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Mechanistic Elucidation, Monitoring, Prediction and Prevention of Adverse Effects of Performance Stressors

Temporal Effect of Hypoxic and/or Hyperoxic Treatments on Organ Injuries and Memory Performance

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14. ABSTRACT
Aerospace professionals frequently experience hypoxia, which is followed by hyperoxic exposure as a compensatory measure. Both conditions have significant impacts on neurological, cognitive and motor functions. Abnormal oxygen levels in the inspired air can induce organ injuries. However, the correlation of these effects with the changes in cognitive functions and memory performance is not completely understood. To address this knowledge gap, this study was designed to investigate the effect of hypoxic and hyperoxic exposure on organ injuries in a systematic manner and correlate these results with impaired memory performance. This interim report presents the results of the investigation of the effects of high (95%) and low (7.5%) oxygen levels on memory performance and organ injuries. Briefly, hypoxic exposure caused disruption of blood-brain barrier and alveolar-capillary barrier, resulting in injuries of these vital organs. Hypoxia also induced memory impairments. Additional molecular and biochemical analyses were performed. These results, as presented in this report, provided important mechanistic insight into the adverse effects of hypoxic and hyperoxic stressors.

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PREFACE

This study was conducted at the Applied Biotechnology Branch, Airman Bioengineering Division, Airman Systems Directorate of the 711th Human Performance Wing of the Air Force Research Laboratory (AFRL / 711 HPW / RHBB), Wright-Patterson AFB, OH. This Interim technical report covers the performance period October 1, 2016 – September 31, 2018 for AFRL Work Unit H0AO.

Contractor employees were working under Cooperative Agreement with the Henry M. Jackson Foundation (FA8650-15-2-6608) or with the Oak Ridge Institute for Science and Education.

This study was conducted under the approved Air Force Research Laboratory Institutional Animal Care and Use Committee, Animal Protocol Number F-WA-2015-0156-A. All experiments involving animals were approved by the Wright-Patterson Air Force Base, Institutional Animal Care and Use Committee (IACUC), and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Research, National Research Council, National Academies Press, 2011, and in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

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1.0 SUMMARY

Aerospace professionals frequently experience hypoxia, which is followed by hyperoxic exposure as a compensatory measure. Both conditions have short- and long-term impacts on neurological and cognitive functions (including the impairment of memory, concentration, working memory and attention) and involuntary muscle movement, resulting in the slowing of motor tasks. Abnormal oxygen levels in the inspired air can induce organ injuries. However, the mechanisms of these changes, especially at the molecular and cellular levels, are not completely understood. In addition, the effect of different oxygen levels on the cell functions in the brain, lung and heart have not been comprehensively studied and correlated with the changes in cognitive functions and performance. To address this knowledge gap, the overall goal of this study was designed to investigate the effect of hypoxic and hyperoxic exposure on organ injuries in a systematic manner and correlate these results with impaired memory performance. This interim report described the results of the first two experiments of this study that focus on the temporal effect of high and low oxygen levels, 95 percent (%) and 7.5%, respectively, on memory performance and injuries of vital organs (i.e., brain, lung and heart). It was found that hypoxic exposure caused disruption of blood-brain barrier and alveolar-capillary barrier, resulting in brain and lung injuries. In addition, hypoxia also induced memory impairments. Molecular and biochemical analyses were subsequently performed using the tissues samples collected from the exposed animal subjects. The results of these analyses provided important mechanistic insight into the adverse effects of hypoxic and hyperoxic stressors. These findings, which are presented in this interim report, also facilitated our design of the remaining experiments of this study that aims to provide an effective means for the monitoring, prediction and prevention of the negative impacts of the hypoxic and/or hyperoxic stressors on health and performance. Since hypoxia and hyperoxia are among the most commonly encountered stressors in aerospace environment, and since these stressors have enormous negative impacts on performance and both short- and long-term health, we expect that the final products of this study will provide significant benefits to US Air Force and Airmen by countering the adverse effects associated with the hypoxic and hyperoxic stressors of the aerospace environment.

2.0 INTRODUCTION

2.1 Adverse Effects of Exposure to Abnormal Oxygen Levels (Hypoxia and/or Hyperoxia)

Hypoxia is a condition in which the tissues or organs in the body are deprived of adequate oxygen supply. The brain is exceptionally sensitive to hypoxic conditions, and the cerebral cortex is most affected by this stressor. Cerebral hypoxia results in devastating neurologic sequelae such as lack of concentration, light headache, dizziness, nervousness, tunnel vision, and panic, resulting in decreased performance. Hypoxia may result from a failure at any stage in the delivery of oxygen to cells, which may be due to decreased partial pressure of oxygen (ppO_2) in the inspired air, problems with breathing rhythm, problems with diffusion of oxygen in the lungs, insufficient hemoglobin in the blood, and/or problems with blood flow to the end tissues. During respiration, oxygen is absorbed in the lung, which depends on the gradient of ppO_2. The ppO_2 at sea level is approximately 159 millimeters of mercury (mmHg). In the lung, it is diluted by carbon dioxide and water vapor down to about 105 mmHg and passively diffuses to the arterial blood in the alveoli. Almost all oxygen in the blood is carried by hemoglobin, with only a very small fraction carried by the plasma. Hemoglobin increases the oxygen-carrying capacity of blood by about 40-fold. In the end tissues, oxygen diffuses into cells through a pressure gradient. Oxygen diffusion becomes rate limiting when ppO_2 falls to 60 mmHg or less, and such condition can be lethal (for reviews, see Sarkar et al., 2003; Clanton 2007; Adams et al., 2009; Cataldi 2010; Joyner and Casey 2014). In humans, hypoxia is detected by the chemoreceptors located in the carotid body that are sensitive to changes in both carbon dioxide and oxygen levels in the blood. Below the normal level of oxygen, the activity of neurons innervating these receptors increases dramatically and can override the signals coming from the central chemoreceptors in the hypothalamus. The signaling within the chemoreceptors is mediated by the release of neurotransmitters (including dopamine, noradrenaline, acetylcholine, substance P, vasoactive intestinal peptide and enkephalins) by the glomus cells (for reviews, see Shimoda et al., 2000; Acker and Acker 2004; Bärtsch and Saltin 2008; De Caro et al., 2010; Teppema and Dahan 2010).

Hypoxia normally causes dyspnea (shortness of breath or breathlessness) that stimulates ventilation. When the arterial partial pressure of oxygen (PaO_2) falls to about 50 mmHg, the oxygen chemoreceptors become stimulated and send impulses to the inspiratory and cardiovascular areas. This leads to autonomic reflex changes in the functions of the respiratory system (breath rate and breath amplitude) and the cardiovascular system (heart rate). Activation of these physiological systems results in an increase in the volume of inspired air and the cerebral blood flow aimed to maintain oxygen delivery to the brain and other vital organs. The increase in alveolar ventilation involves interaction of chemoreceptors, the respiratory control centers in the medulla, the respiratory muscles, and the lung/chest wall systems. Hyperventilation raises the depth and rate of breathing, thereby increasing alveolar ppO_2 to restore the level of PaO_2 toward normal capacity. Constriction of the pulmonary vascular smooth muscle occurs, together with coronary and cerebral vessel vasodilation to increase blood flow to tissues. In the peripheral organs, erythropoietin (EPO) is released by the kidney and liver cells, and vascular endothelial growth factors (VEGFs) are secreted by the parenchymal cells of

multiple organs (for reviews, see Shimoda et al., 2000; Acker and Acker 2004; Bärtsch and Saltin 2008; De Caro et al., 2010; Teppema and Dahan 2010).

At the molecular level, there is an immediate depolarization block by changing potassium, sodium and chloride ion fluxes across the cellular membrane. The metabolic and other cellular pathways are redirected to activate (and alter) the expression of an array of genes, to achieve enhanced cell survival under the hypoxic environment. This involves the up-regulation and/or stabilization of transcriptional factors (such as hypoxia-inducible factor (HIF)) essential for the transactivation of the oxygen responsive genes in a highly coordinated manner. Despite a general inhibition of protein synthesis under hypoxia, HIF-mediated up-regulation of enzymes and growth factors can induce neural stem cell growth, cell survival, angiogenesis, and anaerobic glycolysis in an organ-specific manner. However, certain polymorphisms in the genes involved in the Rapoport-Luebering and the renin-angiotensin pathways can affect the response and adaptation to hypoxia, resulting in genetic predispositions to adverse hypoxic response, including cerebral and pulmonary edema (for reviews, see Oski et al., 1970; Miwa 1982; Nakamura et al., 1986; Tanaka and Zerez 1990; Prchal and Pastore 2004; Cho et al., 2008).

Prolonged hypoxic exposure may lead to cerebral edema, pulmonary edema and other potentially fatal complications. Cerebral edema is a condition in which the brain swells with fluid. Symptoms of cerebral edema include confusion, disorientation, severe headaches, ataxia, fatigue, nausea, and loss of consciousness. Without proper care, cerebral edema can be fatal within 48 hours. Cerebral edema is caused by the breakdown of the blood brain barrier (BBB), allowing excessive fluid to accumulate in the extracellular space in the brain (McCormack et al., 1993; Juurlink 1997; Aschner et al., 2002). Inflammation that compromises the endothelial tight junctional structures is the major underlying mechanism. Damage to the BBB can also cause microvascular permeability and microhemorrhages, resulting in the disruption of white matter metabolism. Hemosiderin deposit (due to vascular permeability) can be detected in the affected areas. Hypoxia may also induce nitric oxide synthase. Nitric oxide induces vasodilation, which in turn can increase vascular permeability and result in vasogenic edema (for reviews, see Fung 2003; Zwingmann and Leibfritz 2003; Kemp et al., 2004; Weir and Olschewski 2006; Fukuda and Warner 2007; Chen et al., 2009; Waypa and Schumacker 2010; Jelkmann 2011; Bennett et al., 2012; Engelhardt et al 2014; Granger and Kvietys 2015)

Hypoxia also results in pulmonary edema. Two processes, pulmonary hypertension and increased vascular permeability, are believed to be important for the onset of pulmonary edema. In most tissues of the body, the response to hypoxia is vasodilation, in which the widening of the blood vessels will allow increased perfusion to the tissues. By contrast, the response to hypoxia in the lungs is vasoconstriction, known as hypoxic pulmonary vasoconstriction, resulting in increased pulmonary arterial and capillary pressures, i.e. pulmonary hypertension (for reviews, see Fagan and Weil 2001; Evans 2006; Sommer et al., 2008; Evans et al., 2011; Mishra et al., 2015).

In the aerospace environment, hypoxia could occur under the condition of low oxygen in the breathing air that might be caused by malfunctions of the on-board oxygen generation system (OBOGS) and the backup/ emergency oxygen supply systems. This could also occur under excessive and sustained g-forces, which can cause the blood to drain away from the brain and pool into the legs and abdomen by the centrifugal force, resulting in cerebral hypoperfusion, hypoxia and G-force induced loss of consciousness (G-LOC). Repeated exposure to the hypoxic stressor could result in neuronal injury and degraded cognitive functions. G-LOC may be prevented by the use of anti-g suits. As the g-force increases significantly, the anti-g suit will inflate and squeeze on the abdomen and the legs to prevent blood pooling to the lower part of the body (for reviews, see Werchan 1991; Bjurstedt 1993; Guillaume et al., 1997; Guillaume et al., 2007).

Intervention of hypoxic conditions normally involves oxygen supplementation, in which breathing air with ppO_2 significantly greater than the normal level is used. However, inspiration of air with ppO_2 higher than 159 mmHg only increases the amount of oxygen available to the tissue very slightly, but this will dramatically increase the chance of atelectasis (i.e., the collapse or closure of hte lung resulting in reduced or absent gas exchange). Exposure of the lungs and other tissues to excess levels of oxygen also result in hyperoxic stresses. Prolonged hyperoxic exposure can lead to oxygen toxicity, with symptoms like irritation, congestion, disorientation, cerebral and pulmonary edema, and even death. The harmful effects of hyperoxia are mainly due to an increased level of reactive oxygen species (ROS), which can damage the cellular constituents (including lipids, proteins, cell membranes and nucleic acids).

Oxygen toxicity depends on the oxygen concentrations in the inspired gas and exposure time. Although the human body has naturally occurring antioxidants to combat these ROS, the protective antioxidant defenses may become overwhelmed, resulting in oxidative stress and injury of the affected tissues and organs. Oxygen toxicity in the central nervous system (CNS) may cause a generalized tonic-clonic seizure – a type of generalized seizure that affects the entire brain and potentially causes a loss of consciousness. Signs of pulmonary oxygen toxicity begin with slight irritation in the throat with mild coughs, followed by greater irritation and worse coughs until breathing becomes quite painful and the coughing becomes uncontrollable. If the hyperoxic exposure is continued, chest tightness, difficulties in breathing and shortness of breath will occur; such respiratory problems can be fatal due to the damage to the lung that eventually makes it impossible for gas exchange to occur in the pulmonary alveoli. Vital capacity (i.e. the amount of air that can be inspired in one large breath) decreases with increasing pulmonary oxygen toxicity. Mild symptoms, which result in a reduction of approximately 2% in vital capacity, are completely reversible and will heal in 2-4 weeks, with no permanent lung damage. Severe symptoms, resulting in a 10% reduction of vital capacity, can prevent individuals from continued breathing, especially in the presence of a gas mixture with high ppO_2.

In the aerospace environment, the level of oxygen in the breathing air provided to the aircrews, especially at high altitudes, is much higher than the normal sea level ppO_2. Such high oxygen levels are intended to provide protections against rapid decompression

at high altitudes, since super-oxygenation of the bloodstream will maximize the time of useful consciousness should rapid decompression occur. In addition to the risk of oxygen toxicity, high oxygen concentrations also induce adverse physiological conditions such as atelectasis as described above. Therefore, an optimal ppO_2 in the breathing air will be needed to ensure that the ppO_2 is sufficient to maximally oxygenate the blood, without causing atelectasis, oxygen toxicity and organ injuries.

2.2 Physiological Incidents of F-22 Pilots

According to the house hearing on the F-22 physiological issues (House Hearing, 112 Congress [H.A.S.C. No. 112-154]: F-22 Pilot Physiological Issues), from 2003 to 2008, six (6) physiological incidents were reported by F-22 pilots, and the number had doubled to 12 between April 2008 and January 2011. From May to September of 2011, the F-22 fleet was temporarily grounded for four months, due to an upward trend in the physiological incidents. After the grounding of the F-22 ended, the pilots were directed to fly in the maximum oxygen production mode, to prevent or preclude any hypoxic conditions. Nonetheless, the number of hypoxia-like events continued to increase. Since the F-22 returned to flying status in September of 2011, there have been 11 hypoxia-like incidents, which were initially reported as unknown cause. Some of the pilots affected have experienced degradation in mental state, nausea, dizziness, confusion, decreased alertness, memory loss, disorientation, and loss of consciousness. Some of them also experienced lingering respiratory problems, fatigue and chronic coughing. Other symptoms include irritability, emotional abnormalities and neurological changes. In some pilots, these symptoms lasted for several days before recovery. Overall, the F-22 has a rate of 27 physiological incidents per 100,000 flight hours, which is eight times higher than that of other warplanes (House Hearing, 112 Congress [H.A.S.C. No. 112-154]: F-22 Pilot Physiological Issues). In July of 2012, the Air Combat Command (ACC) had determined that the cause of the F-22 pilot physiological issues was due to the oxygen content, but not the quality of the air supply delivered to the pilots. After the return to flight, finger pulse oximeters were used to monitor the blood oxygenation in the pilots. However, it was found that this device was not reliable, which suggests that a better approach for monitoring hypoxic exposure in F-22 pilots (and the pilots of other airplanes) is needed.

The oxygen supply in the F-22 is provided by an OBOGS. The system takes the bleed air from the compressor stage of the engine and concentrates it to a higher level of oxygen that matches the required level in the breathing air, based on the cabin pressure and altitude. When the temperature rises, especially at high-altitude with low-power settings, the air cycle machine may reduce or even shut down the output of the bleed air system. When this occurs, the supply of breathing air from the OBOGS to the pilot will decrease or even be completely shut off. Under these situations, the pilot may not receive enough oxygen and become hypoxic. This problem was compounded by the lack of a backup oxygen system in the F-22 (Williams, 2002; Miller, 2005). Although an emergency system could provide adequate oxygen supply should the OBOGS fail completely, the emergency system requires manual activation. However, adequate instrumentation that warns pilots of problems in oxygen supply for timely intervention was not available. As a result, delayed activation of the emergency oxygen supply in the event of a lack of breathing air from the OBOGS could significantly increase the chance of hypoxia (House

Hearing, 112 Congress [H.A.S.C. No. 112-154]: F-22 Pilot Physiological Issues; Department of Defense Inspector General Accident Investigation Board Reports on F-22A Mishaps, 2009 & 2010).

Upon the onset of hypoxia-like symptoms, the pilots are supposed to immediately switch to 100% oxygen. Across multiple flights, pilots are exposed to large variations in oxygen and pressure due to the flight environment and normal functioning of life support systems. While systems are designed to maintain consistent and adequate oxygen, pilots nonetheless may experience variability in levels that could have damaging effects on vital organs, including the brain, heart and lung. Additionally, high concentrations of oxygen in the breathing air that are commonly used in the aerospace environment can result in adverse physiological responses. This is because increasing the oxygen concentration will decrease the amount of inert gases (i.e. nitrogen) in the breathing air. The presence of these inert gases in the breathing air is to hold the alveoli open after the oxygen is absorbed in the lung. Low levels (or absence) of inert gases in the breathing air will result in alveoli deflation and collapse, or even a complete collapse of the lung (atelectasis). Consequently, gas exchange in the alveoli will reduce significantly or even stop completely. Spontaneous physiological responses like coughing will occur, until the alveoli opens up and the atelectasis condition no longer exists. In addition to these acute effects, hyperoxia can result in oxygen toxicity and long-term adverse effects including oxidative stress and organ injuries.

Therefore, one of the goals of this study is to investigate the effect of abnormal levels of oxygen in the inspired air on the cellular functions in vital organs in a systematic manner. Changes in the oxygen level in the inspired air, resulting in hypoxia and hyperoxia, as well as repeated hypoxic-hyperoxic exposure as described above, can induce severe organ injuries and degradation of health and performance. However, the effect of different oxygen levels on the cellular functions of the brain, lung and heart have not been comprehensively studied and correlated with the changes in cognitive functions. Thus, this study is designed to address this knowledge gap by elucidating the molecular underpinning of hypoxia- and hyperoxia-induced organ injuries and cognitive degradation in a systematic manner. Specifically, this interim report will describe the results of the analysis of the temporal effects of hypoxic and/or hyperoxic exposure, including the effects of 5-day and 3x 5-day exposure to hypoxia (7.5% oxygen), hyperoxia (95% oxygen) and oscillatory hypoxia-hyperoxia (10 cycles of 5% ↔ 95% oxygen) on memory performance and injuries of the brain, lung and heart.

3.0 MATERIALS AND METHODS

3.1 Animal Husbandry

This study protocol was reviewed and approved by the Wright-Patterson Air Force Base IACUC and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance. The experiments in this report were conducted in a facility accredited by the AAALAC. All experiments were performed in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals" (National Research Council of the National Academies, Guide for the Care and Use of Laboratory Animals).

The animals used in this study were 6-week old Specific Pathogen Free Sprague Dawley Rats (from Charles River Laboratories, Wilmington, MA). They were socially housed in clear plastic cages (2 rats/cage) and provided tunnels, nesting material, and nylon bones for enrichment. Animals were provided with conventional bedding (CellZorb, Cincinnati Lab Supply, Cincinnati, OH). Food (LabDiet Formulab Diet 5008, Cincinnati Lab Supply) and water were freely available. The animal rooms were climate-controlled (20 – 26 degrees Celsius (°C), 30 – 50 % humidity) and maintained at a 12-h light/dark cycle (on at 0600).

3.2 Study Design

The design of these experiments was to investigate the temporal effects on the injuries of vital organs induced by hypoxia and hyperoxia. Specifcially, organ injuries in the brain, lung and heart, induced by high and low oxygen levels (95% and 7.5%, respectively) were determined and correlated with with the degree of impaired cognitive performance. Blood, brain, lung and heart specimens are collected from the control and treated animals for the evaluation of organ injuries and dysfunctions, using well-established laboratory tests and assays. Cognitive performance of the control and treated animals are assessed using the novel object recognition (NOR) test, which evaluate the formation and retrieval of episodic memory. Therefore, the findings of this study will provide a mechanistic understanding of the adverse effects of the hypoxic and hyperoxic stressors.

3.3 Hypoxic and/or Hyperoxic Exposure

Normobaric exposure to inspired air containing altered oxygen levels were performed in custom-built, clear, polycarbonate chambers approximately 40 cm wide, 35 cm long, and 21 cm high (Coy Laboratory Products, Grass Lake, MI), with real-time control over the internal gas concentrations. The chambers were calibrated daily using compressed, medical grade Nitrogen and Oxygen gases (Airgas, Dayton, OH). The oxygen level was set according to the exposure condition and supplemented with nitrogen to maintain a normobaric atmosphere in the chamber. Sixty animals were divided into five experimental groups (12 animals/group). Group 1 exposed to 21% oxygen, group 2 exposed to 7.5% oxygen, group 3 exposed to 95% oxygen, group 4 exposed to repetitive cycling of hypoxia 5% oxygen/hyperoxia 95% oxygen and group 5 Open Air Control. Due to size limitations of the test chambers, the animal exposures were conducted as follows: Groups of two cohoused animals were transferred to the same chamber daily and

exposed to one of three atmospheres for 5 days. Normal oxygen level exposure was 21% (ppO_2 = 159 mmHg), corresponding to the oxygen level at sea level for 60 minutes. Hypoxic exposure was 7.5% oxygen, corresponding to an oxygen level at altitude of 8200 meters (ppO_2 = 57 mmHg) for 60 minutes. Hyperoxia-treated subjects breathed 95% oxygen (ppO_2 = 722 mmHg) for 60 minutes. The oscillatory hypoxia-hyperoxia group started with 15 minutes at 95% oxygen, followed by the exposure to 10 cycles of hypoxia-hyperoxia (5% oxygen for 3 minutes followed by 95% oxygen for 8 minutes). It normally takes less than 2 minutes to complete each change in oxygen concentration in the chamber, so the total exposure lasted about 2.5 hours. During the treatment, the animals were continuously monitored for signs of serious distress. The Open Air Control group was exposed to normal atmospheric room air, housed in clear plastic cages near the exposure chambers. After the treatments, animals were allowed to recover for 15 minutes in normal open air before returning to their home cages.

3.4 Assessment of Episodic Memory Performance

Learning and memory performance were assessed using the NOR test. This task measures the formation and retrieval of episodic memory. Performance in this task relies on prefrontal cortex and hippocampus (Vogel-Ciernia and Wood, 2014). The advantage of this test is that it is less stressful than other methods, such as the Morris water maze. Since the NOR task relies on the natural preference for novel objects displayed by rodents, no positive or negative reinforcement is needed. The test procedure consists of three phases: habituation, familiarization, and test phase. In the habituation phase, each animal is allowed to freely explore the open-field arena in the absence of objects. During the familiarization phase, an animal is placed in the open-field arena containing two identical sample objects (A + A) for a predetermined time period (typically 5 minutes). After the retention period (from 24 up to 120 hours), the animal is again placed in the open-field arena containing two objects, one is identical to the training object, while the other is a novel one (A + B) (Ennaceur 2010; Ennaceur and Dela-cour, 1988; Gaskin et al., 2010; Hammond et al., 2004; Taglialatela et al., 2009). During the test phase, the animal would normally spend more time exploring the novel object. The strongest novel object preference scores tend to occur early in the test phase while the novel object is still relatively novel (Broadbent et al., 2009). After this initial period, the novel object starts becoming familiar and gradually losing its attractiveness.

Animals underwent NOR testing at the end of the 5-day treatment of altered oxygen levels using a procedure similar to that described by Barnes *et al* (2017). The NOR field was an open-top, 60 cm square, black plastic box with 40 cm walls. Activity was video-recorded and analyzed using Ethovision XT12 tracking software with a three-point body-tracking module (Noldus, Leesburg, VA). Fluorescent tube lights supplied 3 to 6 lux to the NOR field. Habituation consisted of 30 minutes in the testing room and 10 minutes in an empty NOR field each day, starting 3 days before the familiarization phase. Exploration time for familiarization was 10 minutes, and for testing was five minutes. Three similarly sized objects were used (i.e., structures of different shapes made from multicolor Lego blocks), and exploration included a 2 cm buffer around the object. Familiar and novel objects, were assigned randomly while ensuring equal distribution to the groups, with their locations in the NOR field using a counterbalanced design. Objects

were placed in equal distances from opposite corners of the field and held in place by Velcro. Rats with a statistically significantly positive discrimination ratio (DR) (Inostroza et al., 2013) passed the test, while the magnitude of the ratio along with the percent of time exploring each object served to indicate the degree of effect.

3.5 Analysis of Potential Biomarkers of Hypoxic and/or Hyperoxic Exposure

Upon the completion of the NOR test and 2 days of recovery, the animals were euthanized by exsanguination or decapitation under anesthesia (Ketamine/Xylazine). The heart, lung and brain were collected, weighted and stored at -80 °C. Some of these tissues samples were fixed in 4% paraformaldehyde, where applicable. Whole blood was collected in K_3EDTA Vacuette tubes (Greiner, Monroe, NC) for the measurements of hematocrit, hemoglobin, 1,3–bisphosphoglycerate (1.3–BPG), 2,3–BPG, and bisphosphoglycerate mutase (BPGM). Hemoglobin was measured using the Hemoglobin Assay Kit (MilliporeSigma, St. Louis, MO). Blood for the hematocrit was processed using the StatSpin MP (Iris, Chatsworth, CA) and measured using the CritSpin Digital Hematocrit Reader (Iris). Red blood cells (RBCs) were collected by centrifugation and lysed using RBC lysis buffer (Abcam, Cambridge, MA) in order to measure the concentrations of 1,3–BPG and 2,3–BPG and BPGM, using analyte-specific, rat enzyme-linked immunosorbent assay (ELISA) kits (MyBiosource, San Diego, CA). Serum was isolated using centrifugation of post-mortem blood collected in BD Vacutainer SST tubes (BD, Franklin Lakes, NJ) and stored at -80 °C until used for the quantification of the level of angiotensin II, copeptin, S100B, VEGF (MyBiosource), and EPO (Biomatik, Wilmington, DE), using ELISA kits for the respective analytes. The bioassays conducted in this study are outlined in the table below.

Table 1. List of Bioassays Performed Using Samples Collected

Biological Process	Assay	Sample(s)
Brain Injury	Fluoro-Jade staining	Brain
	Glial fibrillary acidic protein (GFAP) staining	
	Cerebral edema	
	S100B (Blood brain barrier damage)	Serum
Lung Injury	Pulmonary edema	Lung
	Hematoxylin and eosin (H&E) staining	
	Hemoglobin concentration	Bronchoalveolar Lavage (BAL)
	Albumin concentration	
	Total white blood cell count	
Heart Injury	H&E staining	Heart
Oxygen Binding Capacity and Affinity	Erythropoietin	Serum
	Hematocrit	Whole Blood
	Bisphosphoglycerate mutase	Erythrocytes
	2,3-bisphosphoglycerate and 1,3-bisphosphoglycerate concentrations & ratio	
Hypoxia-Induced Response	Angiotensin II	Serum
	Copeptin (surrogate for vasopressin)	
	Vascular endothelial growth factor	

3.6 Statistical Analysis

The data were analyzed using Prism 7 for Windows, version 7.05 (GraphPad Software, La Jolla, CA). The means were compared using one-way analysis of variance with alpha = 0.05, followed by the Bonferroni's multiple comparison correction test when a significant effect was identified. The mean for the experimental groups was compared to the 21% Oxygen Control group. The comparison of this Control group to the Open Air Control allows the evaluation of the chamber effect.

4.0 RESULTS

4.1 Experimental Design – Analysis of Temporal Effect of Hypoxic and/or Hyperoxic Exposure on Memory Performance and Organ Injuries

The objective of this experiment is to investigate the temporal effects of hypoxic and/or hyperoxic exposure on memory performance, which will be correlated with the injuries in three vital organs, brain, heart and lung. In this experiment, the baseline memory performance of the animals was determined using the NOR test, prior to the hypoxic and/or hyperoxic exposure. After the baseline NOR test, the animals were subjected to 60-minute hypoxic, hyperoxic or 10 cycles of oscillatory hypoxic-hyperoxic treatment for 5 days or 3x 5 days. Assessment of memory performance was also performed at the end of the entire 5-day or 3x 5-day treatment. After 2 days of recovery, the animals were euthanized, and tissue samples collected for the bioassays as described in the MATERIALS AND METHODS Section. The overall design of this experiment is shown in Figure 1.

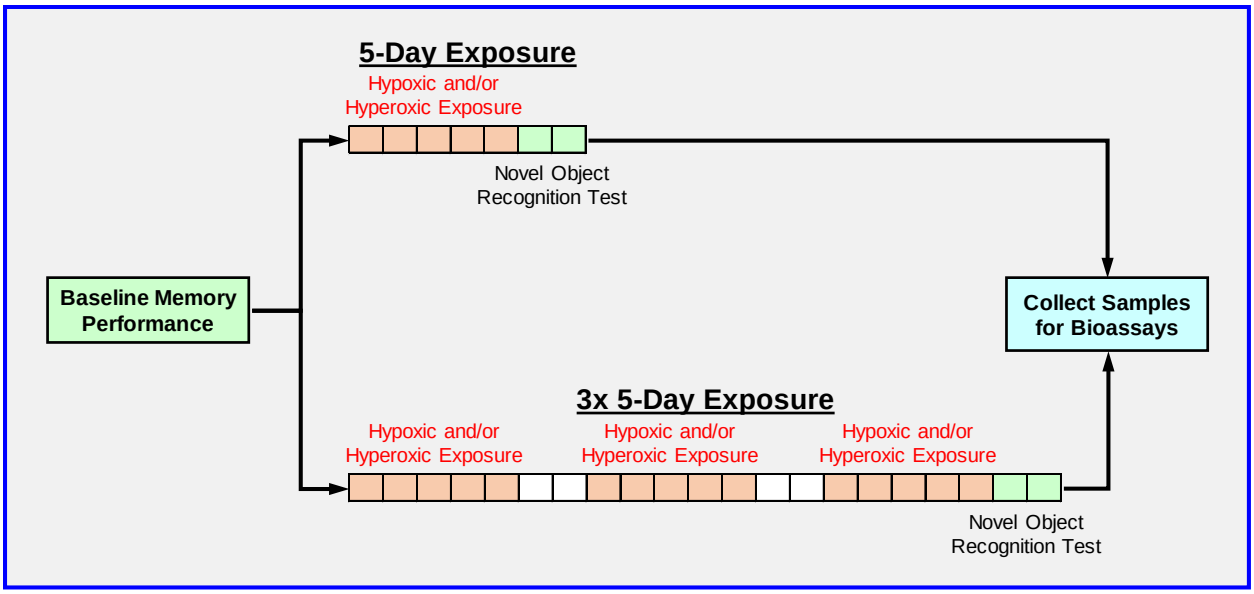

Figure 1. Graphical Representation of Experimental Design

Green boxes indicate the days on when memory performance (NOR) was evaluated. Light red boxes represent the days on which hypoxic and/or hyperoxic treatments were performed. White boxes indicate no treatment (rest days). Animals (n=12) were subjected to normobaric (760 mmHg) hypoxia (7.5% O_2, ppO_2 = 57 mmHg for 60 minutes), normoxia (21% O_2, ppO_2 = 159 mmHg for 60 minutes), hyperoxia (95% O_2, ppO_2 = 722 mmHg for 60 minutes), or oscillatory hypoxia-hyperoxia (5% O_2, ppO_2 = 38 mmHg for 3 minutes, then increased to 95% O_2, 722 mmHg for 8 minutes, repeated for 10 cycles), using the exposure chamber from Coy Lab Product (Grass Lake, MI). Animals in the Open Air Control group were placed in regular rat cages and exposed to normal atmospheric room air for 60 minutes. Prior to the first hypoxic and/or hyperoxic exposure, the baseline memory performance of the animals were determined using the NOR test. Assessment

of memory performance was also performed after the final exposure. At the end of the experiment, the animals were euthanized, and tissue samples collected for various bioassays.

4.2 Effect of Hypoxic and/or Hyperoxic Treatments on Body and Organ Weights

Of the various exposure conditions, the repeated cycles of oscillatory hypoxic-hyperoxic treatment appeared to be the most stressful. Although the increase in body weight after 5-day or 3x 5-day of exposure was observed in the oscillatory hypoxic-hyperoxic treatment group, it is significantly lower than that of the Open Air Control Group. In contrast, neither hypoxic nor hyperoxic exposure alone showed any significant effect on the increase in body weight (Tables 2 & 3). The values in all tables of this report represent Mean±Stand Deviation.

Table 2. Body Weight Changes in Rats Subjected to 5-Day Hypoxic and/or Hyperoxic Treatments

Experimental Group	Body Weight (g)		% of Pre-Exposure Weight
	Pre-Exposure	Post-Exposure	
21% Oxygen	450.3±35.4	480.7±37.7	106.8±3.7
7.5% Oxygen	436.1±41.1	464.1±50.3	106.45±6.7
95% Oxygen	435.4±40.5	460.4±45.4	105.7±2.9
5% ↔ 95% Oxygen	439.8±36.4	457.3±35.4	104.0±2.5*
Open Air	436.9±36.3	464.4±34.9	106.4±3.5

* P=0.02 (compared to 21% Oxygen group)

Table 3. Body Weight Growth in Rats Subjected to 3x 5-Day Hypoxic and/or Hyperoxic Treatments

Experimental Group	Body Weight (g)		% of Pre-Exposure Weight
	Pre-Exposure	Post-Exposure	
21% Oxygen	390.4±26.9	459.3±34.7	117.8±6.6
7.5% Oxygen	426.7±39.2	467.0±48.6	110.2±15.9
95% Oxygen	415.3±30.3	474.8±37.9	114.3±3.3
5% ↔ 95% Oxygen	430.1±16.9	466.4±25.2	108.5±5.2*
Open Air	411.3±16.8	477.6±30.9	116.1±5.2

* P=0.01 (compared to 21% Oxygen group)

However, no significant differences in the total weight of the brain, lung and heart between these experimental groups were not observed (Tables 4 & 5), although a positive trend of reduced heart and lung weights, compared to the Open Air Control Group, were detected after 5-day 95% oxygen and oscillatory hypoxic-hyperoxic treatments,

respectively (Table 4). In contrast, the animals receiving these treatments appeared to be able to maintain the weights of these organs after 3x 5-days of treatment, suggesting that an adaptive response may have occurred after 5 days of exposure.

Table 4. Weight of Selected Organs in Rats Subjected to 5-Day Hypoxic and/or Hyperoxic Treatments*

Experimental Group	Lung (g)	Heart (g)
21% Oxygen	4.04±0.43	1.67±0.20
7.5% Oxygen	3.86±1.27	1.66±0.21
95% Oxygen	3.89±0.61	1.49±0.21[#]
5% ↔ 95% Oxygen	3.48±0.73[#]	1.57±0.28
Open Air	4.00±0.81	1.49±0.30

* The weight of the whole brain was not determined in this experiment.
[#] P<0.1 (compared to 21% Oxygen group)

Table 5. Weight of Selected Organs in Rats Subjected to 3x 5-Day Hypoxic and/or Hyperoxic Treatments

Experimental Group	Brain (g)	Lung (g)	Heart (g)
21% Oxygen	2.08±0.42	3.29±1.26	1.64±0.22
7.5% Oxygen	1.90±0.39	3.74±1.60	1.87±0.61
95% Oxygen	2.19±0.12	3.34±1.51	1.72±0.26
5% ↔ 95% Oxygen	2.11±0.24	3.40±1.42	1.59±0.22
Open Air	2.11±0.27	3.70±1.18	1.72±0.25

4.3 Effect of Hypoxic and/or Hyperoxic Treatments on Brain Injury

Brain injury was evaluated using two parameters, namely cerebral edema and BBB damage. Although the body and organ weight seemed to be impacted by hypoxic and/or hyperoxic exposure, none of these treatments, including the oscillatory hypoxic–hyperoxic exposure, resulted in cerebral edema, as determined by the water content (in percentage) in the right brain, even after the 3x 5-day treatment (Table 6).

Table 6. Cerebral Edema in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	% Water of Total Weight (Right Brain)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	77.24±0.76%	78.56±3.69%
7.5% Oxygen	77.28±0.30%	74.67±1.73%
95% Oxygen	77.36±0.47%	81.12±1.49%
5% ↔ 95% Oxygen	77.27±0.35%	80.04±0.84%
Open Air	77.46±0.76%	78.37±3.90%

S100B has emerged as a candidate peripheral biomarker of BBB permeability and CNS injury. Serum levels of S100B increase in patients during the acute phase of brain damage. Elevated S100B levels accurately reflect the presence of neuropathological conditions including traumatic head injury or neurodegenerative diseases. Interestingly, this analyte significantly increased after 5 days of hypoxic exposure (Table 7), despite that no cerebral edema was detected in this group of animals. This analyte also increased after 3x 5-day exposure to hypoxia and/or hyperoxia, but the change was not statistically significant. A large difference in the serum concentration of S100B was observed between the animals used in these two experiments. The reason for this is not clear. This might be due to different lots of animals used in these experiments, or the age difference at the time of sample collection - due to additional 2 weeks required for the 3x 5-day treatment experiment. Nevertheless, this does not affect the overall conclusion of this result.

Table 7. S100B Concentration in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	S100B Concentration (pg/ml)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	26.69±1.63	159.40±33.30
7.5% Oxygen	34.98±3.32*	248.07±68.64
95% Oxygen	24.11±1.69	323.17±117.64
5% ↔ 95% Oxygen	23.79±2.54	212.49±54.72
Open Air	26.20±1.79	164.93±24.62

* P<0.05 (compared to 21% Oxygen group)

4.4 Effect of Hypoxic and/or Hyperoxic Treatments on Lung Injury

Similarly, lung injury was evaluated using two parameters, namely pulmonary edema and disruption of the alveolar-capillary barrier. The 5-day hypoxic and/or hyperoxic treatments used in this study did not cause pulmonary edema (Table 8). However, prolonging the exposure to 3x 5 days produced a positive trend of reduced water content in the lung tissue of the hypoxic treatment group, which is independent of the changes in the weight of this organ.

Table 8. Pulmonary Edema in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	% Water of Total Weight (Right Lung)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	79.33±1.54%	84.25±3.39%
7.5% Oxygen	79.12±1.10%	78.24±4.50%[#]
95% Oxygen	79.52±2.29%	86.38±3.05%
5% ↔ 95% Oxygen	77.34±2.57%	83.61±3.84%
Open Air	76.13±8.40%	83.33±4.63%

[#] P<0.1 (compared to 21% Oxygen Group)

Disruption of the alveolar-capillary barrier, which is generally associated with increased blood-derived proteins in the bronchoalveolar lavage (BAL), also known as bronchoalveolar washing, was assessed. To evaluate the integrity of the alveolar-capillary barrier, BAL was prepared and used in the assays of hemoglobin, albumin and total white blood cell (WBC) count. No change in the hemoglobin or albumin concentration in the BAL could be detected after 5-day or 3x 5-day hypoxic and/or hyperoxic exposure (Tables 9 & 10). In fact, there is a trend that albumin was decreased in the 7.5% oxygen group. These results thus suggest that the alveolar-capillary barrier appears to be intact in the treated animals. Consistently, there was no increase in the total number of WBCs in BAL from rats undergoing hypoxic and/or hyperoxic treatments (Table 11). Interestingly, a positive trend of chamber effect was observed in the 5-day exposure experiment (Tables 9-11).

Table 9. Hemoglobin Concentration in Bronchoalveolar Lavage (BAL) from Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Hemoglobin Concentration (g/dL)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	14.65±1.83	17.17±1.57
7.5% Oxygen	12.84±1.59	21.75±2.29
95% Oxygen	14.61±1.66	18.26±1.47
5% ↔ 95% Oxygen	15.65±2.38	19.48±1.93
Open Air	9.03±0.74[#]	18.64±2.54

[#] P<0.1 (compared to 21% Oxygen Group)

Table 10. Albumin Concentration in Bronchoalveolar Lavage (BAL) from Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Albumin Concentration (μg/mL)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	18.68±3.89	12.78±2.19
7.5% Oxygen	19.55±3.10	10.13±1.22[#]
95% Oxygen	29.49±13.35	10.74±2.15
5% ↔ 95% Oxygen	20.12±3.16	12.53±2.08
Open Air	15.76±2.65[#]	13.08±1.96

[#] P<0.1 (compared to 21% Oxygen Group)

Table 11. Total Number of White Blood Cells (WBCs) in Bronchoalveolar Lavage (BAL) from Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Albumin Concentration (μg/mL)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	3.00E+06±9.48E+05	1.27E+06±5.55E+05
7.5% Oxygen	3.93E+06±2.20E+06	1.69E+06±8.67E+05
95% Oxygen	1.04E+06±2.79E+05	1.44E+06±8.03E+04
5% ↔ 95% Oxygen	1.84E+06±1.45E+06	1.86E+06±7.86E+05
Open Air	8.09E+05±2.36E+05[#]	4.51E+05±1.75E+05[#]

[#] P<0.1 (compared to 21% Oxygen Group)

4.5 Effect of Hypoxic and/or Hyperoxic Treatments on Oxygen Binding Capacity/ Affinity and Delivery

EPO (also known as hematopoietin or hemopoietin) is a glycoprotein cytokine secreted by the kidney in response to cellular hypoxia. EPO stimulates red blood cell production (erythropoiesis) in the bone marrow. Under hypoxic conditions, EPO production may increase up to 1000-fold. As shown in Table 12, 5-day hypoxic and oscillatory hypoxic-hyperoxic treatments resulted in significant increases in EPO. In contrast, significantly decreased EPO was observed in the hyperoxia-exposed animals. Interestingly, no significantly changes in the EPO was detected in any of the animal groups after 3x 5-day hypoxic/ hyperoxic treatment, suggesting that a negative feedback regulation and adaptation occur during prolonged hypoxic/ hyperoxic exposure. We observed that there is a ~2.3-fold difference in the EPO concentration in the animal groups between the 5-day and 3x 5-day exposure experiments; this however does not affect the overall conclusion of the result.

Table 12. Serum Erythropoietin (EPO) Concentration in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Erythropoietin Concentration (pg/mL)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	261.83±61.52	609.95±41.95
7.5% Oxygen	361.20±132.04*	645.78±70.78
95% Oxygen	218.77±54.53*	745.17±172.24
5% ↔ 95% Oxygen	337.90±108.49*	673.70±89.70
Open Air	258.11±91.03	600.61±43.69

* P<0.05 (compared to 21% Oxygen Group)

To evaluate the effect of increased EPO in these animals, hematocrit was determined after 5-day or 3x 5-day treatment. As shown in Table 13, there ~~is~~ were no significant changes in the hematocrit in any treatment group in the 5-day treatment experiment. On the other hand, it was significantly increased in the 7.5% oxygen group only after 3x 5-day exposure, indicating that there is a lag phase between increased EPO production and the occurrence of increased RBCs in the blood. It also suggests that by this time, the negative feedback control mechanism has been activated and down-regulated the production of EPO.

Table 13. Hematocrit in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Hematocrit (%)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	41.5±3.5	40.42±2.55
7.5% Oxygen	41.9±3.5	45.02±3.09*
95% Oxygen	41.5±3.3	41.98±3.04
5% ↔ 95% Oxygen	40.4±3.1	40.65±2.44
Open Air	39.6±2.6	41.34±2.45

* P<0.001 (compared to 21% Oxygen Group)

Besides increased EPO expression and RBCs production, hypoxic response also involves changes in the oxygen binding affinity of hemoglobin, which depends on the metabolism of BPG and the ratio of 2,3–BPG to 1,3–BPG. 1,3–BPG is a metabolic intermediate in the glycolytic pathway, generated from D-glyceraldehyde 3-phosphate by glyceraldehyde phosphate dehydrogenase. One of its roles is for ATP production in the glycolytic pathway catalyzed by 3-phosphoglycerate kinase. The level of 1,3–BPG will rise when oxygen levels are low, as one of the mechanisms of adaptation. Increased 1,3–BPG levels in turn raises the level of 2,3–BPG, which facilitates the efficiency of

oxygen release from hemoglobin. 2,3–BPG is part of a feedback loop that helps prevent tissue hypoxia to counteract the detrimental effects of this stressor. It is a key factor to regulate the release of oxygen from hemoglobin, by shifting the equilibrium of hemoglobin toward the deoxy-state. 2,3–BPG can fit neatly into the cavity of the deoxygenated hemoglobin beta subunits, which in turn decreases the affinity for oxygen and allosterically promotes the release of the remaining oxygen molecules bound to hemoglobin. Therefore, it can enhance the release of oxygen from RBCs near the end tissues. As shown in Table 14, 5-day hypoxic and/or hyperoxic treatment did not result in any significant change in the levels of 1,3–BPG, 2,3–BPG nor the ratio of these two molecules. On the other hand, a positive trend of increased 2,3–BPG and the ratio of 2,3–BPG to 1,3–BPG was observed after 3x 5-day hypoxic and oscillatory hypoxic-hyperoxic exposure (Table 15).

Table 14. Red Blood Cell Bisphosphoglycerate Concentration and Ratio in Rats Subjected to 5-Day Hypoxic and/or Hyperoxic Treatments

Experimental Group	[1,3–BPG] (nmol/mL)	[2,3–BPG] (nmol/mL)	[2,3–BPG]:[1,3–BPG]
21% Oxygen	4.59±1.85	85.52±33.06	4.59±0.56
7.5% Oxygen	4.64±1.88	74.12±22.69	4.64±0.57
95% Oxygen	3.76±1.79	75.83±23.25	3.76±0.54
5% ↔ 95% Oxygen	4.02±2.36	105.16±36.20	4.02±0.71
Open Air	3.28±1.23	52.47±17.38	3.28±0.37

Table 15. Red Blood Cell Bisphosphoglycerate Concentration and Ratio in Rats Subjected to 3x 5-Day Hypoxic and/or Hyperoxic Treatments

Experimental Group	[1,3–BPG] (nmol/mL)	[2,3–BPG] (nmol/mL)	[2,3–BPG]:[1,3–BPG]
21% Oxygen	3.90±1.34	229.45±24.04	62.69±7.17
7.5% Oxygen	3.63±1.13	314.74±40.38[#]	88.10±10.03[#]
95% Oxygen	3.98±1.46	275.57±18.38	76.46±7.64
5% ↔ 95% Oxygen	3.41±1.69	301.88±40.86	109.72±22.58[#]
Open Air	4.28±1.36	218.92±33.59	60.24±10.21

[#] P<0.1 (compared to 21% Oxygen Group)

The conversion of 1,3–BPG to 2,3–BPG is catalyzed by the enzyme BPGM, which is unique to erythrocytes (and placental cells). Although there is a positive trend of increased 2,3–BPG and the ratio of 2,3–BPG to 1,3–BPG after 3x 5-day hypoxic and oscillatory hypoxic-hyperoxic treatments, a similar degree of increase in BPGM is not observed. Surprisingly, a trend of decreased BPGM was in fact detected in the animal group received 3x 5-day exposure to 7.5% oxygen. This result somewhat confirms the activation of the negative feedback regulation and adaptation.

Table 16. Red Blood Cell Bisphosphoglycerate Mutase (BPGM) Concentration in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Bisphosphoglycerate Mutase Conc. (pg/mL)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	55.64±13.03	30.18±6.67
7.5% Oxygen	63.49±19.34	17.22±2.84[#]
95% Oxygen	63.36±21.19	23.33±3.70
5% ↔ 95% Oxygen	84.54±43.83	19.52±2.07
Open Air	75.53±28.35	23.64±2.67

[#] P<0.1 (compared to 21% Oxygen Group)

Hypoxic response also involves the formation of new blood vessels from pre-existing vasculature (i.e. angiogenesis), a process stimulated by VEGF, which belongs to the platelet-derived growth factor family of cystine-knot growth factors. It is part of the adaptation system to restore the oxygen supply to end tissues under hypoxic conditions when blood supply is inadequate. VEGF production can be induced in cells that does not receive enough oxygen. In cells, HIF1α and HIF1β are constantly being produced, but they are highly labile under aerobic conditions. When the cell becomes hypoxic, the HIF1α/β complex is stabilized, which in turn stimulates the expression and release of VEGF. Circulating VEGF then binds to its receptors on endothelial cells, triggering a tyrosine kinase pathway leading to angiogenesis. As shown in Table 17, 5-day hypoxic and/or hyperoxic exposure resulted in increased VEGF expression, while prolonging the treatment period to 3x 5 days again appears to activate the negative feedback control mechanism, resulting in no significant changes in VEGF.

Table 17. Serum Vascular Endothelial Growth Factor (VEGF) Concentration in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	VEGF Concentration (pg/ml)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	153.74±16.06	105.77±21.40
7.5% Oxygen	188.73±12.82[#]	124.28±11.75
95% Oxygen	203.32±21.04[#]	149.94±14.17
5% ↔ 95% Oxygen	214.84±6.55*	144.62±14.13
Open Air	197.14±13.60	166.85±19.05

* P<0.05; [#] P<0.1 (compared to 21% Oxygen Group)

In most tissues of the body, hypoxic response results in vasodilation – the widening of the blood vessels to improve perfusion and thus oxygen supply to the end tissues. In contrast, hypoxia response involves vasoconstriction in the lung – a process known as hypoxic pulmonary vasoconstriction. Angiotensin is a peptide hormone that causes vasoconstriction and an increase in blood pressure. Angiotensin II acts on the CNS to increase vasopressin production. It also acts on the venous and arterial smooth muscle to cause vasoconstriction. There is a crosstalk between HIF-1 pathway and the angiotensin pathway. Angiotensin II plays an important role in the intracellular accumulation of HIF-1α under hypoxic conditions. It regulates hypoxia-induced VEGF synthesis stimulated by HIF-1α. Angiotensin II is also capable of stimulating HIF-1α accumulation in cells. Interestingly, hypoxic exposure significantly reduced and increased angiotensin II concentration after 5 days and 3x 5 days of hypoxic treatment, respectively (Table 18), suggesting that it may be involved in vasodilation in the near-term and the induction of HIF1α in the long-term.

Table 18. Serum Angiotensin II Concentration in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Angiotensin II Concentration. (pg/ml)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	1941.7±206.0	1433.4±559.0
7.5% Oxygen	1240.3±152.2*	2495.8±746.2*
95% Oxygen	1800.0±266.3	1680.2±940.3
5% ↔ 95% Oxygen	1559.6±133.9#	1547.0±440.2
Open Air	1518.4±250.3	1849.9±1112.5

* P<0.005; # P<0.1 (compared to 21% Oxygen Group)

Angiotensin II can act on CNS to increase the production of arginine vasopressin (AVP), also known as antidiuretic hormone). AVP is synthesized as a peptide prohormone in neurons in the hypothalamus. It is converted to AVP and travels down the axon that terminates in the posterior pituitary. It is released from vesicles into the circulation in response to extracellular fluid hypertonicity (hyperosmolality). AVP causes the kidneys to reabsorb solute-free water and return it to the circulation from the tubules of the nephron, thus restoring the tonicity of the bodily fluids toward normal. AVP also constricts arterioles, which increases peripheral vascular resistance and raises arterial blood pressure. However, measurement of AVP is not practical because of its very short half-life, making it difficult to quantify and obtain a reliable result. On the other hand, copeptin, a 39-amino acid-long peptide derived from the C-terminus of pre-pro-hormone of AVP, neurophysin II and copeptin, can be measured with ease. In response to serum osmolality fluctuations, the kinetics of copeptin are comparable to that of vasopressin. Although there is no known biological functions for copeptin once it is secreted into the bloodstream, it has been used as a reliable surrogate for vasopressin expression. Consistent with the result of angiotensin II, there is a positive trend of increased copeptin

(and thus AVP) expression in animals after 5-day or 3x 5-days of hypoxic exposure (Table 19), further confirming the crosstalk between the HIF-1 and angiotensin pathways.

Table 19. Serum Copeptin Concentration in Rats Subjected to Hypoxic and/or Hyperoxic Treatments

Experimental Group	Copeptin Concentration (pg/ml)	
	5-Day Exposure	3x 5-Day Exposure
21% Oxygen	37.25±6.31	219.59±41.75
7.5% Oxygen	51.86±5.63[#]	311.86±41.30[#]
95% Oxygen	51.84±6.88	191.32±48.03
5% ↔ 95% Oxygen	48.18±5.67	263.59±36.50
Open Air	40.98±4.98	251.14±154.45

[#] P<0.1 (compared to 21% Oxygen Group)

4.6 Effect of Hypoxic and/or Hyperoxic Treatments on Memory Performance

The body's Initial response to lowered blood oxygen is to redirect the blood to the brain to increase cerebral blood flow. If increased blood flow cannot completely correct the problem, symptoms of cerebral hypoxia will begin to appear. Mild symptoms include difficulties with complex learning tasks and reductions in short-term memory. If oxygen deprivation continues, cognitive disturbances, and decreased motor control will result. Since brain cells are extremely sensitive to reduced oxygen levels, they will begin to die off within a few minutes once deprived of oxygen supply. Severe oxygen deprivation can result in fainting, long-term loss of consciousness, coma, seizures, cessation of brain stem reflexes, and brain death. However, we would like to point out that the treatment condition used in this study is relative mild that none of these severe symptoms occurred.

Brain damage can occur both during and after oxygen deprivation. During oxygen deprivation, cell death occurs by the increase of acidity in the brain tissue (acidosis). Additionally, there is buildup of materials that can generate free radicals. When oxygen re-enters the tissue, these materials interact with oxygen to generate high levels of oxidizing agents, which can interfere with the normal brain chemistry and cause further damage – a process known as reperfusion injury. To evaluate the functional effect of hypoxic and/or hyperoxic exposure, the pre– and post–treatment memory performance of the animals was investigated using the novel object recognition test. As shown in Table 20, the animals used in each of these experiments, as a group, (i.e. containing all animals used in the experiment regardless of the treatment groups they were assigned to), showed preference for the novel object and passed the test at baseline (pre-exposure). However, some animals did show position preference during training. While the animals of the third experiment (i.e., Experiment c) of 5-day exposure study showed a positive trend of preferring the familiar object position, the animals of the third experiment (i.e., Experiment c) of the 3x 5-day exposure study showed statistically significant preference

for the novel object position. Despite the position preference during training, all animal groups showed increased and statistically significant Discrimination Index during testing. Since position preference will render the result of the NOR test invalid, animals that showed strong position preference were excluded from the data set during data analysis.

Table 20. Pre-Exposure Baseline Novel Object Recognition Performance of Rats Used in 5-Day and 3x 5-Day Exposure Studies*

Experiment	NOR Task	Discrimination Index (%)		P-Value
		Familiar Object Position	Novel Object Position	
5-Day Exposure (Experiment a, n=20)	Training	47.00±15.65%	53.00±15.65%	0.232
	Testing	45.30±11.71%	54.70±11.71%	0.018
5-Day Exposure (Experiment b, n=20)	Training	47.09±12.26%	52.91±12.26%	0.141
	Testing	41.80±12.71%	58.21±12.71%	0.0002
5-Day Exposure (Experiment c, n=20)	Training	53.22±11.53%	46.78±11.53%	0.085
	Testing	44.75±13.71%	55.25±13.71%	0.020
3x 5-Day Exposure (Experiment a, n=20)	Training	53.74±12.42%	46.26±12.42%	0.115
	Testing	38.08±15.94%	61.92±15.94%	0.00012
3x 5-Day Exposure (Experiment b, n=20)	Training	46.26±15.22%	53.74±15.22%	0.230
	Testing	35.49±15.62%	64.51±15.62%	0.001
3x 5-Day Exposure (Experiment c, n=20)	Training	44.09±13.19%	55.91±13.19%	0.016
	Testing	38.07±18.85%	61.93±18.85%	0.004

* Both the 5-Day and 3x 5-Day Exposure Studies were performed three times, Experiments a-c (n=20 per experiment, 4 per treatment group). The results of these experiments were pooled to generate a data set with a sample size of n=12 per treatment group that will provide the statistical power needed for the analysis of Discrimination Index as shown in Tables 21 and 22.

Reanalyzing the baseline (pre-exposure) data according to the treatment groups (i.e. separating the animals into various groups based on the treatments they received) similarly confirmed that all control and treated groups showed significant novel object preference and passed the test at baseline (Table 21). However, all animals groups subjected to 5-day hypoxic, hyperoxic and oscillatory hypoxic-hyperoxic treatments failed to show novel object preference and did not pass the test (Table 21), suggesting impaired formation and/or retrieval of episodic memory. This finding was confirmed by the result of the 3x 5-day exposure experiment (Table 22), where after 5 days of exposure, the hypoxic, hyperoxic and oscillatory hypoxic-hyperoxic treatment groups failed to show novel object preference (Table 22). In addition, the 3x 5-day hypoxia exposure group also failed the memory test. Unexpectedly, animals receiving 3x 5-day hyperoxic and oscillatory hypoxic-hyperoxic treatments seemed to have recovered and showed novel object preference and passed the test. Only the animal group that received hypoxic treatment for 5 and 3x 5 days continued to show impairment of episodic memory performance and did not pass the test. As described above, the animals, which showed

very strong position preference during training, were excluded from the data set during data analysis.

Table 21. Novel Object Recognition Performance of Rats Subjected to 5-Day Hypoxic and/or Hyperoxic Treatments*

Experimental Group	Timepoint	NOR Task	Discriminative Index (%)		P-Value
			Familiar Object Position	Novel Object Position	
21% Oxygen (n=12)	Pre-Exposure	Training	46.40±15.25%	53.60±15.25%	0.2814
		Testing	45.62±8.40%	54.38±8.40%	0.0240
	5-Day Exposure	Training	48.06±10.94%	51.94±10.94%	0.4144
		Testing	42.00±5.06%	58.00±5.06%	0.0000001
7.5% Oxygen (n=12)	Pre-Exposure	Training	47.46±8.75%	52.54±8.75%	0.1689
		Testing	42.27±13.94%	57.73±13.94%	0.0126
	5-Day Exposure	Training	51.76±11.16%	48.24±11.16%	0.4488
		Testing	49.40±11.95%	50.60±11.95%	0.8077
95% Oxygen (n=12)	Pre-Exposure	Training	51.11±13.23%	48.89±13.23%	0.6852
		Testing	41.87±18.09%	58.13±18.09%	0.0386
	5-Day Exposure	Training	49.59±9.82%	50.41±9.82%	0.8396
		Testing	44.58±15.48%	55.42±15.48%	0.1003
5% ↔ 95% Oxygen (n=12)	Pre-Exposure	Training	52.05±15.40%	47.95±15.40%	0.5208
		Testing	45.36±8.72%	54.64±8.72%	0.0161
	5-Day Exposure	Training	50.76±11.27%	49.24±11.27%	0.7453
		Testing	46.64±11.09%	53.36±11.09%	0.1516
Open Air (n=12)	Pre-Exposure	Training	46.31±9.91%	53.69±9.91%	0.0818
		Testing	44.52±8.55%	55.49±8.55%	0.0047
	5-Day Exposure	Training	47.68±5.85%	52.32±5.85%	0.0780
		Testing	39.65±7.60%	60.35±7.60%	0.000001

* Results of Experiments a-c were pooled to generate a single data set.

Table 22. Novel Object Recognition Performance of Rats Subjected to 5 and 3x 5-Day Hypoxic and/or Hyperoxic Treatments*

Experimental Group	Timepoint	NOR Task	Discriminative Index (%)		P-Value
			Familiar Object Position	Novel Object Position	
21% Oxygen (n=12)	Pre-Exposure	Training	57.54±12.35%	42.46±12.35%	0.0067
		Testing	39.67±16.16%	60.33±16.16%	0.0048
	5-Day Exposure	Training	47.43±19.94%	52.58±19.94%	0.5335
		Testing	40.33±12.01%	59.67±12.01%	0.0020
	3x 5-Day Exposure	Training	53.56±25.66%	46.44±25.66%	0.5040
		Testing	41.23±18.16%	58.77±18.16%	0.0445
7.5% Oxygen (n=12)	Pre-Exposure	Training	48.97±18.73%	51.03±18.73%	0.7996
		Testing	41.40±17.26%	58.60±17.26%	0.0300
	5-Day Exposure	Training	54.04±22.86%	45.96±22.86%	0.4172
		Testing	42.83±24.02%	57.17±24.02%	0.1985
	3x 5-Day Exposure	Training	45.95±20.58%	54.05±20.58%	0.3675
		Testing	44.72±18.26%	55.28±18.26%	0.1899
95% Oxygen (n=12)	Pre-Exposure	Training	45.10±15.99%	54.90±15.99%	0.1475
		Testing	34.73±18.00%	65.27±18.00%	0.0004
	5-Day Exposure	Training	45.25±15.13%	54.75±15.13%	0.1382
		Testing	43.67±23.69%	56.33±23.69%	0.2038
	3x 5-Day Exposure	Training	46.29±21.71%	53.71±21.71%	0.4116
		Testing	41.29±15.00%	58.71±15.00%	0.0131
5% ↔ 95% Oxygen (n=12)	Pre-Exposure	Training	46.25±15.82%	53.75±15.82%	0.2579
		Testing	39.48±17.67%	60.52±17.67%	0.0080
	5-Day Exposure	Training	50.39±21.01%	49.61±21.01%	0.9281
		Testing	44.19±20.02%	55.81±20.02%	0.1693
	3x 5-Day Exposure	Training	52.90±18.47%	47.10±18.47%	0.4498
		Testing	42.20±15.73%	57.80±15.73%	0.0307
Open Air (n=12)	Pre-Exposure	Training	50.00±12.46%	50.00±12.46%	0.9992
		Testing	36.65±16.16%	63.35±16.16%	0.0017
	5-Day Exposure	Training	47.82±15.52%	52.18±15.52%	0.5173
		Testing	43.38±13.63%	56.62±13.63%	0.0338
	3x 5-Day Exposure	Training	43.88±16.51%	56.12±16.51%	0.1147
		Testing	35.04±14.19%	64.96±14.19%	0.0001

* Results of Experiments a-c were pooled to generate a single data set.

5.0. DISCUSSION

In this report, the effects of 5-day and 3x 5-day hypoxic, hyperoxic and oscillatory hypoxic-hyperoxic treatments on organ injuries and episodic memory function were described. The results of bioassays suggested that these treatments, especially the oscillatory hypoxic-hyperoxic exposure caused significant physiological changes, as well as organ injuries. For example, the increase in body weight was significantly reduced even after only 5 days of oscillatory hypoxic-hyperoxic exposure, and a more profound effect was detected after 3x 5-day treatment. However, there was no significant weight change in other treatment groups.

As expected, hypoxic treatment stimulated EPO expression, which in turn stimulated erythropoiesis and increased the percentage of packed cell volume of RBCs in the blood (hematocrit) after 5-day and 3x 5-day treatments, respectively. Unlike EPO, VEGF seemed to be stimulated by both hypoxic and hyperoxic treatments, but only the 5-day oscillatory hypoxic-hyperoxic group showed a significant change. While the 5-day treatment groups showed a positive trend, the 3x 5-day treatment did not show any changes, suggesting the negative feedback control mechanism might have been activated at some point during the 3x 5-day treatment period. On the other hand, altered expression of Angiotensin II appeared to be hypoxia-specific, where the 5-day treatment exhibited a significantly reduced of Angiotensin II concentration. In contrast, the levels of Angiotensin II were significantly increased after 3x 5-day hypoxic treatment.

Brain damage is one of the major consequences of hypoxic exposure, which can occur both during and after oxygen deprivation. Because of this reason, this study was designed to include an experimental group that receives oscillatory hypoxic-hyperoxic treatment. The results of NOR test suggested both hypoxic and hyperoxic (including the oscillatory hypoxic-hyperoxic) treatments can functionally affect episodic memory formation and/or retrieval. This may be due to the death of neurons and other brain cells, or due to the damage of the BBB. The result of S100B analysis however suggested that only the 5-Day hypoxic group had BBB damage. Although increases in this marker were detected in all treatment groups of the 3x 5-day exposure experiment, they did not pass the thresholds for statistical significance or a positive trend. We are currently performing histopathological and immunohistochemistry analyses to investigate the correlation between brain injury and cognitive degradation.

Of the bioassays described in the RESULTS Section of this report, only a few of them showed significant changes after hypoxic and/or hypoxic treatments. This is probably due to the treatment conditions employed in this experiment, since only relatively mild conditions that do not cause any severe symptoms (such as fainting, long-term loss of consciousness, coma, seizures, cessation of brain stem reflexes, and brain death) were approved for the use in this study. Despite this limitation, this study did identify a number of analytes (including S100B, EPO, VEGF and Angiotensin II) that could be

further developed into a panel of sensitive biomarkers for rapid detection of hypoxic and/or hyperoxic exposure.

6.0. CONCLUSION

In this report, the temporal effects of hypoxic, hyperoxic, and oscillatory hypoxic–hyperoxic exposure on organ injuries and episodic memory performance were described. Although relatively mild conditions were used, statistically significant results were obtained in some bioassays, as well as the behavioral test for episodic memory formation and retrieval. These results form the foundation essential for subsequent experiments of this study that aim to prevent brain injury and cognitive degradation using specific neuroprotective peptides, as well as to develop novel biomarkers based on neuronal electrical signals for rapid and sensitive detection of hypoxic and/or hyperoxic exposures.

7.0. REFERENCES

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	Percent
AAALAC	Association for the Assessment and Accreditation of Laboratory Animal Care
AVP	Arginine vasopressin
BAL	Bronchoalveolar lavage
BBB	Blood–brain barrier
BPG	Bisphosphoglycerate
BPGM	Bisphosphoglycerate mutase
CNS	Central nervous system
EPO	Erythropoietin
G-LOC	G-force induced loss of consciousness
HIF	hypoxia-inducible factor
IACUC	Intuitional Animal Care and Use Committee
NOR	novel object recognition
OBOGS	On-board oxygen generation system
PaO_2	Arterial partial pressure of oxygen
ppO_2	Partial pressure of oxygen
RBCs	Red blood cells
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor
WBC	White blood cell