real world" conditions where a somewhat steady concentration of CO would be delivered over time. The exposure chamber was designed with this in mind. The CO was mixed with atmospheric air in an upper plenum, and drawn downwards into a lower plenum where it was exhausted from the chamber. This kept the CO concentration within the chamber constant, and it removed any exhalants, particularly CO_2, from the rats (Reboulet, Lear et al. 2009).

Optimization of CO Exposure at 1000 ppm

This phase of the experiment began under the following assumptions: 1) The "Benignus-Annau" COHb formation curve was accurate across rat strains; and 2) The half-life of COHb in mammalian systems is 4-6 hours as demonstrated in humans (Haldane 1895; Allen and Root 1957; Benignus and Annau 1994). According to Benignus and Annau, the rats should have reached a COHb level of at least 50% after 1 hour of exposure at 1000 ppm CO in air (Benignus 1994). The rats were expected to achieve syncope as well (Piantadosi, Zhang et al. 1997; Gilmer, Kilkenny et al. 2002). The results of the optimization exposures at 1000 ppm CO appeared to conflict both these assumptions. Our first exposure at 1000 ppm CO lasted for 75 minutes and produced a COHb level of 43.3%. The rats remained conscious the entire time and showed no obvious sign of lethargy when removed from the exposure chamber. Increases in exposure time did not always correlate to increased levels of COHb, although that was the overall trend (See Results, Table). Syncope was not achieved at any exposure time, nor was an average COHb level above 50% measured. A direct correlation between exposure time and COHb level was expected as was syncope.

Possible explanations for these observations included: 1) weight differences in the rats, 2) unequal distribution of CO in the exposure chamber, 3) genetic differences in the out bred rats. There were no observed correlations between COHb levels and body weight. The exposure had been characterized for uniform distribution of CO, so an insufficient or uneven exposure to was ruled out (Reboulet, Lear et al. 2009). The rats were out bred to mimic the genetic variability seen in human populations, so perhaps this was the reason for the difference. Further review of the experimental data compared to the Benignus-Annau curve revealed that what we were observing was exactly what was predicted by Benignus and Annau. After approximately 1 hour of exposure to 1000 ppm CO the rats were reaching a "steady state" plateau. However, COHb levels of approximately 55% should have been obtained per the curve(Benignus and Annau 1994). It was hypothesized that the observed COHb levels did not correlated with those predicted were 1) the rats being out bred, and 2) Beningus' data was generated with Long-Evans rats, so perhaps there is a small difference in CO tolerance between the strains (Benignus and Annau 1994). It was also hypothesized that the rats might be eliminating the COHb more quickly than expected. This hypothesis was considered remote at best, as we had found no current peer reviewed literature documenting this.

To test the hypothesis that COHb was being eliminated by the rats more quickly than expected, seven naive rats were exposed to 1000 ppm CO in air for 120 minutes. These rats were allowed to recover from the exposure in room air for 120 minutes before measuring their COHb level. The exposed rats were expected to have a COHb level of approximately 38%. This was assuming the COHb half-life was 4- 6 hours, and the animals had achieved a COHb level of approximately 50% as data from previous 120 minute exposures at 1000 ppm CO indicated. The average COHb level was 5.8% (n=2) 120 post exposure. At 240

minutes post exposure, COHb levels were less than the control rats (control = 4.1% COHb, exposed = 3.0 %). This result was completely unexpected. Possible reasons for these results included: equipment failure, and/or the CO was delivered mixed with 100% O_2 , not atmospheric air as requested. Thorough inspection and testing of all the equipment (CO monitor, COOximeter, exposure chamber) revealed no malfunctions. The CO supplier was contacted and they provided documentation verifying that they had 10% CO in atmospheric air as requested. Testing of the CO gas in did not show more than 20% O2. This data places the COHb half-life at approximately 20-45 minutes *in vivo* for the Sprague Dawley rat! A further current literature search yields only two sources documenting the *in vivo* COHb half-life in rats. The first by Kimmel, Carpenter et al. indicates an *in vivo* COHb half-life in male, F-334 rats at approximately 2 hours. The in vivo COHb concentrations were measured for only one hour post exposure to 500 ppm Co in air for 1 hour (Kimmel, Carpenter et al. 1999). The second, by Andersen et al., exploring the kinetics of dichloromethane (CO is a metabolite of dichloromethane), shows an *in vivo* COHb half-life of approximately 20 minutes (Andersen, Clewell et al. 1991)(See Figure 13). This was after

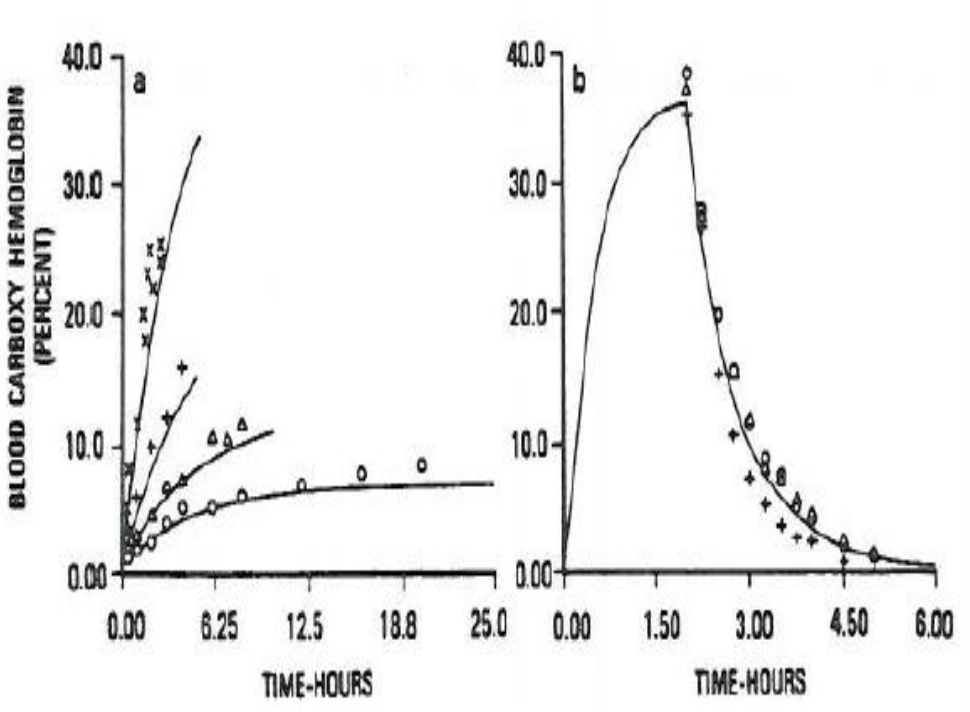


Figure 14: Blood COHb concentrations vs time in humans(a) and male, F334 rats (b). Human dat was produced by RD Stewart and refenced in by Andersen et al. (Stewart 1975; Andersen, Clewell et al. 1991). This graph clearly show the *in vivo*,20 minute half-life of COHb in F334 rats vs the established 4-6 hour COHb half-life in humans.

an exposure of 500 ppm CO for 2 hours (Andersen, Clewell et al. 1991). Review of the raw data from the Andersen paper reveals an unpublished calculation of rat COHb half-life at 26 minutes (Andersen, Clewell et al. 1991). A possible reason for this rapid elimination of COHb from the rat is the rate of respiration. A typical rat has respiratory rate of 70-115 breaths per minute (Sharp and Regina 1998). By comparison a normal human respiratory rate is about 12 breaths per minute (Guyton and Hall 2000).

Despite this very surprising result, the study continued as originally planned, as CO induced DNS develops long after COHb levels return to baseline (1.0-3.0%).

A final pilot exposure for 180 minutes at 1000 ppm CO was performed. The first exposure for 180 minutes yielded an average COHb level of 51.5 % (n=2). Still, the rats remained conscious during the entire exposure and showed no obvious signs of lethargy when removed from the exposure chamber. This exposure procedure was repeated on the remaining rats from this lot (n=4) obtaining an average COHb of 46.8%. This lower COHb result was attributed it to the recently discovered short half life. If the time for the CO to clear the exposure chamber (5 min) and for the anesthesia to take affect before the heart stick was performed (5 minutes) is factored in, COHb levels are in easily in excess of 50%. Although the rats did not reach syncope, an indicator of DNS development in humans, it was postulated that a 180 minute exposure would be sufficient to generate the predicted, observable neurobehavioral effects (Choi 1983; Myers, Snyder et al. 1985; Lee and Marsden 1994; Thom, Taber et al. 1995; Mimura, Harada et al. 1999; Lee, Mak et al. 2001; Gilmer, Kilkenny et al. 2002; Weaver, Valentine et al. 2007).

DNS Rat Model Development at 1000 ppm CO

No standard animal model for CO induced DNS exists. In fact most major studies on CO intoxication have been performed on different strains of rat. All rats survived the 180 minute exposure at 1000 ppm CO in air. As expected the animals neither reached syncope, nor were lethargic when removed from the exposure chamber. We observed the rats closely for any adverse affect of the CO exposure, but their behavior post exposure was indistinguishable from the control animals. Because there is no clear description of the clinical signs of, or FOB for DNS in rats in the literature, both control and CO exposed rats were video recorded during the open field neurobehavioral testing. This was to capture clear physical signs of the CO induced DNS. The symptoms of CO induced DNS are readily apparent in humans (shaking, disorientation, incontinence, etc.), and it was assumed that rats would exhibit the same clinical signs (Garland and Pearce 1967; Lee and Marsden 1994; Choi, Kim et al. 1995; Mimura, Harada et al. 1999). Thus there would be a reference source for an FOB for the clinical signs of CO induced DNS in male, Sprague-Dawley rats for future studies.

The following assumptions were made regarding the development of our DNS model: 1) the rats would all develop DNS at the same time, and 2) an exposure time of 180 minutes would be sufficient induce DNS. These were broad assumptions. The litraure suggests that if DNS develops, their appearance/manifestation depends on the individual (Garland and Pearce 1967; Choi 1983; Ginsberg 1985; Gibson, Davis et al. 1991; Benignus 1994; Piantadosi, Zhang et al. 1997; Ernst and Zibrak 1998; Lee, Mak et al. 2001; Raub and Benignus 2002; Brunssen, Morgan et al. 2003; Scheinkestel, Jones et al. 2004; Handa and Tai

2005; Prockop and Chichkova 2007; Weaver, Valentine et al. 2007; Hampson and Hauff 2008) .

As documented in the results section, the 180 minute exposure to 1000 ppm CO in air yielded no observable, measurable neurobehavioral sings of DNS. The protocol was modified to increase the concentration of CO to 3000 ppm

Optimization of CO Exposure at 3000 ppm

Given the COHb half-life data from the 1000 ppm exposure, the COHb levels in the CO exposed rats at 60 minute time points post exposure. Prior to the increase in CO concentration, all equipment was rechecked and calibrated, ensuring that the CO was equally distributed within the exposure chamber. As there was only one literature source for the half-life of CO in rats(Andersen, Clewell et al. 1991), it was hypothesized that CO concentration would have an effect on rate COHb elimination. Also should the data be similar to what was observed at 1000 ppm, it would strongly suggest that the previous post exposure COHb measurements were not an aberration. As planned for rats to 3000 ppm CO in air for 90 minutes. All 4 rats survived, remained conscious and exhibited no signs of lethargy when removed from the exposure chamber. The rats attained a COHb level of approximately 51.6 % (n=1). We then measured the COHb in the remaining three rats at 60 minute time intervals. The results mirrored those observed after an exposure to 1000 ppm CO (see results figure). By three hours post exposure, the COHb level had returned to base levels (exposed = 1.9%; control = 1.7%). The data strongly suggests that rate of elimination of COHb appeared to be independent of CO concentration and exposure time.

The final pilot exposure at 3000 ppm CO continued until the animals reached syncope. This specific end point was never attained at any point during all of the1000 ppm CO exposure.

At the 135 minute time point one of the rats had died, and the surviving two began to seize. The exposure was stopped at 135 minutes. The two surviving rats never reached true syncope, but were very lethargic when removed from the exposure chamber. They were not responsive to toe pinch, thus anesthesia was not required prior to heart stick. The rats attained an average COHb level of 55.4% (n=2) with one of the animals attaining 57.6%. The original endpoint of COHb > 55% had finally been acheivd. Although syncope remained elusive, and exposure time of 135 minutes was set as the exposure time for the 3000 ppm CO exposure.

DNS Rat Model Development at 3000 ppm CO

Sixteen rats were exposed to 3000 ppm CO in air for 135 minutes. During the exposure, four rats died in chamber with 7-10 minutes exposure remaining. This was regarded as significant, because no rats had died in the exposure chambers. Of the surviving rats several were appeared disoriented when removed. All rats were lethargic when removed from the exposure chamber. Two of the surviving rats were very lethargic when removed from the exposure chamber, and their survival was in question. The CO exposed rats were observed very closely for 1 hour post exposure. After 24 hours all surviving rats exhibited no futher signs of lethargy.

Blood samples from the four deceased rats established an upper threshold value past which the rats would not recover. The average COHb from the deceased animals was 71.8%. During this exposure rats the time between syncope and death was extremely short, too short to stop the exposure, evacuate the CO, and the remove them. Basically, if the rats reached syncope, they died.

At 24 hours post exposure we performed the Open Field tests on the control and CO exposed animals, as previously described. Unlike the 24 hour point for the 1000 ppm exposure there was a noticeable difference in activity levels between the control and Co exposed rats. This "performance gap" continued to widen, becoming statistically significant on day 7 post exposure, and reaching a zenith on day 14 post exposure. By day 28 post exposure, the gap in performance had become statistically insignificant, and was narrower than the gap at the 24 hour testing point. The data were established as the model for DNS, as the observed pattern established followed the classic description of DNS: starting small, becoming more severe, and then going away.

Based on these results, the master plan of the experiment was modified in two key ways: 1) the neurobehavioral testing would end at 14 days post CO exposure and/or treatment, and 2) the delay in treatment would be set at 4 hours post CO exposure in order to harvest and analyze the brains of the rats during DNS. At four hours post exposure, the rats would have completely eliminated any excess COHb caused by exogenous CO; any further delay would be superfluous. The original philosophy behind the experiment was to mimic in an animal model what was usually seen in case of CO intoxication in an ER. Our estimate was that by the time CO intoxication had been verified in a human patient, one at least one COHb half-life had occurred.

Incident at 3000 ppm CO in Air

During the 3000 ppm exposure that was to precede treatment in the hyperbaric chamber an unforeseen incident occurred. Between 5-10 minutes after reaching 3000 ppm CO in the exposure the first rat died. This was unusual, because a rat had not died in the exposure chamber that quickly before. A 25% mortality rate was expected, however, so there was no

concern. The second and third rats died at 75 minutes into the exposure. This was what was observed during the optimization trials of the 3000 ppm exposure. Again, this was not a cause for concern, there were still 60 minutes remaining in the exposure and the rats had been extremely tolerant of the CO in all the past exposures. Within the next 5 minutes 6 animals died without warning, leaving only 5 surviving rats. Four of the remaining rats died within the next 20 minutes. The last remaining rat died at the 2 hour point, fifteen minutes before any of them should have. As mentioned before, the rats had been extremely tolerant of the CO up this point. This was quite the dilemma. A major exposure/treatment group had just been lost and fifty rats were waiting for CO exposure over the two days. All equipment was rechecked, and all equipment checked out. Everything was functioning as it should be. Euthanizing the remaining rats was an option of last resort, and there was no longer adequate time to start over. The only endpoint from the exposure optimization study that would still apply to the reaming rats was the 25% mortality rate. Given all the data we had accumulated to this point, we divided the remaining rats into three groups of 16 as originally designated, room air, HBOT, and NBOT (6 control and 10 exposed). The rats would be exposed to 3000 ppm CO in air. The exposure would continue until 2 rats died (25% mortality). This way statistical power, would be retained, the experiment would continue on schedule, and 49 rats would not be needlessly euthanized. The time point determined by this first exposure would be the exposure time for all remaining exposures in the experiment. After the first exposure the exposure time was determined to be 80 minutes. It was also decided that three rats from the next lot would be exposed to 3000 ppm of CO for 80 minutes to ensure that they would be able to survive.

Nothing in the literature, or observations of previous exposures had prepared us for this. Exposures for 135 minutes at 3000 ppm CO had been twice repeated with only a 25%

mortality. It was hypothesized that the cause of this discrepancy in CO tolerance was the out breeding of the rats. The rats in this lot of animals lacked genetically what the previous lot had. This was the only explanation, as the rats were the only thing that was different during this exposure. There was also concern that the animals would not be exposed to the CO long enough to produce DNS. This was a valid concern. The exposure at 1000 ppm for 180 minutes did produce DNS, and given the rapid rate at which the rats eliminate COHb from their blood anything shorter than a 135 minute exposure would not be sufficient to reproduce the results seen in the 3000 ppm exposure. A repeatable end point was required to keep conditions equal for the neurobehavioral tests. The adjustment in exposure time was not without risks. Comparing the neurobehavioral results to the results garnered from the development of the DNS model would be tenuous at best since the times spent in the exposure chamber would differ, and environmental factors would be unequal.

The next 3000 ppm CO exposure lasted for 75 minutes. At that time point 2 rats had died, and the exposure was ended as previously determined. The deceased rats were removed from the cages and the surviving rats were placed into the hyperbaric chamber. The surviving rats were conscious, though lethargic when removed from the placed into the hyperbaric chamber for treatment. This exposure time was repeated for the next two exposures in the immediate treatment group. Two rats died at the end of the two exposures remaining in the immediate treatment group at the 75 minute time point.

Three rats of the next lot were exposed to 3000 ppm CO for 75 minutes. One rat died 4 minutes 25 seconds into the "ramp-up" period of the exposure where the CO level was approximate 1000 ppm. If this lot of rats (n=64) was hyper sensitive to CO, then the entire study was in jeopardy. One of the main goals of this study was to establish a treatment

"window", we needed the 75 minute exposure to compare the results from the immediate treatment groups. Fortunately the two remaining rats survived to the end of the 75 minute exposure. These two "sentinel" rats were lethargic (slow response to toe pinch) at the end of the exposure. They were observed for 1 hour post exposure and survived, all of the data we had about the CO exposed rats thus far indicated that if the rats survived the exposure they would recover, and live through the end of the neurobehavioral tests. After one hour of close observation, the CO exposed, surviving rats were euthanized per the protocol. In hindsight this was short sighted. Given the past inconsistencies with the rats a recovery of 24 hours would have been more prudent.

Fortunately, CO hypersensitivity was not an issue for this lot of rats. There was only one death, over the next three CO exposures (Room Air, HBOT, and NBOT subgroups). The one mortality occurred 70 minutes into the exposure indicating that these rats were perhaps similar in their sensitivity to CO. On the other hand, 83% less mortalities also indicates that perhaps these rats would have been more tolerant of the CO, and survived a longer exposure. Unfortunately, the neurobehavioral data from this group (delayed treatment) was to be compared to the previous groups (immediate treatment) so all environmental conditions were kept the same.

As mentioned above, all but one of the rats in this group survived the CO exposure. After a four hour delay (during which the rats were removed to their cages with food and water *ad libitum*) all rats spent equal time in the hyperbaric chamber (2 hours total), and then returned to their cages, observed for 1 hour and returned to the vivarium. All the exposed rats appeared to have returned to normal activity during the four hour delay before treatment. Given previous data their COHb levels should have returned to background levels by that

time point. All animals (both control and CO exposed) were alert and active when removed from the treatment chamber. This was the case regardless of treatment type (room air, HBOT, or NBOT). This was not surprising as the same results were seen in the immediate treatment group. This result also lead to fears that the neurological tests for this group would not yield the same results we had obtained when we developed the neurobehavioral model for DNS.

Neurobehavioral Testing

As expected (feared) the neurobehavioral test results for both groups (immediate treatment and delayed treatment) did not yield any statistically significant ($p<0.05$) neurobehavioral differences from control rats in all subcategories regardless of treatment. Statistical analyses between treatment subgroups and across groups (i.e. immediate HBOT vs. delayed HBOT) yielded nothing. In fact the results for all treatment subgroups resembled the neurobehavioral results obtained from the 1000 ppm CO exposure. Most telling was that there were no differences in the measured behaviors between the rats allowed to recover in room air versus those with either oxygen treatment. The most plausible reason for this is the reduced CO exposure time in combination with the Sprague-Dawley rat's demonstrated ability to eliminate COHb, and the ability for this rat strain to function with 50% of their blood oxygen carrying capacity comprimised.

An equally plausible explanation for our lack of repeatable results is that we were simply measuring the wrong behaviors. CO intoxication is well known to affect both learning and memory (Piantadosi, Zhang et al. 1997). The simple Open field tests do not measure either of those endpoints

TUNEL

As expected form the negative neurobehavioral results, no signs of apoptosis occurring "in process" were detected in the basal ganglia, cortex, or hippocampus in any of the rats, either CO exposed or control. This was true regardless of exposure or time of death. When compare to a positive control, the tissue slices showed no apoptotic activity whatsoever. We did not encounter any false positives as is sometimes encountered with TUNEL assays in lipid rich tissue (Whiteside, Cougnon et al. 1998; Whiteside and Munglani 1998; Ribble, Goldstein et al. 2005). The kits were functioning correctly as the positive controls correctly label damaged DNA (See Results Pictures 4 – 9).

H&E

The brain tissue samples from CO exposed rats also show no signs of necrosis when compared to control. The tissue itself displayed evidence of *post mortem* damage done by cryotome slicing and tissue preparation. Again this result was not surprising as the exposed rats did not exhibit any neurobehavioral deficits when compared to control or between exposure and/or treatment groups (See Results Pictures 10 – 15).

CONCLUSIONS

No measurable evidence of CO induced DNS was not produced in any of the experimental rats at the 14 day post Co exposure time point, thus the hypotheses put forth cannot be proven or disproven by this data.

Time of Sacrifice

Although we had previously measured peak neurobehavioral differences between control and CO exposed rats at 14 days post exposure, given the mercurial nature of DNS, it is entirely possible that the rats were sacrificed too early. DNS in humans can appear anywhere from 1 to 6 weeks post CO exposure (Garland and Pearce 1967; Lee and Marsden 1994; White 2008). By shortening the observation period to 14 days, it is entirely possible. The two lots of rats used for Phase III were clearly different from the lot on which the model used was based (see Results). It is logical to assume that the rats would develop neurobehavioral deficits at a different time point. Perhaps waiting for the clinical signs of DNS to appear (if at all) and sacrificing individual animals at individual time points would prove a better approach.

Perhaps a Biochemical Trigger

A recent publication suggests that Xanthine oxidoreductase (XOR) activity is responsible, at least in part, for CO induced DNS (Han, Bhopale et al. 2007). This would rule out increased apoptosis or necrosis the cause of CO induced DNS as we hypothesized. The critical element would be examining the correct pathway at the time the DNS were occurring.

Different Animal Model

Although our results show a wide variability in CO tolerance in the Sprague-Dawley rat (as one sees in humans), the study demonstrated that this particular rat strain can eliminate COHb very rapidly, roughly one sixth the time it take in a human (Haldane 1895; Haldane 1895; Pace, Strajman et al. 1950; Allen and Root 1957; Peterson and Stewart 1970; Sasaki 1975). If this rat strain is doing in room air what it takes a human an hour in 3 atmospheres of 100% oxygen, then this strain should not be used as model for CO induced DNS. Or, in order to mimic the clinical situation more accurately, treat the rats at a timepoint that reflects the same number of half lives of COHb in the rat as would be the case clinically. Although a neurobehavioral deficit was achieved in CO exposed rats, the result was not repeatable. The effects of the CO we observed may not have been DNS. There was no real period of recovery, just a widening behavioral deficit that receded over time. This model is more in sync with the lingering post exposure effects of CO, rather than true DNS as observed in humans (Choi 1983; Myers, Snyder et al. 1985; Lee and Marsden 1994; Choi, Kim et al. 1995; Thom, Taber et al. 1995; Mimura, Harada et al. 1999; Lee, Mak et al. 2001; Gilmer, Kilkenny et al. 2002; Han, Bhopale et al. 2007; Weaver, Valentine et al. 2007). Also these rats would

die when they achieved syncope. This being said, perhaps an animal with CO sensitivity similar to a human, a more terrestrial animal such as a guinea pig, should be used.

Different NB Testing

Due to time a personnel constraints, we chose more simplistic neurobehavioral tests. This was perhaps short sited as previous studies were able to measure deficits in CO exposed rats using more robust test such as the radial arm maze (Piantadosi, Zhang et al. 1997; Gorisch and Schwarting 2006; Han, Bhopale et al. 2007). This testing method measure both learning and memory, two behavioral deficits that are most like seen in human victims of acute CO intoxication (Garland and Pearce 1967; Lapresle and Fardeau 1967; Choi 1983; Ginsberg 1985; Benignus 1994).

Change CO Exposure

Our exposure methods were unique from what has been done, in that the exposure was held constant over time. Other studies involving CO induced DNS use variable Co concentrations followed by a bolus dose of pure CO to achieve syncope in the rats (Benignus and Annau 1994; Piantadosi, Zhang et al. 1997; Han, Bhopale et al. 2007). Our results are consistent with previous observations that syncope is a strong indicator of DNS development (Choi 1983; Lee and Marsden 1994; Thom, Taber et al. 1995; Prockop and Chichkova 2007; Weaver, Valentine et al. 2007; White 2008). The rats did not achieve syncope as a result of our chosen exposure method (see methods), thus it was unlikely the rats would develop DNS as planned.

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