ABSTRACT

Investigating the Efficacy of a Novel Therapeutic to Mitigate Traumatic Brain Injury: Contributions of Environmental Exposures to Overall Healing

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Traumatic brain injury (TBI) is a leading cause of disability and premature death among both civilians and military personnel. Morbidity and deaths are mainly caused by several secondary processes that exacerbate brain dysfunction in the minutes to days following the primary injury when blood vessels and tissues are torn, stretched, or compressed. In previous studies, proper oxygen supply has been shown to help brain cells to grow and repair, remove the obstruction in blood flow, and alleviate brain edema to prevent secondary injury. OX-66, a novel therapeutic, potentially provides an efficient supply of oxygen to the cells. This therapeutic was investigated in this study to determine its cytotoxicity and potential mechanism of cellular repair in invitro-injured rat brain cells. The effects of exposure to polycyclic aromatic hydrocarbons (PAH) on TBI patients and the corresponding restorative influence of OX-66 were also evaluated.

Investigating the Efficacy of a Novel Therapeutic to Mitigate Traumatic Brain Injury: Contributions of Environmental Exposures to Overall Healing

by

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CHAPTER ONE

Introduction

There are multiple insults and injuries faced by military personnel when serving the U.S in various combat scenarios. Due to the wide use of explosive devices at the forefront of war, a large number of U.S. military service personnel endure traumatic brain injuries (TBIs) caused by blasts and explosions. This is especially pertinent in modern wars such as Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF). Due to the high frequency of the occurrence and the seriousness of the adverse effects, TBI is regarded as the leading cause of morbidity and mortality in combat settings (Cheng et al., 2010). The Department of Defense and Defense and Veteran's Brain and Injury Center (DVBIC) estimated that about 22% of all combat casualties from OIF and OEF and 12% of all Vietnam War veterans were reported to have some level of TBI.

TBI is also a significant cause of disability and premature death for civilians. According to the reports from Centers for Disease Control and Prevention, there are nearly 1.7 million people in the United States who suffer from TBI every year. TBI occurs every 23 seconds in the U.S.A. (Faul et al., 2010). Approximately 54-60 million sustain a TBI each year on a global scale, which means that every second two people in the world sustain a TBI (Feigin et al., 2013). The major causes of TBI include motor vehicle crashes, falls, acts of violence, and sports injuries (Andriessen et al., 2011; Ragnarsson, 2006).

Although mortality from severe traumatic brain injury has reduced from 50% to about 25% due to better out-of-hospital and emergency care over the past 30 years, it has

not provided TBI patients with a better quality of life (Zink, 2001). Conventional treatment has had little impact on TBI outcomes (Erickson et al., 2010). In order to address these life-threatening issues and alleviate the pain of wounded soldiers and civilians, multiple novel treatment strategies have been investigated. Hyperbaric oxygen treatment (HBOT), calcium channel inhibitors (Muehlschlegel et al., 2009), and application of magnesium and minocycline (Homsi et al., 2009) are just a few examples of treatment regimens enlisted to address TBI. However, no treatment has been approved for standard medical care of TBI (Jain, 2008; Narayan et al., 2002; Vink and Nimmo, 2009). Currently, there is no approved medical intervention for TBI that provides neuroprotection or neuro-restoration following moderate or severe TBI.

In critical care medicine, maintenance of adequate tissue oxygenation is considered as one of the primary objectives (Maloney-Wilensky et al., 2009a) following brain injury. Due to the limited oxygen and glucose reserves within the brain, continuous and adequate blood flow delivery is required to meet its metabolic demands. A brain injury can disrupt regular blood flow, decrease oxygen delivery, and accelerate brain metabolism, leading to secondary injury which dramatically increases brain damage and is associated with an unfavorable outcome and mortality (Stiefel et al., 2005). In fact, about 90% of the death in TBI patients is caused by the secondary injury occurring in minutes to days after the primary injury. Several investigations have shown that reestablishing oxygen delivery via microcirculation during periods of cellular edema, inflammation, and tissue damage results in favorable outcomes (Brain Trauma et al., 2007; Maloney-Wilensky et al., 2009a; Nangunoori et al., 2012; Narotam et al., 2009; Rosenthal et al., 2009).

Considering the important role of oxygen in healing brain injury, there is one such proprietary therapeutic—OX-66, a polyoxygenated aluminum hydroxide that could play a substantial role in treatment. Different from the conventional modes of oxygen delivery, OX-66 presents as a solid-state compound due to combing oxygen with aluminum atoms in a clathrate structure. This formulation is stable, easily reformulated to allow a wide range of application scenarios, and economical.

Due to complex combat scenarios, TBI occurring on the battlefield is affected by various factors, such as the exposure to sulfur dioxide, nerve agents, sarin gas, depleted uranium, and other environmental and chemical hazards that carry potential health risks (Kang et al., 2000; Proctor et al., 1998). One example of the inter-related nature of brain injury and environmental exposures is Gulf War Syndrome (GWS), more recently designated as Gulf War Illness (GWI). In the aftermath of the 1991 Gulf War, up to one quarter of the nearly 700,000 U.S. veterans reported diverse symptoms and health problems that did not easily conform to standard medical diagnoses (Smith et al., 2004; Smith et al., 2003). The new diagnostic entity was described as GWS. It is associated with different functional symptoms integrating the neurosensory, digestive, and musculoskeletal systems with different types of endpoints such as chronic headaches, cognitive difficulties, chronic diarrhea, psychiatric conditions, Parkinson disease, etc. (Iversen et al., 2007). The potential causes of the symptoms included toxic exposures to oil-well fire smoke, depleted uranium, sarin gas, pesticides, etc. (Kang et al., 2009). During the catastrophic oil fires set at the end of the Gulf War, large amounts of PAHs were generated and released into the air contaminating water and soil in Kuwait, thereby creating a complex mixtures exposure scenario for military personnel in those regions.

Since the toxicity of OX-66 and its potential efficacy in treating TBI have not been explored, a preliminary in vitro study was needed. Specifically, this thesis evaluated immortalized rat hippocampal cells and astrocytes, which are two essential cell types that take charge of maintaining functions of spatial navigation and memory storage, and supporting blood-brain barrier, respectively. This study had three specific aims: 1) to characterize the toxicological effects of pristine OX-66 on non-injured rat brain cells; 2) to evaluate the restorative effects of pristine OX-66 on rat brain cells following an in vitro TBI model; 3) to examine the effect of exposure to PAHs on TBI on rat brain cells exposed to PAHs mixture prior to injury process.

CHAPTER TWO

Literature Review

Mechanisms and Pathophysiology of TBI

TBI is defined as disruption to the function of the brain resulting from external mechanical force, impact, blast waves, or penetration by a projectile. When an external force applied to the brain results in stretching, compression, or tearing of tissues and blood vessels, *primary injury* occurs and consequently alters neurological functioning or consciousness (Taber, 2010). Primary brain injury such as cerebral contusions, lacerations and diffuse axonal injury often induces irreversible damage to brain tissue (Maas et al., 2008). Secondary brain injury occurs in minutes or days after trauma due to flow-metabolism mismatches, in which the increased metabolic needs of the injured brain tissue is at odds with the decreased delivery of O_2 and nutrients to damaged areas caused by decreased blood flow. Intracranial causes of secondary brain injury include mass lesions, focal/diffuse brain swelling, seizures, infection, vasospasm, and intracranial hypertension. Extracranial causes of secondary brain injury include hypotension, hypoxia, hyper/hypocapnia, hyper/hypoglycemia, anemia, pyrexia, infection, coagulopathy, and electrolyte abnormalities. Secondary brain injury can exacerbate the damage from primary injury by exacerbating cerebral dysfunction, increasing inflammation, creating free radical overload, excitotoxicity, dysfunction of mitochondria, and the influx of calcium and sodium ions into neurons (Park et al., 2008).

TBI can be classified into penetrating brain injuries and closed brain injuries based on mechanism of insult. A penetrating injury is defined as one caused by the

penetration of a foreign object, such as bone chips and fragments of munitions, while any injury to the brain tissue caused by a force that leaves the skull intact is categorized as a closed-head injury. Closed-head injury includes focal and diffuse brain damage. Focal brain damage results from contact injury types that cause laceration, contusion, and intracranial hemorrhage, while diffuse injury is considered to be caused by acceleration/deceleration injury types that lead to brain swelling or diffuse axonal injury (Baethmann et al., 1998; Marshall, 2000; McIntosh et al., 1996; Nortje and Menon, 2004). Contrecoup injury is the most common focal injury and is often observed in frontal regions. Several biomechanical explanations of contrecoup injury have been raised (Pudenz and Shelden, 1946; Shaw, 2002), including shear deformation, brain rotation, skull vibration, pressure waves, and cavitation, but because of the practical difficulty of measuring brain deformation in humans, the actual biomechanics remain unknown.

Different lobes with varying densities rotate at different speeds causing whole-brain shearing, which is known as diffuse axonal injury, generating more widespread effects. Diffuse axonal injury (DAI) occurs at the grey-white matter junction of the brain to cause damage of the brain stem or corpus callosum, which is the collection of axons connecting the right and left sides of the brain. Mechanism of DAI is similar in severe trauma, as axons are stretched beyond a physiological injury threshold (Graham et al., 1995). Although the subject of TBI has been under scrutiny for decades, biomechanical explanations of TBI are still poorly understood.

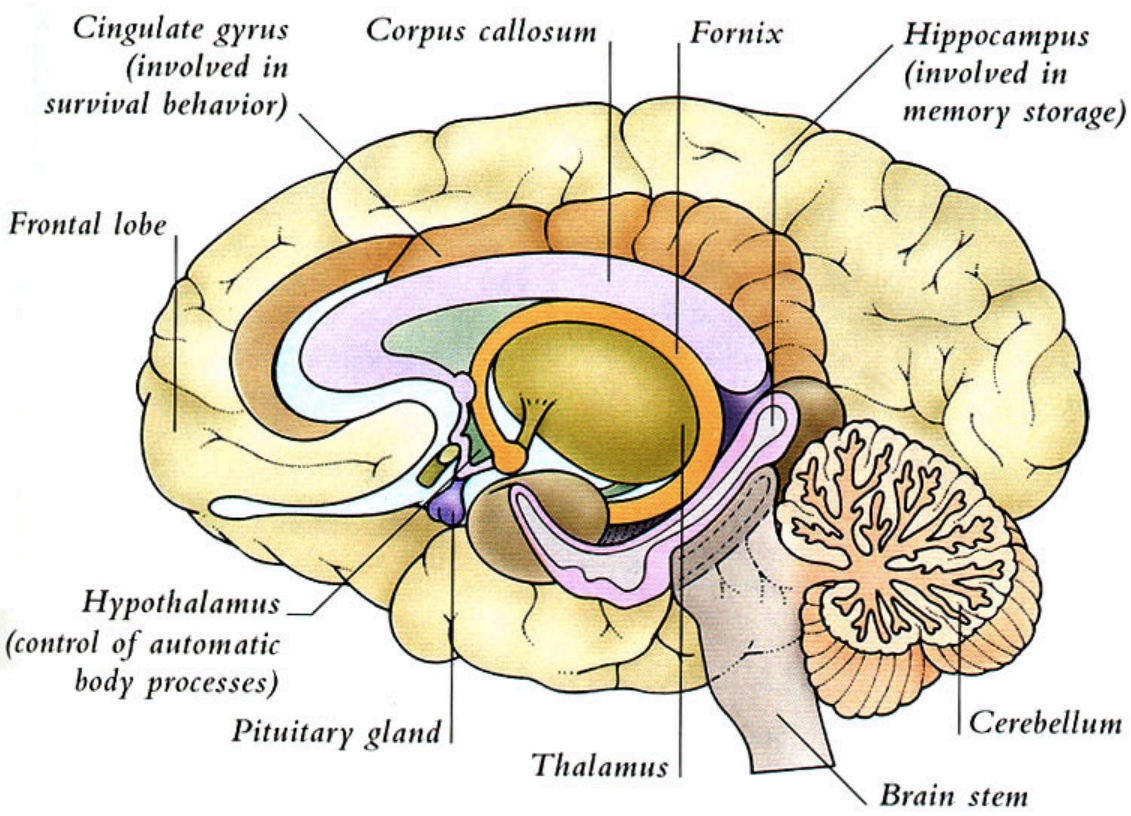


Figure 1 Cerebral Lobes and their Functions (Source: Adapted from www.educarer.org, 2006)

On the battlefield, TBI is the most common injury, usually caused by blast events, gunshot wounds, motor vehicle crashes, and falls. Among these, blast events cause the most casualties due to the widespread use of high-explosive arms. This unique type of TBI our military personnel face is also known as blast-related TBI (BR TBI). BR TBI involves three principal causes termed primary, secondary, and tertiary injury. Primary blast injury results directly from exposure to the blast wave, which can be more than 300m/s (Cernak et al., 2001) with subsequent changes in atmospheric pressure causing cavitation, air emboli, and electromagnetic fields (Tsokos et al., 2003). Objects put into motion by a blast, such as shrapnel and debris, strike the head, causing secondary blast injury. This can result in either be a closed-head injury through blunt force trauma or a

penetrating head injury if the skull is breached by these materials. Tertiary blast injury occurs when a body is put into motion and accelerated through the air by the kinetic energy of the explosion and lands on ground or other solid objects. Once the body stops, the brain continues to move in the direction of the force, hitting the interior of the skull and bouncing back into the opposite side, causing a coup-contrecoup injury (Wang et al., 2011). Thus, most BR TBIs are a culmination of multiple injury mechanisms (Goodrich et al., 2013).

The first stage of the pathophysiological cascade of cerebral injury is more likely an "ischaemia pattern", characterized by direct tissue damage and impaired regulation of cerebral blood flow (CBF) and metabolism. Further, inadequate O_2 supply to the traumatized brain results in the conversion of aerobic metabolism to anaerobic metabolism. This causes lactic acid accumulation and acidosis in consequence, which results in depletion of stored ATP leading to the failure of maintaining the homeostasis of energy-dependent membrane ion pumps. In the second stage, several events occur, including terminal membrane depolarization, excessive release of excitatory neurotransmitters, activation of Ca^{2+}-Na^{+} channels, etc. These events and the subsequent activation of lipid peroxidases, proteases, caspases, translocases and endonucleases increase the intracellular concentration of free fatty acids and free radicals, initiatiate progressive structural changes of biological membranes and nucleosomal DNA, and eventually the degradation of vascular and cellular structures, resulting in cell death via necrosis or apoptosis.

One of the main syndromes of TBI is imbalance between cerebral oxygen delivery and cerebral oxygen consumption. This imbalance is induced by vascular and

hemodynamic mechanisms ending in brain tissue hypoxia. Even when presenting normal cerebral perfusion pressure (CPP) or intracranial pressure (ICP), oxygen deprivation can still occur in the secondary phase of injury (Jaeger et al., 2006; Johnston et al., 2005; Lang et al., 2003; Leal-Noval et al., 2006; Menzel et al., 1999; Rose et al., 2006; Stiefel et al., 2005).

Military/ Medical Needs

TBI is a major cause of death and disability, especially in military. BR injuries mostly occur on the battlefield, usually caused by blast or explosion. Due to the wide usage of the improvised explosive devises (IED), mines, rocket-propelled grenades, mortars, and other high-explosive munitions, TBI has become the leading cause of injuries among military personnel (Goodrich et al., 2013). A study from Navy-Marine Corps Combat Trauma Registry reported that about 88% of the BR TBIs were related to explosions (Galarneau et al., 2008). In the conflicts over the past decades, the number of BR injuries increased (OEF/OIF 81%, Vietnam 65%) whereas the number of gunshot wounds has decreased (OEF/OIF 19%, Vietnam 35%) (Owens et al., 2008). According to a related study, about 60% of the wounded soldiers injured by explosion had a TBI (Okie, 2005). From January 1997 to December 2006, there were 110,392 military members who had at least one TBI-related symptom. Among them, 15,732 hospitalizations were associated with TBI (Cameron et al., 2012). From 2000 through 2011, more than 233,000 military members were reported to have sustained a TBI (Goodrich et al., 2013). During the wars in Iraq and Afghanistan, approximately 10%-20% of all veterans among more than 2,000,000 U.S military personnel have experienced mild TBI (Jorge et al., 2004).

Traumatic brain injury is known to cause multiple neurologic, vision, and other symptoms and related health problems. TBI has been demonstrated to cause a period of loss of or alteration in consciousness, such as confusion, and disorientation; neurological deficits, such as weakness, loss of balance, and change in vision; and loss of memory (amnesia). A set of studies from Veterans Affairs (VA) Polytrauma Rehabilitation Center showed that 97% of the combat-injured personnel receiving inpatient care at the center had a TBI. More than half of them had mental health issues, such as symptoms of depression (36%) and posttraumatic stress disorder (PTSD) (35%). All the patients had pain-related issues, including headache (52%), musculoskeletal (48%), and neuropathic (14%) (Cifu et al., 1996; Sayer et al., 2009). Eighty-five percent of OEF/OIF Veterans with BR TBI (40% mild, 20% moderate, 40% severe) have reported hearing and/or visual impairments (Lew et al., 2009).

According to the current model developed by the Department of Defense and the Department of Veterans Affairs, TBI can be classified into mild, moderate, and severe injuries. This model system uses three criteria: Glasgow Coma Scale after resuscitation, duration of post-traumatic amnesia, and loss of consciousness. Also, changes that are visible on neuroimaging, such as focal lesion, swelling, or diffuse injury have been used as other methods of classification. Mild TBI, also known as concussion, involves a Glasgow Coma Scale of 13-15, a loss of consciousness of less than 30 minutes, an temporary alteration of consciousness for less than 24 hours, and posttraumatic amnesia whose duration is less than 1 day. Thus, mild TBI typically presents with transient mental symptoms and normal structural imaging. Moderate TBI is associated with a Glasgow Coma Scale of 9-12, a loss of consciousness of more than 30 minutes but less than 24

hours, an alteration in consciousness of more than 24 hours, and a posttraumatic amnesia of more than 1 day but less than 7 days. A variety of physical, emotional, and cognitive symptoms are typically involved in moderate TBI patients, which may last for months. These patients may require medication and/or psychotherapy intervention to alleviate their symptoms. Severe TBI requires a Glasgow Coma Scale of less than 9, a loss of consciousness of more than 24 hours, an alternation of consciousness of more than 24 hours, and a posttraumatic amnesia of more than 7 days (Taber, 2010). Severe TBI may result in different levels of physical or cognitive disability and long-term disturbance of brain functioning. Therefore, in order to optimize recovery potential for the wounded with severe TBI, rapid evacuation, early intervention, and specialized treatment are usually necessary (Jaffee et al., 2009).

Civilian Needs

As same as in military personnel, TBI is the leading cause of death and disability in civilians. An estimated 1.7 million civilian TBI cases occur annually in the United States. It contributes nearly 30.5% of all injury-related deaths each year. More than 80,000 individuals are left with life-long disability from TBI. By 2006, there were 5.3 million people living with a brain injury in the United States, according to reports from CDC. Even though the number is extremely large, many TBI cases often remain unreported and underestimated due to the absence of or mild physical impairments after TBI, while the common cognitive and behavioral impairments associated with TBI are often misdiagnosed (Krause et al., 1999). The leading causes of TBI within the civilian population include falls (28%), motor vehicle-traffic crashes (20%), struck by/against

events (19%), and assaults (11%). Children aged 0-4 years, adolescents ages 15-19, and seniors aged over 65 years are the ages most likely to sustain a TBI.

Children account for a large proportion of civilian TBI cases. Approximately, 475,000 TBI occur annually in children younger than 14 years old, and about 64,000 children are hospitalized for a TBI every year (Langlois et al., 2005). TBI in children is associated with an estimated $1 billion annually. Even though minor intracranial injuries without neurological damage are more common in this group, severe TBI resulting from unintentional trauma remains one of the primary causes of death among U.S youths. According to studies by the National Youth Sports Safety Foundation, Inc, about 2 out of 5 TBI in children are related to sports and recreational activities (Rivara, 1994). A survey conducted in 2009 indicated that cycling, football, baseball/softball, basketball, skateboards/scooters, water sports, soccer, powered recreational vehicles, winter sports, and trampolines are the top 10 sports and recreational activities contributed to the highest number of TBIs among children aged 14 and younger. Other causes include baby shaking and falls. Falls contribute to 50% of the TBI among children, but rarely lead to severe brain injuries when occurring at home or when falling from less than 5 feet. The effects of TBI depend on the age of the child when injury occurs. Children aged 7 or younger at injury can encounter increased risk of long-term difficulties on existing abilities as well as deficits in the development of emerging abilities such as learning abilities, complex problem solving, cognitive development, and behavioral development.

Following children and young adults, the elderly (>65 years) are the group suffering from the second highest incidence of TBI. Researchers suggest that falls are the most frequent cause of TBI in older persons. Several factors can result in falls, including

cognitive impairment or dementia, physical limitations, vision impairments, and side effects from taking medications. It has been demonstrated that increased age of elders is related to comparatively poorer outcomes after TBI (LeBlanc et al., 2006). And as the aging population increases greatly, TBI-caused disabled elders will increase correspondingly requiring medical and custodial care (Susman et al., 2002).

Sports-related injuries are another leading cause of TBI, leading to approximately 300,000 of 1.54 million mild to moderate TBIs every year. It can occur at any level of sport, ranging from school games to professional athletics. Direct impact to the head from a ball or another player is one of the most common causes, as well as acceleration-deceleration forces during practices or games. Research has shown that concussions account for 5.5% of all high school athletic injuries and 6.2% of all collegiate athletic injuries, and there has been about approximately 0.41 concussions in every NFL game. Ongoing efforts have been made to enact legislation which regulates play guidelines for sports-related TBIs.

Retrospectives of TBI Therapy

Treatments for TBI

Stem cells are one of the restorative tools that have been investigated with regards to TBI treatment. Within the Central Nervous System (CNS), stem cells that exist in the hippocampus and subventricular zone naturally generate neurons, astrocytes and oligodendrocytes, which are able to repair damage and restore lost function after trauma or disease occurs. In order to enhance production of neural tissue by stem cells, there are two means that have been explored and utilized: increasing endogenous creation and

transplanting in vitro-derived stem cells (Castillo-Melendez et al., 2013). Results of many animal researchers showed that neural cells produced by implanted adult stem cells migrate to the area of injuries, often associated with functional improvement. This may indicate the contribution of stem cells on neurological function improving (Chen et al., 1997; Jeong et al., 2003; Lee et al., 2007). However, there remains many and varied unresolved issues before stem cells can be safely used in clinical translation studies (. The barriers include purifying stem cell cultures, in vitro production of neural stem cells for transplantation, optimizing the efficiency of delivery systems, and overcoming rejection of foreign cells (Castillo-Melendez et al., 2013).

Thorough neuropsychological assessment for TBI in humans usually includes blood oxygen and pressure measurements, Glasgow Coma Scale measurement, and pupil examination in pre-hospital period; then neurobehavioral test, the anxiety test, mood and sleep scale evaluation, simple reaction time, and continuous performance test for concussion. For assessment in the battlefield, since the austere tactical environment does not allow comprehensive assessment or thorough evaluation, some quick methods have been used for detecting and evaluating TBI. One of them is Military Acute Concussion Evaluation (MACE). It provides a gross score on four cognitive domains—orientation, concentration, immediate memory, and memory recall—by using the Standardized Assessment of Concussion. Combining blood pressure, pupillary status, GCS score and other information, MACE can help make decisions regarding therapeutic interventions and transport destinations. Brief Traumatic Brain Injury Screen is another tool under the TBI screening and assessment systems established by the military and VA. This assessment tool can identify patients suspected of TBI who are not exhibiting symptoms

to prevent a delayed diagnosis. It also can facilitate appropriate care for patients with ongoing symptoms.

Treating TBI requires experts and clinicians from different fields to work as an interdisciplinary team to provide appropriate therapeutic approaches. For mild TBI, appropriate medication must be selected by clinicians to mediate and or alleviate symptoms related to headache, vision problems, balance issues, hearing deficits, sleep disruption, irritability, and cognitive difficulties, while avoiding side effects and possible drug interactions. Medicines that can reduce pain, fever, inflammation (such as ibuprofen, naproxen, and compazine) and depression (such as sertraline), or treat hypertension, panic, anxiety (such as propranolol), epilepsy, seizure (such as gabapentin), insomnia and other brain disorders (such as zolpidem and trazodone) have been used in current treatment for mTBIs (Marklund et al., 2006). Treatments for moderate and severe TBI involve more complicated interdisciplinary approaches. During the prehospital period, the management of airway, ventilation, oxygenation, fluid resuscitation, and cerebral herniation are key issues. In the initial treatment, neurology, psychiatry, neuropsychology, physical medicine and rehabilitation, and neurosurgery compose the core medial disciplines. Afterward, ancillary services must be involved to improve the functional recovery. These include physical therapy, audiology, cognitive rehabilitation, occupational therapy, optometry, and case management (Jaffee et al., 2009).

Oxygen in Healing

Our body is dependent on oxygen as a catalyst and energy source for many cellular functions including maintenance, metabolism, and repair. Other than the function in the electron transfer oxidase system in cellular metabolism, oxygen plays an essential

role in neovascularization, fibroblast proliferation, angiogenesis, epithelialization, and collagen deposition (Huang et al., 2012). The U.S. military now uses a multi-stage system to determine how care is given. The first stage is basic first aid that is given on the battleground. Those who are more seriously injured are taken to a mobile hospital so that emergency surgery can be performed. Those needing extensive care after the surgery are initially taken to Landstuhl Regional Medical Center in Germany, then taken to the U.S. Army Institute of Surgical Research (USAISR) (D'Avignon et al., 2011). In this entire process of transferring care, oxygen has been considered as the critical component for pain management, mitigating infection and subsequent healing to maintain the stability of the patients.

In the early postinjury stage, of critical importance is the transport to a medical facility in the so-called "Golden Hour" as well as the initial hours of in-hospital care. During this period, ensuring proper oxygen supply, maintaining adequate cerebral blood flow, and controlling raised intracranial pressure (ICP) are the primary concerns. In injury management, the main aim is to prevent secondary brain damage that occurs in the hours, days, and weeks after the primary insult. Primary injury causes tissue distortion and destruction, while secondary cerebral injury is associated with hypoxemia. Hypoxemia is a strong predictor of outcome in TBI patients. In a study conducted by Chesnut et al. (Chesnut et al., 1993), hypoxemia (when arterial hemoglobin oxygen [SaO_2]<60mmHg) was demonstrated to have higher mortality on the outcome of patients, especially when associated with hypotension (26.9% for no occurrence of hypoxemia and hypotension, 28% for hypoxemia alone, and 57.2% for both occurrence). SaO_2 saturation less than 90% should be avoided. Table 1 shows the relationship between the oxygen

saturation and rates of mortality and severe disability in TBI patients during a process of transportation by helicopter. According to the study by Maloney-Wilensky et al. (Maloney-Wilensky et al., 2009a), there is a 20%-40% mortality rate due to severe TBI within the first 48 hours post-trauma, as well as 20% of patients remain severely disabled, most of which is due to secondary cerebral injury (Maloney-Wilensky et al., 2009a, b). Both animal and human studies in the last three decades have shown that neuronal survival after TBI depends on an adequate supply of oxygen and glucose. Consequently, how to ensure the adequate oxygen supply is the most important issue in TBI treatment.

Table 1 Relationship between oxygen saturation, mortality, and severe disability.

Oxygen saturation	Mortality	Severe Disability
>90%	14.3%	4.8%
60-90%	27.3%	27.3%
<60%	50%	50%

Oxygen Treatments

In the prehospital period, bag mask ventilation, rapid sequence intubation (RSI) or endotracheal intubation, laryngeal mask airways, and other airway adjuncts are often used individually or in varying combination to provide oxygen. According to related studies, the safety and efficacy of RSI remains undetermined (Davis et al., 2003).

Hyperbaric oxygen is another treatment that has been proposed to minimize brain damage by improving the oxygen supply to the brain. It provided an increased pressure of oxygen, which is at least 1.4 times higher than atmosphere absolute pressure (1 ATA, equal to 760mmHg), to increase the oxygenation of the blood and tissues of the patient, so that mitochondrial metabolism and tissue oxygenation will be improved. Two to three ATA of the oxygen for 90-120min is often used for clinical HBOT treatment, but the

total number of the treatments, the dose, and the interval between treatments on each patient varies on clinical indication. The HBOT treatment is based on the theory that the metabolic or electrical pathways of the functionally retrievable neurons around severely damaged or dead neurons may be reactivated by exposure to hyperbaric oxygen so that their function may be recovered or partially recovered. However, limited evidence has proven the reduction of death rate and the improvement of GCS after HBOT and its effectiveness on the recovery of brain injuries still remains uncertain. There are also some concerns regarding the potential side effects of this therapy, such as barotrauma to the middle ear or sinuses, temporary myopia, claustrophobia, and oxygen toxicity seizure (Wolf et al., 2012).

Oxygen Toxicity

Even though oxygen is necessary for cell metabolism, short exposure to high partial pressures of oxygen under hyperbaric conditions can cause central nervous system toxicity (Bitterman, 2004). High concentration of oxygen increases the formation of oxidative stress and consequently causes damage to cell structures by initiating a damaging chain reaction of lipid peroxidation in the unsaturated lipids within cell membranes, and also increases the formation of other free radicals that harm DNA and other biomolecules (Djurhuus et al., 1999; Freiberger et al., 2004; Thom and Marquis, 1987). The oxygen toxicity on central nervous system is characterized by seizures and brief periods of rigidity followed by convulsions and unconsciousness (Acott et al, 1999).

OX-66

Ox-66 is a novel material that may improve or mitigate the complications associated with those conditions resulting from low oxygen levels in tissues. Ox-66, a polyoxygenated aluminum hydroxide, is approximately 66% oxygen. Different from the conventional modes of oxygen supply, OX-66 presents solid state due to combing with the aluminum atoms. In the critical situations of extreme blood loss, TBI, and tissue injury, nano-sized OX-66 molecules have the potential of providing oxygen microcirculatory benefits to the red blood cells, in the pre-hospital and acute treatment, to satisfy the body's need for large amounts of oxygen. It would not only be more stable and convenient for the application, but also has the potential to reduce the expenses.

Preliminary data has identified rehabilitating characteristics on the abraded skin of a guinea pig as well as increased proliferation of liver cells that were injured via ethanol overdose (data not shown). However, elucidating the full potential of this compound has not been realized and the unintended consequences are not known.

In vitro TBI Modeling

Through in vivo animal studies, significant understanding of trauma has been achieved. However, disadvantages also emerged with the use of in vivo models. For instance, it is comparatively difficult to assess the effect of trauma on individual cell types, and furthermore, in vivo models cannot provide precise measurement of tissue motion. Therefore, several in vitro models have been employed by using tissue slices or cells in culture. To introduce mechanical disruption to the cells, Regan and Choi (Regan and Choi, 1994) scratched off the cells in a culture flask by stylet to study the response of the damaged cells adjacent to those removed. In 1991, Shepard et al. stimulated TBI on

human astrocytes by placing the culture dishes in a fluid-filled chamber that was connected to a standard fluid percussion device (Shepard et al., 1991). In 1993, another study conducted by Murphy et al. placed cultured cells in a compression chamber exposing cells to up to 20atm air pressure for up to 10 min. Although these models have not been widely used due to various reasons, such as the expensive cost, the limitation on certain type of cells, or the difficulty of providing enough cell mass for reliable measurements, they have reached useful results on simulating in vitro TBI. For this study, the goal was to apply a simple in vitro model that could reliably and consistently simulate TBI on large cell mass in culture.

Potential Effects of Environmental Contaminant Exposures on TBI

Deployed military personnel can be exposed to various chemicals, including nerve agents, pyridostigmine bromide pills (given to protect troops from the effects of nerve agents), depleted uranium, mustard gas, and pesticides to name a few. During the 1990-1991 Gulf War, due to the combustion of over 700 oil wells, a large amount of a composite smoke plume of gaseous constituents, volatile organic compounds, and polycyclic aromatic hydrocarbons (PAHs) was produced and inhaled by deployed troops (Lange et al., 2002). According to a 2010 U.S. Department of Veterans Affairs (VA) sponsored study, 250,000 of the 696,842 U.S. returned veterans and civilian workers continue to suffer from a chronic multi-symptom illness including neurological problems, chronic diarrhea, memory problems, and headache known as Gulf War Illness (GWI) or Gulf War Syndrome (GWS). Oil-well fire is considered as one of the potential causes of GWI, and PAHs were one of the major pollutants contained in the smoke plumes. According to a telephone study of 10,051 Gulf War veterans in 2007, 96% reported

breathing or being enveloped in the oil fire smoke, 90% had been within clear visual area of the oil fires, 72% worked in, lived in, or made travel through the burning oil fields, 68% washed in water with an oily sheen, 66% reported an oily taste to their food, and 65% had oil-tasting water (Iversen et al., 2007).

However, the effects of exposure to oil fire smoke on soldiers who suffered from TBI remains unknown. In study, due to the consideration that PAHs are one of the synergistic potential causes of GWI, we evaluated whether the exposure to PAHs mixtures is able to adversely affect TBI. Based on previous studies of the concentrations of different PAHs in the environment and biota in the Kuwait area, phenanthrene, fluoranthene, and benzo[a]anthracene were the three PAHs that had the highest concentration detected in biota and air (Al-Hashem et al., 2007; Gevao et al., 2006; Saeed et al., 1996). Benzo[a]anthracene and fluoranthene were both found to possess carcinogenic properties in animal studies (Busby et al., 1985). Fluoranthene was also found to be genotoxic in studies of mice (Hoover et al, 2014). In this study, the effect of a mixture of phenanthrene, fluoranthene and benzo[a]anthracene on in vitro-simulated TBI was evaluated. The restorative effect of OX-66 was also evaluated in cells after exposure to both PAHs and stretch-induced injury. This investigation was the first step in screening a therapeutic that can be administered in the first stage of care and be continued through the treatment process.

Specific Aims

The primary objective of this study was to characterize the toxicological and restorative effects of pristine OX-66 on healthy and in vitro injured rat brain cells. Hippocampal cells (H19-7/IFG-IR), which play an important role in maintaining spatial

navigation function and consolidating information from short-term memory to long-term memory storage, and astrocytes (DI-TNC1), which are the most abundant cells in mammal brains and provide biochemical support for endothelial cells that form the blood-brain barrier are both evaluated. The influence of PAHs on the simulated TBI in vitro and the subsequent OX-66 treatment were also investigated. This data will then be used to optimize formulations of OX-66 for future use in the next stage of this research, in vivo animal studies, and eventually human clinical trials.

The hypotheses tested in this study include 1) OX-66 is not cytotoxic to H19-7 and DI-TNC1 cells at a range of dosing concentrations; 2) OX-66 has restorative effects on both H19-7 and DI-TNC1 cells injured via in vitro-simulated TBI (cell stretch-strain injury); 3) exposure to PAHs prior to the injury process in vitro exacerbates the adverse effects on injured H19-7 and DI-TNC1 cells; 4) OX-66 can mitigate these injuries and heal the cells.

Cell viability and plasma membrane integrity were chosen as the two endpoints evaluated in this study, since, instead of specific endpoints, these two general cytotoxicity measurements allow the consideration of various alterations that the exposures to OX-66 or PAHs made on the cells. Correspondingly, the specific aims, as mentioned in the introduction, are first, to evaluate general cytotoxicity for OX-66 via 5-carboxyfluorescein diacetate-acetoxymethyl ester (5-CFDA-AM) assays and lactate dehydrogenase (LDH) assays. 5-CFDA-AM is a lipophilic substrate that can permeate most cell membranes. It is cleaved by intracellular esterases and produces fluorescent carboxyfluorescein (CF) which is retained by live cells when the cell membrane is intact. Therefore, the conversion to CF by the cells indicates integrity of the plasma membranes

(Gorokhova et al, 2012) and cell viability. LDH is a stable enzyme, present in all cell types, and is rapidly released into the cell culture medium upon damage of the plasma membrane. LDH release was measured spectrophotometrically, with the intensity directly indicating the number of lysed cells. The second objective is to induce a cell stretch injury model to simulate an in vitro TBI and investigate the restorative effects of OX-66 on the damaged cells via the 5-CFDA-AM viability assay. The last objective is to dose cells with PAHs before the injury process to evaluate whether it adversely affects cell viability via loss of plasma membrane integrity. The restorative effects of OX-66 following exposure to PAHs and in vitro TBI will also be tested.

CHAPTER THREE

Materials and Methods

Cell Lines

Rat hippocampal cells (H19-7/IGF-IR) and rat astrocytes (DI-TNC1) were purchased from ATCC (American Type Culture Collection, Manassas, VA). H19-7/IGF-IR cell line was isolated from hippocampi of embryonic day 17 Holtzman rat embryos. They were immortalized by retroviral transduction of temperature sensitive tsA58 SV40 large T antigen. DI-TNC1 cell line was established from primary type 1 astrocytes cultured from brain diencephalon tissue of 1-day-old rats. Three days after the initial plating, the primary cultures were transfected with a DNA construct containing the early region of SV40 under the transcriptional control of the human GFAP promoter (pGFA-SV-Tt). Upon receipt, the frozen vials of H19-7 and DI-TNC1 cells were stored in liquid nitrogen. Cells were subcultured before being used for experimental purposes to ensure high level of viability and ample cell stocks.

Phase I: General Cytotoxicity Analysis

Cell Culture

Before subculturing H19-7 cells, culture flasks and plates were first coated with poly-l-lysine (Sigma-Aldrich, ST, Louis, MO), a positively charged amino acid polymer that increases the number of positively charged sites, in order to enhance electrostatic interaction between negatively charged ions of the cell membrane and the culture surface to promote cell adhesion. Sterile tissue culture water was added to poly-l-lysine to make

0.1mg/ml solution. Flask surface was coated with 1 ml per 25 cm^2 of solution by gently rocking to ensure even coating. After 5 minutes, solution was removed by aspiration. Culture flasks and plates were then air-dried for two hours at room temperature before introducing cells and medium. DI-TNC1 cells were seeded on non-coated flasks.

Complete H19-7 cell growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) -high glucose (Sigma-Aldrich, St. Louis, MO), sodium bicarbonate, 0.2mg/ml G418, 0.001mg/ml puromycin, and 10% fetal bovine serum (FBS). DI-TNC1 cell medium had similar formula, including DMEM-high glucose, sodium bicarbonate, 1% penicillin-streptomycin (PS), and 10% FBS. Before retrieving the frozen vials, culture medium was warmed for approximately 30 minutes at 37°C (Since the medium was slightly cooled down before it was used, the difference of the temperature between 37°C and 34°C, which was needed by H19-7 cells, was ignored). T-75 flasks were flamed on the neck before and after opening for sterilization and filled with 17ml warm medium. The frozen vials were retrieved from the liquid nitrogen tank and were placed into warm water collected from the water bath. When thawed, the vials were quickly wiped with an alcohol-sprayed paper towel and the cell content was immediately transferred to the prepared T-75 flask. The newly seeded flasks were then moved into the incubator at 5% CO_2 and 34°C (for H19-7) or 37°C (for DI-TNC1) after being observed under microscope. Cell medium was replaced every 3-4 days as needed. Over a period of approximately 6-7 days, the H19-7 cells were grown to 80-85% confluent before being split onto 96-well plates. For DI-TNC1 cells, it took 2-3 days to reach confluence. As detailed above, 96-well plates were coated with poly-l-lysine before seeding H19-7 cells and non-coated plates were used for DI-TNC1 cells. Before the cell-splitting procedure, phosphate-

buffered saline (PBS-Free, Sigma-Aldrich, Saint Louis, MO), trypsin-EDTA solution (Sigma-Aldrich, Saint Louis, MO) and culture medium were placed in the waterbath for approximately 30 minutes prior to use. The flasks were taken out from the incubator and flamed on neck before and after opened. Medium was then removed from the flasks and replaced with 18ml PBS-Free. After 2 minutes, PBS-Free was removed and replaced with 3ml trypsin. The flask was then placed into the incubator for about 4 minutes until the cells detached from the bottom of the flask. Immediately, 7ml medium was added to each flask and the cell suspension was transferred to a 15 ml conical tube. For cell counting, a 20μL aliquot was taken and placed in a cell counter vial with 20ml isotonic saline solution. The conical tube then was placed in the centrifuge to spin for 3 minutes at 3000rpm. Cells formed a pellet after centrifugation. Based on the amount of cells counted, the supernatant medium was removed and fresh medium was added to make a concentration of 1 million cells/ml. Then the tube was vortexed until the cells became resuspended without clumps (usually 20-30 seconds). When ready, 1.2ml of H19-7 cell suspension was taken and mixed with 8.8ml fresh medium in a sterilized basin for each coated 96-well plate, and similarly, 0.3ml of DI-TNC1 cell suspension was taken and mixed with 9.7ml fresh medium. For each cell line, one clear and one black 96-well plate was seeded for LDH and CFDA assays, respectively. The calculation assumed that each well had 12,000 H19-7 cells or 3,000 DI-TNC1 cells, which was determined by the previous cell density tests on 96-well plates, from which 12,000 cells/well was approved to be the best initial concentration for H19-7 cells to reach 80-85% confluent after 72 hours, and 3,000 cells/ well was the best concentration for DI-TNC1.

Cell Dosing

24 hours after seeding, the cells were dosed with OX-66 solution at various concentrations. To prepare OX-66 dosing solution, 2.5mg OX-66 was measured and dissolved in 20ml fresh medium to make 125ppm stock solution, which was then diluted to the concentrations ranging from 15.65ppb to 125 ppb. Each plate was set with one negative control column containing only fresh medium, one positive control with 0.1% Triton X solution in fresh medium, and four different concentrations of OX-66 solution at 15.65, 31.5, 62.5, and 125ppb. After establishing a dose-response curve, the most significantly different concentration range from the negative control group for each cell line was picked and the dosing tests were done again with narrowed-down concentrations of 62.5, 80, 95, and 110ppb for H19-7 cells and 2, 5, 10, 12.5ppm for DI-TNC1 cells.

Fluorescent Staining and LDH Leakage Detection

24 hours after dosing, cell viability and cell membrane integrity were measured via the CFDA and LDH assays, respectively.

To make CFDA dye solution, 11 μL 5-CFDA-AM dye was added in 11ml ACAS medium for each plate (1). Before doing CFDA assay, 50μL cell medium was taken and transferred to clear 96-well plates, which was then stored in the 34℃ incubator to keep warm for use in LDH assay. Then the remaining cell medium was removed from each well using a multi-channel pipettor, and PBS++ solution (2) was added to rinse the cells twice. After all the PBS++ was aspirated and the wells were dried, CFDA dye solution was placed on the monolayers and the plate was stored in the incubator at 34℃. After 30 minutes, the dye solution was aspirated and cells were rinsed by ACAS medium twice to remove the excess dye. Since this fluorescein is light sensitive, the whole process was

performed in a dark environment. The plate was then covered with aluminum foil and read by Fluoroskan Ascent FL (Thermo Scientific, Waltham, MA) with filter pair at excitation/emission wavelengths of 485/538nm (3).

LDH solution consisted of 5ml TRIS, 5ml Li lactate, 4.6ml NAD solution mixed with 200 μL of INT solution and 200 μL of PMS solution. The prepared plate was retrieved from the incubator, and then 150 μL LDH solution was added into each well by multi-channel pipettor. Five minutes later, 50 μL stop solution was added in each well and the plate was read by EL×800 Absorbance Microplate Reader (Biotek, Winooski, VT) with filter at wavelength of 490nm.

The CFDA assay was used to evaluate cell viability. A 0.1% concentration of Triton X, a highly toxic detergent, was used as positive control to evaluate the efficacy of the assay. Cell viability was measured as normalized cell viability, obtained by dividing the fluorescence intensity value in each group by the mean value of the control samples. Likewise, LDH release was measured as normalized LDH release, which was obtained by dividing the LDH released in each group by the mean value of the control samples.

Phase II: Post-Injury Cell Viability Recovery Analysis

Cell Culture

Following the same cell culture procedure as mentioned in the process of Phase I, H19-7 cells and DI-TNC1 cells were subcultured until achieving 80-85% confluence. Cells were then rinsed, detached, and counted following the cell splitting procedure as in Phase I. After counting, 1.5 million H19-7 cells were harvested and seeded in a laminin-coated six-well BioFlex Culture Plate (Flexcell International Corp., Burlington, NC) to

ensure the cell density of 25,000 cells/well. About 0.42 million DI-TNC1 cells were harvested and seeded in a non-coated six-well BioFlex Culture Plate (Flexcell International Corp., Burlington, NC) to ensure the cell density was 7,000 cells/well. Twenty-one plates were set for injury scenarios of mild, moderate, and severe in 24 hours, respectively, for each cell line. Each scenario included 7 plates.

In vitro TBI Model

After 24 hours, approximately 30-35% confluent H19-7 and DI-TNC1 monolayers growing in six-well Flex Plates were subjected to injury using a Cell Injury Controller II (Custom Design and Fabrication, Inc., Sandston, VA). Among the seven plates for each injury scenario, only one plate remains uninjured, which was considered as the control group. CIC II is an electronically controlled pneumatic device that allows the study of morphologic, physiologic, and biochemical responses of tissue-cultured cells to trauma. An inlet on the Cell Injury Controller was connected to a tank of compressed gas. The Cell Injury Controller regulated the pressure and duration of a pulse of air that was delivered through a closed tube system to an adapter fitted with an airtight seal into the top of each tissue culture well in the Flex Plate. A valve and timer (1-100 msec) on the Cell Injury Controller unit controlled the exact millisecond duration of the valve opening and the air pressure pulse. The air pressure pulse was delivered by pressing a trigger on the controller. The unit is designed to ensure that once the air pulse is delivered the air between the unit and Flex Plate is immediately vented to the atmosphere. This provided a rapid deformation and rebound of the deformed silastic membrane in the individual wells of the BioFlex Plate.

The peak injury pressures were chosen according to the standard provided by the Cell Injury Controller II operation manual. Table 2 correlates peak well pressure to empirical data on the resulting injury severity obtained with the CIC Model. In order to more accurately evaluate the response of injured cells to OX-66 treatment, one lower and one higher peak injury pressure were chosen for each injury level to simulate a milder and a more severe scenario under each category of injury. Therefore, 1.8psi and 2.4 psi, 3.3 psi and 4.0 psi, 4.5psi and 8.0psi were chosen to represent a mild, moderate, or severe injury scenario, respectively. Under each injury scenario, each injury pressures were applied on the three cultured BioFlex plates for each cell line, respectively. The pulse duration for all peak injury pressures employed was 50 msec. After exposure to injury pressures, cells were immediately taken to microscope and examined by light microscopy.

Table 2 BioFlex Injury Settings.

Injury Level	Approximate Membrane Stretch	Membrane deformation (dry reference well)*	CIC II-BioFlex equivalent injury peak pressure
Mild	120-135%	5.5-6.5 mm	1.8-2.7 PSI
Moderate	135-155%	6.5-7.5 mm	2.7-4.0 PSI
Severe	>155%	>7.5 mm	>4.0 PSI

*The deformation data is the measured distension of a dry reference well. The corresponding peak pressure values are based on the anticipated injury deformation with the addition of 2ml of fluid media in the BioFlex well.

Cell Dosing

In order to more accurately evaluate cellular response to OX-66, one low concentration and one high concentration at which cell viability was most improved were selected based on the results from Phase I—62.5ppb and 110ppb for H19-7 cells and 5ppb and 12.5ppb for DI-TNC1 cell. Twenty-four hours after the injury process, within

each injury scenario for each cell line, medium was aspirated and 2ml of each selected concentration of OX-66 medium solution was placed on one lower-pressure injured plate and one higher-pressure injured plate. Fresh medium (2ml) was, likewise, applied onto one lower-pressure injured plate and one higher-pressure injured plate as well as the control plate.

Overall, at each injury scenario of mild, moderate and severe, there were seven experimental conditions (shown in Figure 2):

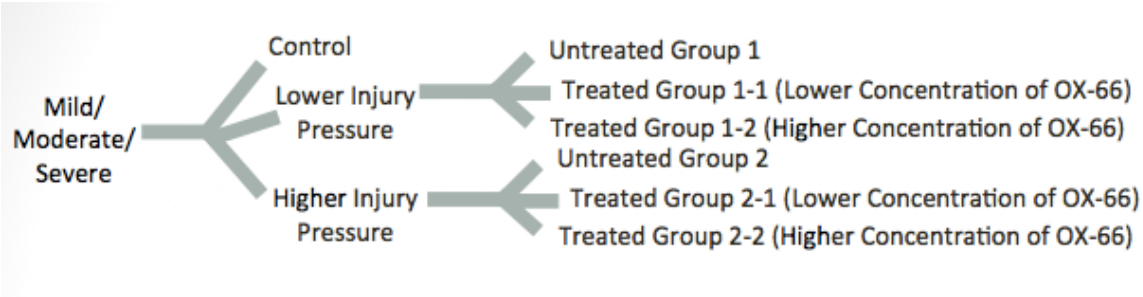


Figure 2. Diagram illustrating experimental groups (mild/ moderate/ severe)

1) Control—cells did not receive injury pressure or OX-66 treatment; 2) untreated group 1 (low pressure)—cells injured by the lower injury pressure with no post-injury OX-66 treatment; 3) OX-66 treated group 1-1 (low pressure/low conc.)—cells injured by the lower injury pressure and were dosed with lower concentration of OX-66 at 24h post injury; 4) OX-66 treated group 1-2 (low pressure/high conc.)—cells injured by the lower injury pressure and were dosed with higher concentration of OX-66 at 24h post injury; 5) untreated group 2 (high pressure)—cells injured by the higher injury pressure with no post-injury OX-66 treatment; 6) OX-66 treated group 2-1 (high pressure/low conc.) 2—cells injured by the higher injury pressure and were dosed with lower concentration of OX-66 at 24h post injury; 7) OX-66 treated group 2-2 (high pressure/high conc.) 2—cells

injured by the higher injury pressure and were dosed with higher concentration of OX-66 at 24h post injury. There were six replicates for each group.

Fluorescent Staining and LDH Leakage Detection

Again, cell viability and cell membrane integrity were evaluated by CFDA and LDH assays at 24 hours after dosing with OX-66. The CFDA and LDH solutions were prepared following the same protocols in Phase I, 50μl of the supernatant in each well of the BioFlex plate was drawn out and placed into a clear 96-well plate. The plate was then stored in the incubator to remain warm for use in the LDH assay later. The rest of the medium was aspirated and replaced with 2.5ml of PBS++ to rinse the monolayers. After two rinses, 2.5ml of the CFDA solution was placed in each well and the plate was placed in the corresponding incubator for each cell line for 30 minutes. During this time, LDH assay was performed by adding 150 μl of the mixed LDH solution in each well of the prepared supernatant-containing plate. Five minutes afterwards, 50 μl stop solution was added to accomplish the assay. The plate was then read using EL×800 Absorbance Microplate Reader at a wavelength of 490nm.

While the EL×800 Reader was running, the BioFlex Plates were taken out from the incubator to accomplish the CFDA assay. The dye solution was aspirated and replaced with 2.5ml of ACAS medium in each well to remove the excess dye. After rinsing twice, the same amount of ACAS medium was placed in and left in the wells. The whole process was performed under a dark environment. Cells were viewed using a Zeiss microscope with a total epifluoresence magnification of 400×. All areas of the elastic membrane are stretched, thus it is assumed that all the areas of the silastic membrane were injured similarly, based on the mechanical design of the Cell Injury Controller II.

Fluorescent images were taken for each tissue culture well. The first being in the center of the well and the four remaining fields being directly above, below, right and left-hand side. The fields were randomly chosen without being viewed to avoid bias. Cell fluorescence was counted for each picture by image analysis software ImageJ (Jerkins, CA) and injury was expressed as the corrected total cell fluorescence (CTCF). Under the ImageJ operating environment, the cell of interest was selected by using a freeform drawing tool to measure the integrated density, area, and mean value of fluorescence. A region that has no fluorescence next to each cell was then selected to register the background readings. The CTCF was then calculated by using the formula below:

CTCF = Integrated Density – (Area of selected cell × Mean fluorescence of background readings)

Phase III: Post-Injury Cell Viability Recovery Analysis after Exposure to PAHs

Dose-response tests of a mixture of three PAH compounds was conducted first to determine the dosing concentration for each cell line. Benzo[a]anthracene, phenanthrene and fluoranthene were diluted in fresh cell media from 5000ppm stock solution to 9 different concentrations ranging from 0.2ppb to 16ppm with 1% DMSO, mixed at ratio of 1:1:1. The dosing solution of each concentration was then applied on two columns of the cells in 96-well plates (16 replicates), with two columns of control (fresh media) and solvent control (fresh media and 1% DMSO). These three PAHs were the most frequently identified in the air, lake sediment, and animal tissues in the Kuwait area according to several studies of Gulf War oil fires and their environmental impact (Al-Hashem et al., 2007; Gevao et al, 2005; Saeed et al, 1996).

When the dosing concentration was decided for each cell line, the same protocol as mentioned in Phase II was applied for culturing the cells on BioFlex plates. Cells were divided into three groups: 1) those only received injury; 2) those exposed to PAHs mixture 24h prior to injury; 3) those exposed to PAHs mixture 24h prior to injury and treated with OX-66 24h post injury. Cells in each group were injured at the six injury pressures employed in the previous phase—1.8 and 2.4psi (mild), 3.3 and 4.0psi (moderate), 4.5 and 8.5psi (severe). There was one control included in each group as well, in which cells had no injury, PAHs exposure or OX-66 treatment. After cells reached 20-25% confluency, all cell media was aspirated and 2ml fresh media was applied in group 1) and the controls in group 2) and 3), while media in the rest of the wells in group 2) and 3) was replaced with 2ml mixed PAH solution. After 24h, 1.8, 2.4, 3.3, 4.0, 4.5 and 8.0psi were employed in each group to mimic mild, moderate, or severe injury, to injury wells, except for the controls. Each pressure had three replicates (three wells). After another 24h, all cell media was aspirated again and only the injured wells in group 3) received 2ml OX-66 treatment, while others had the same amount of fresh media added. Based on the results of Phase II, only one of the two OX-66 concentrations for each cell line was chosen to use in this phase, which are 110ppb for H19-7 cells and 12.5ppb for astrocytes, based on the cellular response in Phase II. Plates were incubated for twenty-four hours, then cell viability and cell membrane integrity were measured by CFDA and LDH assays following the same procedure in Phase II.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Dunnett's test was performed to determine the statistical significance among the mean values.

Computational analysis was accomplished using SigmaPlot and JMP 8.0 statistical program (SAS Institute, Inc.). All numerical data were presented as mean values $\pm$SEM (standard error of the mean). Statistical significant differences were determined at α=0.05. A p value less than 0.05 was considered significantly different. Increasing significance are set at $p<0.05$, $p<0.01$, and $p<0.005$

CHAPTER FOUR

Results

General Toxicity of OX-66

In Figure A.1, CFDA illustrates that there were a 96.7% decrease of viability in H19-7 cells and 96.6% decrease of viability of DI-TNC1 cells followed by death within 24 hours if no intervention was applied. Figure A.1 shows that the viability of the dosed H19-7 cells changed as the concentration of OX-66 dosing varied. Among the seven treated groups, six of them displayed significantly higher percentage of cell viability than the control group with the exception of the lowest concentration (15.65ppb). At concentrations of 31.5, 62.5, 80, 95, 110, and 125ppb of OX-66 treatment, statistically significant improvements in cell viability were observed. Approximately 14.8%, 46%, 310.1%, 444.5%, 434.6%, 426.7% improvements, as compared to the control group were observed (Figure A.1). Figure A.2 indicates the dose-dependent change of DI-TNC1 cell viability. The mean viability of cells treated with OX-66 at concentrations of 5ppb and 125ppb was 13.4% and 13.5% higher than the control group, respectively. Cells dosed with other concentrations of OX-66 did not show statistically significant increases in cell viability.

The LDH assay was used to detect cell death by quantifying LDH release due to plasma membrane damage. Again, positive control groups dosed with 0.1% Triton X solution showed a 70.3% increase of LDH leakage in H19-7 cells and a 28.7% increase of LDH leakage in DI-TNC1 cells, indicated that the LDH assay was effective. The larger amount of LDH release indicated more plasma membrane damage. As Figure A.3

presents, cells treated by 15.65, 31.5, 62.5, 95, and 110ppb of OX-66 had 13.9%, 16.7%, 27.4%, 7.2%, and 6.7% less LDH release than the control group, respectively representing a statistically significant response. Figure A.4 presents the results of DI-TNC1 cells that illustrates, at concentrations of 2, 5, 10, 12.5, 31.25, 62.5 and 125ppb, the amount of LDH release was 8.7%, 12.3%, 9.3%, 14.3%, 5.7%, 7.2%, and 4.3% significantly higher than the control group.

Injury Recovery Study

H19-7 Cells

Figure A.5 shows the recovery of H19-7 cells at 48h after receiving low/high mild peak injury pressures followed by OX-66 treatments. The CTCF measured by CFDA assay was significantly decreased after injury at 1.8psi (mild-low pressure) and treatment with 110ppb of OX-66, indicating a significant decrease of cell viability. However, cells that were stretched by 2.4psi (mild-high pressure) and treated with 62.5ppb OX-66 displayed significantly increased CTCF. The rest of the treated groups did not differ significantly from the non-treated groups. Cells treated with 62.5ppb in the 1.8psi injury section had a 1.6% slight decrease in fluorescence intensity, whereas those treated with 110ppb in the 2.4psi section had a 21.1% increase of CTCF. In Figure A.6, only cells that stretched by 1.8psi and treated with 110ppb displayed a significant decrease in amount of LDH leakage. All other mildly injured groups showed a trend of decline on their LDH release, but no significant difference.

As shown in Figure A.7 and A.8, moderately injured cells appeared to have increasing CTCF as the OX-66 concentration increased. With 3.3psi injury (moderate-

low pressure), cells displayed 5.4% and 19.1% increase of fluorescence intensity, and 1.2% and 2.8% decrease of LDH release. Stretched cells injured by 4.0psi (moderate-high pressure) present a similar trend of change—15.2% and 42.8% increased CTCF, 2.6% and 3.9% decreased LDH release, but none of them differed in a statistically significant manner.

Likewise, in 4.5psi (severe-low pressure) injured group, the CTCF did not statistically increase in either OX-66 treated groups, although 34.0% and 39.8% higher fluorescence intensity were displayed (Figure A.9). A 9.5% increase was observed in 8.5psi (severe-high pressure) stretched cells after treatment with 62.5ppb OX-66, but with 110ppb, the fluorescent intensity weakened. Significant decrease was observed in LDH assay. As shown in Figure A.10, cells that were stretched at 4.5psi and treated with 62.5ppb OX-66, stretched by 8.5psi and treated with 62.5ppb OX-66, and stretched by 8.5psi and treated with 110ppb OX-66 showed 4.5%, 4.0% and 5.2% significant decrease of LDH release, respectively.

DI-TNC1 Cells

In Figure A.11, the mildly injured astrocytes showed minor decline on fluorescent intensity on all treated groups except for 1.8psi injury/12.5ppb OX-66. A minor increase of CTCF was shown in 5ppb OX-66 treated cells after a 3.3psi injury, but slightly declined when OX-66 concentration was increased to 12.5ppb. A 52% significant recovery was observed among cells treated with 5ppb OX-66 at 24h after injury by 4.0psi and 46% recovery in 12.5ppb treated group, although the improvement was not statistically significant (Figure A.13). According to results from LDH assays (Figure A.12 and A.14), dosed with 5ppb of OX-66, cells injured at 1.8psi, 2.4psi and 4.0psi

produced 17.6%, 29.7% and 23.9% less LDH release, respectively. Cells injured at 1.8psi, 2.4psi, 3.3psi, and 4.0psi displayed 30.3%, 51.8%, 18.1%, and 14.0% significant decline on the amount of LDH release, respectively, after treatment with 12.5ppb of OX-66.

As displayed in Figure A.15, cells that received lower injury pressure—4.5psi—in severe injury group showed a trend of increased cell viability in both OX-66 treated groups. The increase was 25.6% and 3.6% for 5ppb and 12.5ppb, respectively. No increase, but a slight trend of decline of cell viability was observed in severe-high pressure (8.5psi) injured cells. In Figure 17, all treated groups exhibit a declined amount of LDH release compared to their corresponding untreated group. At 4.5psi, the decrease was 13.7% and 34.5% for 5ppb and 12.5ppb, while at 8.5psi, 12.5% and 12.3% for 5ppb and 12.5ppb, respectively. The group of 4.5psi injury/12.5ppb OX-66 displayed a statistically significant difference.

Injury Recovery After Exposure to PAHs

Phenanthrene, fluoranthrene and benzo[a]anthracene were selected to simulate a simple PAHs mixture in a ratio of 1:1:1. Dose-response curves in Figure A.17 and A.18 showed that H19-7 and DI-TNC1 started displaying significant decreases in cell viability when the concentration of the PAHs mixture reached 20ppb and 2ppb, respectively. In order to measure the cellular response without the interruption of observable adverse effect from PAHs, therefore, 2ppb and 0.2ppb were selected for H19-7 and DI-TNC1, respectively, as the final dosing concentration of the PAHs mixture before injury.

Based on the results from Phase II, 110ppb and 12.5ppb were selected as the dosing concentrations of PAHs mixture for H19-7 and DI-TNC1 in Phase III, respectively based on the cellular response in Phase II. Figure A19, A.21, A.23 and A.25

compare the results of CFDA and LDH assays for cells that exposed to PAHs mixture prior to injury process with those only had injury but no PAHs exposure to determine the effect of PAHs on injured cells. Figure A.20, A.22, A.24 and A.26 compare the cellular response of injured cells that were exposed to PAHs prior to injury process and treated with OX-66 post injury process with those that were exposed to PAHs but had no OX-66 treatment to evaluate the repair effect of OX-66 on injured cells after PAHs exposure.

In Figure A.19, the CFDA results shows that H19-7 cells exposed to the PAHs simple mixture prior to 2.4, 3.3 and 4.0psi resulted in a 17.2%, 27.9%, and 22.5% lower fluorescence intensity than those that had no PAHs exposure prior to injury. Cells stretched at 1.8, 4.5 and 8.0psi also showed a decline in CTCF, but none of them were statistically significant. As Figure A.20 displays, after exposure to PAHs, cells stretched by 3.3psi and treated by OX-66 had 24.9% significant recovery from the non-treated group. Other groups had higher CTCF values when compared to corresponding non-treated groups, except for those injured at 8.0psi, but no significant difference was shown.

LDH results in Figure A.21 illustrate that exposure to the PAHs mixture caused H19-7cells that were injured at 3.3psi to have a 19.1% significantly less LDH release than those that had no PAHs exposure. No significant difference was shown in other groups, but all injured groups that were exposed to PAHs displayed higher levels of LDH release than those without PAHs exposure. In Figure A.22, the amount of LDH released by cells treated with OX-66 after PAHs exposure and stretch injury at 4.0 and 4.5psi was 21.1% and 17.1% less than their corresponding non-treated groups, and the decline was statistically significant. All other groups also had less, but not significantly different, LDH leakage.

As for injured DI-TNC1 cells, shown in Figure A.23, CTCF declined in all groups that were exposed to PAHs 24h prior to injury. The viability of cells that experienced injury at 1.8 and 3.3psi after exposure to PAHs was 16.3% and 18.4% lower than their corresponding non-exposed groups, respectively. And the decline was statistically significant. Groups injured by 2.4, 4.0, 4.5, 8.0psi had 13.4%, 11.9%, 7.3% and 2.7% decrease on CTCF, respectively. With the same PAHs exposure and stretch, as illustrated in Figure A.24, groups with OX-66 treatment illustrated 20.9% and 17.8% recovery on mildly injured cells by 2.4psi and moderately injured cells by 4.0psi over the non-treated group, respectively. CTCF also increased in all of the mild and moderate injury groups after treatment with OX-66—11.5% and 11.7% for cells injured by 1.8psi and 3.3psi—but not significantly different.

In Figure A.25, 21.9% more LDH release showed in DI-TNC1 cells stretched by 3.3psi with PAHs exposure before injury than those not exposed to PAHs. The amount of LDH released by cells injured at 1.8, 2.4, 4.0 and 4.5psi were 19.4%, 15.4%, 20.1% and 7.6% higher than the corresponding non-exposed groups, respectively. After treatment with OX-66, as shown in Figure A.26, cells exposed to PAHs and injured by 4.0psi displayed a 25.7% decrease than those with no OX-66 treatment. Most of the treated groups displayed higher quantity of LDH leakage except for those severely injured by 4.5psi. The increases of LDH released were 11.4%, 10.1%, 12.4% and 1.5% for cells injured at a pressure of 1.8, 2.4, 3.3 and 8.0psi, respectively. Group of 4.5psi displayed a slight decrease of 4.9%.

CHAPTER FIVE

Discussion

General Cytotoxicity of OX-66

Based on the results of general cytotoxicity in Phase I, within the employed concentration ranges, no toxicity was reflected from any tested cellular responses for both hippocampal cells and astrocytes. Instead, results of CFDA and LDH assays demonstrated the improvement of cell viability and mitigation of cell membrane damage for both cell types. At all the dosing concentrations except for 15.6ppb, the improvement of cell viability of H19-7 hippocampal cells increased in a concentration-dependent manner. Especially at concentrations ranging from 80 to 125ppb, the improvement appeared to be significant. The highest cell viability reached to 444.5% over the control group. While cell viability indicates the ability of cells to maintain or recover viability, the release of LDH is a marker of plasma membrane damage, which also significantly decreased at all the concentrations except for 80 ppb and 125ppb. Different from the results of cell viability, the highest decrease of LDH release occurred at 62.5ppb and concentrations at lower ranges (from 15.6 to 62.5ppb) appear to be able to more significantly enhance the plasma membrane integrity. OX-66 also demonstrated the capacity of improving cell viability of astrocytes, but compared to hippocampal cells, astrocytes only showed this significant difference at two concentrations, 5ppb and 125ppb. More promising results were found on cell membrane repair. Lower concentrations ranging from 2 to 12.5ppb seemed more effective at reducing LDH release. Several studies have shown that hippocampal cells are remarkably sensitive to

environmental context and variables (Muller and Kubie, 1987; O'Keefe and Conway, 1978; Thompson and Best, 1989), whereas glial cells, such as astrocytes, are generally less vulnerable than neurons (Silva et al., 2002). Neurons have higher energy demands than glial cells and can produce glutamate, which is the most common excitatory neurotransmitter. It acts as an excitotoxin in excessive amounts when depolarization of the neuronal membrane occurs due to lack of energy. OX-66 is able to provide oxygen for oxidative phosphorylation of glucose in the process of ATP production to meet the energy needs, so for hippocampal cells, a higher concentration is needed for their higher energy demand. It would explain why cell viability of H19-7 (neurons) was more improved at higher concentrations of OX-66 than DI-TNC1 (astrocytes). According to CFDA results, cell viability did not appear to be improved as plasma membrane integrity was enhanced. It may be due to the function of organelles or enzymes, such as mitochondria and disruption of enzymatic activity. Different from the CFDA assay, results of LDH release for both cell types presented a similar trend.

Restorative Effects of OX-66 on Injured Cells

The goal of Phase II was to evaluate the restorative effect of OX-66 on rat hippocampal cells and astrocytes injured at different levels of injury pressure. Ellis et al. (1995) employed a pneumatically driven device, Cell Injury Controller Model 94A (Commonwealth, VA), to deliver a controlled mechanical insult to cultured rat neurons and glial cells. This model has been extensively used to induce injury to simulate TBI in in vitro studies (Floyd et al., 2004; Weber et al., 2001; Weber et al., 1999). CIC Model 94A used the Flex I tray (Flexcell International Corporation, VA), of which the elastic culture area was molded in the tray and provided consistent displacement of the biaxial

stretch. In this study, Cell Injury Controller II was employed instead, and the major difference was the BioFlex tray used in CIC II model. Instead of being molded in the tray, the elastic culture area was held by a two-piece clamp so that it decreases membrane slippage making displacement more consistent at the pressures used to create cell injury. Another advantage of this model is that cells in various areas of the silastic membrane are able to be similarly injured. The Flex Plate system has been used to study the effect of dynamic motion on cell function, structure, and biochemistry. Furthermore, its efficacy has been proven by previous studies (Banes et al., 1990; Ellis et al., 1995) to accurately evaluate the underlying pathology associated with TBI.

According to results from Phase II under the treatment of OX-66 at selected dosing concentrations, cell viability did not show any improvement at 1.8psi, but plasma membrane damage presented a decreasing trend. At 2.4psi, the higher pressure in mild injury group, H19-7 treated with 5ppb of OX-66 appeared to have the best outcome with a 40.1% recovery on cell viability. Cells treated with 12.5ppb also showed a trend of improvement on cell viability. Plasma membrane integrity of cells injured at 2.4psi was also enhanced after OX-66 treatment. Plasma membrane integrity of all moderately injured groups had decreased after treatment with OX-66, while the cell viability was improved as well. In the severe injury group, OX-66 remarkably improved the cell membrane integrity. Significant difference was only not shown among cells that injured at 4.5psi and treated with 62.5ppb of OX-66. The cell viability showed a potential of being improved as well, but at the maximum injury pressure of 8.5psi, a decline of cell viability was exhibited after treatment with 110ppb of OX-66. Overall, the treated groups with mildly, moderately, or severely injured cells all showed a decreasing trend of or

significantly decreased plasma membrane damage. Among these twelve treated groups, four showed statistically significant differences in LDH assay, including one mildly injured and three severely injured groups, indicating that OX-66 may have a significant potential for repairing cellular membrane integrity in severely injured hippocampal cells. Correspondingly, most of the treated groups presented increased fluorescent intensity, indicating that cell viability presented an improving trend with the exception of cells that injured at the lowest pressure (1.8psi) and treated with 110ppb and those injured at the highest pressure (8.5psi) and treated with 110ppb.

The difference between results of 5-CFDA-AM and LDH assays in some treated groups indicated that even though the integrity of plasma membrane was enhanced for all treated groups, some intracellular damage such as organelle dysfunction or enzyme deactivation may have occurred causing a decrease in fluorescence detection. The nonpolar, non-fluorescent substance CFDA is taken up by diffusion and converted to the fluorescent dye CF via cytoplasmic esterase-catalyzed hydrolysis. As a result of the mitochondrial dysfunction caused by injury, substrates can be depleted and the activity of esterase can be transiently impaired, and consequently cause the failure of conversion from CFDA to CF. In addition, the concentration of OX-66 applied may not have been high enough to recover the reversible intracellular damage, therefore, even though the plasma membrane integrity was significantly enhanced, the fluorescence intensity was still lower than the non-treated group. For the cells severely injured at 8.5psi, since the pressure was extremely high, even though the cellular membrane damage significantly recovered, the intercellular damage may not be reparable. An in vivo study is needed to verify this assumption. In the two applied concentrations of OX-66, 62.5ppb improved

the cell viability for five out of six treated groups (83.3%) at this concentration, of which one group (16.7%) was significantly different (2.4psi). It displayed a better restorative effect than 110ppb, which improved cell viability for four out of six treated groups (66.7%) but no significant difference performed. By contrast, almost every injured group treated with 110ppb of OX-66 exhibited less LDH release than those treated with 62.5ppb of OX-66, indicating that 110ppb may have a better capability of healing damaged plasma membranes. For mildly injured rat hippocampal cells, OX-66 may have a better healing ability on cell viability when the injury pressure is higher. Since hippocampus, which plays an important role in the formation of episodic memory and is part of a larger medial temporal lobe memory system responsible for general declarative memory (Squire, 1992), is selectively sensitive to TBI, memory and cognitive impairment have been demonstrated to be one of the most common symptoms among patients with brain trauma (Whiting and Hamm, 2008). Lyeth et al. (Lyeth et al., 1990) suggested that this impairment may be due not only to cell death, but also to a disruption of neuronal functioning, especially within the hippocampus. The restorative outcome of OX-66 on injured rat hippocampal cells, therefore, demonstrated that OX-66 might have the capability to alleviate post-trauma cognitive impairment.

Astrocytes are the most numerous cell type in the central nervous system. They provide structural, trophic, and metabolic support to neurons as well as endothelial cells that form the blood-brain barrier (BBB), which has been considered as a target for therapeutic intervention (Chodobski et al., 2011). Normally functioning BBB is key to recovering brain homeostasis and to creating an optimal microenvironment for neuronal repair. It may also allow for more reliable delivery of therapeutic agent. Therefore, to

evaluate the repair of astrocytes is critical for determining the beneficial effects of a novel therapeutic on TBI. OX-66 seemed to have a remarkable capability of mitigating the plasma membrane damage for mildly injured DI-TNC1 astrocytes, and the most significant decrease (53.1%) of LDH release occurred at 2.4psi treated with 12.5ppb OX-66. For both mild injury pressures, a concentration of 12.5ppb of OX-66 seemed to have a better outcome than 5ppb on enhancing the integrity of cell membrane, whereas, based on CFDA results, the trend of improvement of cell viability of mildly injured astrocytes only occurred at 1.8psi/12.5ppb. Cell membrane integrity of moderately injured astrocytes was also enhanced after treatment with OX-66. Only those injured at 3.3psi and treated with 5ppb OX-66 was a significant difference was not shown, indicating that OX-66 also performed a significant restorative effect on healing the damaged cellular membrane of moderately injured astrocytes. All treated groups under moderate injury exhibited higher cell viability than their corresponding non-treated groups except for those injured at 3.3psi and treated with 12.5ppb OX-66. However, there was only one significant difference, showed among cells injured at 4.5psi and treated with 5ppb OX-66. In this group, the lowest amount of LDH release was also presented, indicating the statistically significantly largest improvement of cell viability and plasma membrane integrity by OX-66 in moderately injured groups. The quantity of damaged plasma membrane of severely injured astrocytes after treatment with OX-66 was also lower than the corresponding non-treated groups and the significant difference was shown in 4.5psi-injured cells after treatment with 12.5ppb OX-66. But only those injured at 4.5psi exhibited improved cell viability, and no such increase was shown in the 8.0psi-injured groups. Overall, all the twelve treated groups displayed lower LDH release than their

corresponding untreated groups. Eight of them (66.7%) had statistically significant differences, including 100% of the mildly injured groups, 75% of the moderately injured groups and 25% of the severely injured groups. However, the fluorescence intensity did not show a promising result as only half of the treated groups showed increased CTCF, and one of them (8.3%) exhibited a significant difference, which occurred in a moderate injury group. According to several studies, CF, the product of the enzyme-catalyzed reaction, may not be a reliable index of cellular vigor. Petroski et al (1994) stated that, even though both astrocytes and neurons exhibited high fluorescence intensity within minutes of CFDA loading, astrocytes rapidly lost the cellular fluorescence following a brief rinse of buffered saline. In this study, astrocytes were rinsed with ACAS cell media twice in the absence of CFDA, which may cause the decline of fluorescence intensity.

In the two employed concentrations of OX-66, 12.5ppb performed a better capability of healing the cellular membrane, by presenting a lower amount of LDH release than those treated with 5ppb for every injury level, only except for the groups injured at 4.5psi. Since only two concentrations were tested for these two cell lines, the best concentration for cell recovery from in vitro injury still needs to be determined. Future study with a wide range of OX-66 concentrations is required to determine dose response for cells injured at all levels.

TBI after PAHs Exposure and the OX-66 Treatment

Without OX-66 intervention, exposure to PAHs seemed to have adverse effects on mildly or moderately injured hippocampal cells. Cells that were exposed to PAHs 24h before injury by 2.4, 3.3, 4.0psi resulted in cell viability that was significantly decreased, while those that were injured at 3.3psi significantly increased the quantity of damaged

plasma membrane. All of the other groups also had different levels of declines in cell viability and plasma membrane integrity after PAHs exposure, indicating PAHs and stretch injury together have synergistic effects on rat hippocampal cells. The adverse effects of PAHs, according to both CFDA and LDH results, were most significantly reflected in H19-7 injured at pressures from 2.4-4.0psi. PAHs seemed to also synergistically affect the cell viability and cell membrane integrity of mildly and moderately injured DI-TNC1 astrocytes. There was a significant decrease of cell viability in astrocytes injured by 1.8psi and 3.3psi, while plasma membrane damage significantly increased at 3.3psi. Other groups under mild and moderate injury scenarios also appeared to have declined cell viability and increased LDH leakage. A slight decrease of CFDA fluorescence intensity occurred in the group of 4.5psi, indicating that PAHs in combination with a severe injury increase the severity of injury to DI-TNC1, however the slight decrease of LDH at 8.0psi seemed to contradict this result. There is experimental evidence from previous studies that support that PAHs have a synergistic effect with TBI on both hippocampal neurons and astrocytes. Morley et al. (Morley and Seneff, 2014) and Lange et al. (Lange et al., 2002) demonstrated that exposure to environmental chemicals, such as PAHs and pesticides, may interrupt the immune response to brain injury. In response to exposure to these environmental toxicants, higher concentrations of inflammatory cytokines and excitotoxins such as glutamate and aspartate would be produced by glial cells and neurons. Calcium channels are controlled by N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors of glutamate, so that massive influxes of calcium would be caused by excessive glutamate release. Abnormal intracellular calcium homeostasis appears to be

linked to several toxicity mechanisms that can majorly impact TBI patients suffering from secondary injury by causing further injury to the soma, the dendrites and the synaptic connections of neurons. In the study of Gesto et al. (2006), exposure to PAHs may cause the brain neurotransmitter system to be targeted in the PAHs-induced neuroendocrine disruption.

After treatment with OX-66, cell viability of H19-7 increased in mildly and moderately injured cells. Considering the results of both of these assays, the restorative capability of OX-66 on H19-7 was most significantly reflected on moderately injured cells. At the lowest pressure, 1.8psi, and the highest pressure, 8.0psi, the restorative effects were least significant. These results are promising in terms of the clinical signs that might be reflected in patients treated with OX-66 following a moderate TBI. Moderate injury scenarios lend themselves to observations of significant improvements in quality of life should a therapeutic intervention be successful in this injury category. Further, testing is still needed in a rodent model to verify these observations in vivo. Cells severely injured at 4.5psi showed a slightly higher CTCF value than the corresponding non-treated group, but at 8.0psi, the highest level of injury, cell viability did appear to be improved by OX-66 as demonstrated by its lower CFDA fluorescence intensity when compared to the non-treated group. Cell membrane integrity of all the groups appeared to be enhanced by OX-66, and significant difference were observed at injury levels of 4.0psi and 4.5psi. OX-66 intervention also showed a better restorative effect on mildly and moderately injured DI-TNC1 astrocytes after exposure to PAHs. Cell viability of those injured by 2.4 and 4.0psi were significantly improved, and plasma membrane of astrocytes stretched by 4.0psi was significantly more damaged than those

with no OX-66 treatment. Again, severely injured astrocytes showed slight changes in cell viability and plasma membrane integrity, thus no restorative effect by OX-66 could be inferred.

Assumptions on the Healing Mechanism of Action

After the in vitro traumatic injury, the excitotoxicity of glutamate is induced due to damage of the cell membrane, enhanced glutamate release and impaired glutamate reuptake. A fall in extracellular glucose is accompanied in response to the release of excitatory amino acid neurotransmitters (Chen et al., 1997; Kawamata et al., 1995). Ionotrophic glutamatergic receptors are then activated and result in the opening of sodium channels—an influx of Na^+ ions (and Ca^{++} ions) and an efflux of K^+ ions. This leads to an ionic imbalance and cell membrane depolarization (Walker et al., 2013) in the brain tissue. According to Lifshitz et al. (Lifshitz et al., 2004), neurons and glial cells following brain injury retain swollen mitochondria due to the excessive hydrated ion influx into the organelle. Golgi apparatus and rough endoplasmic reticulum may also be disrupted following TBI (Paschen and Doutheil, 1999; Singleton et al., 2002), causing a limited energy supply. This limited energy supply may be unable to meet the energy demands required by the accelerated metabolism caused by the restoration of dissipated membrane potentials. Membrane depolarization also causes the opening of calcium channels and subsequently increases the calcium influx. The intracellular calcium accumulation results in mitochondrial depolarization (Sullivan et al., 1999) and inhibition of ATP synthesis (Bindokas et al., 1996; Hillered et al., 1983), and initiates many cellular pathways including the activation of phospholipases, proteases, transcription factors, nitric oxide synthases and DNA degrading endonucleases. The over-production of any of

these molecules can lead to degradation of cytoskeletal components, oxidative stress, and pro-apoptotic gene activation to further cause the death of cells (Werner and Engelhard, 2007).

To repair the imbalance of the limited energy supply and higher energy demand for maintaining ionic homeostasis as well as restoring the membrane potential and cellular functions, hyperbaric oxygen treatment (HBOT) is one therapy that is used. It administers 100% oxygen at environmental pressures greater than 1 atmosphere absolute (ATA) in an airtight vessel for respiration (Bennett et al., 2012). Results show that HBOT appears to restore mitochondrial redox potential by increasing oxygen delivery diffusion gradient and increasing cerebral ATP levels (Daugherty et al., 2004; Zhou et al., 2007). Studies have shown that cell proliferation is enhanced by in vitro simulated oxygen therapy in hyperbaric chamber (Tompach et al., 1997). Nonetheless, the hyperoxia environment created by HBOT can easily accelerate the rate of formation of reactive oxygen species (ROS) such as peroxides and free radicals. This occurs via disturbance of the normal redox state of cells. Studies have shown that brain cells are especially vulnerable to free radicals due to their limited ROS scavenging ability and poor catalase activity (Hall and Braughler, 1989; Palzur et al., 2008). Pressures greater than 3.0 ATA typically cause increased formation of ROS and secondary lipid peroxidation that would damage cellular membranes (Harabin et al, 1990; Jamieson et al, 1989; Noda etla, 1983; Puglia et al, 1984).

Normobaric hyperoxia (NBH), another therapeutic method that increases the fraction of inspired oxygen (FiO_2) to 100% at normobaric pressure, is easily administered. However, the improvement on mitochondrial function was only present for a short

duration in one study. Results from this study were not as robust as HBOT (Tisdall et al., 2008). Zhou et al. also showed the increase of neurons in the hippocampus and improvement of neuronal behavior post HBOT, which was not produced by NBH.

Comparatively, the property of a solid-state administration directly into the blood plasma demonstrates that utilization of OX-66 can be much easier, and this study resulted in no oxygen toxicity. Studies have shown that elevated levels of tissue oxygen may repair mitochondrial function by favorably influencing the binding of oxygen in the mitochondrial redox enzyme systems after injury (Rockswold et al., 2010). This can further result in an improvement on glucose utilization and oxidative metabolism. Energy can be produced more efficiently to support the restoration of the ionic homeostasis. However, since OX-66 is a novel therapeutic, its mechanisms of action for healing TBI are not fully understood and testing the assumptions of its mechanism are dependent on the next set of in vitro studies and future in vivo studies.

CHAPTER SIX

Conclusion

In conclusion, based on the results from Phase I tests, there were no observed toxicity due to OX-66 on healthy H19-7 and DI-TNC1. Rather, at certain concentrations, cell viability and cell membrane integrity were improved (24h post treatment).

In Phase II, OX-66 had various degrees of restorative effects on H19-7 hippocampal cells at different levels of injury when evaluated at 24h post injury. The concentration of 62.5ppb tends to have an overall positive effect on cell viability of H19-7. It appears to be able to improve the viability of mildly injured cells more efficiently than 110ppb, but less efficiently than 110ppb when cells received a moderate injury and the lower severe injury pressure. When the injury pressure was at the maximum level, 62.5ppb showed a positive restorative effect on cell viability when compared to 110ppb. The most significant observations in improvement of cell viability over all of the OX-66 treated groups was at a mild injury pressure of 2.4psi with concentration of 62.5ppb. OX-66 also had a positive effect on enhancing plasma membrane integrity of H19-7, and the significant mitigation of membrane damage occurred at 110ppb at the lowest (1.8psi) and highest (8.5psi) injury pressures. Similar to H19-7 hippocampal cells, the integrity of cellular membrane of DI-TNC1 astrocytes appeared to be able to be mitigated by OX-66 at all levels of injury, and the significant change was presented among mildly, moderately, and lower-pressure severely injured cells. This result is significant for mild traumatic brain injuries which are very hard to diagnose and do not appear in traditional MRI imaging. In addition, the symptoms of mild TBI can be diffuse and most are not treated.

OX-66 also showed a potential capability of improving the cell viability of some moderately and severely injured astrocytes, but the result was not as promising, possibly due to the fact that CFDA was not as affective in measuring the cell viability for this cell type, even though this is used to measure cell viability.

Between the two concentrations applied on hippocampal cells, the higher concentration (110ppb) appeared to be more efficient than the lower concentration (62.5ppb) on enhancing the integrity of plasma membrane, especially at a severe injury level. But this advantage was not as obvious in cell viability improvement. As for astrocytes, the concentration of 12.5ppb had better outcome on cellular membrane recovery for mildly injured astrocytes than 5ppb, but this difference was not significant among moderate and severe injury groups.

As assumed, the exposure to PAHs at no observable adverse effect level 24h prior to injury adversely affected cell viability and plasma membrane integrity of H19-7 and DI-TNC1 cells. Greater influence was reflected among moderately and mildly injured hippocampal cells and astrocytes. Severely injured cells did not seem to have a significant additional affect when exposed to PAHs. OX-66 also had a better therapeutic effect on moderately injured H19-7 and DI-TNC1 that were exposed to PAHs before injury.

It is likely that biochemical or functional changes have occurred to cause little or no change in CFDA fluorescence intensity in some cells that presented lower amounts of LDH release than non-treated group. Since only general endpoints were evaluated in this study, more specific cellular mechanisms will need to be explored in the continuing research. Additionally, a wider range of concentrations of OX-66 and more time points

post injury and treatment need to be added in order to evaluate the recovery process more accurately and efficiently. More appropriate cytotoxicity assays are also required in the future studies. Currently, an in vivo study is being run to evaluate further the efficacy of this therapeutic in whole organisms. The results of the present study establish supportive preliminary data for the next phase of research on OX-66.

APPENDICES

APPENDIX A

Figures

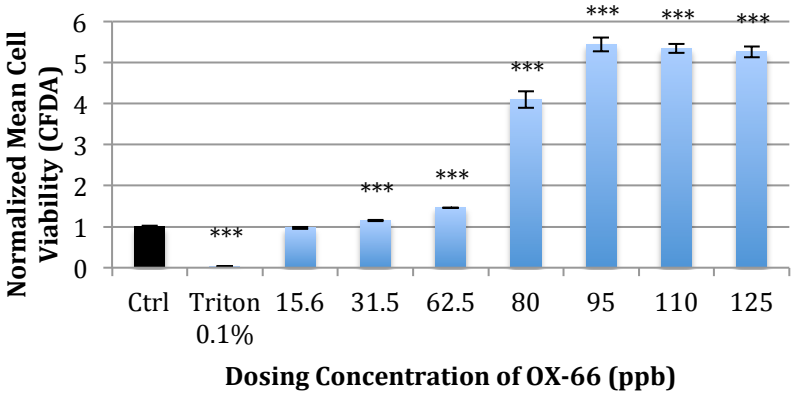


Figure A.1. Normalized cell viability of H19-7 cells after exposure to OX-66 for 24 h (CFDA). All values are expressed as mean±S.E.M (n=16). Asterisk (*) indicates statistically significant difference compared to control. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

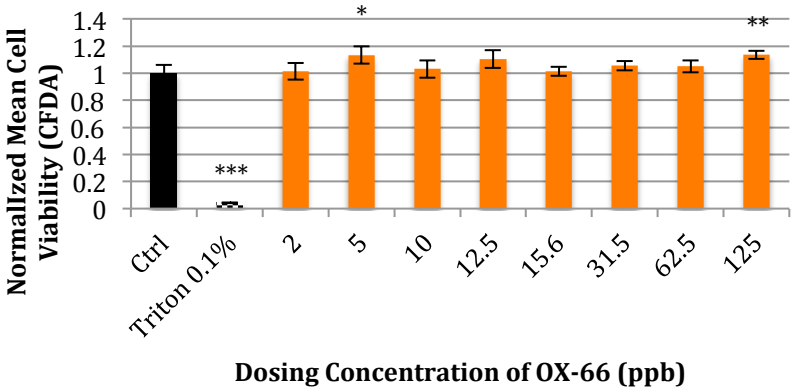


Figure A.2. Normalized mean cell viability of DI-TNC1 cells after exposure to OX-66 for 24h (CFDA). All values are expressed as mean±S.E.M (n=16). Asterisk (*) indicates statistically significant difference compared to control. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

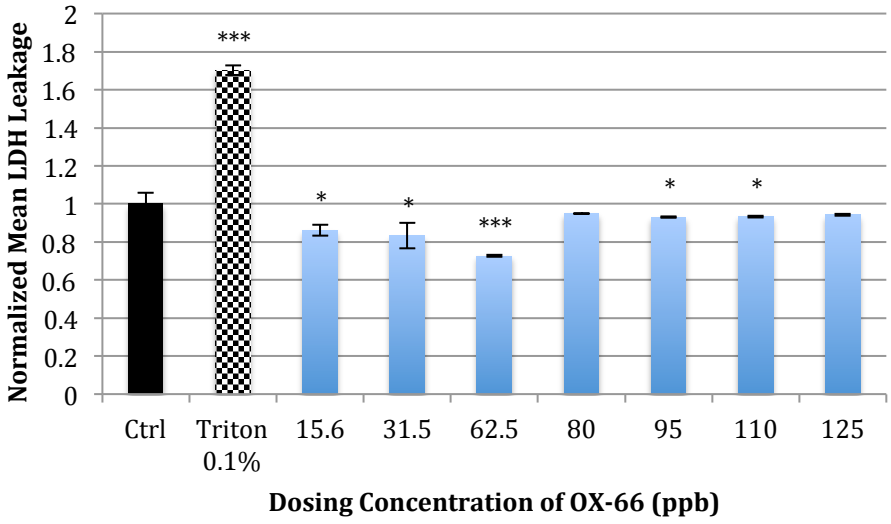


Figure A.3. Normalized mean LDH release of H19-7 cells after exposure to OX-66 for 24h. All values are expressed as mean±S.E.M (n=16). Asterisk (*) indicates statistically significant difference compared to control. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

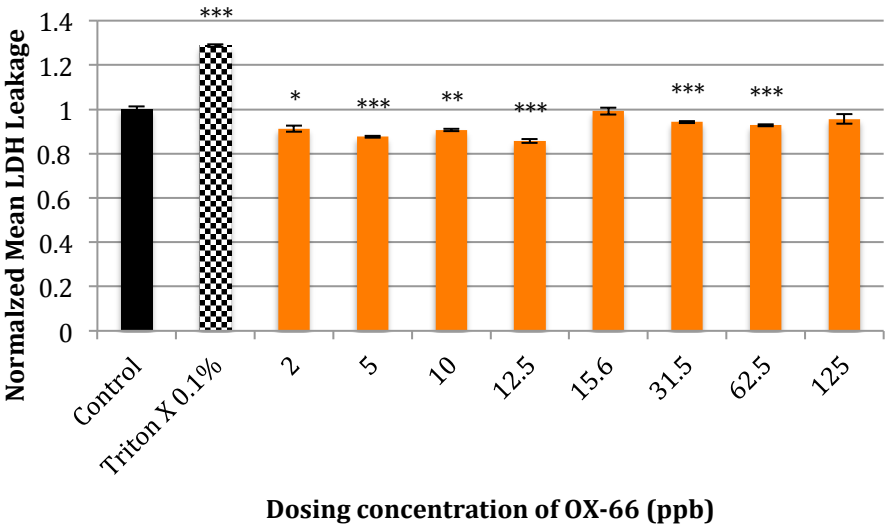


Figure A.4. Normalized mean LDH release of DI-TNC1 cells after exposure to OX-66 for 24h. All values are expressed as mean±S.E.M (n=16). Asterisk (*) indicates statistically significant difference compared to control. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

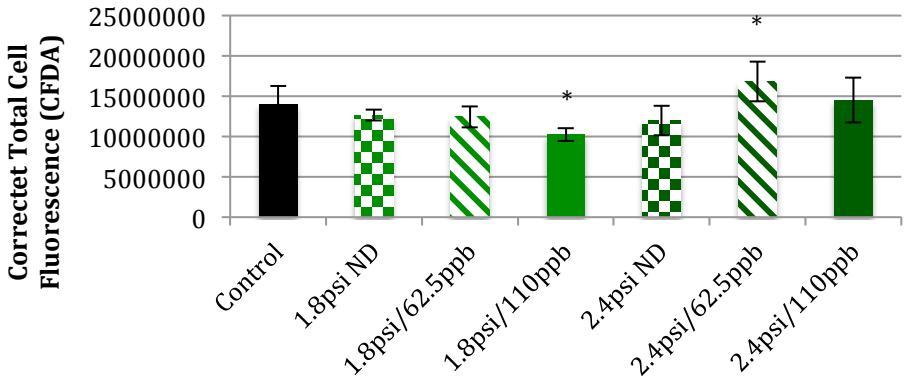


Figure A.5. Corrected total cell fluorescence (CTCF) of mildly injured H19-7 cells after treatment with OX-66 (CFDA). From left to right are 1) control; 2) untreated group 1 (1.8psi—low-mild pressure/No Dose (ND)) 3) treated group 1-1 (1.8psi/62.5ppb); 4) treated group 1-2 (1.8psi/110ppb); 5) untreated group 2 (2.4psi—high-mild pressure/ND); 6) treated group 2-1 (2.4psi/62.5ppb); 7) treated group 2-2 (2.4psi/110ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

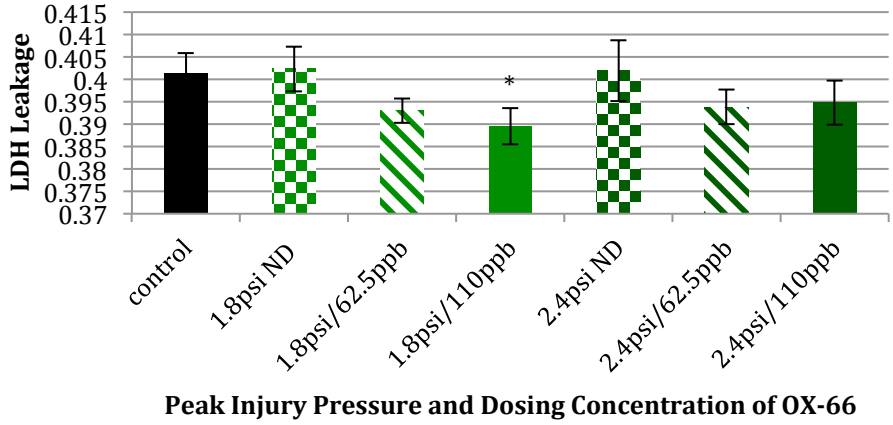


Figure A.6. LDH release of mildly injured H19-7 cells after treatment with OX-66. From left to right are 1) control; 2) untreated group 1 (1.8psi—low-mild pressure/ND) 3) treated group 1-1 (1.8psi/62.5ppb); 4) treated group 1-2 (1.8psi/110ppb); 5) untreated group 2 (2.4psi—high-mild pressure/ND); 6) treated group 2-1 (2.4psi/62.5ppb); 7) treated group 2-2 (2.4psi/110ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

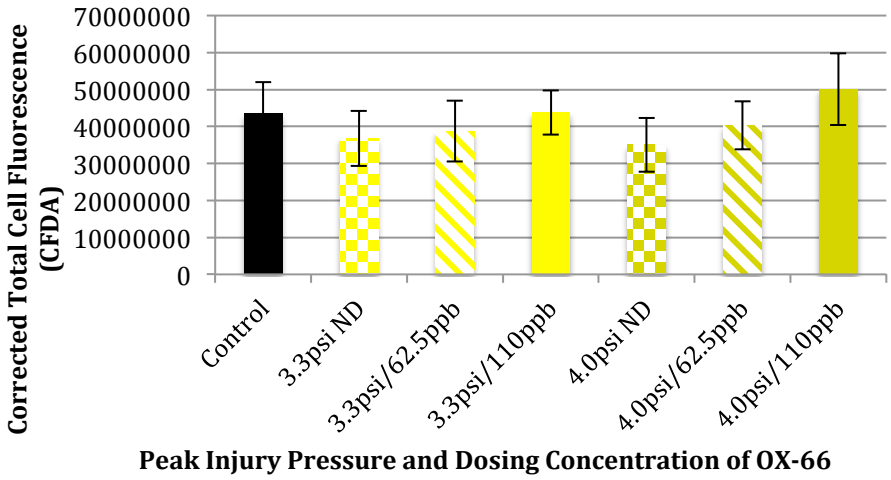


Figure A.7. CTCF of moderately injured H19-7 cells after treatment with OX-66 (CFDA). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (3.3psi—low-moderate pressure/ND) 3) treated group 1-1 (3.3psi/62.5ppb); 4) treated group 1-2 (3.3psi/110ppb); 5) untreated group 2 (4.0psi—high-moderate pressure/ND); 6) treated group 2-1 (4.0psi/62.5ppb); 7) treated group 2-2 (4.0psi/110ppb).

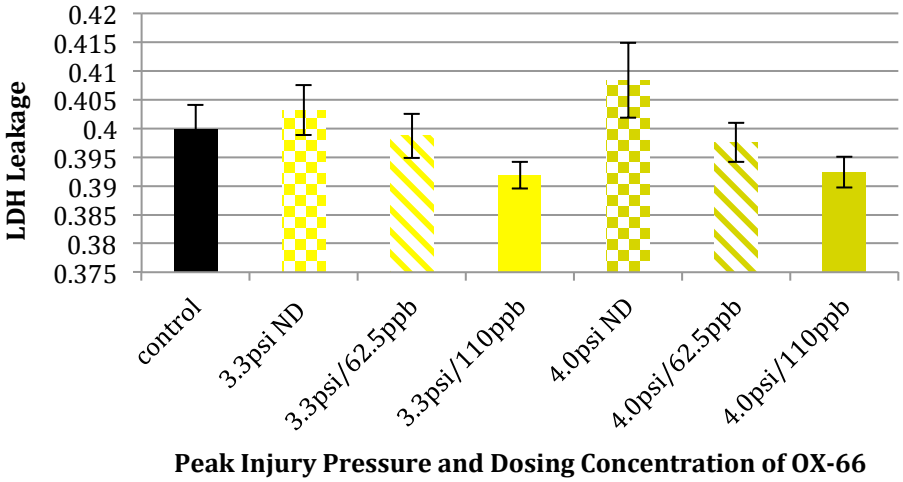


Figure A.8. LDH leakage of moderately injured H19-7 cells after treatment with OX-66 (LDH). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (3.3psi—low-moderate pressure/ND) 3) treated group 1-1 (3.3psi/62.5ppb); 4) treated group 1-2 (3.3psi/110ppb); 5) untreated group 2 (4.0psi—high-moderate pressure/ND); 6) treated group 2-1 (4.0psi/62.5ppb); 7) treated group 2-2 (4.0psi/110ppb).

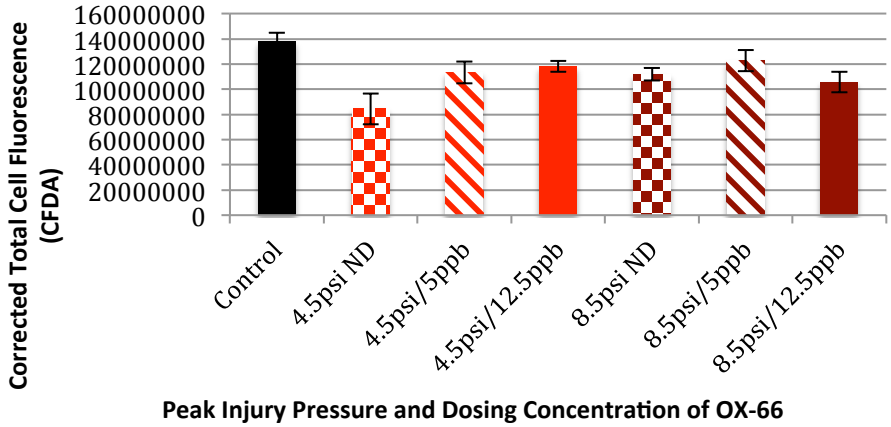


Figure A.9. CTCF of severely injured H19-7 cells after treatment with OX-66 (CFDA). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (4.5psi—low-severe pressure/ND) 3) treated group 1-1 (4.5psi/62.5ppb); 4) treated group 1-2 (4.5psi/110ppb); 5) untreated group 2 (8.5psi—high-severe pressure/ND); 6) treated group 2-1 (8.5psi/62.5ppb); 7) treated group 2-2 (8.5psi/110ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

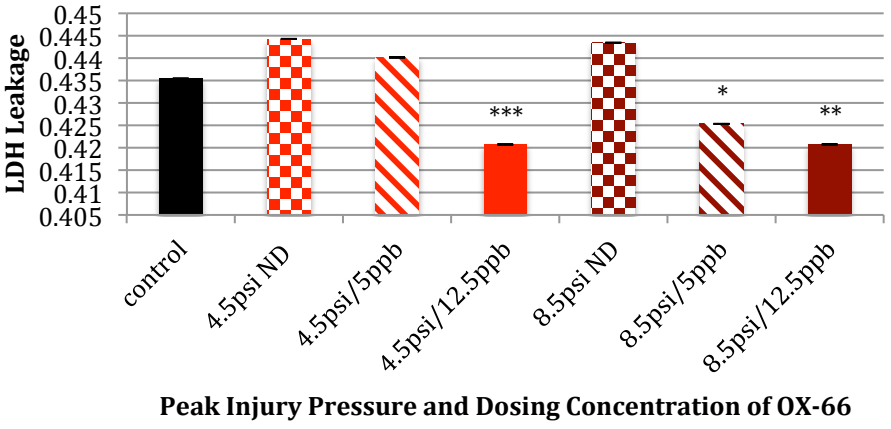


Figure A.10. LDH leakage of severely injured H19-7 cells after treatment with OX-66. Seven experimental groups from left to right are 1) control; 2) untreated group 1 (4.5psi—low-severe pressure/ND) 3) treated group 1-1 (4.5psi/62.5ppb); 4) treated group 1-2 (4.5psi/110ppb); 5) untreated group 2 (8.5psi—high-severe pressure/ND); 6) treated group 2-1 (8.5psi/62.5ppb); 7) treated group 2-2 (8.5psi/110ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

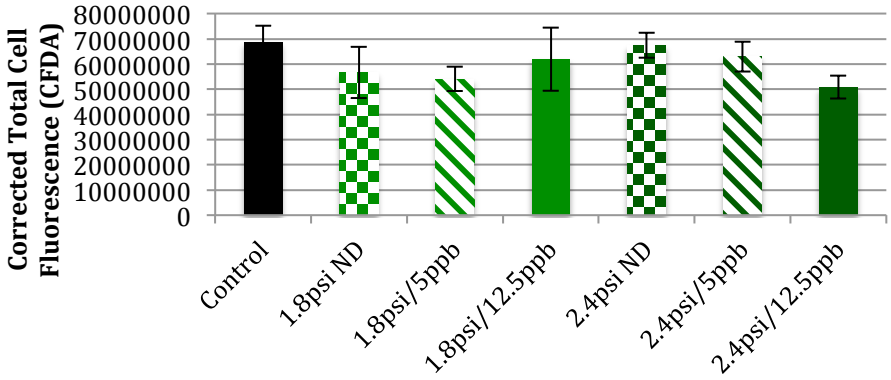


Figure A.11. CTCF of mildly injured DI-TNC1 cells after treatment with OX-66 (CFDA). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (1.8psi—low-mild pressure/ND) 3) treated group 1-1 (1.8psi/5ppb); 4) treated group 1-2 (1.8psi/12.5ppb); 5) untreated group 2 (2.4psi—high-mild pressure/ND); 6) treated group 2-1 (2.4psi/5ppb); 7) treated group 2-2 (2.4psi/12.5ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

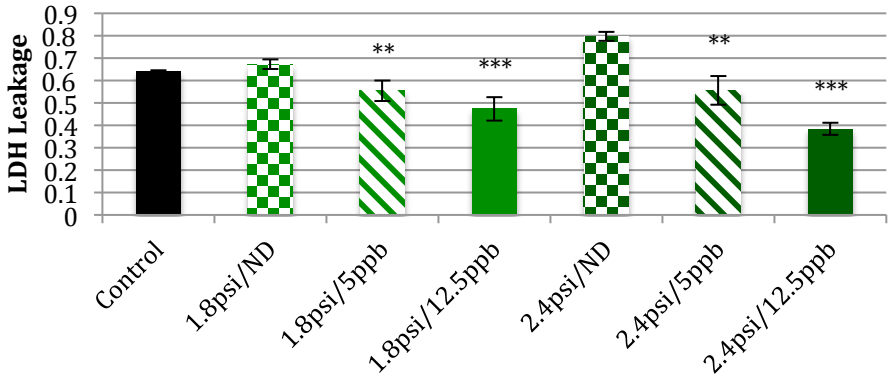


Figure A.12. LDH leakage of mildly injured DI-TNC1 cells after treatment with OX-66. Seven experimental groups from left to right are 1) control; 2) untreated group 1 (1.8psi—low-mild pressure/ND) 3) treated group 1-1 (1.8psi/5ppb); 4) treated group 1-2 (1.8psi/12.5ppb); 5) untreated group 2 (2.4psi—high-mild pressure/ND); 6) treated group 2-1 (2.4psi/5ppb); 7) treated group 2-2 (2.4psi/12.5ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

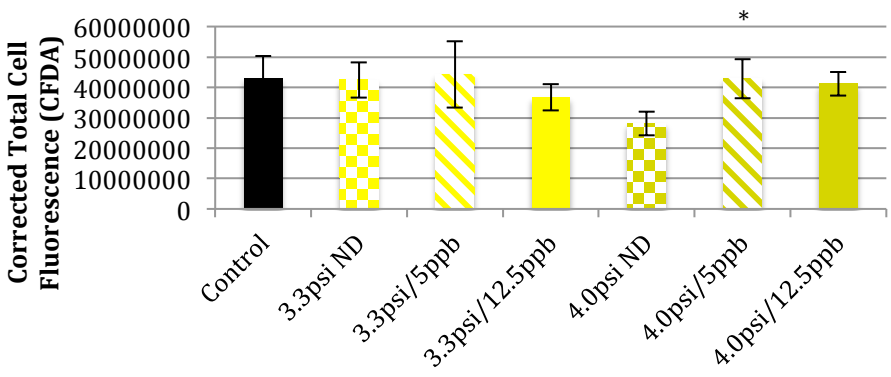
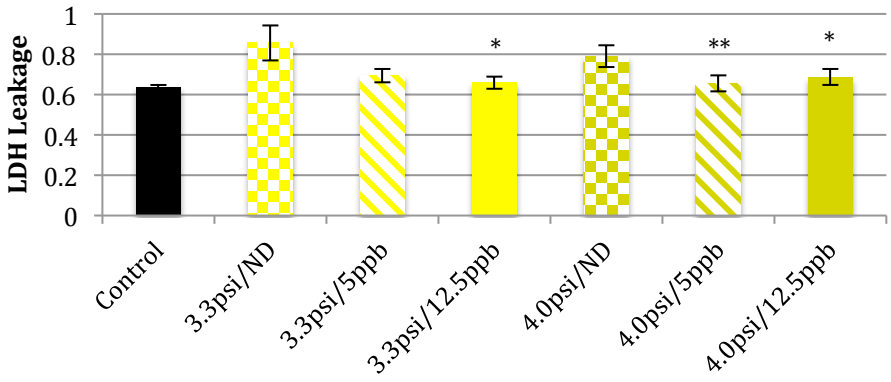


Figure A.13. CTCF of moderately injured DI-TNC1 cells after treatment with OX-66 (CFDA). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (3.3psi—low-moderate pressure/ND) 3) treated group 1-1 (3.3psi/5ppb); 4) treated group 1-2 (3.3psi/12.5ppb); 5) untreated group 2 (4.0psi—high-moderate pressure/ND); 6) treated group 2-1 (4.0psi/5ppb); 7) treated group 2-2 (4.0psi/12.5ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).



Feature A.14. LDH leakage of moderately injured DI-TNC1 cells after treatment with OX-66. Seven experimental groups from left to right are 1) control; 2) untreated group 1 (3.3psi—low-moderate pressure/ND) 3) treated group 1-1 (3.3psi/5ppb); 4) treated group 1-2 (3.3psi/12.5ppb); 5) untreated group 2 (4.0psi—high-moderate pressure/ND); 6) treated group 2-1 (4.0psi/5ppb); 7) treated group 2-2 (4.0psi/12.5ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

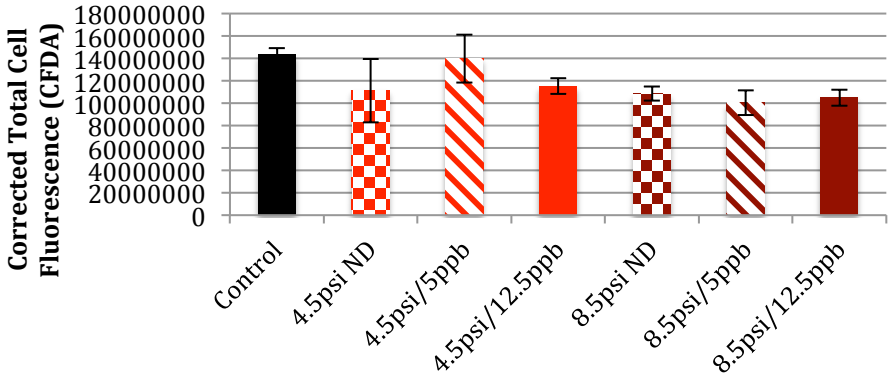


Figure A.15. CTCF of severely injured DI-TNC1 cells after treatment with OX-66 (CFDA). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (4.5psi—low-severe pressure/ND) 3) treated group 1-1 (4.5psi/5ppb); 4) treated group 1-2 (4.5psi/12.5ppb); 5) untreated group 2 (8.5psi—high-severe pressure/ND); 6) treated group 2-1 (8.5psi/5ppb); 7) treated group 2-2 (8.5psi/12.5ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

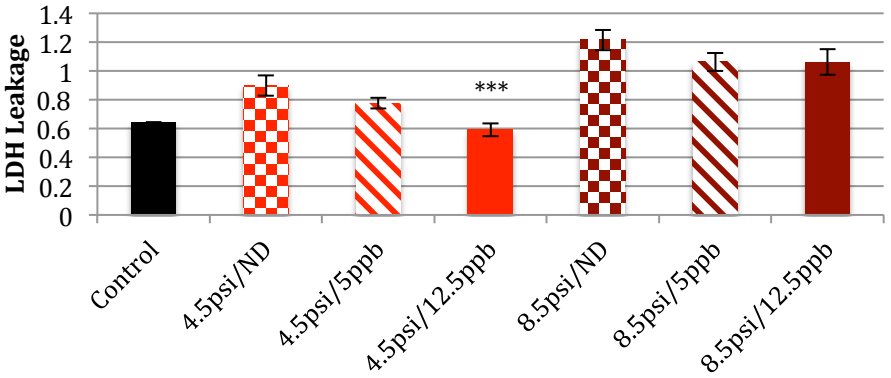


Figure A.16. LDH leakage of severely injured DI-TNC1 cells after treatment with OX-66 (LDH). Seven experimental groups from left to right are 1) control; 2) untreated group 1 (4.5psi—low-severe pressure/ND) 3) treated group 1-1 (4.5psi/5ppb); 4) treated group 1-2 (4.5psi/12.5ppb); 5) untreated group 2 (8.5psi—high-severe pressure/ND); 6) treated group 2-1 (8.5psi/5ppb); 7) treated group 2-2 (8.5psi/12.5ppb). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

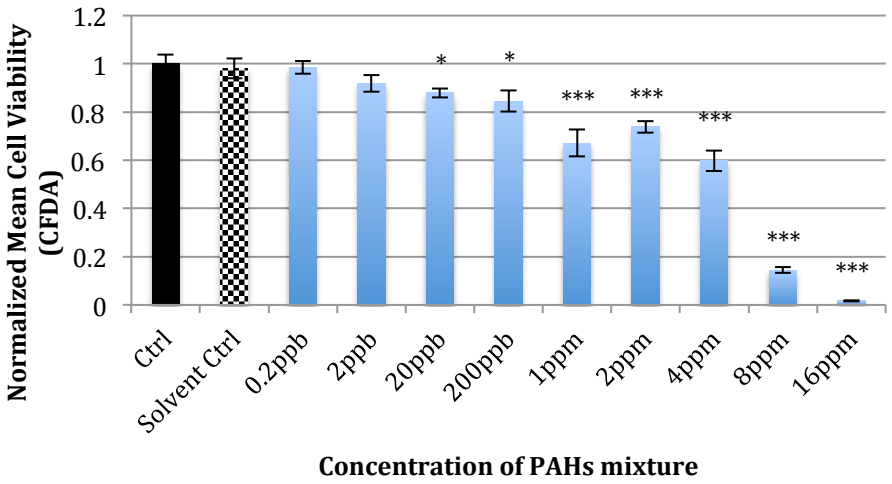


Figure A.17. Dose-response of H19-7 cells exposed to PAHs mixture of phenanthrene, fluoranthene, and benzo[a]anthracene at ratio of 1:1:1 for 24h (CFDA). 1.0% DMSO solution was solvent control. Asterisk (*) indicates statistically significant difference compared to control. *, **, *** indicated p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

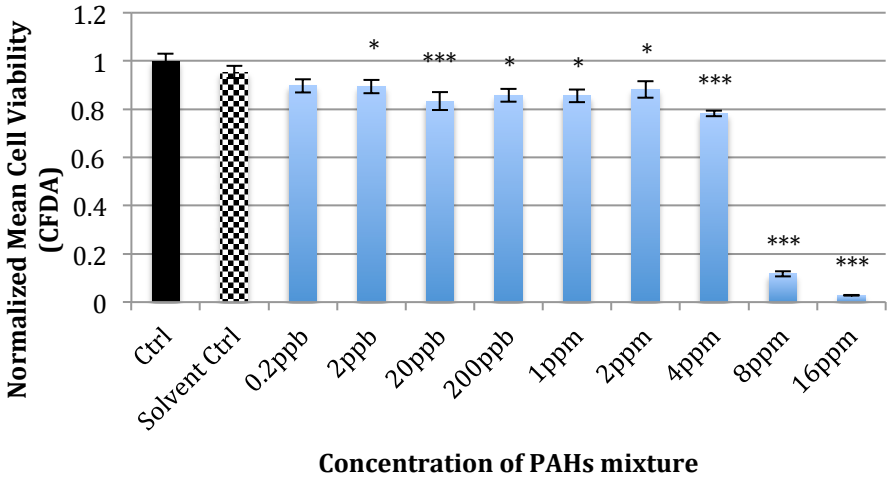


Figure A.18 Dose-response of DI-TNC1 cells exposed to PAHs mixture of phenanthrene, fluoranthene, and benzo[a]anthracene at ratio of 1:1:1 for 24h (CFDA). 1.0% DMSO solution was solvent control. Asterisk (*) indicates statistically significant difference compared to control. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

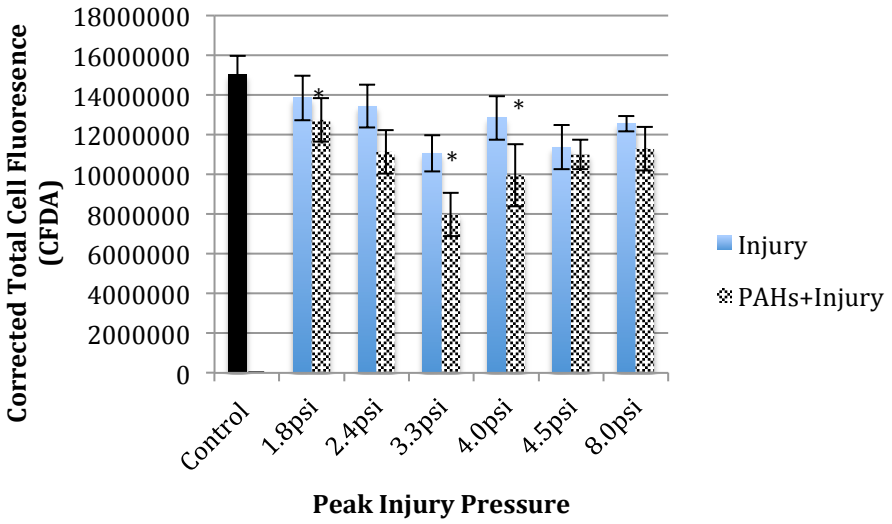


Figure A.19. Effect of exposure to PAHs mixture on cell viability following the injury in H19-7 cells (CFDA). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

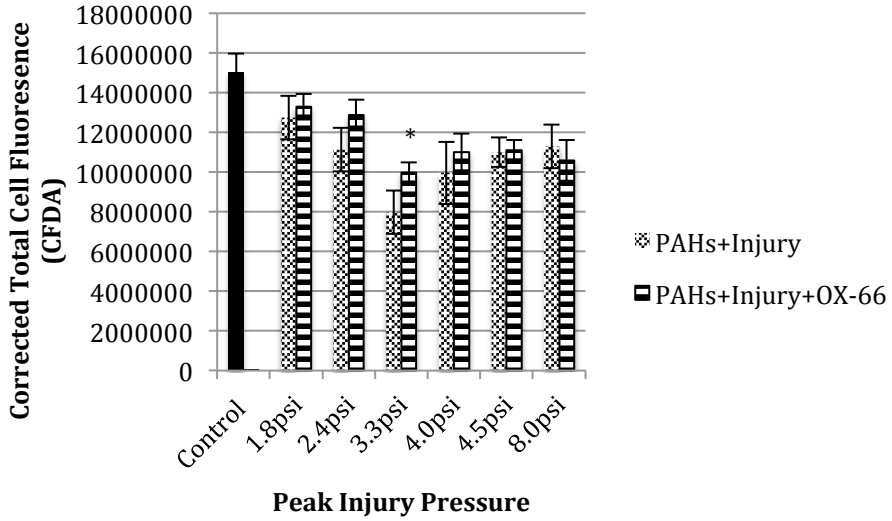


Figure A.20. Restorative effect of OX-66 treatment on cell viability following PAHs exposure and injury in H19-7 cells (CFDA). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

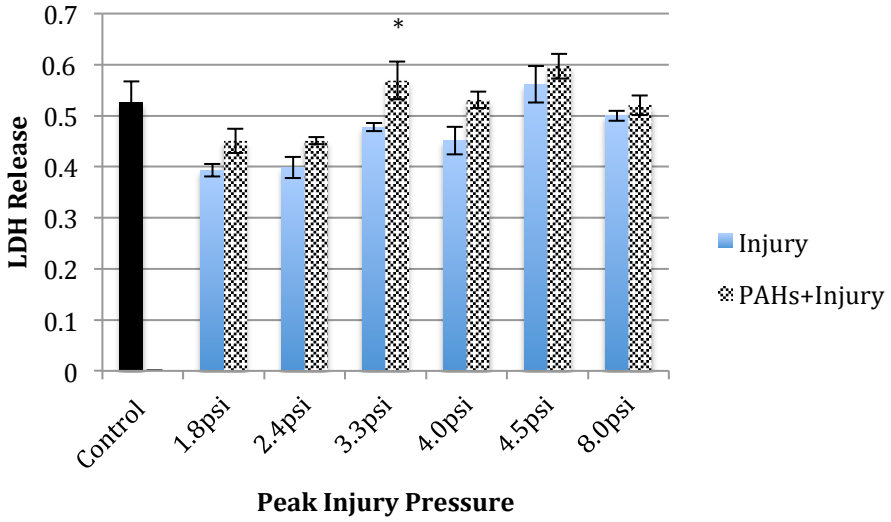


Figure A.21. Effect of exposure to PAHs mixture on the LDH release following injury in H19-7 cells. Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

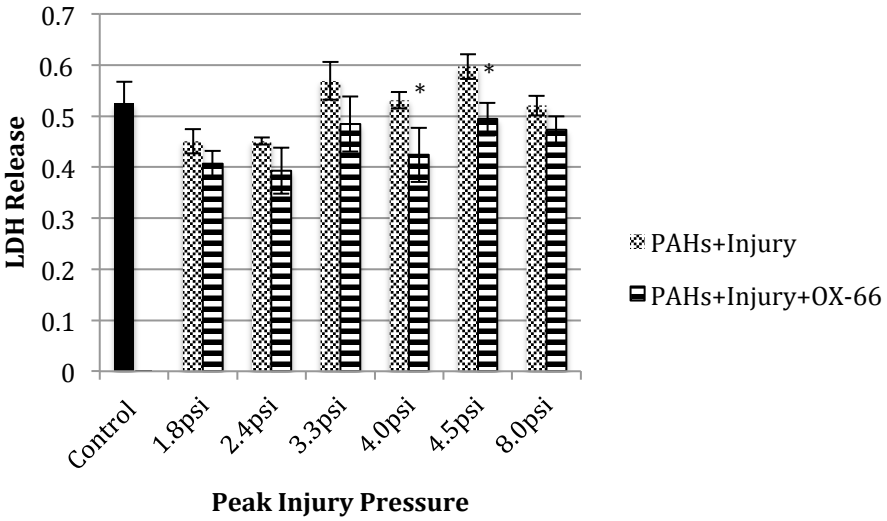


Figure A.22. Restorative effect of OX-66 treatment on the LDH release following PAHs exposure and injury in H19-7 cells. Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

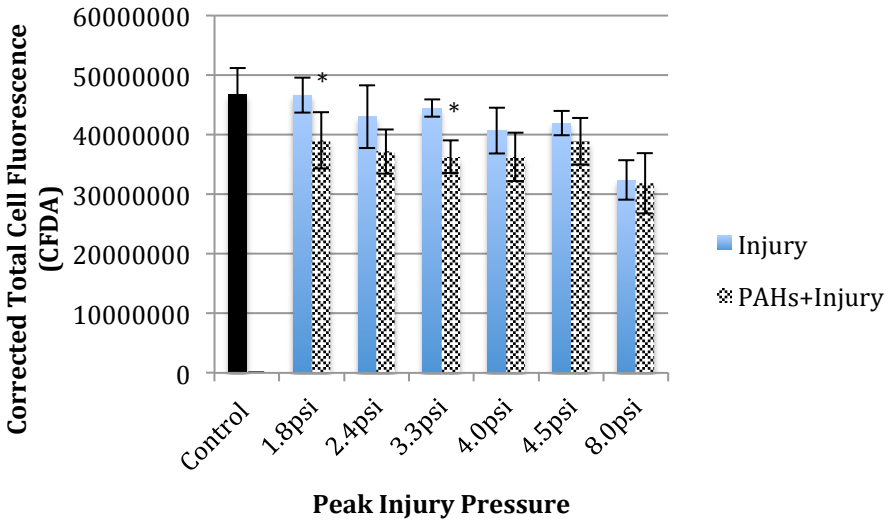


Figure A.23. Effect of exposure to PAHs mixture on cell viability following the injury in DI-TNC1 cells (CFDA). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

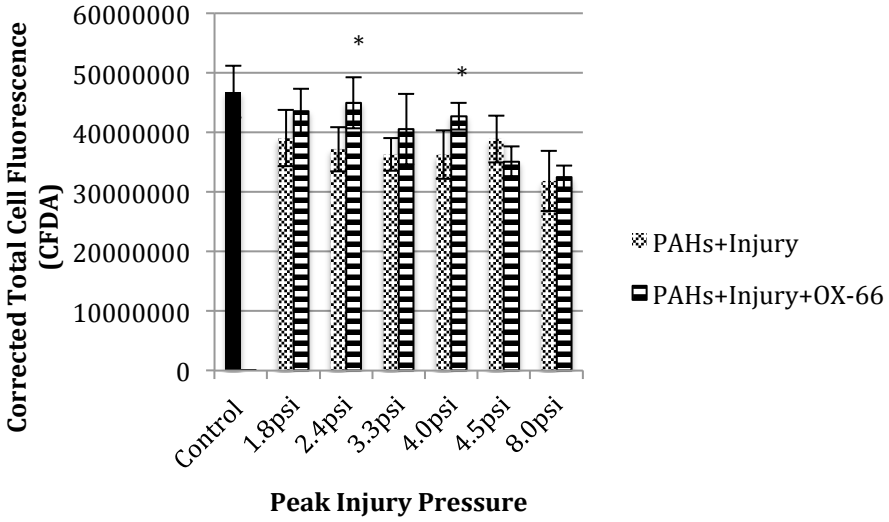


Figure A.24. Restorative effect of OX-66 treatment on cell viability following the PAHs exposure and injury in DI-TNC1 cells (CFDA). Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

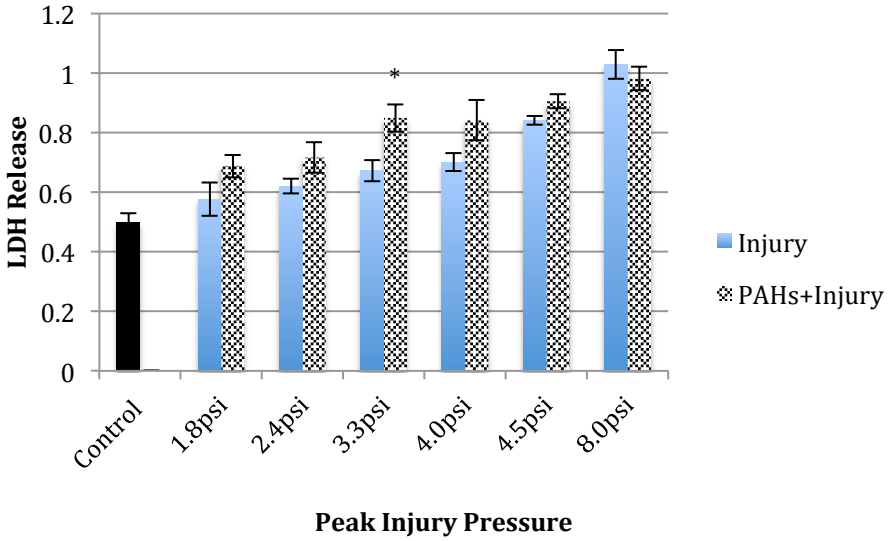


Figure A.25. Effect of exposure to PAHs mixture on the LDH release following the injury in DI-TNC1 cells. Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

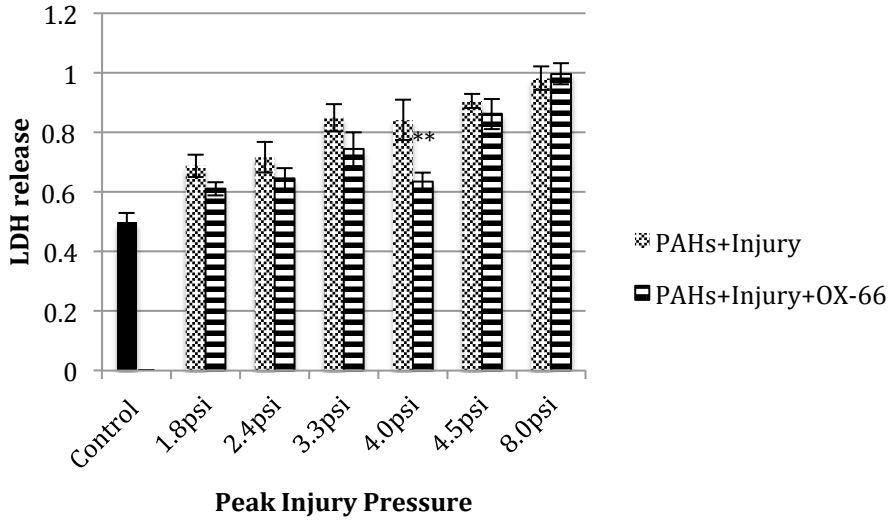


Figure A.26. Restorative effect of OX-66 treatment on the LDH following the PAHs exposure and injury in DI-TNC1 cells. Asterisk (*) indicates statistically significant difference compared to the corresponding experimental group. *, **, *** indicates p value less than 0.05, 0.01 and 0.005, respectively (Dunnett's test).

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