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The Effects of Mild Concussions and Pomegranates on Behavior

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by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

TBI	Traumatic brain injury
MRI	Magnetic resonance imaging
mTBI	Mild traumatic brain injury
rmTBI	Repeated mild traumatic brain injury
CHI	Closed head injury
rmCHI	Repeated closed head injury
Dpi	Days post injury
CCI	Controlled cortical impact
FPI	Fluid percussion injury
DAI	Diffuse axonal injury
PED-SOD	Polyethylene glycol-conjugated superoxide dismutase
RA	Repeated anesthesia
OF	Open field
BB	Beam balance
FF	Foot fault
RR	Rotarod
ZM	Zero maze
TST	Tail suspension test
WM	Water maze

ABSTRACT OF THE DISSERTATION

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Nikita M. Bajwa

Doctor of Philosophy, Graduate Program in Experimental Psychology
Loma Linda University, June 2016
Dr. Richard E. Hartman, Chairperson

The neuropathological effects of mild concussions can accumulate, leading to the development of motor and cognitive deficits. Although there are currently no treatments that can restore lost function after repetitive concussion, our laboratory has shown that dietary supplementation with pomegranate polyphenols can improve Alzheimer's-like pathology, protect mice from the effects of proton irradiation, and protect humans from post-operative cognitive deficits. This study investigated the behavioral and neuropathological effects of repetitive concussions in mice and the use of dietary supplementation with pomegranate to ameliorate these effects. Adult mice were given dilute pomegranate juice or control water for 1 week, followed by anesthesia, repeated anesthesia (3 days apart), or repeated concussive injury (a single closed-head injury to each hemisphere 3 days apart). They were then maintained on the pomegranate juice or control water for 2 additional weeks. Behavioral testing was administered 1, 3, 5, and 7-11 days post injury to assess cognitive, motor, and affective functions. Repeated concussion, but not repeated anesthesia, induced motor and learning deficits, some of which were significantly reduced by pomegranate juice. These data suggest that the model of mild repeated concussive injury in mice might be used to test neuroprotective treatment in future studies.

CHAPTER ONE

INTRODUCTION

Traumatic brain injury (TBI) can be caused by external force to the head or body. TBIs present a serious and emerging medical problem and are the leading cause of death and disability in combat and civilian circumstances, with an estimated 1.7 million people affected annually (H. Yu, Watt, & Mohan, 2014). Falls are the leading cause of TBI in the United States (U.S.) (Faul, Likang, Wald, & Coronado, 2010), and other causes include motor vehicle accidents, sports injuries, and violence (H. Yu et al., 2014). Survivors may experience ongoing and permanent changes to brain structures, leading to long-term cognitive, motor, and personality changes that ultimately increase the risk of developing future neurological disorders. The U.S. military has recently reported that an estimated 22% of all combat wounds in recent military conflicts were brain injuries, and that TBIs are now referred to as the "signature wound" (H. Yu et al., 2014). This phenomenon affects all ages and socioeconomic classes, with millions being hospitalized for one or more TBIs. In addition, TBI also has a large economic impact with annual costs in the U.S. totaling an estimated $60 billion and continuing to rise each year (Langlois, Rutland-Brown, & Wald, 2006). TBI is a major public concern, and despite the high incidence of TBI, few preventative and treatment options are available (Chauhan, Gatto, & Chauhan, 2010).

TBI may be classified based on severity (e.g. mild, moderate, or severe), injury location, time of lost consciousness (H. Yu et al., 2014), and clinical imaging techniques such as magnetic resonance imaging (MRI) are used to classify injuries. Impact injuries

produce damage when the head makes contact with an object, and penetrating injuries occur when an object passes through the protective covering of the skull resulting in direct parenchymal damage (Smith, 2013). Injuries can range from mild concussions, with symptoms lasting from seconds, to more severe injuries with symptoms lasting years or even causing death. The recoveries of motor and cognitive functions are dependent on the severity of injury, with larger injuries corresponding to longer recovery time (Huh, Widing, & Raghupathi, 2011).

The principal mechanisms of TBI are classified as either focal brain damage caused by a contact injury resulting in contusion, laceration, and intracranial hemorrhage, or diffuse brain damage caused by acceleration and/or deceleration, resulting in diffuse axonal injury or swelling (Werner & Engelhard, 2007). The outcome from head injury is determined by two substantially different stages: primary and secondary injury. Primary injury occurs immediately at the moment of trauma and results in the displacement of the structures in the brain. These injuries include damage to vasculature and cortical tissue that may be diffuse or focal in nature. Secondary injuries occur following the primary injury, in which the consecutive pathological processes initiated at the moment of injury remain heightened over time and lead to delayed clinical presentation of symptoms. These include the activation of inflammatory responses associated with brain swelling that may cause further injury to regions that were not initially impacted.

Mild TBI (mTBI) accounts for 75% of all TBIs in the U.S. each year (Gerberding & Binder, 2003) and may result in cognitive, motor, and emotional deficits that lead to social problems, disability and/or unemployment (Baratz et al., 2011; Bazarian et al., 2005). The risk of experiencing more severe brain injuries and long lasting behavioral

deficits increase with each repeated mild TBI (rmTBI). Individuals who have experienced one brain injury are three times more likely to experience a second brain injury (Gerberding & Binder, 2003).

Behavioral Impairments after TBI

Mild (concussion; brief loss of consciousness) to moderate TBI (1 – 24 hours of loss of consciousness) leads to impairment in executive processing, long-term memory consolidation (Miotto et al., 2010), strategy and decision-making (Miotto et al., 2010; van Noordt & Good, 2011), episodic memory, and verbal recognition and verbal episodic memory tasks (Miotto et al., 2010). Deficits from mild TBI typically resolve within a short amount of time, whereas moderate TBI recovery may take several weeks to months (Ajao et al., 2012). Severe TBI (> 24 hours of loss of consciousness) leads to significant cognitive and motor deficits, long lasting behavioral impairments and permanent functional damage as a result of extensive tissue degeneration.

Closed head concussive injuries (CHIs) have been gaining significant attention within the research community. Typically, people with CHIs experience a variety of symptoms, including headache, dizziness, nausea, confusion, abnormal balance, cognitive impairments, and sleep disruption (Slobounov, Gay, Johnson, & Zhang, 2012). Conventional wisdom has been that typical recovery following a concussion is rapid and full, with no residual physical or cognitive impairments. However, there is growing evidence that suggests CHIs may be far more detrimental than previously thought, with cognitive, affective, and underlying neural changes persisting for months or even years post-injury (Slobounov et al., 2012), including an increased risk for neurodegenerative

diseases such as Alzheimer's disease and chronic traumatic encephalopathy (Petraglia et al., 2014).

Concussive-type of injuries are rotational and more diffuse in nature, characterized by extensive lesions in the white matter tracts in the brain, and lead to widespread damage that occurs with increasing severity (Adams et al., 2011). The disruption in neuronal communication throughout the brain may lead to subsequent tissue damage and behavioral deficits (Kinnunen et al., 2011). In a recent study published by our lab, we used a mild closed head injury (mCHI) model to characterize long-term behavioral deficits in young adult mice with a single mCHI and repeated mild CHI (rmCHI). We found that this model produced consistent mild rotational injuries that led to the emergence of late-appearing (90 days post-injury) motor and affective deficits (Bajwa, et al., 2016).

Because the risk of experiencing more severe brain injuries and the resulting behavioral deficits increases with each rmTBI, collaborators have recently used a mild TBI model to better investigate the temporal development of neuropathology using MRI to assess the effects of rmTBI by administering 1 impact to each cortical hemisphere (Donovan et al., 2012). They found tissue damage to be exacerbated following the second mTBI, especially when the injuries were seven days apart. These studies were also extended to investigate white matter and found abnormalities as late as 60 days post injury (dpi) (Donovan et al., 2014). Similarly, our group has shown that brain injury early in life induced long-term white matter abnormalities, delayed development, and persistent behavioral deficits (Ajao et al., 2012). Mice that were exposed to 3 concussive injuries 24 hours apart experienced prolonged loss of consciousness and impaired spatial learning in

behavioral tasks (Creeley, Wozniak, Bayly, Onley, & Lewis, 2004). Another rmTBI model, in which repeated impact to the cranium was delivered to an unrestrained mouse, induced hyperactivity and motor coordination deficits (Kane et al., 2012). An rmTBI model in which rats were given 1, 3, or 5 mild injuries spaced five days apart induced short (24 hr) and long-term (8 weeks) cognitive impairments (Shultz et al., 2012). These studies suggest that the brain remains vulnerable to a subsequent injury for a period of time after injury, increasing the probability of behavioral deficits.

Rodent Models of TBI

The pathophysiological heterogeneity observed in patients with TBI may arise from the nature and severity of the primary injury and any preexisting combination of factors that include, but are not restricted to, age, overall health, gender, medication, and genetics (Margulies & Hicks, 2009). Animal models of TBI are intended to produce a relatively homogenous type of injury, with age, gender, medication, and genetic background and all injury parameters controlled (Xiong, Mahmood, & Chopp, 2014). These models are also essential to study the biochemical, cellular and molecular aspects of human TBI that cannot be assessed in a clinical setting, in addition to developing and characterizing therapeutic interventions (Xiong et al., 2014). Experimental models of TBI are categorized as open- or closed-head, depending on whether the skull is broken or not (Obenaus et al., 2012). The degree of injury can be easily manipulated by various parameters, and more severe injuries generally induce larger locomotor and cognitive deficits (Brody et al., 2007; Obenaus et al., 2012).

Controlled Cortical Impact

The controlled cortical impact (CCI) model allows for independent control of many mechanical parameters and produces many features found in human TBI (Obenaus et al., 2012; S. Yu et al., 2009). This model uses a pneumatic or electromagnetic impact device to drive a motorized piston through a unilateral craniectomy onto the exposed intact dura, and produces cortical tissue loss, acute subdural hematoma, axonal injury, concussion, and blood brain barrier dysfunction (Brody et al., 2007; Chauhan et al., 2010; V. Donovan et al., 2012; Virginia Donovan et al., 2014; Huang et al., 2013; Obenaus et al., 2007; D. H. Smith et al., 1995).

The controlled impact breaks the dura and results in the deformation of the underlying cortex. Although this model delivers a focal injury, further evaluation has revealed that the damage can include other distant areas including the acute cortical region, hippocampus, and thalamus (Hall et al., 2005). Behavioral deficits are evident in motor tasks and learning and memory (Ajao et al., 2012; Brody et al., 2007; Scheff, Baldwin, Brown, & Kraemer, 1997; S. Yu et al., 2009). The advantage of the CCI model compared to others is that TBI severity can be manipulated by varying the piston's speed and/or depth, producing consistent injuries for mild (including concussion), moderate, and severe TBI. Larger depth and speed induce more locomotor and cognitive deficits (Brody et al., 2007).

Fluid Percussion Injury

The fluid percussion injury (FPI) model is one of most widely used TBI animal models that produces features found in human TBI. In this model, the injury is inflicted

by a pendulum striking a water-filled acrylic cylinder with high-pressure tubing that is connected to the injury hub glued to the craniectomy site (along the midline or laterally over the parietal bone; McIntosh et al., 1989) on the mouse. A fluid pressure pulse is delivered to the intact dura and generates a combination of focal and diffuse tissue injury that can cause intracranial hemorrhage, brain swelling, and progressive axonal damage (Graham, McIntosh, Maxwell, & Nicoll, 2000). This model is commonly used, because it produces cognitive and motor deficits (Shultz et al., 2012) that are seen in patients with TBI.

However, there are several limitations in the FPI models. Mortality is very high compared with other models because it is invasive, requires longer surgery, and there is minimal biochemical control of the insult, with only one adjustable mechanical parameter. Thus, injury reproducibility is a large problem in this model. Adjustments to the model have been made to the delivery mechanism and shorter surgical duration, but limited to rats only (Kabadi, Hilton, Stoica, Zapple, & Faden, 2013).

Weight Drop

The weight drop model is an impact acceleration model that uses a free falling, guided weight to fall onto the exposed rodent skull (with or without craniotomy) to produce motor and behavioral deficits seen in TBI (Creeley et al., 2004; Kane et al., 2012; Kilbourne et al., 2009; Marmarou et al., 1994). This model was developed by Marmarou et al. (1994) to produce diffuse axonal injury to mimic human TBI by falls or motor vehicle accidents. This simple model produced a graded, rotational injury in rodents without extensive changes in blood pressure or brain stem damage, and injury

severity can be simply altered with the adjustment of weight and height from where the object is released (Marmarou et al., 1994).

One of the disadvantages of this model is the high variability in injury severity and skull fractures. This presents a critical problem in consistency among results and reproducibility difficulties. In a recent modification of this model (the "Maryland model"), the impact force is applied to the anterior part of the cranium and produces a rotational TBI inside the intact cranium (Kilbourne et al., 2009). Injury features are characterized by an absence of cortical contusions, skull fractures, prolonged apnea, and an absence of mortality, but demonstrate mild subcutaneous hemorrhages and diffuse axonal injury (DAI) (Kilbourne et al., 2009). This model will need to be studied further for consistency and characterization.

Blast Waves

Many military personnel exposed to shock waves from a blast (explosive device) without any penetrating external injuries have been diagnosed with TBI (Benzinger, Brody, Cardin, Curley, & Mintun, 2009; Warden, 2006). Blast injuries cause diffuse tissue injury that lead to chronic neuroinflammation (Goldstein et al., 2013), axonal tissue damage (Garman et al., 2011), learning and memory deficits (Budde et al., 2013), and other motor and behavior abnormalities (Budde et al., 2013; Goldstein et al., 2013). To recreate the effects of primary blast waves on the central nervous system, several animal models of blast TBI have been established, mainly in rodents (Cernak et al., 1996; Cheng et al., 2010; Long et al., 2009; Risling & Davidsson, 2012; Y. Wang et al., 2011) and swine (Bauman et al., 2009; Lanerolle et al., 2011) that use a compression-driven shock

tube to induce an indirect TBI.

One of the disadvantages of these models is the high variability in injury severity
and skull fractures (Xiong et al., 2014). This presents a critical problem in the
reproducibility of the studies and further standardization of methods needs to be
established.

Closed Head Injury (CHI)

CHI is a rodent model of TBI that is more representative of mTBI/concussive
conditions than the more invasive CCI and FPI models that require craniectomy, which
may itself lead to cortical inflammation and behavioral changes (Mouzon et al., 2012). In
the CHI model, the pneumatic or electromagnetic impact device drives a motorized
piston to impact the skull directly. This model produces a mild injury that prevents
cortical contusions, skull fractures, prolonged apnea, and also keeps mortality to a
minimum (Mouzon et al., 2012). The skull and dura remain intact, and the brain rotates
inside the skull, causing more diffuse cortical damage that leads to significant motor and
behavioral deficits. We have recently characterized a mild CHI (mCHI) and repeated
mild CHI (rmCHI) model that produced long-term behavioral and affective deficits
(Bajwa et al., 2016). Mice were placed on a foam base to allow for head rotation after
impact with a motorized piston. MRI at 90 days did not reveal cortical depression and
confirmed the mild feature of our model. We believe the rotational nature of this model
resulted in widespread tissue damage that lead to the emergence of deficits.

Biological Responses after TBI

The pathophysiology of TBI is complex and multifactorial, with several pathways involved in the damage of the brain tissue. Primary injury occurs from a direct external mechanism force at the moment of trauma that leads to skull fractures, brain contusions, lacerations, diffuse axonal injuries, vascular tearing, and intracranial hemorrhages. This impact directly damages neural tissue and causes drastic chemical changes (e.g. excitotoxicity, increased Ca^{2+}) in the brain (Povlishock & Christman, 1995). Secondary injury is induced immediately after primary injury and is mediated by several mechanisms, including raised intracranial pressure, disruption of the blood brain barrier, brain edema, decreased cerebral blood flow, cerebral hypoxia, and ischemia (Graham et al., 2000). The blood brain barrier is compromised after TBI, which triggers a potent inflammatory response with a cascade of molecular, neurochemical, and cellular responses such as such as excitotoxicity (over activation of neurotransmitters), disturbance of cellular homeostasis, oxidative stress, cytoskeletal and mitochondrial dysfunction, inflammation, and apoptosis (cell death) (Povlishock & Christman, 1995). This secondary response dramatically increases the neuronal damage from the primary injury, inducing white matter abnormalities (Virginia Donovan et al., 2014; Huang et al., 2013) and behavioral deficits (Ajao et al., 2012; Brody et al., 2007; Hartman, 2012; Kamper et al., 2013; Lekic et al., 2011).

Neuroinflammation after TBI

Activation of the immune system in the central nervous system has become increasingly recognized as a key component of the normal process of aging, but also the

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pathological onset and progression of many neurological disorders, including TBI and neurodegenerative diseases. The initial inflammatory response after TBI results in neuronal injury and the permeation of the blood-brain barrier (Smith et al., 1997) and the activation of several injury cascades. This impact to the central nervous system results in several factors (blood products, tissue debris, prostaglandins, reactive oxygen and nitrogen species; Dardiotis et al., 2012) triggering the secondary injury response with the activation of resident cells (e.g., microglia and astrocytes), migration and recruitment of leukocytes (white blood cells), and the release of inflammatory mediators.

Microglia

Once the immune response is initiated, microglial cells become activated within minutes and take on a predominantly pro-inflammatory role. They release pro-inflammatory chemokines and also produce other neurotoxic products after injury such as nitric oxide and superoxide free radicals that generate reactive oxygen species and reactive nitrogen species. The initial pro-inflammatory response is to promote the termination of invading pathogens, remove dead cells, and clean the surrounding area. Thereafter, the response is shifted to an anti-inflammatory state, in which debris clearance, extracellular matrix deposition, and new capillary blood vessel growth are promoted (Cherry et al., 2014). TBI can further exacerbate microglial activation and promote long-term tissue damage and subsequent behavioral impairments. In a midline fluid percussion injury model, the diffuse injury leads to the sensitization of microglia and the inflammatory system, which contributed to the depressive-like behavior found in the tail suspension test at 72 hours post TBI dpi (Fenn et al., 2013). Microglia also

remained primed and reactive up to 30 dpi, as a result of the prolonged secondary injury response (Fenn et al., 2013). In several other animal models of brain injury, microglia also remain activated for at least 1 year (Jacobowitz, Cole, McDaniel, Pollard, & Watson, 2012; Jin, Ishii, Bai, Itokazu, & Yamashita, 2012; Nagamoto-Combs, McNeal, Morecraft, & Combs, 2007; D. H. Smith et al., 1997). In humans, long-term inflammation is considered to be a contributing factor to sustained damage following brain injury, with activated microglia and white matter degeneration persisting for years after a single TBI in humans (Johnson et al., 2013), as well as up to 17 years after TBI in the subcortical regions (thalami and putamen) distant from the site of injury (Ramlackhansingh et al., 2011). These studies suggest that the detrimental effect of chronic inflammation following TBI is mediated by microglia in the brain.

Protective Effects of Antioxidants in Brain Injury Induced Deficits

Since each patient with brain injury has a unique and complex pattern of tissue damage, developing pharmacological intervention strategies that target the multiple cellular and molecular events in the neuroinflammatory cascade is difficult. Injury results in increased oxidative stress, cellular dysfunction, and other factors may ultimately result in neuronal death. Despite advances in prevention measures, surgical, and diagnostic techniques, there have been few successful outcomes in human clinical trials of drugs developed to treat TBI. There have been few changes in the way TBI patients are managed, and most drugs and techniques have aimed to reduce brain pressure and swelling. So far, there is no pharmacological treatment currently available for clinical use that specifically targets secondary brain injury mechanisms to improve outcome

(Woodcock & Morganti-Kossmann, 2013). There are several different types of antioxidant/anti-inflammatory items found, including those in drugs, natural compounds (e.g., vitamins), and those that are found in fruits and vegetables (i.e., phytochemicals). Some have been found to be more effective in reducing brain injury induced deficits, and these effects are reviewed in the next sections.

Antioxidant Drugs

There are few pharmacological interventions available for TBI and these include the administration of psychostimulants, antidepressants, antiparkisonian drugs, and anticonvulsants (Talsky et al., 2011). Unfortunately, these are only implemented after the injury has occurred to control adverse symptoms, which may be too late to prevent significant neurodegeneration in the brain. Antioxidant therapies have been directed towards the inhibition of the secondary injury cascade in acute TBI by the pharmacological targeting of a single oxidative damage mechanism. Several experimental models of brain injury in rats have found *N*-Acetylcysteine to attenuate glutamate related excitotoxicity and reduce infarct size (Krzyzanowska, Pomierny, Budziszewska, Filip, & Pera, 2016), decrease apoptosis and neuronal degradation in the tissue surrounding the lesion (Gunther et al., 2015), and decrease oxidative stress and apoptosis in the hippocampus (Naziroglu, Senol, Ghazizadeh, & Yuruker, 2014). Treatment with deferoxamine has also attenuated neuroinflammation (Zhang, Cao, Zhang, Li, & Mi, 2015), cognitive deficits (Zhang et al., 2013; Zhang et al., 2015), and reduced white matter degeneration (Xie et al., 2014) in several experimental animal models. Though several types of anti-oxidant compounds have been tested in animal models, few have

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found success in clinical trials. Polyethylene glycol-conjugated superoxide dismutase (PED-SOD) was found to act as a scavenger of free radicals and this prevented post-traumatic microvascular dysfunction (Muizelaar et al., 1993). However, when PEG-SOD was administered in moderate and severe TBI patients within the first 8 hours of injury, results failed to show consistency or improved survival and neurological outcomes in the phase 3 clinical trial (data not published; discussed in Muizelaar et al., 1993), despite short lived success in the phase 2 clinical trial (Muizelaar et al., 1993). Another drug called Tirilazad (21-aminosteroid lipid peroxidation inhibitor) inhibited free radical lipid damage and stabilized membranes and has been found to lessen post-traumatic blood barrier opening in diffuse concussive head injury models (Hall, Yonkers, Andrus, Cox, & Anderson, 1992). However, when taken in clinical trials within 8 hours of injury, the drug failed to show a beneficial effect after either moderate or severe TBI (Marshall et al., 1998).

Naturally Occurring Antioxidants

Many studies suggest that diets high in antioxidant properties may reduce oxidative damage and attenuate neuronal degradation via multiple pathways. Vitamin E (α-tocophernol and α-tocotrienol) and vitamin C (L-ascorbic acid or L-ascorbate) are naturally occurring antioxidants that have shown to counteract the effects of TBI. Rats pretreated with vitamin E before mild FPI injury had lower levels of oxidative stress that improved synaptic plasticity (communication between neurons) and learning performance when tested in the water maze (Wu, Ying, & Gomez-Pinilla, 2010). Similarly, vitamin E administered before brain injury reduced oxidative stress levels in a mouse model of

Alzheimer's disease (Conte et al., 2004) and guinea pigs (Inci et al., 1998). Despite the results from animal research, no clinical studies have been conducted to test the effect of vitamin E in TBI and other clinical studies with various other forms of brain trauma (e.g. stroke) have generated mixed results. Neither animal nor clinical studies have examined the potential neuroprotective effects of vitamin C on TBI, and clinical studies with other mechanisms of brain trauma (e.g. stroke) did not find a neuroprotective effect.

Phytochemicals

Phytochemicals are chemical compounds that occur naturally in plants and their antioxidant properties may have neuroprotective and anti-degenerative functions by reducing cellular apoptosis and slowing the progression of neuronal cell loss. Studies have shown that phytochemicals reduce oxidative stress by the activation of the Nrf2 transcription factor (regulates expression of antioxidant proteins) and scavenging free radicals. Sulforaphane, an organosulfur compound that is found in cruciferous vegetables (e.g broccoli), decreased edema following TBI in rats (Zhao, Moore, Redell, & Dash, 2007) and up-regulated neuroprotective genes (Nrf2 driven genes), maintained blood brain barrier integrity (Zhao, Moore, Clifton, & Dash, 2005), and improved performance in the water maze (Dash, Zhao, Orsi, Zhang, & Moore, 2009).

The antioxidant compounds in green tea (catechins and epicatechins) protected neurons against mitochondrial dysfunction (Chen, Lin, Liu, & Lin-Shiau, 2008), prevented neuronal damage in the hippocampus (Park et al., 2009), and reduced both glial swelling (Panickar, Polansky, & Anderson, 2009) and oxidative damage (Etus, Altug, Belce, & Ceylan, 2003).

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Polyphenols

Polyphenols are water soluble, organic chemicals found in spices and fruits have antioxidant and anti-inflammatory properties. Supplementing polyphenols into diets may be more effective in the treatment of neuroinflammation, injury related deficits, and slowing progression neurological diseases as compared to pharmaceutical medications. Curcumin (a polyphenol found in the spice turmeric) reduced oxidative protein levels and improved synaptic plasticity and cognition (learning and memory tasks) in rats with mild TBI (Wu, Ying, & Gomez-Pinilla, 2006). Similarly, treatment with curcumin after mild TBI also protected against injury-induced fine motor deficits and hippocampal synaptic dysfunction (Wu, Zhing, Schubert, & Gomez-Pinilla, 2011). Curcumin administration after CHI also reduced acute activation of microglia and other inflammatory processes in mice (Zhu et al., 2014). Similar findings were observed in a model of intracerebral hemorrhage (Yang, Zhao, Zou, Zhang, & Feng, 2014).

Fruit Polyphenols

Fruits contain powerful antioxidant and anti-inflammatory properties that provide neuroprotection against oxidative damage in the brain (Ates et al., 2007). The antioxidant polyphenols found in fruits have been shown to lessen age related deficits in rodents (Joseph et al., 1998; Shukitt-hale, Carey, Jenkins, Rabin, & Joseph, 2007; Shukitt-Hale, Carey, Simon, Mark, & Joseph, 2006; Shukitt-Hale, Cheng, & Joseph, 2009). These have also been shown to attenuate several age-related diseases including Alzheimer's disease (Hartman et al., 2006; Joseph et al., 2003). Therefore, these dietary antioxidant and anti-

inflammatory compounds may protect against TBI-induced inflammation and associated behavioral deficits, and ultimately improve prognosis.

The polyphenols found in fruits have been shown to protect against neuroinflammation (Dulcich & Hartman, 2013; Hartman et al., 2006; Milardi, Stringaro, Colone, Bonincontro, & Risuleo, 2014; Shukitt-Hale et al., 2008; West, Atzeva, & Holtzman, 2007) by increasing the antioxidant and free-radical scavenging properties (Priyadarsini, Khopde, Kumar, & Mohan, 2002; Singleton et al., 2010), though the exact mechanisms of neuroprotective activities remain unclear. Blueberries have been shown to decrease cortical microglial activation (Lau, Bielinski, & Joseph, 2007; Y. Zhu, Bickford, Sanberg, Giunta, & Tan, 2008) and oxidative stress (Goyarzu et al., 2004; Shukitt-Hale et al., 2008). Data from our lab suggests that AD transgenic mice pretreated with a grape-enriched diet significantly reduced lesion volume after mild TBI (preparing submission). Resveratrol (a polyphenol found in grape skin) has also been shown to decrease hippocampal oxidative damage (de Almeida et al., 2008; Singleton, Yan, Fellows-Mayle, & Dixon, 2010) and cortical microglial activation (Gatson et al., 2013). Resveratrol injected in 7-day-old rat pups immediately after blunt head trauma increased exploratory activity to that of controls 14 days after injury, whereas injured pups without treatment had lower activity levels and increased anxiety behaviors (Sönmez, Sönmez, Erbil, Tekmen, & Baykara, 2007). Resveratrol injected in adult rats as 3 post-injury doses protected against contusion volume and hippocampal neuronal loss (Singleton et al., 2010). Supplementation post-TBI may alone dramatically increase individual motor and cognitive prognosis.

Pomegranate Polyphenols

Pomegranate is an important source of bioactive compounds, and different parts of it have been used in traditional medicine for many centuries. The healing properties of pomegranates can be attributed to the fruit's high polyphenol content and have been associated with its antioxidant and anti-inflammatory properties (Dorsey & Greenspan, 2014; Ismail, Sestili, & Akhtar, 2012; Seeram et al., 2005; Vegara et al., 2014). All parts of the fruit contain polyphenols, including the peal, the inner membranes, and the arils; although the peel and membrane are reported to contain the highest concentrations (Gil et al., 2000). Pomegranate juice contains 2 major classes of polyphenolic compounds: hydrolyzable tannins (break down in water) and flavonoids. The hydrolyzable tannins are the most abundant polyphenols in the juice and include ellagitannins, gallotannins, and gallagoyl esters (Gil et al., 2000). The flavonoids consist of anthocyanidins (pigments that color the juice), flavanols, and flavanol glucosides (Gil et al., 2000). The activity of the polyphenolic components of pomegranate juice have been reported to decrease oxidative stress and act as scavengers for free radicals (Priyadarsini et al., 2002). Ellagic acid alone has demonstrated its ability to reduce free radicals, oxidative stress, reactive nitrogen species (Priyadarsini et al., 2002), and protein modification (Dorsey & Greenspan, 2014). Pretreatment of ellagic acid (daily doses 7 days prior to TBI induction) significantly reduced hippocampal mediated memory impairments and inflammatory markers (IL-1β and IL-6 cytokines) in rats (Farbood et al., 2015). Clinical studies have reported pomegranate juice to have anticancer (Pantuck et al., 2006) and cardiovascular effects (Aviram et al., 2004; Aviram & Dornfield, 2001; Rosenblat et al., 2006; Sumner et al., 2005).

Neuroinflammation and behavioral deficits resulting from TBI are similar to those seen in Alzheimer's disease. Anti-inflammatory polyphenols found in pomegranate juice (0.2-1.0% polyphenols; PomWonderful) have been found to attenuate Alzheimer's-like pathology in mice (Hartman et al., 2006). In this study, Hartman et al. used a 1:20 diluted concentration of pomegranate juice and found significant improvements in behavior and neuropathology. This dilution was roughly equivalent to two 8-ounce glasses of juice per day for humans. Mice that consumed the pomegranate-enriched water for 6 months improved in spatial learning tasks (the water maze) and had significantly reduced hippocampal amyloid beta plaques. In another study, maternal supplementation with pomegranate juice influenced mice pup outcome after brain injury (Loren, Seeram, Schulman, & Holtzman, 2005). Mouse pups subjected to neonatal stroke had less cortical tissue loss than controls when exposed in utero to pomegranate juice, regardless of dose. Also, pomegranate juice supplementation (1:20 dilution) for 10 weeks ameliorated irrdiation (2 Gy) induced depression-like behavior and improved motor performance in male mice (Dulcich & Hartman, 2013) and increased neurogenesis in non-irradiated mice (unpublished), demonstrating the neuroprotective properties of pomegranate juice.

Pomegranate juice has higher polyphenolic contents than apple, black cherry, Concord grape, cranberry, and pineapples juices (Dorsey & Greenspan, 2014). Research suggests that the anti-inflammatory and antioxidant properties found in pomegranates can be attributed to the synergistic effect of the multiple polyphenols found in the juice, peel, pulp, and seeds. The whole juice has been found to have more anti-apoptotic properties than any individual polyphenol substrate in pomegranates (Seeram et al., 2005). More recently, pomegranate extract attenuated the release of a pro-inflammatory cytokine, TNF

alpha, from microglial cells (Jung et al., 2006), validating the anti-inflammatory potential of pomegranates. Altogether, research suggests that the polyphenols found in pomegranates could be an effective therapy against inflammation in the brain. No studies to date have looked at the protective effects of pomegranates in concussions and this fruit may be beneficial in ameliorating the negative effects of brain injury.

Aims of the Study

The present study will analyze possible long-term behavioral and neuropathological changes in adult male C57BL/6 mice following a mild concussive brain injury (mCHI) or repeated concussive brain injury (rmCHI) at acute (1, 3, 5, and 7 dpi) and long-term (30 dpi) time points. In addition, the neuroprotective effects of pomegranate juice against injury-induced deficits will be analyzed.

Aim 1

Explore the behavioral effects of mild concussions in adult male mice.

Hypothesis 1: Mice with repeated concussions will demonstrate greater behavioral deficits compared to mice with single anesthesia and repeated anesthesia.

Hypothesis 2: Mice with repeated anesthesia will demonstrate greater behavioral deficits compared to mice with single anesthesia.

Aim 2

Explore microglial activation after mild concussions.

Hypothesis 1: Mice with concussions will have higher levels of activated microglia compared to single anesthesia and repeated anesthesia mice.

Hypothesis 2: Mice with repeated anesthesia will have higher levels of activated microglia compared to single anesthesia mice.

Aim 3

Explore the effects of diet on behavior and neuropathology following mild concussions.

Hypothesis 1: Concussions will lead to deficits on behavioral tests, and pomegranate juice will protect against these deficits.

Hypothesis 2: Concussions will lead to higher levels of activated microglia, and pomegranate juice will protect against activation.

Hypothesis 3: Repeated anesthesia will lead to deficits on behavioral tests compared to single anesthesia mice, and pomegranate juice will protect against these deficits.

Hypothesis 4: Repeated anesthesia will lead to higher levels of activated microglia, and pomegranate juice will protect against activation.

Aim 4

Explore interactions between effects of mild concussions and diet on behavioral and microglial activation, as well as behavioral/biomarker correlations.

Hypothesis 1: Microglial activation will be correlated with deficits in behavioral tests.

CHAPTER TWO

METHODS

Experimental Design

Male C57BL/6 mice (3 months old) were separated into 5 groups
(pomegranate/no-pomegranate, anesthesia/double anesthesia/rmCHI), yielding a total of
6 groups (see Table 1). Twenty-two animals started the pomegranate juice diet
immediately and continued on this until time of sacrifice. The pomegranate juice was
administered in the animal's drinking water at a 1:20 ratio. This concentration of
pomegranate juice reduced Alzheimer's pathology and improved radiation induced
deficits (Dulcich et al., 2013; Hartman et al., 2006). Twenty animals did not receive the
pomegranate preparation, but instead received filtered drinking water as control
treatment. Water treatments continued until time of sacrifice.

Table 1. *Animal group breakdown.*

	Pomegranate Juice	Water
Anesthesia	n = 6	n = 6
Repeated Anesthesia	n = 8	n = 7
Repeated Closed Head Injury	n = 8	n = 7

Animals were allowed to acclimate in to the animal facility for 2 days following delivery. After acclimation, animals started on either the pomegranate or control treatment. After 1 week, animals were subjected to anesthesia, repeated anesthesia (RA), or repeated closed head injury (rmCHI). Thereafter, animals underwent a battery of behavioral tests at 1, 3, 5, 7-11 days post injury (dpi). These tests assessed cognitive, motor, and learning abilities. After behavioral testing at 11 dpi, mice were deeply anesthetized with isoflurane and perfused through the heart with 4% paraformaldehyde until euthanization. Once euthanization was confirmed, brains were removed and placed in paraformaldehyde for 24 hours, then stored in saline until further tissue preparation or were removed and immediately frozen in liquid nitrogen for protein analysis. See Figure 1 for timeline of experimental design.
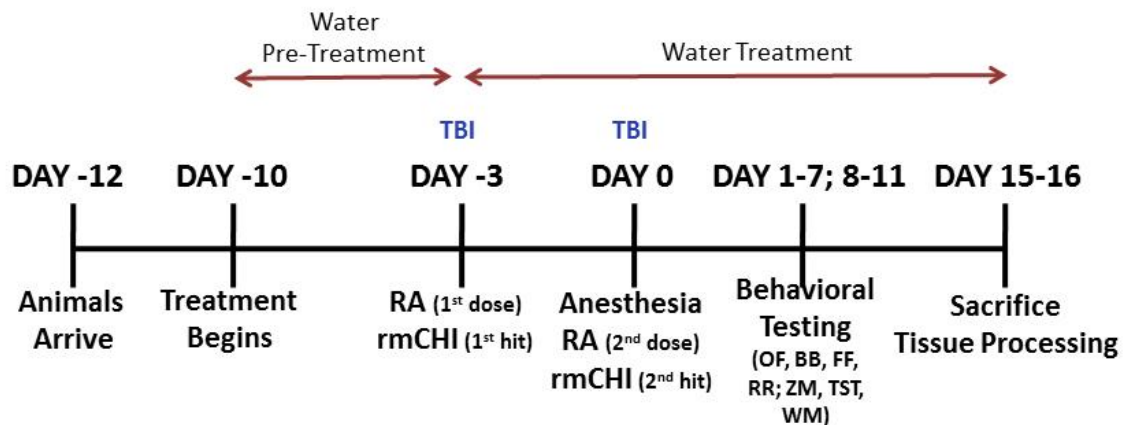
Figure 1. Timeline of the experimental design.

Animals

All protocols and procedures were approved by the Institutional Animal Care and

Use Committee of Loma Linda University and complied with the principles and

procedures of the Guidelines for the Care and Use of Experimental Animals. Adult 3-

month-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed

individually in cages on a 12-hr light-dark cycle at constant temperature and humidity,

and fed *ad libitum.* All experimental animals were randomly assigned to groups that

included anesthesia (n=12), RA (n=15), or rmCHI (1 hit to each hemisphere spaced 3

days apart; n=15). See Table 1 for detailed group numbers.

Pomegranate Preparation

Pomegranate juice (PomWonderful) was diluted 1:20 into filtered water. Full

strength pomegranate juice consists of 84% water, 14% carbohydrates, 0.48% citric acid,

0.1% protein, 0.02% fat, and 1% other, including polyphenols (phenolic acids and

flavonoids). Phenolic acids include 115 ppm ellagic acid and 5 ppm gallic acid.

Flavonoids include 1880 ppm hydrolysable tannins (e.g. gallotannins, ellagitannins,

punicalagin) and 369 ppm anthocyanins, and their glycosides (e.g. cyanidin, delphinidin,

pelargonidin). PomWonderful pomegranate juice was purchased from local grocery

stores and kept refrigerated at 4° C throughout the experiment. The solution was prepared

as needed (as often as the animals' water needed changing), and was administered by

diluting the solution into the animal's drinking water. Animals had *ad libitum* access to

their water bottles. Animals that did not receive the pomegranate juice treatment received

filtered drinking water.

Anesthesia

Mice were anesthetized (isoflurane: 3% induction and maintenance; Webster

Veterinary Supply, Inc., Sterling, MA) for 30 minutes in a chamber with a heating pad

that maintained body temperature at 37°C. Animals were placed in a recovery chamber

before they were returned to their home cages. Repeated anesthesia (RA) mice underwent

similar procedures with an additional anesthesia dose 3 days later.

Repeated Closed Head Brain Injury

Mice were deeply anesthetized with isoflurane (isoflurane: 3% induction, 1-2%

maintenance) and placed on a cushioned foam base (Foam to Size Inc., Ashland, VA).

Anesthesia was monitored throughout the surgery and body temperature was maintained

at 37°C with a heating pad (except during injury). Lidocaine (0.01 mg/kg; dilution

0.01/mL) was injected subcutaneously for post-surgery pain relief from the surgical

procedure. Following a midline incision and retraction of the skin overlying the skull,

injury was induced to the right temporal-parietal cortex of the skull using a 4-mm

rounded plastic tip (Delrin) affixed to an electromechanical impactor (Leica

Microsystems Company, Richmond, IL). The injury was delivered using impact duration

of 200 milliseconds at a velocity of 4 m/sec and resulted in a rotational head

displacement of 2 mm. The center of the injury was located 2.5 mm below Bregma and

2.5 mm to the right of the Sagittal midline at ~9-11° angle, perpendicular to the cortical

surface. The surgical site was sutured after recording of any bleeding or fractures (n = 5).

Intraperitoneal buprenorphine (0.01 mg/kg; dilution 0.01/mL) injections were

administered to mice for post-surgery pain relief before the animals are returned to their

home cages. Mice underwent similar surgical procedures with an additional injury over the left temporal-parietal cortex 3 days after the initial impact. The center of the injury was located 2.5 mm below Bregma and 2.5 mm to the left of the sagittal midline.

Behavioral Testing

Behavior was tested at 1, 3, 5, 7-11 dpi. All behavioral tests at each time point were carried out within the 12-hour light cycle and groups were interleaved in testing sequence. Experimenters blinded to the treatment groups completed testing. Tests administered on 1, 3, 5, and 7 dpi were completed in the following order: open field, foot fault, beam balance, and rotarod. Behavioral tests at 8-11 dpi were completed in the following order: elevated zero maze, tail suspension, and water maze. The orders of tests were determined based on the degree of difficulty, with easy tests administered first and more rigorous, challenging tests administered last.

Open Field Activity

Open-field testing assessed general exploratory behavior and activity levels. Mice were placed in a 49 cm x 36 cm opaque open-topped plastic bins and allowed to explore, unrestricted, for the duration of 30 minutes. Movements of each animal was recorded by an overhead camera and analyzed by a computerized tracking system (Noldus Ethovision; Information Technology, Inc., Leesburg, VA). Various parameters were used as a measure of overall activity level including total distance traveled, percentage of time spent moving, percentage of time in the center, and percentage of time in the periphery.

Beam Balance

Fine motor coordination and balance were assessed using the beam balance test. A square acrylic glass beam balance (61 cm length, 0.65 cm width) labeled in 2.5-cm increments was used. Mice were placed at the midpoint of the beam and were allowed to walk unrestricted in either direction for 60 seconds. The number of falls, total time spent on the beam, distance traveled, left and right turns, and the numbers of left or right paw slips were recorded. Each animal was given 2 trials, approximately 30 min apart.

Rotarod

The accelerating rotarod is a test of sensorimotor coordination and balance that consists of a 3-cm diameter rotating horizontal cylinder (Rotamex-5; Columbus Instruments, Columbus, OH). Mice were placed on the cylinder and continuously walked forward to avoid falling. Latency to fall was recorded. Mice were tested with three blocks of two consecutive trials per day. Blocks consisted of two stationary trials (at 5 RPM steady), two trials that started at 5 RPM and accelerated 3 RPM every 5 seconds, and two trials that started at 5 RPM and accelerated 3 RPM every 3 seconds. Each trial lasted up to 60 seconds with approximately 30 minutes between each block. Performance over days of testing was used as a measure of motor learning.

Elevated Zero Maze

The elevated zero maze was used to assess exploratory behaviors in an anxiety-provoking environment. The maze consisted of a plastic ring, with a 100 cm outer diameter, 10 cm wide. The two opposing quadrants were enclosed with 35 cm walls. The

room was dimmed, and halogen lights directly illuminated the open spaces of the maze. Animals were placed in the center of one of the open spaces and allowed to freely explore the zero maze for 5 minutes. The percentage of time spent in the enclosed quadrants was calculated. Spending time in the enclosed spaces is generally associated with anxiety-like behavior.

Tail Suspension Test

The tail suspension test was administered to assess depression-like behaviors. Mice were suspended by the tail with adhesive tape that was attached approximately 1.5 cm from the tip of the tail. The other end of the tape was wrapped around a hook that was embedded in the center of the ceiling of a wooden box (19 cm x 21 cm x 40 cm). Once suspended, the animal's rostral end was approximately 20 cm from the floor of the box. The box was enclosed on all sides except for the viewing side, and lighting and sound in the room was kept at a minimum. Each animal received one 6-min trial and was rated on mobility and agitation for the duration of 6 minutes. The time that the animal remained immobile during the final 4 minutes of the trial was recorded. Immobility was defined as the complete lack of movement by the mouse, even if it was swinging back and forth from a previous struggle or if it is curled up while holding its paws (as long as it was not struggling or moving otherwise).

Water Maze

Learning and memory was assessed with the water maze. This test of spatial navigation required an animal to learn the location of a hidden platform in a pool of water

using the visual cues from around the room. The water maze consisted of a metal pool

(110 cm diameter) filled with water that was colored opaque with white tempura paint.

The pool contained a moveable platform (11 cm diameter) that the animal could step onto

to escape the water. Animals were given a total of 10 trials per day for 5 consecutive

days. For each trial, animals were released into the pool, with their nose against the wall,

at one of the four release points and were allowed to swim to the platform. The trials

lasted a maximum of 60 seconds. If mice do not find the platform in the allotted time,

they were manually guided to the platform. An overhead camera recorded the swim

paths, which gathered data for the quantification of distance, latency, proximity to target,

and swim speed by a computerized tracking system (Noldus Ethovision). Cued learning,

which is a control task for assessing sensorimotor and/or motivational deficits that may

affect performance during the spatial phase, was assessed on day 1 of the water maze

protocol. The surface of the escape platform was visible (5 mm above the surface of the

water) and a pole was placed on top of the platform to make its location more obvious.

The location of the platform varied from trial to trial. Animals were released into the pool

opposite the location of the platform and will be allowed to remain on the platform for 5

seconds after finding it. As performance improves, escape latency and swim path length

generally decrease.

Spatial learning was assessed on days 2 and 3 of the water maze protocol. In this

phase of testing, the mice had to find the platform based on its relationship to the spatial

cues around the room, rather than direct visualization. The escape platform was

submerged 1 cm below the surface of the opaque water, and the location of the platform

was changed each day. After finding the platform, animals were allowed to remain on

there for an additional 5 seconds. A probe trial was administered on day 3. In the probe trial, the platform was removed from the pool, allowing the animal to search for the platform for 60 seconds. The amount of time the animal spent in the probe quadrant was measured as well as the total number of times the animal crossed over the former location of the platform. After an hour, the platform was placed back into the pool at a new location, and the next sets of 10 trials were administered.

Animal Euthanasia and Tissue Preparation

Mice were prepped for perfusions to have their brains used for immunohistochemistry or western blot analysis after behavioral testing was completed (11 dpi). Mice allotted to immunohistochemistry (n = 26) were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde in phosphate buffered saline (PBS). After euthanization, brains were extracted from the skull immediately and immersed in 4% paraformaldehyde in 0.1 M PBS solution at 4º C for 24 hours. Remaining mice (n = 16), were allotted to western blot analysis. These mice were deeply anesthetized with isoflurane and perfused through the heart with PBS. Brains were extracted and cut into 5 sections (left anterior cortex, right anterior cortex, left posterior cortex, right posterior cortex, and cerebellum) over ice, frozen with liquid nitrogen, and stored at -80º C for further processing. Additional mice (n = 8) were gifted from Dr. André Obenaus to be used towards western blot analysis. These mice received dietary treatment and injuries similar to other mice from this study, but did not undergo behavioral testing. Mouse brains were processed as mentioned above.

Western Blot

Western blotting is a technique used in cell and molecular biology to detect specific proteins in a sample of tissue homogenate. Gel electrophoresis is used to separate proteins by structure or length of the peptide. Proteins are then transferred to a membrane and stained with antibodies specific to the target protein and visualized.

Protein Extraction

Brain were removed from -80º C, weighed, cut into 4-5 sections and placed in a glass test tube that was kept over ice. 500 µl of lysis buffer (10 mM Tris, pH 7.4, 100 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate) was added to each glass tube. Tissue was homogenized (pulverized; TissueRuptor, Hilden, Germany) and contents were transferred to Eppendorf tubes (Fisherbrand^{TM}, Waltham, MA) kept over ice. Cells were further lysed with 27 gauge needles (BD PrecisionGlide^{TM}, Franklin Lakes, NJ) and tubes were placed on a rotator at 4º C for 2 hours to further agitate cells. Tubes were placed in a centrifuge at 4º C and spun at 12,000 rpm for 20 minutes. The supernatant (solution with cytosolic contents) was removed and divided between aliquots and stored at -80º C. 250 µl of lysis buffer was added to each tube, mixed, and the contents were lysed with 27 gauge needles. Tubes were placed in a centrifuge at 4º C and spun at 16,000 rpm for 30 minutes. The supernatant was removed and divided between aliquots and stored at -80º C.

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Determination of Protein Concentration

To ensure that samples are in proper range of detection for the assay and can be compared equally, the concentration of total protein in each sample must be determined. A protein assay kit (Pierce^TM BCA protein assay kit; ThermoFisher Scientific; Waltham, MA) was used to determine the total protein concentration for each sample. Samples were removed from -80º C and placed on ice to thaw. Standard protein solutions (diluted bovine serum albumin in PBS) were made to determine the linear range of the protein assay and 10 μl of each solution was added to one column of the 96-well plate. Since the lysis buffer reacted with the BCA assay, diluted (1:2 or 1:5) lysis buffer was added to the wells (3 wells per dilution) to correct protein absorbance values. Samples (supernatant) were diluted (1:2 or 1:5) in PBS and 10 μl was added into the wells using a pipette. To correct for pipette variation and error, each sample was added a duplicate, meaning 2 wells with 10 μl in each. Two hundred μl of BCA solution was added to each well using a pipette. The plate was placed on a warmer at 37º C for 15 minutes to accelerate the chemical process (purple color) and placed into a cell absorption reader. The color change that resulted was proportional to the amount of protein in the sample. The absorbance of the lysate solution was set to 562 nm. Protein concentration is determined by comparison of the target samples to a known standard. Values were extracted and the standard curve was determined. The absorbance values for each sample and lysis buffer were averaged. Absorbance values were determined and plotted on the equation generated from the standard curve to yield the protein concentration of each sample.

Protein Determination

The western blot gel package was removed from 4º C and opened over the sink. The tape and comb were removed and rinsed with dH^2O. The western blot chamber was assembled and filled to the top with running buffer (20X MOPS + ddH^2O; used to separate medium to large size proteins). The outer chambers were filled halfway with running buffer. The samples of interest were removed from -80º C and placed on ice to thaw. Based on the protein concentrations of each sample, the volumes of each sample and PBS were calculated and added to an Eppendorf tube. Seven µl of a master mix (reducing agent and loading buffer) was added to the tubes to help denature and unfold proteins. This was done to reveal the epitope site for antibodies to bind to. Total volume of solution to load into the gel was 27 µl per sample. The samples were heated for 15 minutes at 65º C and immediately placed on ice for 1 minute. A 10 µl ladder was added to the first lane. The ladder, also known as molecular weight markers, was a premade mixture of proteins with known molecular weights that span a range from 10 kDa to 200 kDa. Ladders are used to determine the progress while the gel is running, checking transfer efficiency, and for orienting the blot. Twenty-seven µl of each sample was loaded into a specific lane. The chamber ran on a power supply at 200 volts (400mA current threshold) for 57 minutes.

Fifteen minutes before the gel was finished running, sponges were pre-soaked in transfer buffer (methanol and transfer buffer). The PVDF membrane was soaked in methanol alone for 2-3 minutes. The gel was removed from the case and floated into a trap with transfer buffer. The transfer cassette was layered as follows: 2 sponges, filter paper, gel, PVDF membrane, filter paper, and 3 sponges. Air bubbles were removed by

rolling over with a pipette tip. The cassette was fitted and locked into the chamber and transfer buffer was added to the inner chamber. The outer chambers were filled with dH^20 and placed on ice. The transfer occurred for 1 hour 45 minutes at 30 V (400mA current threshold). The membrane was removed and placed in blocking for 1 hour in TBST-M (tris-buffered saline, tween 20, and non-fat milk powder) to reduce non-specific binding of the antibody. Primary antibodies (Iba1, Wako, Richmond, VA, dilution 1:1000; GFAP, EMD Millipore, Billerica, MA, dilution 1:1000) were added to the primary buffer (TBST-M and TBST) and incubated overnight at 4° C on a rotator.

The next day, the membrane underwent 3- 10-minute washes in TBST and blocked with TBST-M for 10 minutes. The secondary antibodies were added (dilution 1:5000) and incubated for 2 hours at room temperature. The membrane underwent 3- 10-minute washes in TBST and imaged.

There have been several western blot experiments performed and analysis is pending.

Statistical Analysis

All statistics were computed using IBM SPSS 21. After data screening and preliminary analyses, it was determined that data would be appropriately analyzed as two separate comparisons, not all three groups compared to one another, as double anesthesia is the comparative sham for rmCHI. Thus, data was analyzed as injury (repeated anesthesia, rmCHI) vs. treatment (control, pomegranate) and injury (anesthesia, repeated anesthesia) vs. treatment (control, pomegranate).

One-way ANOVA's (Injury: Double Anesthesia/rmCHI; Anesthesia/Double Anesthesia) and univariate ANOVA's were conducted for all behavioral tests with 2 between subjects groups with two levels each (Injury: Repeated Anesthesia/rmCHI; Anesthesia/Repeated Anesthesia, Treatment: Control/Pomegranate). Repeated measures (Injury: Repeated Anesthesia/rmCHI; Anesthesia/Repeated Anesthesia vs. Test Day) were conducted on OF, BB, FF, RR, and WM data. Swim data from the water maze will be analyzed by averaging trials into 5 blocks of 2 trials each. Probe swim data will be analyzed by averaging all trials. To control to sphericity and compound symmetry due to repeated measures in the test-day (trials), the Greenhouse-Geisser correction to the degrees of freedom was used to determine the p-value. In cases where normality was violated, a square root transformation was conducted and run on the univariate ANOVA. Correlations between behavioral and biological variables were determined using the Pearson product-moment coefficient. An α-level of 0.05 will be used for all statistical significance tests.

CHAPTER THREE

RESULTS

Repeated Anesthesia (RA) vs. rmCHI

There were no weight or treatment differences between RA ($M = 26.32$, $SD = 2.69$; $M = 275.67$, $SD = 75.78$) and rmCHI ($M = 27.58$, $SD = 2.03$; $M = 314.20$, $SD = 97.39$) mice, respectively.

General Activity: Open Field

Generalized locomotor activity was recorded at 3, 5, and 7 dpi. There were no differences in activity found between RA and rmCHI mice on days of testing, (Figure 2 and 3). Water treatment did not influence activity levels in RA or rmCHI mice throughout days of testing (Figure 4 and 5).
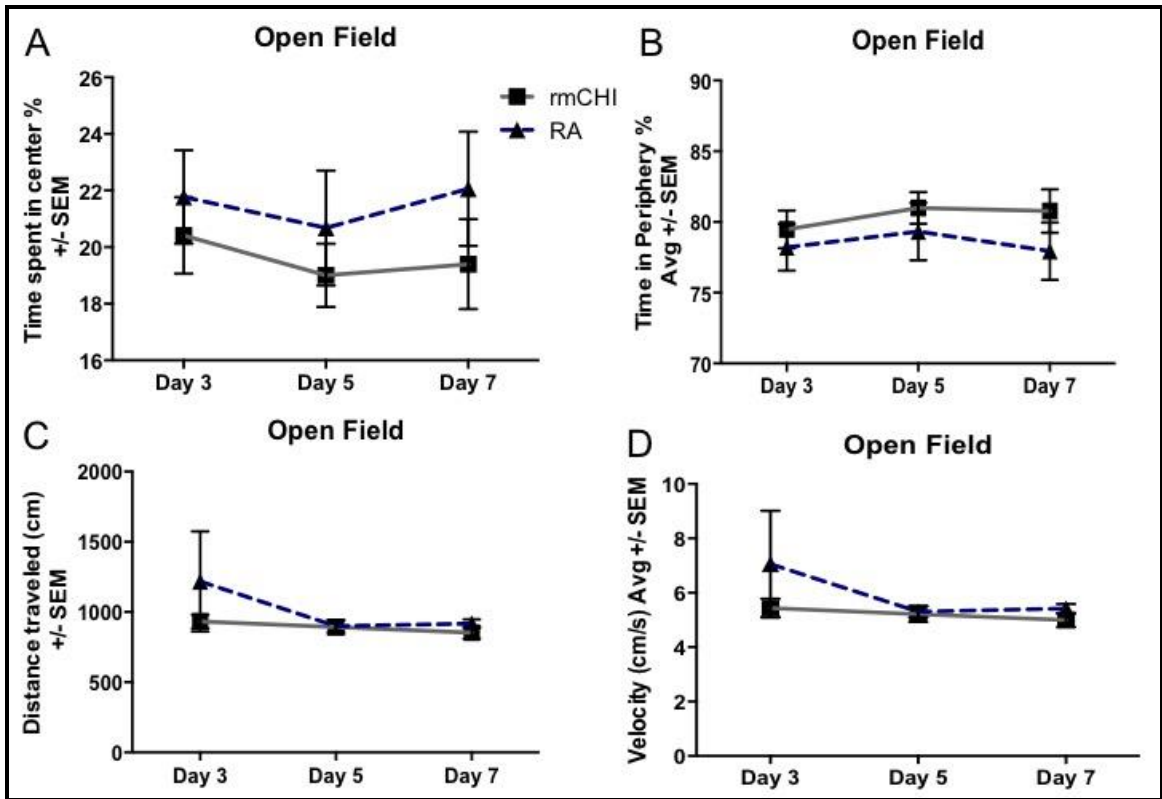
Figure 2. (A, B, C, D) There were no significant differences found between groups across days of open field testing.
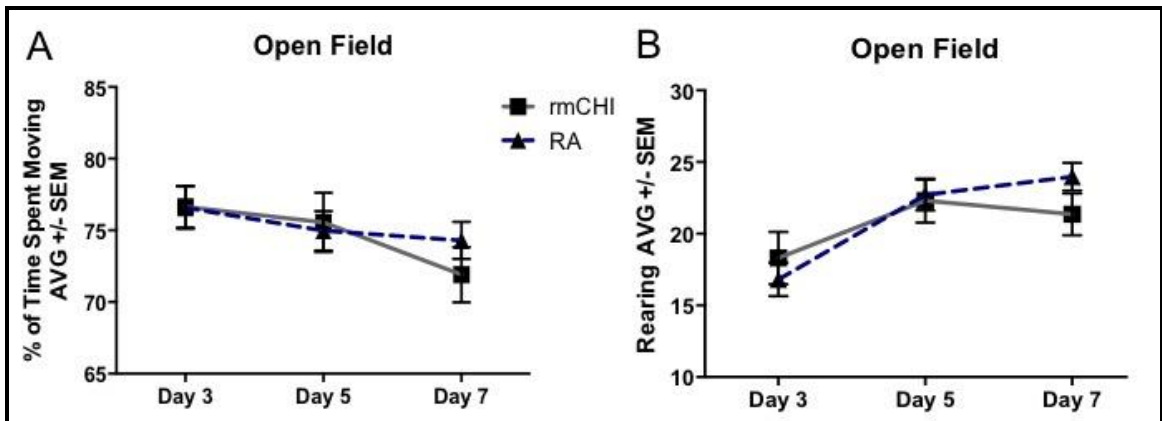
Figure 3. (A & B) There were no significant differences found between groups across days of open field testing.
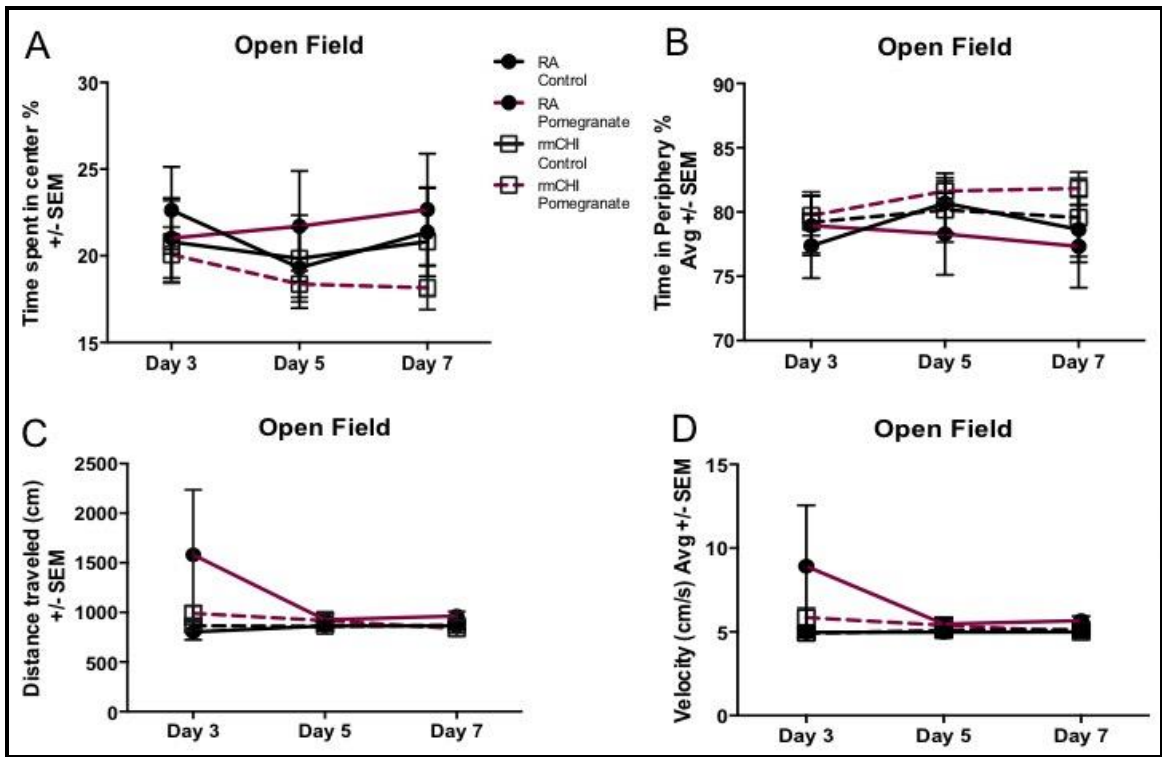
Figure 4. (A, B, C, D) Pomegranate treatment did not affect exploratory activity levels across days of testing in the open field test ($p > .05$).
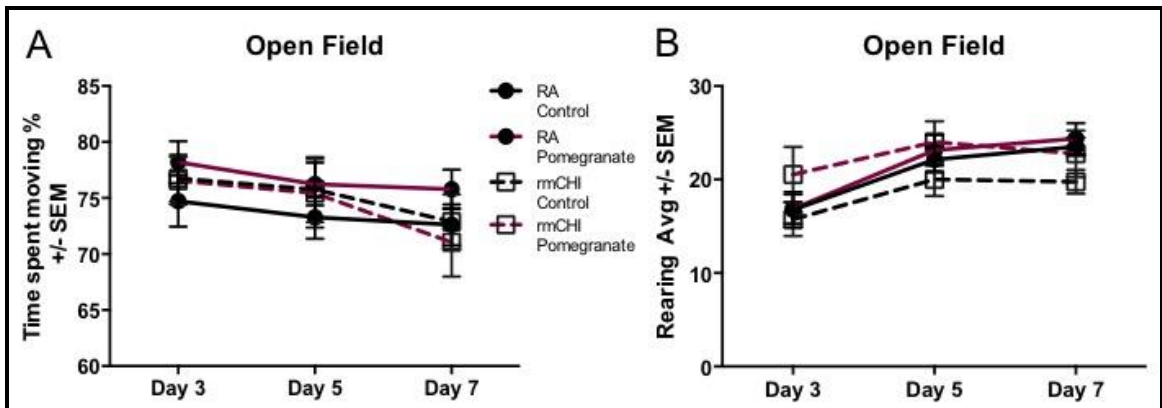
*Figure 5*. (A & B) Pomegranate treatment did not affect exploratory activity levels across days of testing in the open field test ($p > .05$).

Motor Skill: Foot Fault

The foot fault test was administered 1, 3, 5, and 7 dpi in which the number of faults and distance traveled were recorded. A repeated measures ANOVA was conducted to analyze faults across days. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 29.33$, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .62$). Mice showed evidence of faulting less across days, ($F(1.87, 48.56) = 71.55$, p $= .00$, partial $\eta^2 = .73$, power $= 1.00$; Figure 6A). There was a main effect of injury ($F(1, 26) = 12.18$, p $= .00$, partial $\eta^2 = .32$, power $= .92$; Figure 6A). Further analysis revealed that rmCHI mice displayed motor deficits by faulting significantly more than RA mice on 1 dpi ($F(1,28) = 6.57$, $p = .02$) and 3 dpi (Welch's $F(1,24.36) = 6.57$, $p = .02$, $\omega^2 = .16$; Figure 6A).

A repeated measures ANOVA was conducted to analyze distance across days of testing. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 27.93$, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .71$). Mice showed evidence of faulting less across days, ($F(2.11, 54.96) = 8.36$, p $= .00$, partial $\eta^2 = .24$, power $= .96$; Figure 6B). There was a main effect of injury ($F(1, 26) = 5.79$, p $= .02$, partial $\eta^2 = .18$, power $= .64$; Figure 6B). Further analysis revealed that rmCHI mice traveled a greater distance than RA mice at 3 dpi ($F(1,28) = 5.95$, $p = .02$).
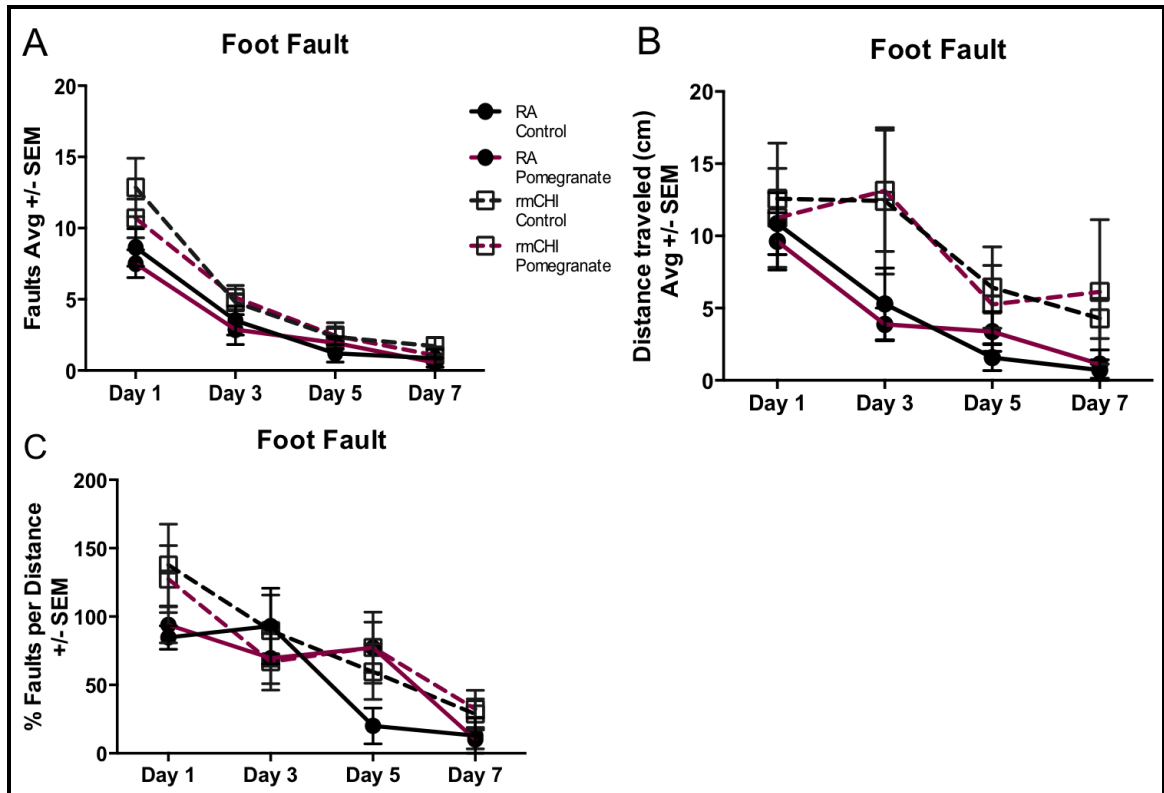
A repeated measures ANOVA was conducted to analyze the percentage of faults per distance across days of testing. Mice showed evidence of faulting less across days, ($F(3, 78) = 15.41$, p $= .00$, partial $\eta^2 = .37$, power $= 1.00$; Figure 6C). There was trend
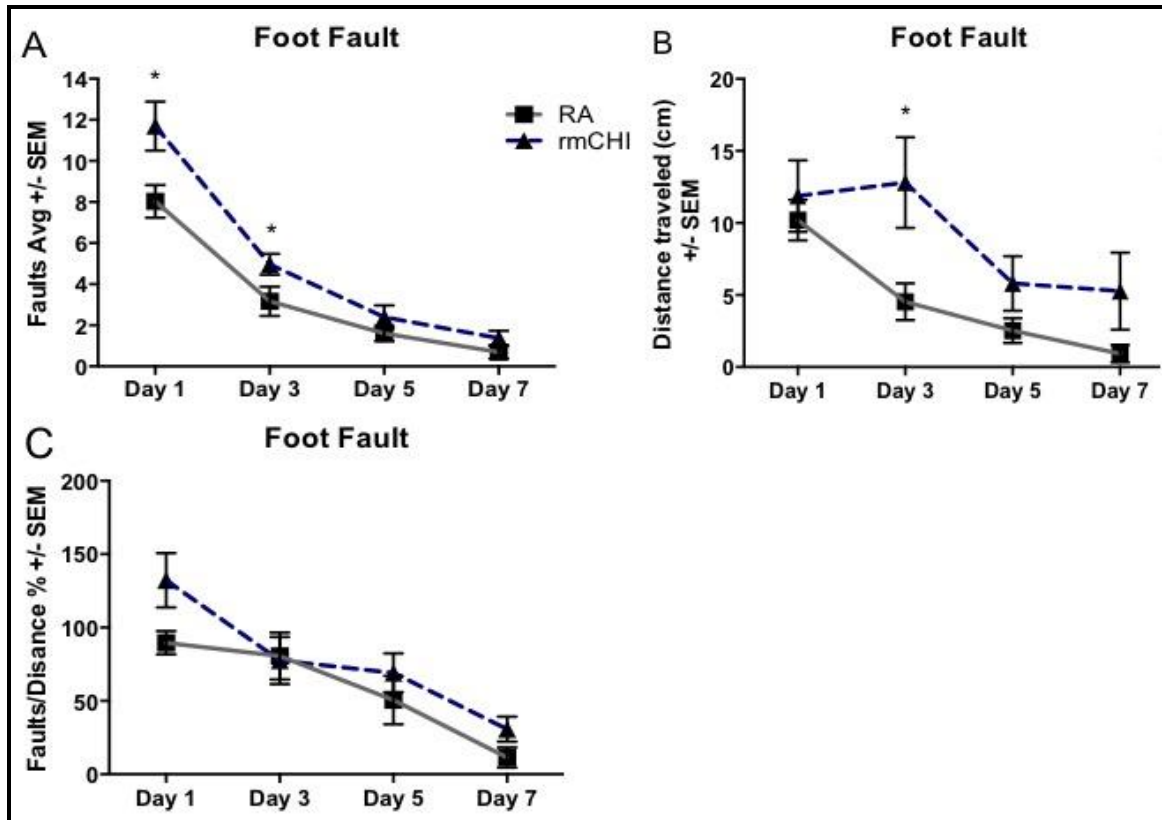
Figure 6. (A) Mice with repeated concussions faulted significantly more on 1 and 3 dpi compared to repeated anesthesia mice (*$p < .05$). (B) Mice with repeated concussions traveled significantly more on 3 dpi compared to repeated anesthesia mice (*$p < .05$). (C) All mice reduced the percentage of faults per distance across days ($p < .01$).

towards a main effect of injury ($p = .06$); however these results did not reach statistical

significance (Figure 6C).

Pomegranate treatment did not reduce the number of faults ($p > .05$; Figure 7A),

distance ($p > .05$; Figure 7B), or the percentage of faults per distance across days of

testing ($p > .05$; Figure 7C).

Figure 7. (A, B, C) Pomegranate treatment did not affect performance in the foot fault task ($p > .05$).

Motor Skill: Beam Balance

Motor skills and coordination were assessed with the beam balance at 1, 3, 5, and 7 dpi. Repeated measures ANOVA was conducted to analyze whether there were differences in left sided slips across days. Mauchly's test indicated that the assumption of sphericity had not been violated $\chi^2(5) = 6.60$, $p = .25$, therefore sphericity was assumed. The number of left sided slips decreased across days of testing, ($F(3, 78) = 4.04$, p = .01, partial $\eta^2 = .13$, power = .82; Figure 8A).

Another repeated measure ANOVA was conducted to analyze whether there were differences in right-sided slips across days. Mauchly's test indicated that the assumption

of sphericity had not been violated $\chi^2(5) = 10.31$, $p = .07$, therefore sphericity was assumed. The number of right-sided slips decreased across days of testing, ($F(3, 78) = 6.88$, p = .00, partial $\eta^2 = .21$, power = .97; Figure 8B).

Repeated measure ANOVA was conducted to analyze whether there were differences in distance traveled across days. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 13.93$, $p = .02$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .76$). The distance mice traveled decreased across days of testing, ($F(2.27, 58.95) = 3.32$, p = .04, partial $\eta^2 = .11$, power = .64; Figure 8C).

Mice did not differ in the number of times they fell off the beam across days of testing ($p > .05$; Figure 8D).
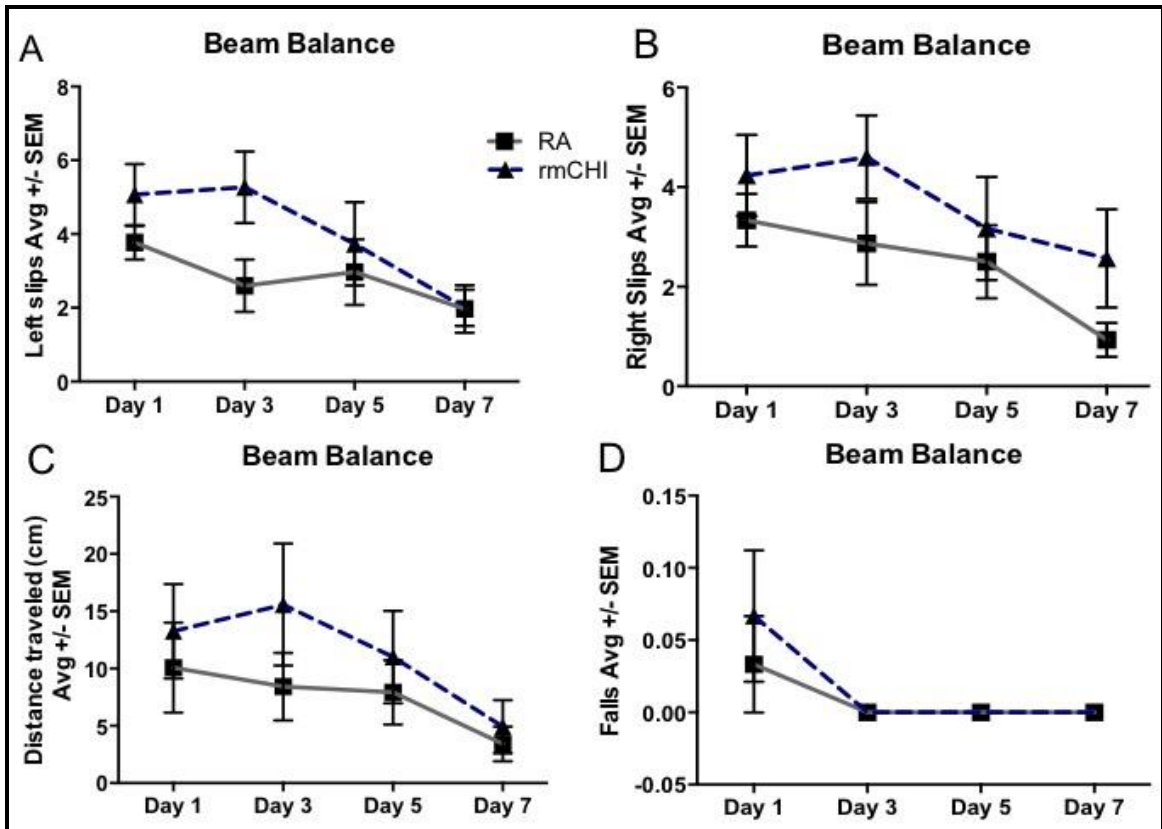
Figure 8. (A, B, C) Mice decreased the number of left-sided slips, right-sided slips, and distance traveled across days of testing on the beam balance, regardless of injury ($p <$ .05). (D) Mice did not differ in the number of times they fell off the beam across days of testing ($p >$ .05).

Another repeated measure ANOVA was conducted to analyze whether there were differences in left turns across days. Mauchly's test indicated that the assumption of sphericity had not been violated $\chi^2(5) = 7.12$, $p = .21$, therefore sphericity was assumed. The number of left turns decreased across days of testing, ($F(3, 78) = 5.19$, p = .00, partial $\eta^2 = .17$, power = .91; Figure 9A).

Another repeated measure ANOVA was conducted to analyze whether there were differences in right turns across days. Mauchly's test indicated that the assumption of sphericity had not been violated $\chi^2(5) = 9.08$, $p = .11$, therefore sphericity was assumed.

The number of right turns decreased across days of testing, ($F(3, 78) = 2.93$, p = .04, partial $\eta^2 = .10$, power = .68; Figure 9B).
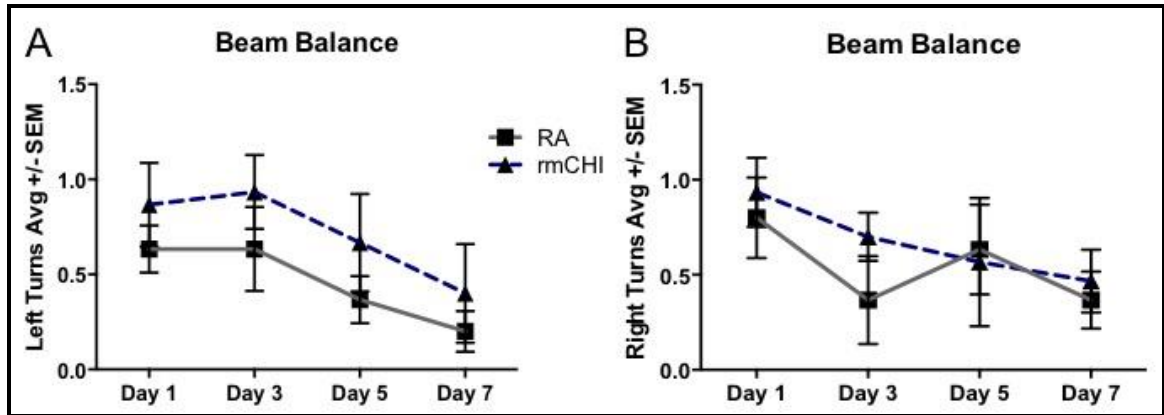
*Figure 9.* (A & B) Mice decreased the number of left turns and right turns across days of testing on the beam balance, regardless of injury ($p < .05$).

Pomegranate treatment influenced performance in the beam balance. There was a significant interaction between injury and treatment across days of left-sided slips, ($F(3,78) = 3.41$, $p = .02$, partial $\eta^2 = .12$, power = .75;Figure 10A). Further analysis revealed a main effect of injury at 3 dpi ($F(1,26) = 5.81$, $p = .02$, partial $\eta^2 = .18$, power = .64; Figure 10A) and a significant interaction between treatment and injury at 5 dpi, ($F(1,26) = 5.95$, $p = .02$, partial $\eta^2 = .19$, power = .65; Figure 10E). rmCHI mice on the pomegranate treatment made fewer left-sided slips compared to those on the control diet, whereas RA mice on the control diet made fewer left-sided slips compared to those on the pomegranate treatment (Figure 10E). The pomegranate treatment seemed to improve performance in rmCHI mice.

Pomegranate treatment did not influence right-sided slips ($p > .05$; Figure 10B). There was a significant interaction between injury and treatment across days of testing, ($F_{(2.27, 58.95)} = 3.52$, p = .03, partial $\eta^2 = .12$, power = .67; Figure 10C). Further analysis revealed that pomegranate treatment reduced deficits in rmCHI mice and induced deficits in RA mice at 5 dpi, ($F_{(1,26)} = 3.13$, $p = .09$, partial $\eta^2 = .11$, power = .40; Figure 10F). Pomegranate treatment did not influence falls on the beam balance ($p > .05$; Figure 10D).
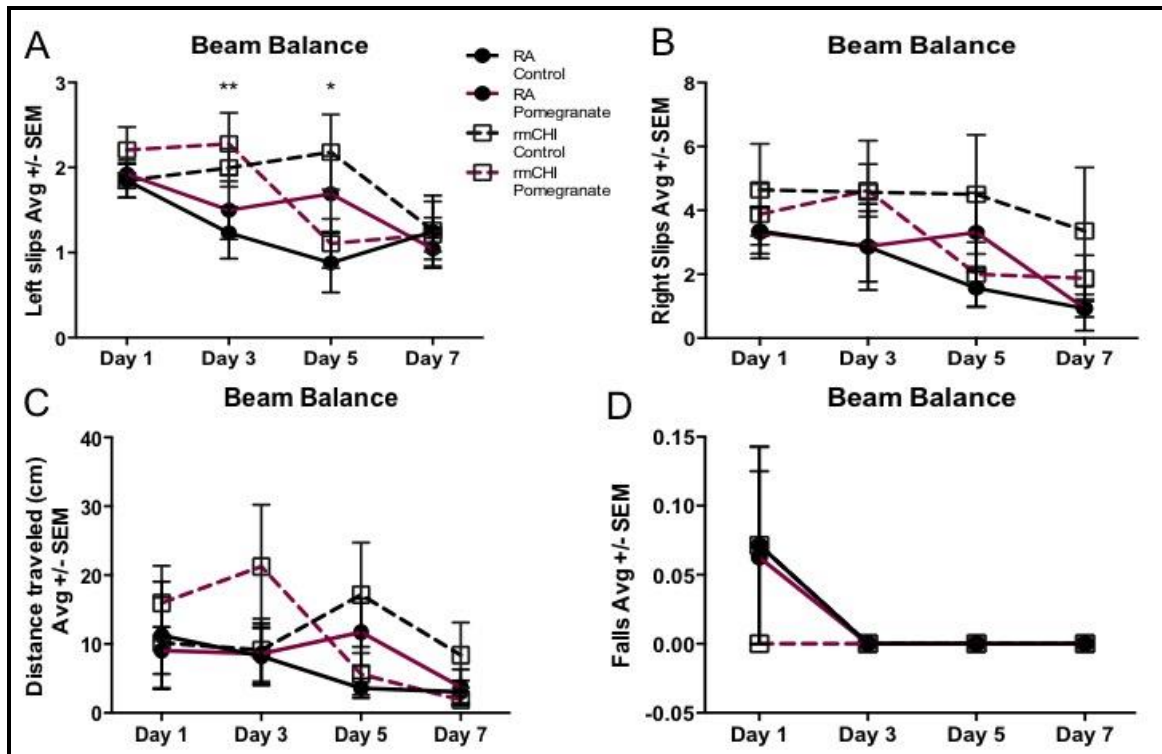
Figure 10. (A) Injured mice made more left-sided slips compared to controls at 3 dpi, regardless of diet (**$p < .05$). There was a significant interaction at 5 dpi (*$p < .05$). (B) Pomegranate treatment did not influence right-sided slips across days of testing on the beam balance ($p > .05$). (C) Pomegranate treatment may negatively impact beam balance performance in RA mice at 5 dpi, but may improve performance in rmCHI mice. (D) Pomegranate treatment did not influence the number of falls across days of testing ($p > .05$).
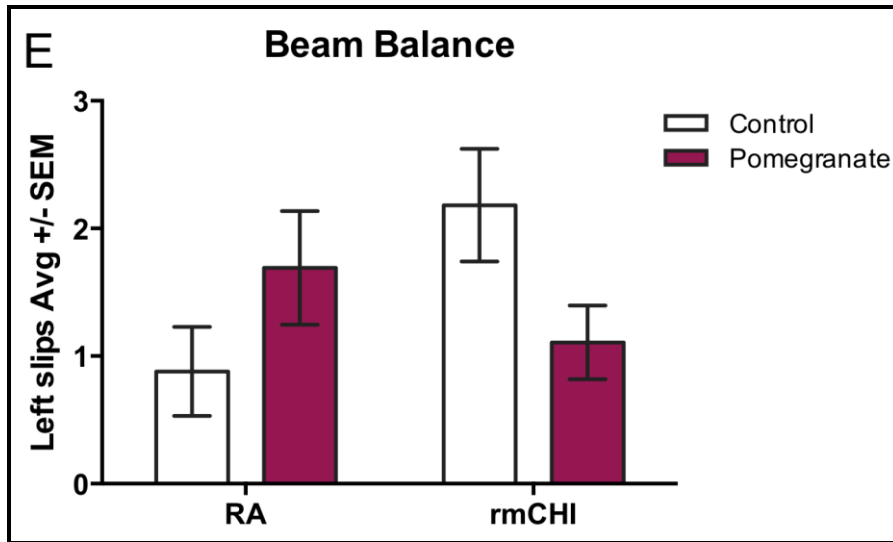
Figure 10E. Pomegranate treatment significantly influenced performance on the beam balance in rmCHI mice at 5 dpi. Injured mice on the pomegranate treatment made fewer left-sided slips compared to those on the control diet, whereas RA mice on the control diet made fewer left-sided slips compared to those on the pomegranate treatment ($p < .05$).
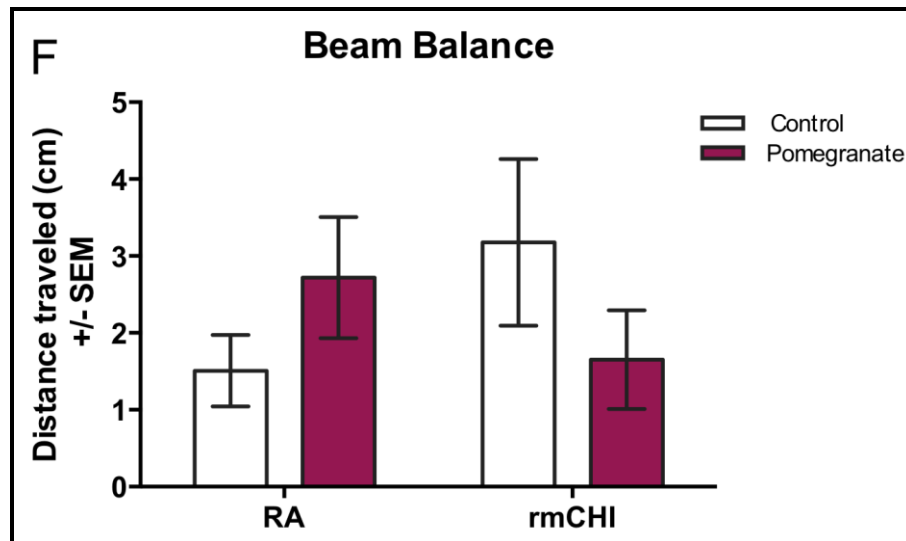
Figure 10F. Pomegranate treatment may negatively impact beam balance performance in RA mice at 5 dpi, but may improve performance in rmCHI mice.

Pomegranate treatment did not influence the number of left turns or right turns made across days of testing ($p > .05$; Figure 11 A and B, respectively).
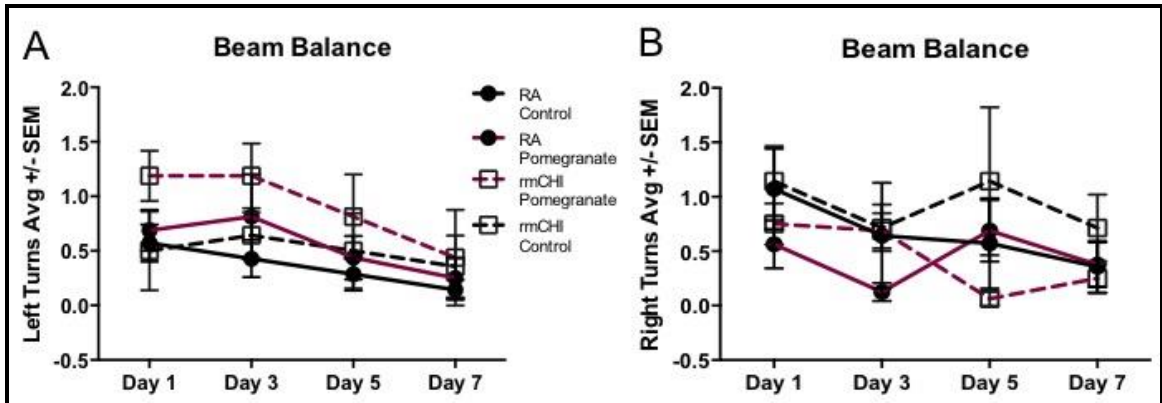
Figure 11. (A & B) Pomegranate treatment did not influence the number of left or right turns mice made across days of testing ($p > .05$).

Motor Skill: Rotarod

Rotarod performance was assessed at 1, 3, 5, and 7 dpi. Repeated measures ANOVA was conducted to analyze whether there were differences in performance during the steady speed (5 rpm) across days. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 12.23$, $p = .03$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .80$). Performance on the rotarod improved across days of testing, regardless of injury; ($F(2.40, 67.31) = 33.93$, p = .00, partial $\eta^2 = .55$, power = 1.00; Figure 12A). There were no main effects or significant interactions. Another repeated measures ANOVA was conducted to analyze whether there were differences in performance testing during the more challenging speed

where 3 rpm would increase every 5 seconds (accelerating) across days. Mauchly's test

indicated that the assumption of sphericity had not been violated $\chi^2(5) = 6.95$, $p = .23$.

Performance on the rotarod improved across days of testing, regardless of injury; ($F(3,

84) = 9.08$, p $= .00$, partial $\eta^2 = .25$, power $= 1.00$; Figure 12B). There were no main

effects or significant interactions. Another repeated measures ANOVA was conducted to

analyze whether there were differences in performance testing during the most

challenging speed where 5 rpm would increase every 3 seconds (fast) across days.

Mauchly's test indicated that the assumption of sphericity had not been violated $\chi^2(5) =

1.91$, $p = .86$. Performance on the rotarod improved across days of testing, regardless of

injury; ($F(3, 84) = 12.68$, p $= .01$, partial $\eta^2 = .13$, power $= .85$; Figure 12C). There were

no main effects or significant interactions.
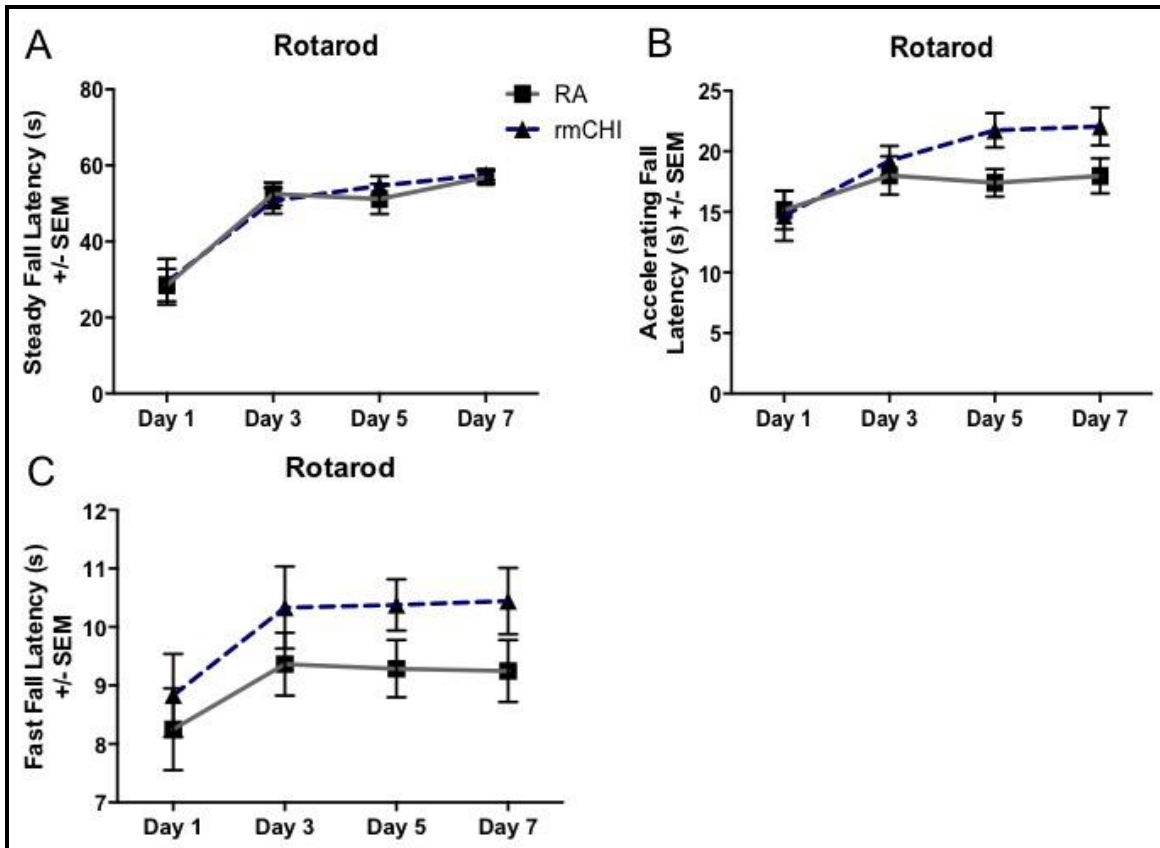
Figure 12. (A, B, C) Mice improved their performance on the rotarod test across days of testing, regardless of injury ($p < .05$).

Pomegranate treatment did not influence performance across days of testing

(Figure 13).
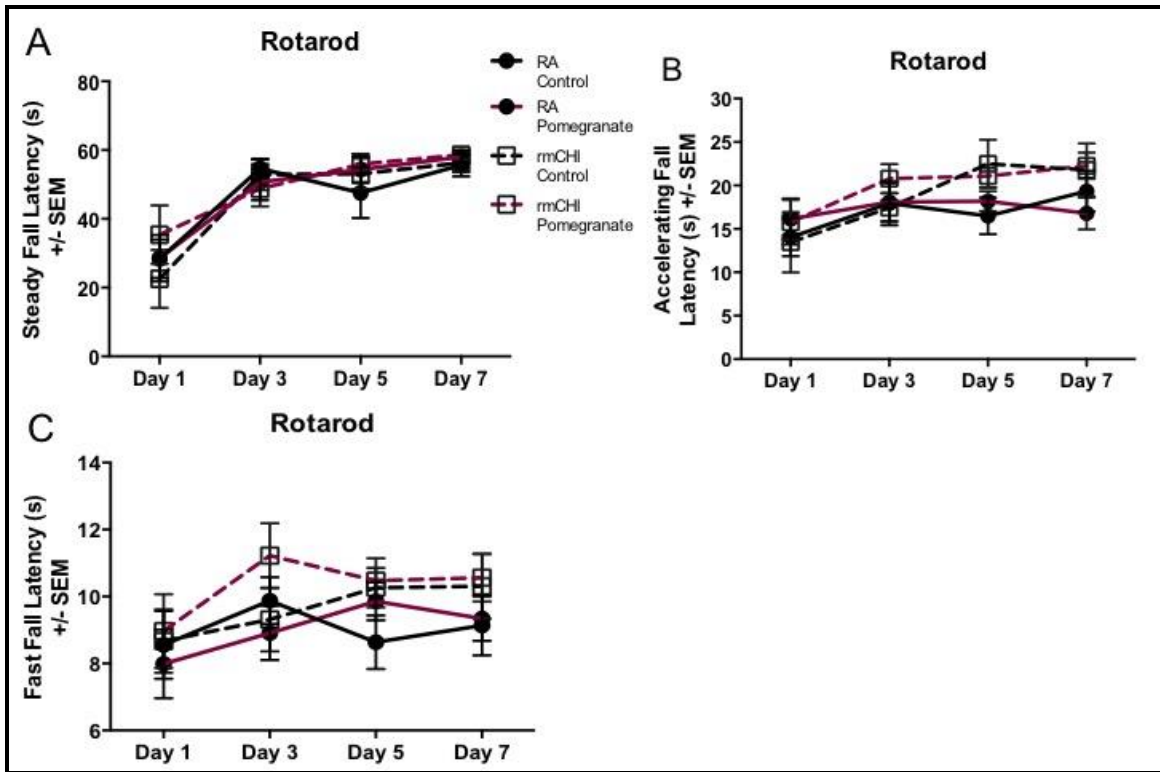
Figure 13. (A, B, C) Pomegranate treatment did not improve motor performance in the steady, accelerating, or fast trials of the rotarod, ($p > .05$)

Anxiety-like Behavior

Anxiety-like behavior was assessed with the elevated zero maze. RA mice and rmCHI mice did not differ in the percentage of time spent in the darker quadrants (anxiety behavior; ($p > .05$; Figure 14A). RA mice also did not differ in the percentage of time spent in the darker quadrants ($p > .05$; Figure 14B).
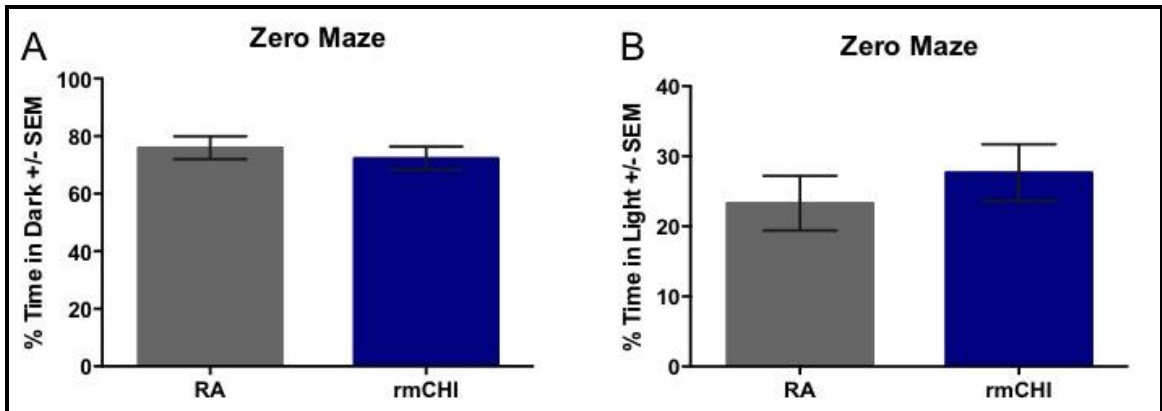
Figure 14. (A & B) RA and rmCHI mice did not differ in the percentage of time spent in the darker quadrants or lighter quadrants in the elevated zero maze test ($p > .05$).

Water treatment did not affect RA or rmCHI mice in the percentage of time spent in the darker or lighter quadrants, (Figure 15 A & B, respectively).
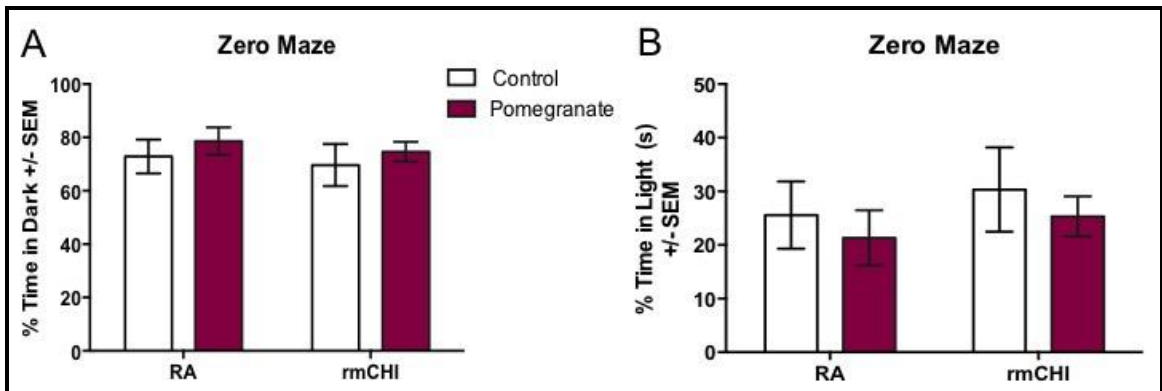
Figure 15. (A & B) Water treatment did not affect the percentage of time spent in the darker quadrants or lighter quadrants in the elevated zero maze test, ($p > .05$).

Depression-like Behaviors

Depression-like behaviors were assessed with the tail-suspension test. RA and rmCHI mice did not differ in the time they first stopped struggling (latency; Figure 16A) or the time they spent mobile after the first latency (Figure 16B). RA and rmCHI also did not differ in the percentage of time they spent immobile in the test (Figure 16C). Thus, neither group exhibited depression-like behaviors.
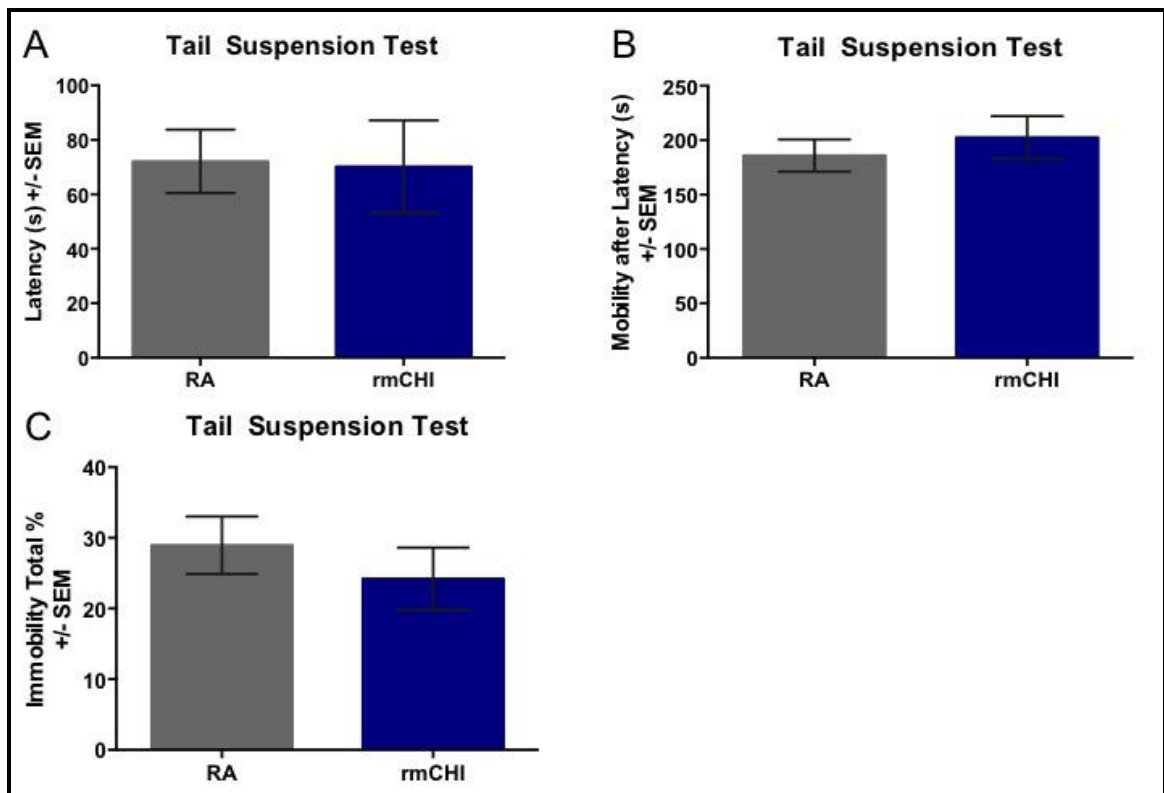
Figure 16. (A, B, C) RA and rmCHI mice did not differ in latency, mobility after latency, or the percentage of time spent immobile in the tail suspension test ($p > .05$).

Pomegranate treatment did not affect latency or mobility after the first latency (Figure 17 A & B, respectively). Percentage of time spent immobile failed to meet

assumptions of equal variances for the factorial ANOVA. To better understand the

relationship between treatment and injury groups on these variables, a square root

transformation was applied, however, failed to correct for the assumptions that were not

previously met. There was a trend towards a significant interaction between treatment

and injury in percentage of time mice spent immobile, ($F(1,26) = 2.88$, p = .10, partial η^2

= .10, power = .37; Figure 17C). The control treatment may have reduced depression-like

behavior in rmCHI mice, whereas the pomegranate treatment may have made rmCHI

more depressed, ($F(1,26) = 3.52$, p = .08; Figure 17C).
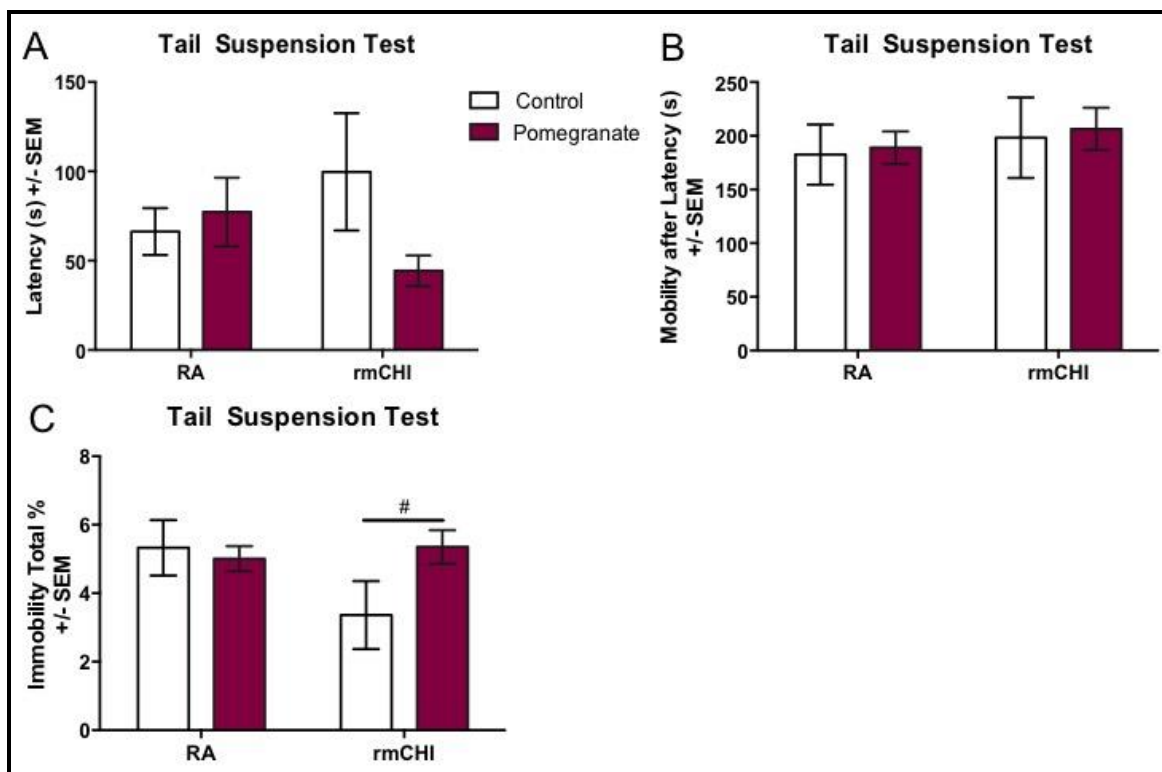
Figure 17. (A & B) Pomegranate treatment did not reduce depression-like behaviors on the tail suspension test (*p* > .05). (C) rmCHI mice on the pomegranate treatment trended towards worse performance, compared to rmCHI mice on the control treatment (#*p* < .10).

Learning/Memory

Learning and memory were assessed with the water maze. A repeated measures ANOVA was conducted to analyze cued water maze performance across blocks. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(9) =$ 59.09, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .46$). Mice showed evidence of learning the locations of the visible platform during the cued training water maze trials by finding the platform quicker across blocks, ($F(1.82, 51.08) = 32.08$, p = .00, partial $\eta^2 = .53$, power = 1.00; Figure 18A). There were no significant differences in performance between RA and rmCHI mice in the cued water maze (Figure 18A).

Overall, mice spent less time searching for the submerged platform across blocks in the spatial 1 water maze ($F(4, 112) = 4.98$, p = .00, partial $\eta^2 = .15$, power = .96; Figure 18B), however, significant deficits emerged in rmCHI mice by block 5, (Welch's $F(1,16.63) = 8.98$, $p = .01$, $\omega^2 = .21$; Figure 18B).

RA and rmCHI mice showed evidence of learning the new location of the submerged platform across blocks on spatial day 2, ($F(4, 112) = 13.63$, p = .00, partial η^2 = .33, power = 1.00; Figure 18C).
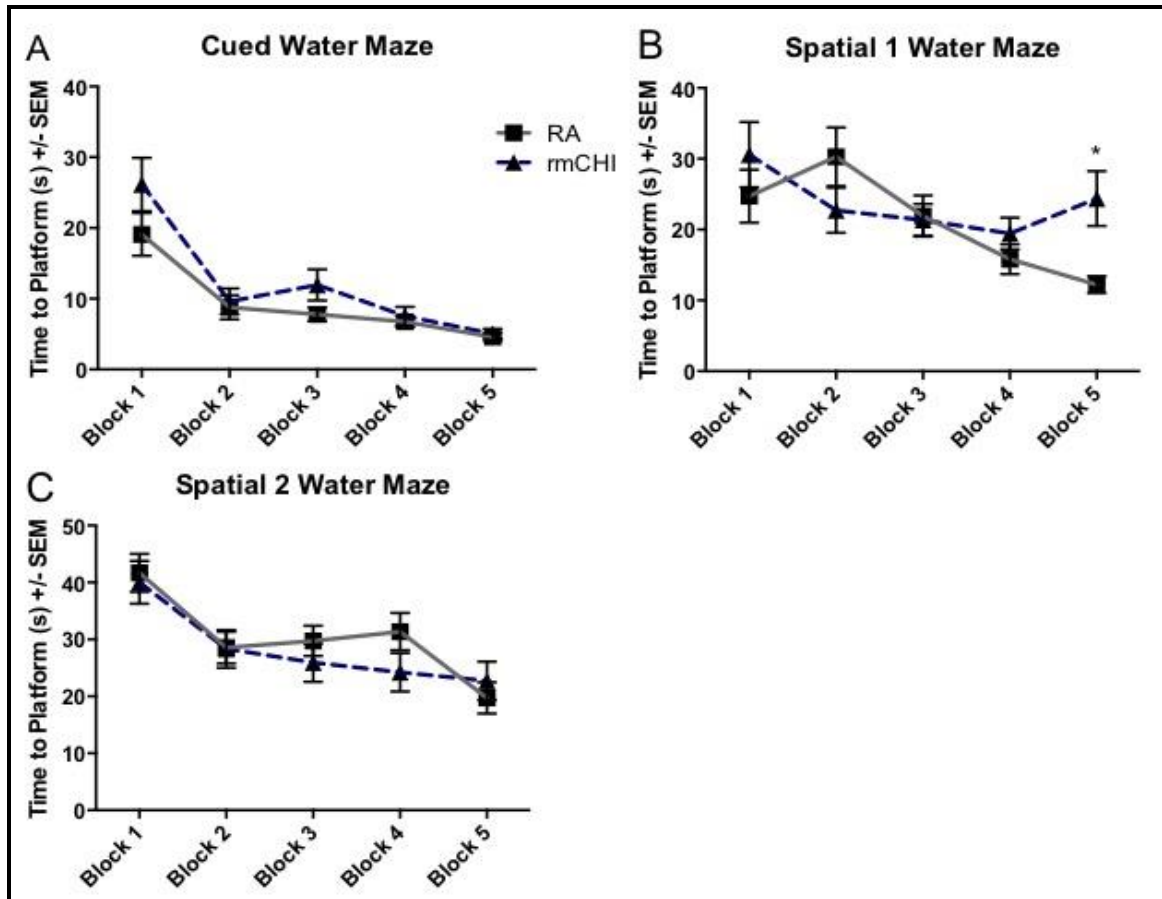
Figure 18. (A) RA and rmCHI mice did not differ in amount of time to locate the visible platform during the cued water maze ($p > .05$); however, all mice learned the location of the platform by block 5 ($p < .05$). (B) RA mice showed evidence of learning the location of the submerged platform on the spatial 1 water maze, whereas rmCHI mice showed no evidence of locating the platform by block 5 (*$p < .05$). (C) Both RA and rmCHI mice showed evidence of learning the location of the submerged platform on day 2 of the spatial water maze.

Further analysis revealed differences in swim behavior, such that 95% confidence intervals indicated that RA mice had a significant tendency to swim to the left while searching for the submerged platform ($p < .05$, Figure 19C). There were no differences in swim behavior in the cued, spatial 1, or in probe water maze trials ($p > .05$; Figure 19A, B, & D).
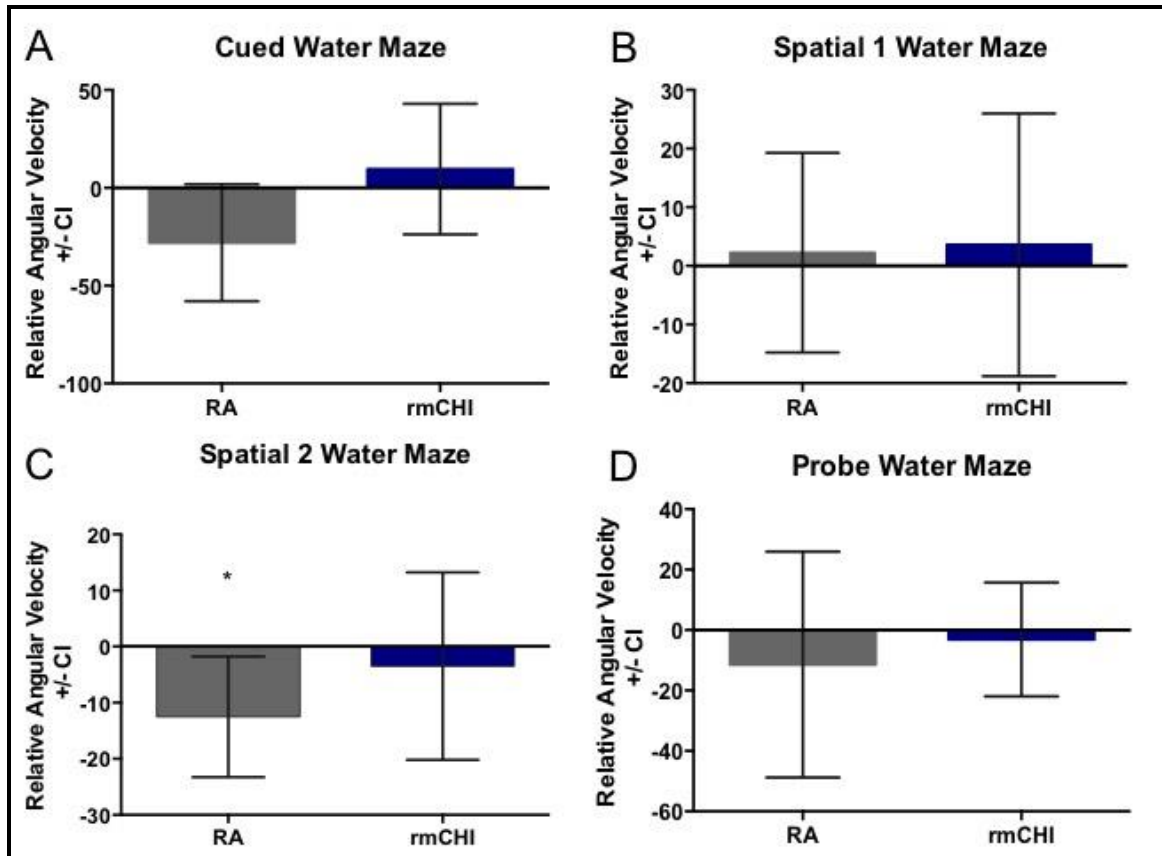
Figure 19. (A, B, & D) There were no differences in swim behavior in the cued, spatial 1, or probe water maze, respectively ($p > .05$). (C) In day 2 of the spatial water maze, RA mice tended to swim to the left compared to rmCHI mice (*$p < .05$; mean +/- 95% CI).

A repeated measures ANOVA was conducted to analyze the effects of injury and treatment in water maze performance in the cued trials. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(9) = 58.01$, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .45$). Mice showed evidence of learning the locations of the visible platform during the cued training water maze trials by finding the platform quicker across blocks, ($F(1.80, 46.82) = 30.78$, p = .00, partial $\eta^2 = .54$, power = 1.00; Figure 20A). Pomegranate treatment did not affect water maze performance in the cued trials (Figure 20A).

Spatial 1 water maze performance improved across blocks, ($F(4, 104) = 5.02$, p = .00, partial $\eta^2 = .16$, power = .96; Figure 20B). In addition, performance of each injury group differed across blocks, ($F(4, 104) = 2.99$, p = .02, partial $\eta^2 = .10$, power = .78; Figure 20B). These differences became statistically significant in block 5, ($F(3, 26) = 3.39$, p = .03). Tukey's HSD post hoc analysis revealed that pomegranate treatment impaired performance in rmCHI mice, taking took longer to find the platform compared to RA mice on the control (p < .05) and pomegranate (#p = .06) treatments. There were no differences between rmCHI and water treatments.

Mice improved performance across blocks ($F(4, 104) = 12.96$, p = .00, partial η^2 = .33, power = 1.00; Figure 20C), though treatment and injury did not interact with spatial 2 water maze performance. Data from the probe trial revealed no significant differences. Both RA and rmCHI mice swam the maze at random, spending a similar amount of time in each quadrant.
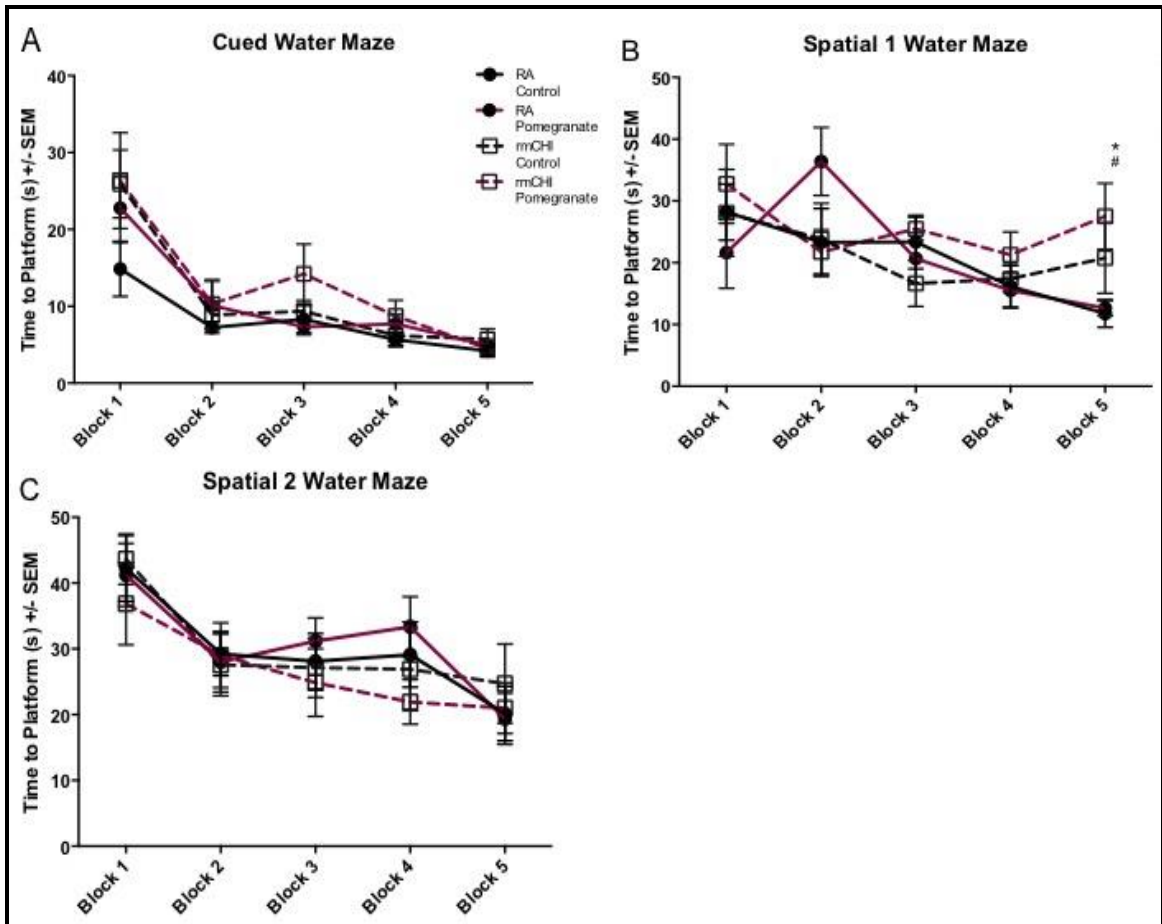
Figure 20. (A) Mice learned the location of the visible platform by block 5 ($p < .01$); however, water treatment did not influence performance in the cued water maze. (B) Performance in the spatial 1 water maze improved across blocks; however, deficits began to emerge in rmCHI nice with pomegranate treatment by block 5. rmCHI mice with the pomegranate treatment took longer to find the submerged platform than RA mice on the control treatment (*$p < .05$) and pomegranate treatment (#$p = .06$). (C) Treatment and injury did not interact with performance in day 2 of the spatial water maze. All mice improved in locating the submerged platform by block 5.

Table 2. *Summary of significant behavioral findings and their associated p-values.*

Test	Summary of Significant Behavioral Findings	p-value
Foot Fault	rmCHI faulted more than RA at 1 dpi	< .03
	rmCHI faulted more than RA at 3 dpi	< .03
	rmCHI traveled a greater distance than RA at 3 dpi	< .04
Beam Balance	rmCHI made more left-sided slips at 3 dpi	< .03
	rmCHI with pomegranate treatment made fewer left-sided slips than RA at 5 dpi	< .03
	RA with control treatment made fewer left sided slips than RA with pomegranate treatment at 5 dpi	< .03
	rmCHI mice with pomegranate treatment traveled less than RA with pomegranate treatment at 5 dpi	< .04
Water Maze	rmCHI mice took longer to find the platform in spatial 1	< .02
	rmCHI mice with pomegranate treatment took longer to find the platform in spatial 1 than rmCHI on control treatment	< .05
	RA mice had left turn bias in spatial 2	< .05

Behavioral Correlations

rmCHI mice with pomegranate treatment had both increased depression-like behaviors and motor deficits across tests. Specifically, rmCHI mice that consumed more of the pomegranate treatment spent a higher percentage of time immobile in the tail suspension test, suggesting increased depression-like symptoms from pomegranates, $r = .84$, $p = .01$ (Figure 21). rmCHI pomegranate treatment mice that had increased activity in the open field also increased the distance explored in the foot fault test, $r = .71$, $p = .05$ (Figure 22). Pomegranate treated rmCHI mice that traveled the beam balance more spent less time searching for the platform in the target quadrant in the probe water maze, $r = -$

.74, $p = .04$ (Figure 23). Lastly, rmCHI mice treated with pomegranate that took longer to first give up (latency) in the tail suspension test also spent more time in the darker quadrants in the elevated zero maze ($r = .82$, $p = .01$; Figure 24), indicative of normal behavior.
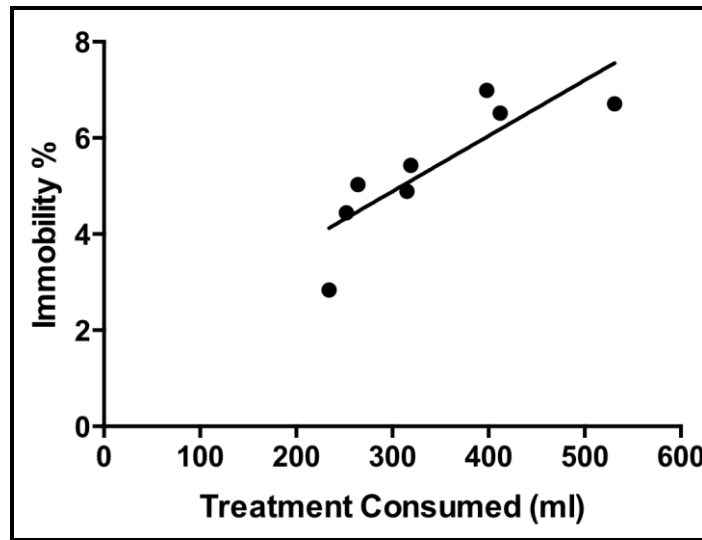
Figure 21. rmCHI mice that consumed more of the pomegranate treatment spent a higher percentage of time immobile in the tail suspension test, $p < .01$.
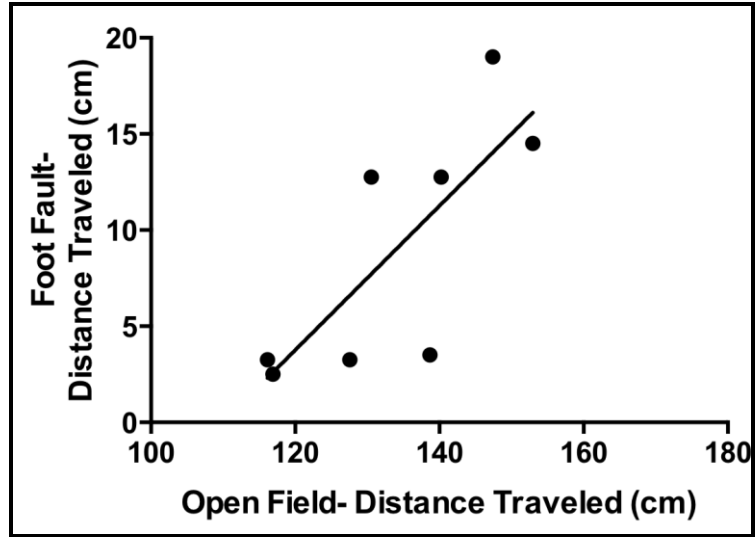
Figure 22. Pomegranate treated rmCHI mice that had increased activity in open field testing also had increased exploratory behavior in the foot fault test, $p = .05$.
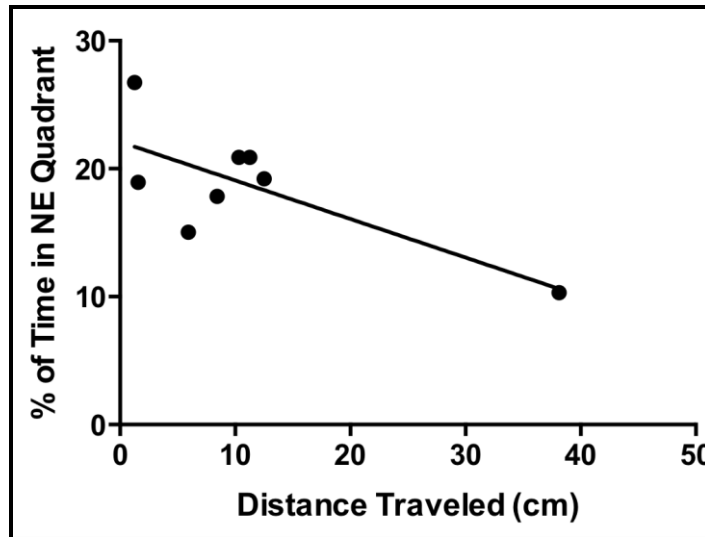
Figure 23. Pomegranate treated rmCHI mice that had reduced activity in the beam balance spent more time in the target quadrant during the probe water maze trial, $p < .05$.
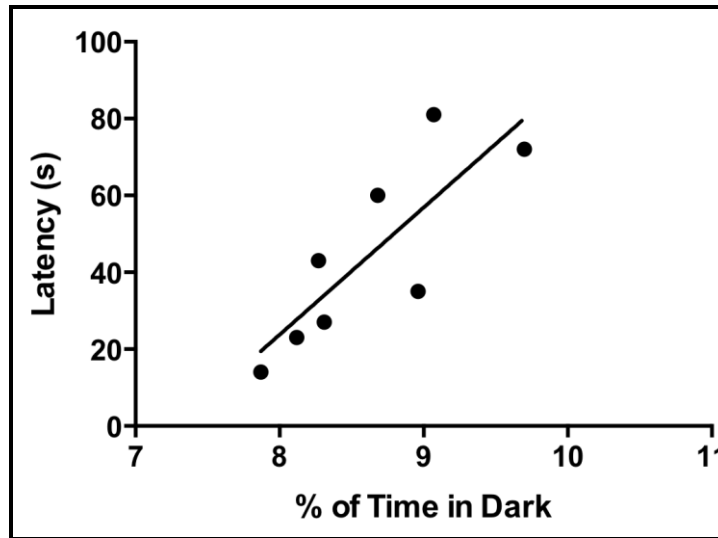
Figure 24. Pomegranate treated rmCHI mice that too longer to fist give up in the tail suspension test also spent more time in the darker quadrants in the elevated zero maze, *p* < .05.

Interestingly, the pomegranate treatment seemed to improve motor performance in RA mice across tests. Mice that consumed more pomegranate treatment had a reduction in the number of faults per distance in the foot fault test, $r = -.71, p = .05$ (Figure 25). More pomegranate treatment consumption in RA mice also led to better average performance on the rotarod (stayed on the test longer), especially in the most challenging speed of the test, $r = .73, p = .04$ (Figure 26). In addition, pomegranate treated RA mice that spent more time in the perimeter of open field had better performance on the rotarod, especially in the most challenging speed of the test, $r = .74, p = .04$ (Figure 27).
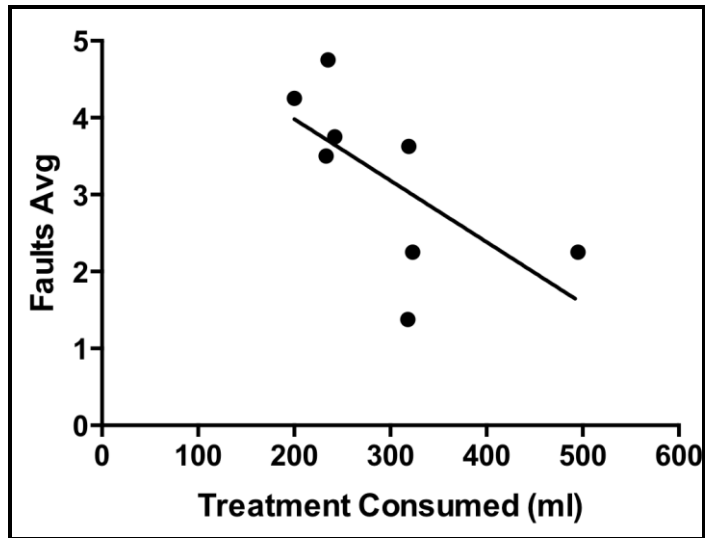
Figure 25. RA mice that consumed more pomegranate treatment made fewer faults in the foot fault test, $p = .05$.
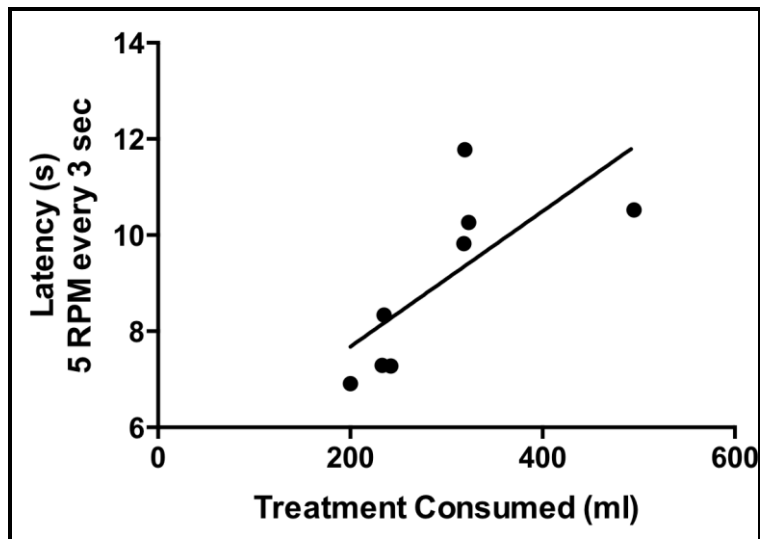
Figure 26. RA mice that consumed more pomegranate treatment had improved performance on average in the most challenging rotarod trials, $p = .05$.
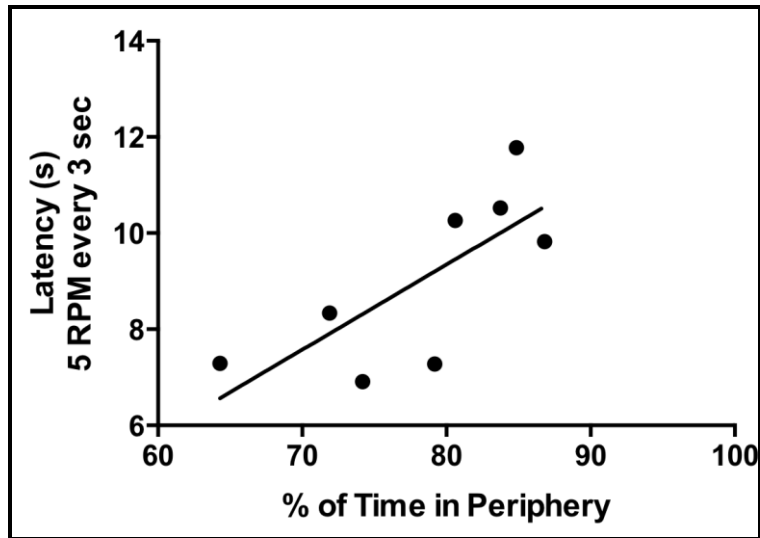
Figure 27. Pomegranate treated RA mice that spent more time in the periphery in open field testing had improved performance on average in the most challenging rotarod trials, $p < .05$.

Anesthesia (SA) vs. Repeated Anesthesia (RA)

There were no weight or treatment differences between SA ($M = 26.95$, $SD = 2.53$; $M = 270.92$, $SD = 63.50$) and RA ($M = 26.32$, $SD = 2.69$; $M = 275.67$, $SD = 75.78$) mice, respectively.

General Activity: Open Field

Generalized locomotor activity was recorded at 3, 5, and 7 dpi. Repeated measures ANOVA was conducted and Mauchly's test indicated that the assumption of sphericity had not been violated and equal variances was assumed. There were no differences in activity found between SA and RA mice across days of testing, (Figure 28, Figure 29A). Both SA and RA mice increased rearing behavior across days of testing,

($F_{(2, 28.41)}$ = 11.85, p = .00, partial η^2 = .24, power = .99; Figure 29B), that may have

indicative of anxiety-like behavior.
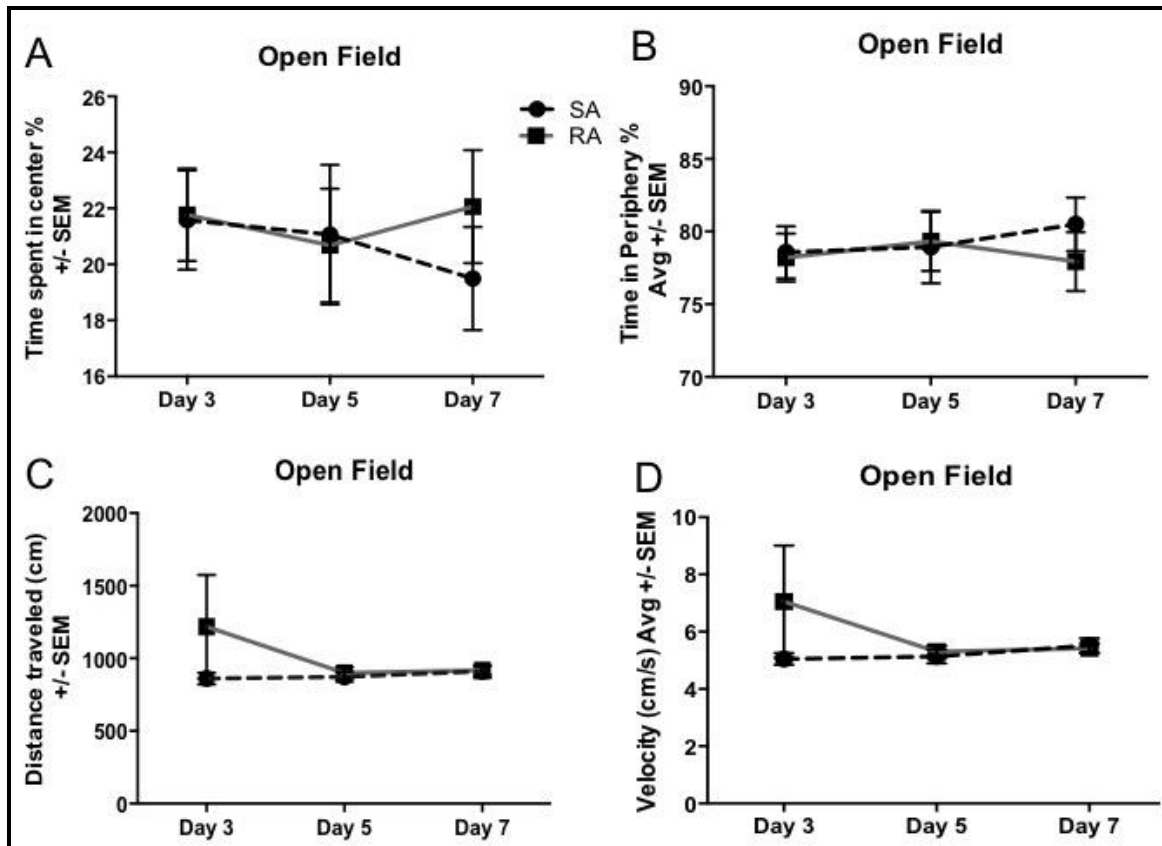
Figure 28. (A, B, C, D) There were no significant differences found between groups across days of open field testing (*p* > .05).
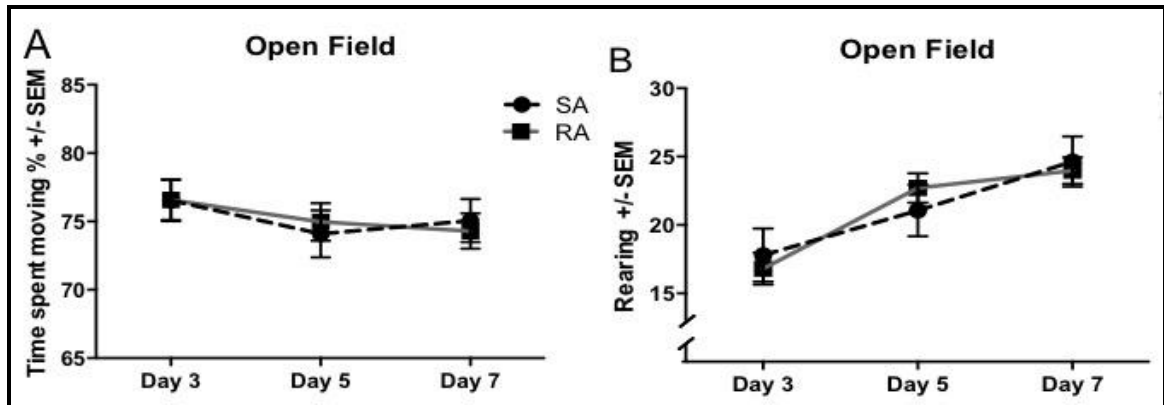
Figure 29. (A) SA and RA mice did not differ in the percentage of time they spent exploring the test ($p > .05$). (B) SA and RA mice increased rearing behavior across days of open field testing ($p < .01$).

Water treatment did not influence the percentage of time mice spent in the center,

periphery, distance traveled, or velocity across days of testing, ($p > .05$; Figure 30).

Water treatment also did not influence the distance traveled or rearings across days of

testing, ($p > .05$; Figure 31).
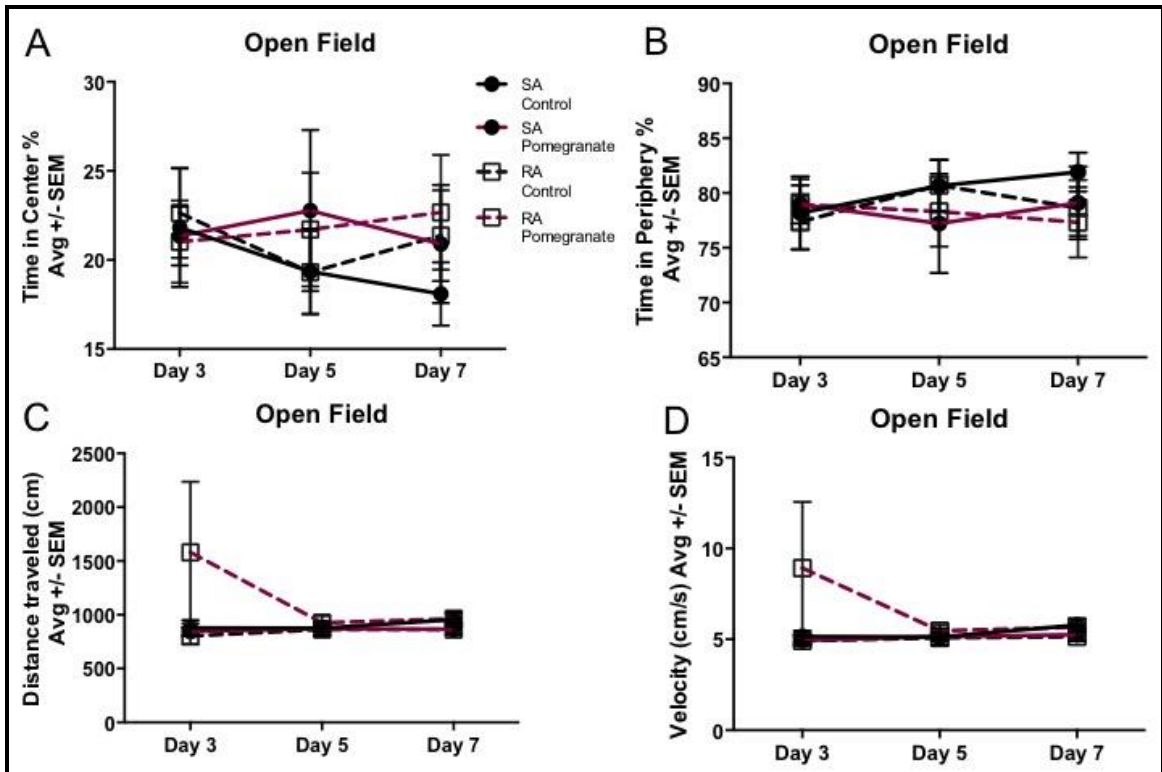
Figure 30. (A, B, C, D) There were no significant differences found between groups across days of open field testing ($p > .05$).
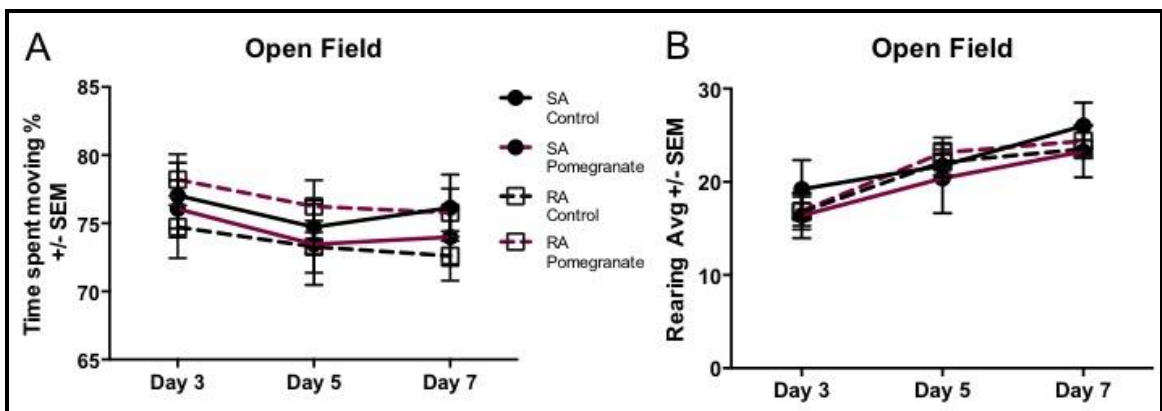
Figure 31. (A & B) There were no significant differences found between groups across days of open field testing ($p > .05$).

Motor Skill: Foot Fault

The foot fault test was administered 1, 3, 5, and 7 dpi in which the number of faults and distance traveled were recorded. A repeated measures ANOVA was conducted to analyze the effects of anesthesia on the number of faults. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 15.15$, $p = .01$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .78$). Overall, mice reduced the number of faults across days of testing, ($F(2.35, 54.00) = 68.88$, p = .00, partial $\eta^2 = .75$, power = 1.00; Figure 32A). There were no significant main effects or interactions.

Another repeated measure ANOVA was conducted to analyze the effects of anesthesia on the distance traveled in the foot fault test. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 14.45$, $p = .01$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .76$). Overall, mice reduced the number of faults across days of testing, ($F(2.27, 52.25) = 21.28$, p = .00, partial $\eta^2 = .48$, power = 1.00; Figure 32B). There were no significant main effects or interactions.

Another repeated measures ANOVA was conducted to analyze the effects of anesthesia on the percentage of faults per distance. Mauchly's test indicated that the assumption of sphericity had not been violated and equal variances were assumed. Mice reduced the percentage of faults per distance across days of testing, ($F(3, 69) = 12.61$, p = .00, partial $\eta^2 = .35$, power = 1.00; Figure 32C). There were no significant main effects or interactions.
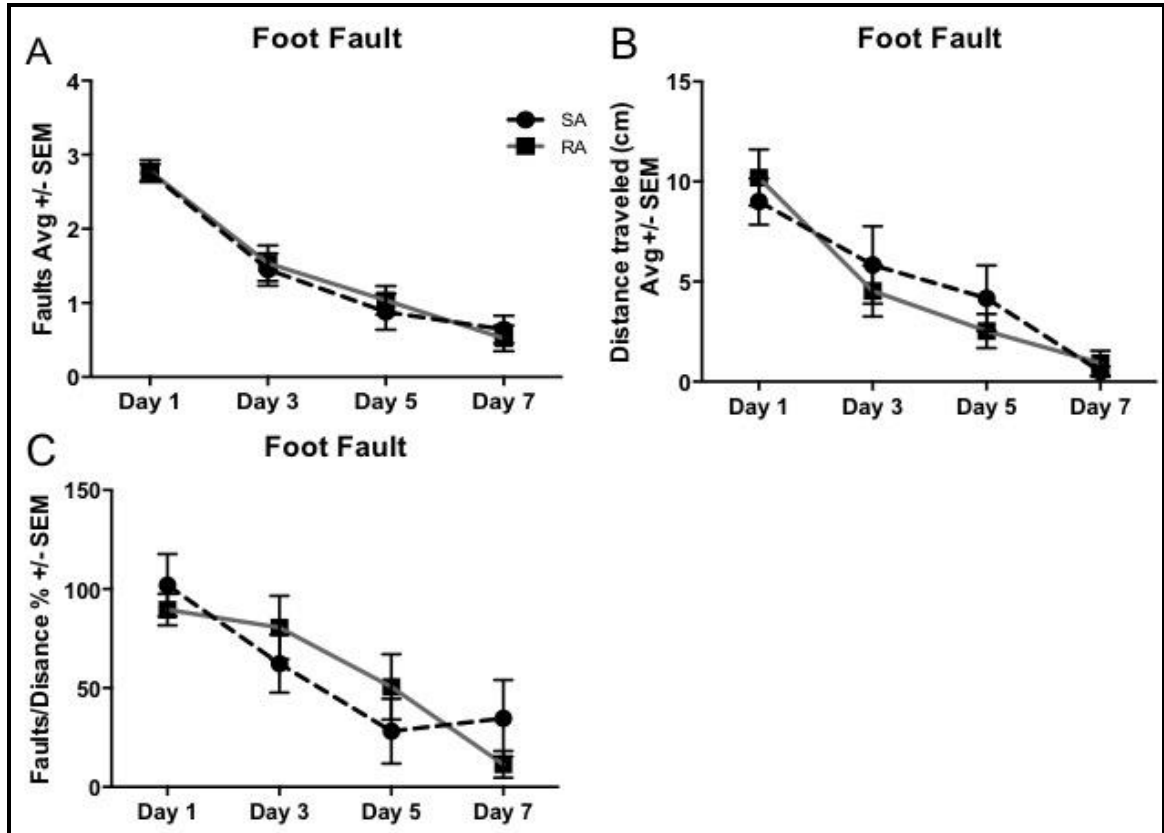
Figure 32. (A) SA and RA mice reduced faults across days of foot fault testing ($p < .01$). (B) SA and RA mice traveled a shorter distance across days of testing ($p < .01$). (C) SA and RA mice made fewer faults per distance across days of testing ($p < .01$).

Pomegranate treatment did not reduce the number of faults ($p > .05$; Figure 33A), distance ($p > .05$; Figure 33B), or the percentage of faults per distance across days of testing ($p > .05$; Figure 33C).
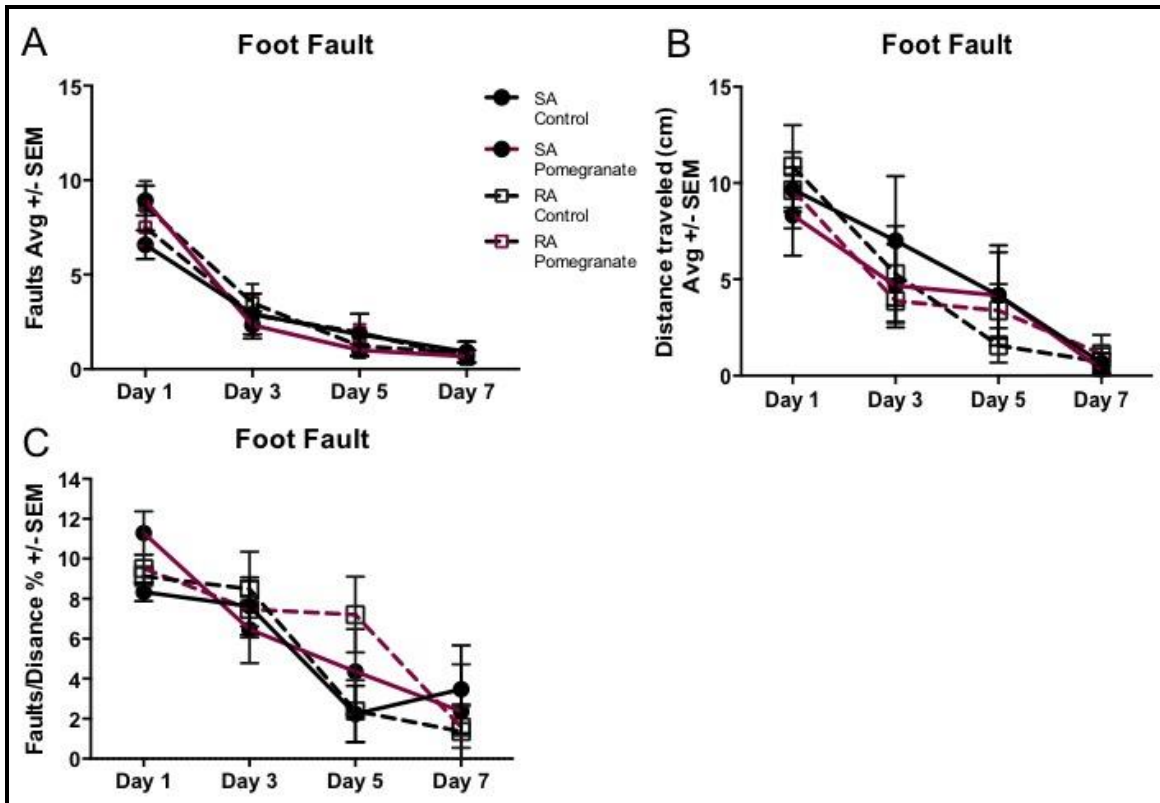
Figure 33. (A, B, C) Water treatment did affect performance in the foot fault test ($p >$ .05).

Motor Skill: Beam Balance

Motor skills and coordination were assessed with the beam balance at 1, 3, 5, and 7 dpi. Overall, RA mice trended towards motor deficits with more left sided slips compared to SA mice, ($F(1,25) = 3.20$, $p = .09$; Figure 34A).
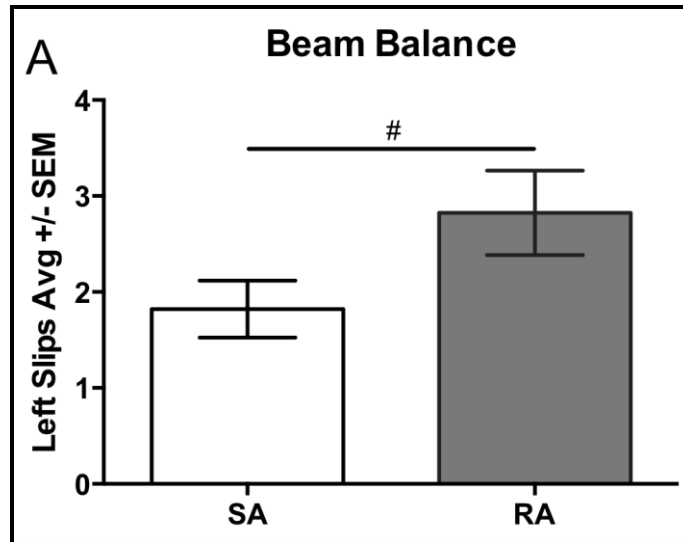
Figure 34A. RA mice trended towards more left sided slips compared to SA mice in the beam balance, (*p* = .09).

A repeated measures ANOVA was conducted to investigate whether anesthesia affected left-sided slips. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 13.67$, *p* = .02, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = .70). Overall, mice reduced the number of left-sided slips across days of testing, ($F(2.11, 48.51) = 4.92$, p = .01, partial η^2 = .18, power = .80; Figure 35A). There were no significant main effects or interactions.

Another repeated measures ANOVA was conducted to investigate whether anesthesia affected right-sided slips. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 13.82$, *p* = .02, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = .74). Overall, mice reduced the number of right-sided slips across days of testing, ($F(2.23, 51.27) = 4.64$, p =

.01, partial η^2 = .17, power = .79; Figure 35B). There were no significant main effects or interactions.

Another repeated measures ANOVA was conducted to investigate whether anesthesia influenced the distance mice traveled in the beam balance. Mice did not differ in the distance traveled across days of testing ($p > .05$; Figure 35C). There were no significant main effects or interactions.

Another repeated measures ANOVA was conducted to investigate whether anesthesia affected the number of times mice fell off the beam. Mice did not differ in the number of times they fell off the test ($p > .05$; Figure 35D). Also, there were no significant main effects or interactions.
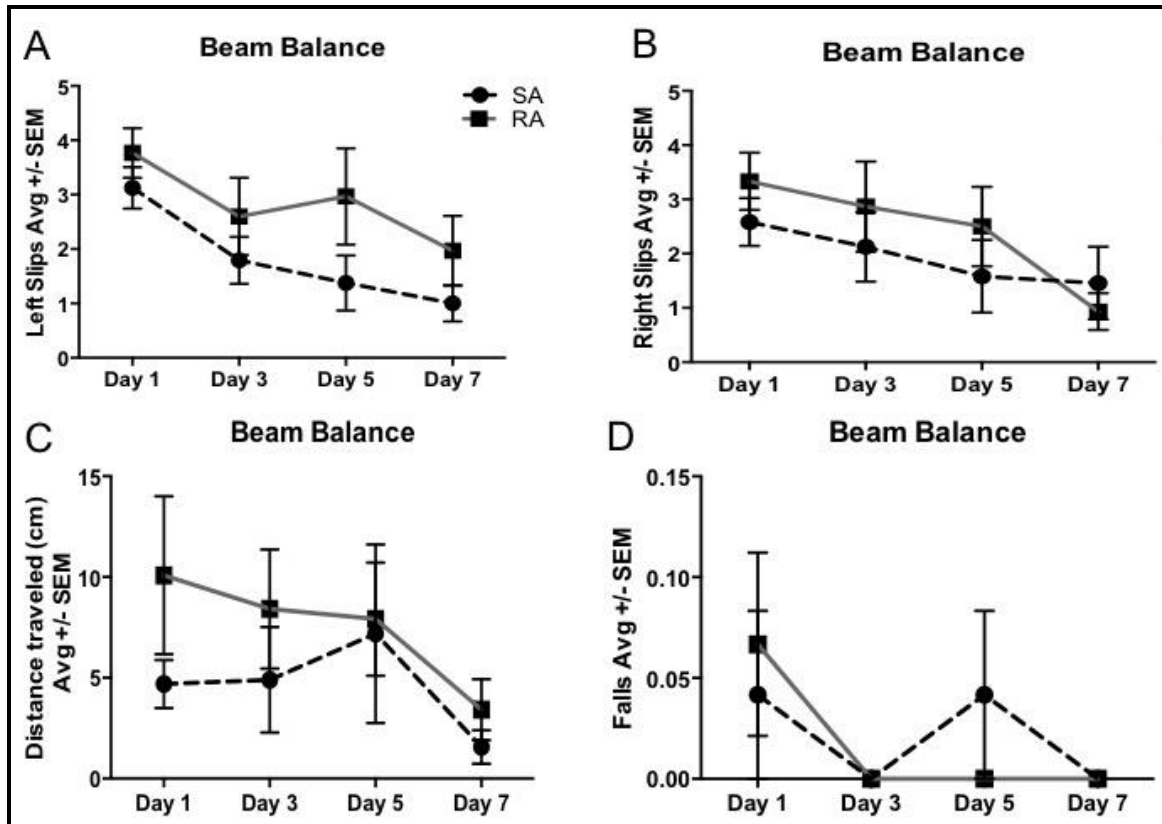
Figure 35. (A) Mice made fewer left-sided slips across days of testing on the beam balance ($p < .05$). (B) Mice made fewer right-sided slips across days of testing ($p < .05$). (C) Mice did not differ in the distance traveled across days of testing ($p > .05$). (D) Mice did not differ in the number of times they fell off the beam across days of testing ($p > .05$).

A repeated measures ANOVA was conducted to investigate whether anesthesia affected left turns on the beam balance. Mice reduced the number of left turns across days of testing, ($F(3, 69) = 4.23$, p = .01, partial η^2 = .16, power = .84; Figure 36A). There were no significant main effects or interactions.

Another repeated measures ANOVA was conducted to investigate whether anesthesia affected right turns on the beam balance. Mice did not differ in the number of right turns made across days of testing, ($p > .05$; Figure 36B).
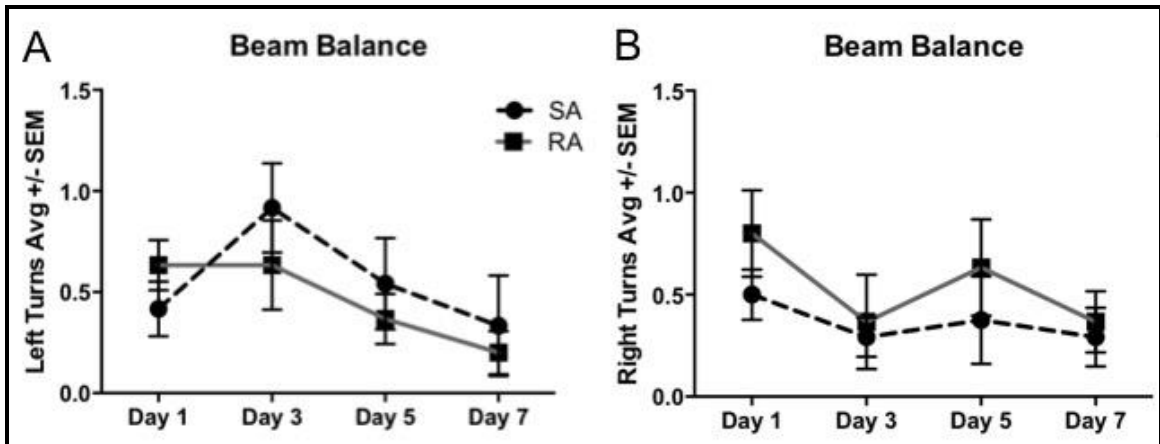
Figure 36. (A) Mice made fewer left turns across days of testing on the beam balance (*p* < .05). (B) Mice did not differ in the number of right turns across days of testing (*p* < .05).

Pomegranate treatment and anesthesia did not affect performance in the beam balance (*p* > .05; Figure 37 & 38).
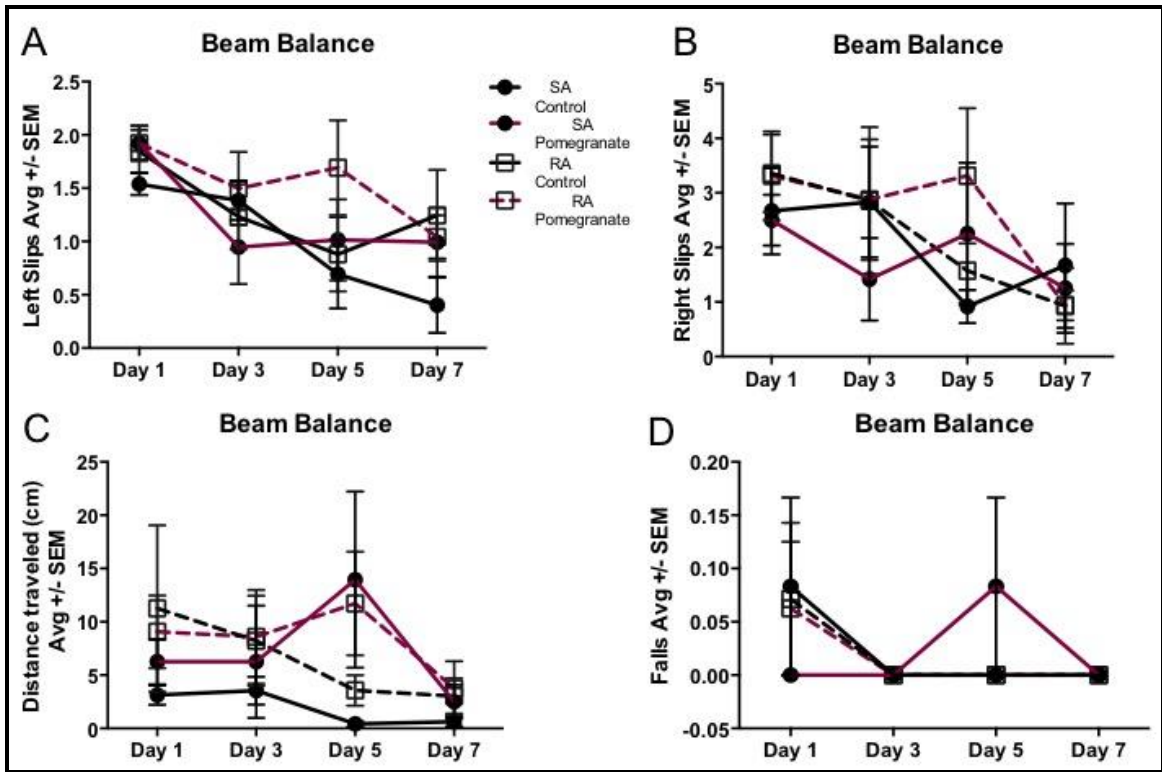
Figure 37. (A, B, C, D) Water treatment did affect performance in the beam balance test (*p* > .05).
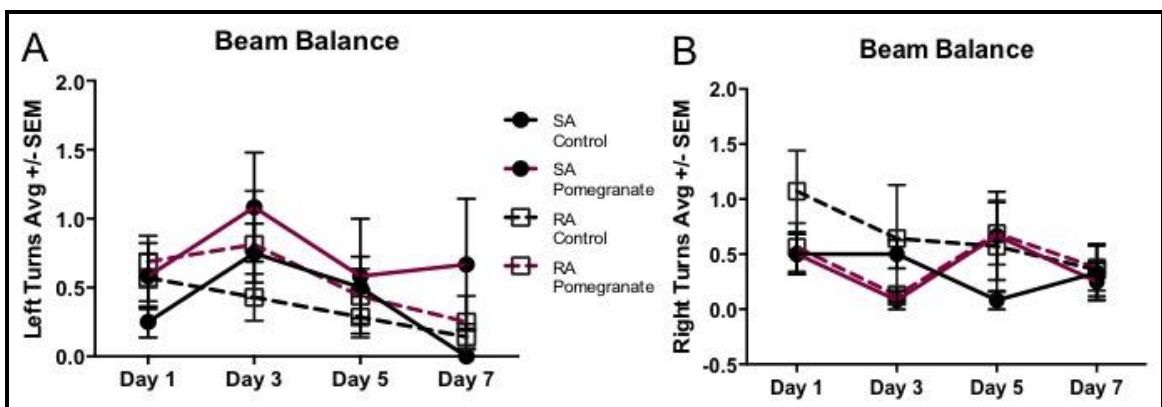
Figure 38. (A & B) Water treatment did affect performance in the beam balance test (*p* > .05).

Motor Skill: Rotarod

Rotarod performance was assessed at 1, 3, 5, and 7 dpi with repeated measures ANOVAs. Mauchly's test indicated that the assumption of sphericity had not been violated. Performance on the rotarod improved (mice stayed on longer) across days of testing in the steady rotarod trials, ($F(3, 69) = 34.50$, p = .00, partial η^2 = .60, power = 1.00; Figure 39A). There were no significant main effects or interactions.

A repeated measures ANOVA was conducted to analyze the effects of anesthesia and motor performance in the accelerating rotarod trials. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 18.25$, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .63$). Overall, mice improved across days of testing, ($F(1.88, 43.32) = 5.45$, p = .01, partial η^2 = .19, power = .81; Figure 39B). There were no significant main effects or interactions.

Another repeated measures ANOVA was conducted to analyze the effects of anesthesia and motor performance in the fast rotarod trials. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(5) = 12.91$, $p = .02$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .71$). Overall, mice improved across days of testing, ($F(2.12, 48.84) = 3.84$, p = .03, partial η^2 = .14, power = .69; Figure 39C). There were no significant main effects or interactions.
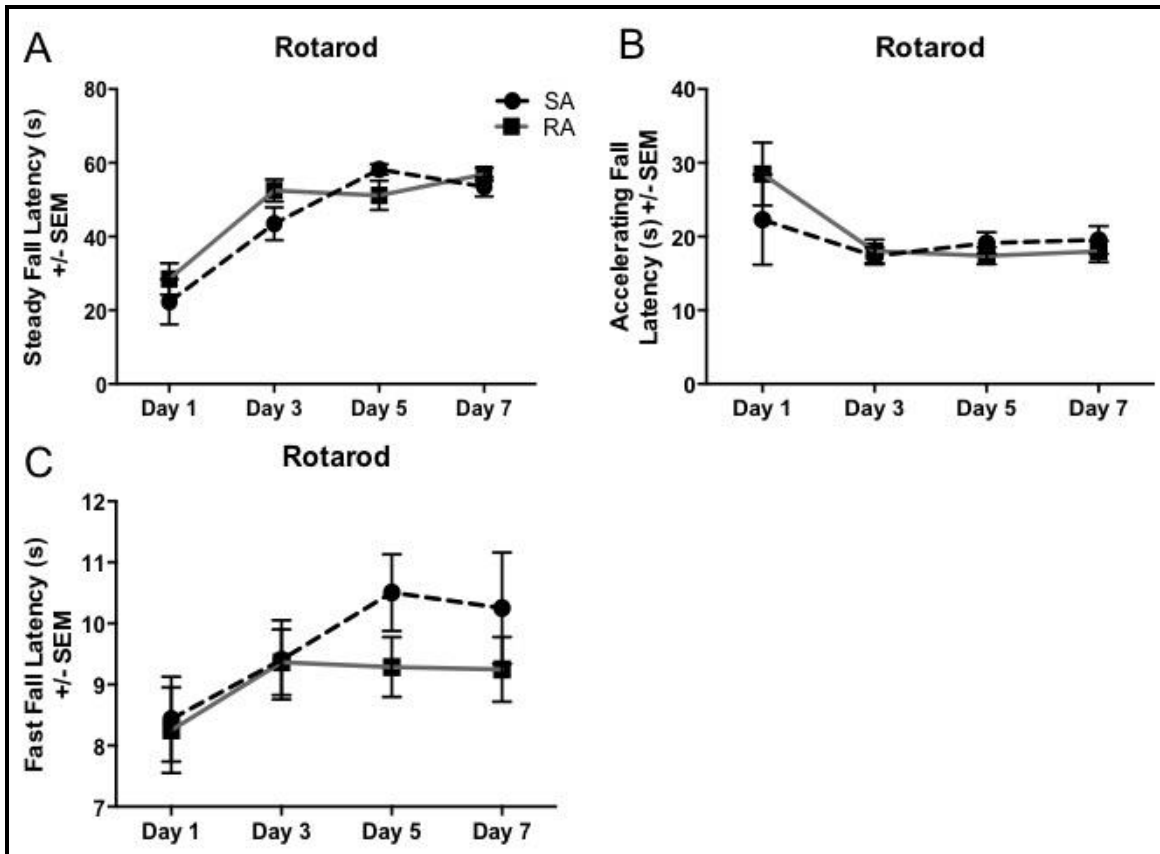
Figure 39. (A, B, C) Mice improved their performance on the rotarod test across days of testing, regardless of injury ($p < .05$).

Water treatment did not influence performance in the rotarod across days of testing (Figure 40; $p > .05$).
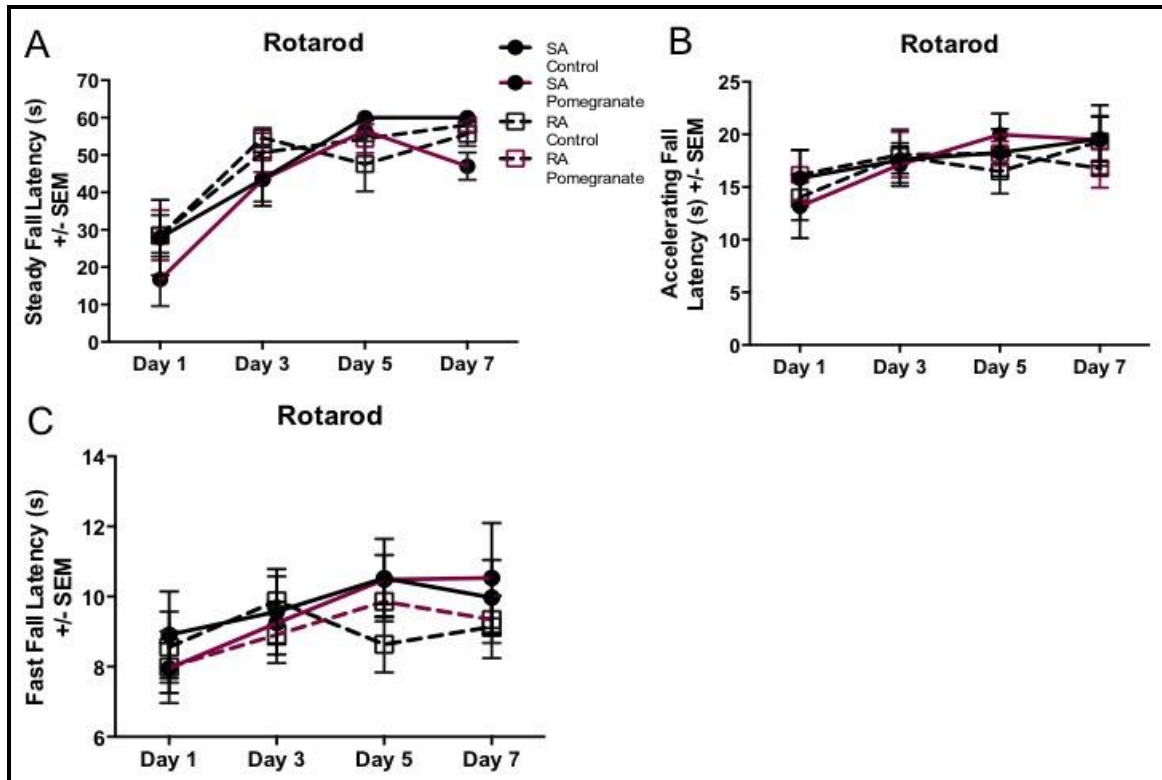
Figure 40. (A, B, C) Water treatment did not influence performance in the rotarod across days of testing ($p > .05$).

Anxiety-like Behavior

Anxiety-like behavior was assessed with the elevated zero maze. Mice did not differ in the amount of time spent in the darker quadrants of the maze ($p > .05$; Figure 41A). SA mice trended towards spending higher percentages of time in the lit quadrants compared to RA mice, suggesting more anxiety-like and risk taking behaviors, (Welch's $F(1,15.69) = 4.01$, $p = .06$, $\omega^2 = .10$; Figure 41B).
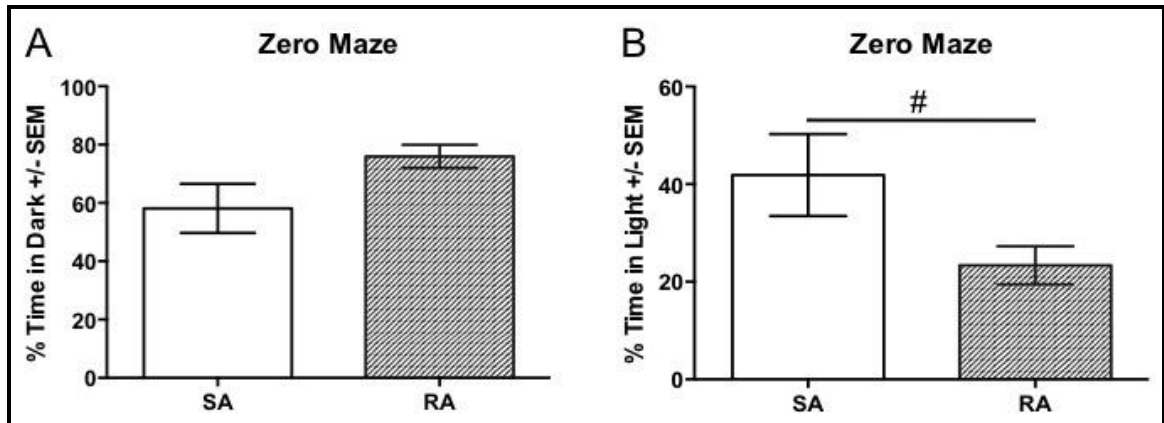
Figure 41. (A) Mice did not differ in the amount of time spent in the darker quadrants of the elevated zero maze test. (B) SA mice trended towards more risk taking and anxiety-like behaviors compared to RA mice in the elevated zero maze test (#p = .06).

A factorial ANOVA was used to test the relationship between treatment and anesthesia on anxiety-like behaviors. Anesthesia and pomegranate treatment did not influence anxiety-like behaviors (time mice spent in the darker quadrants; $p > .05$; Figure 42A). However, there was a trend towards a main effect of anesthesia in the time spent in the lit quadrants, ($F(1,23) = 4.17$, $p = .05$, partial $\eta^2 = .15$, power = .50; Figure 42B), but no significant interaction was found.
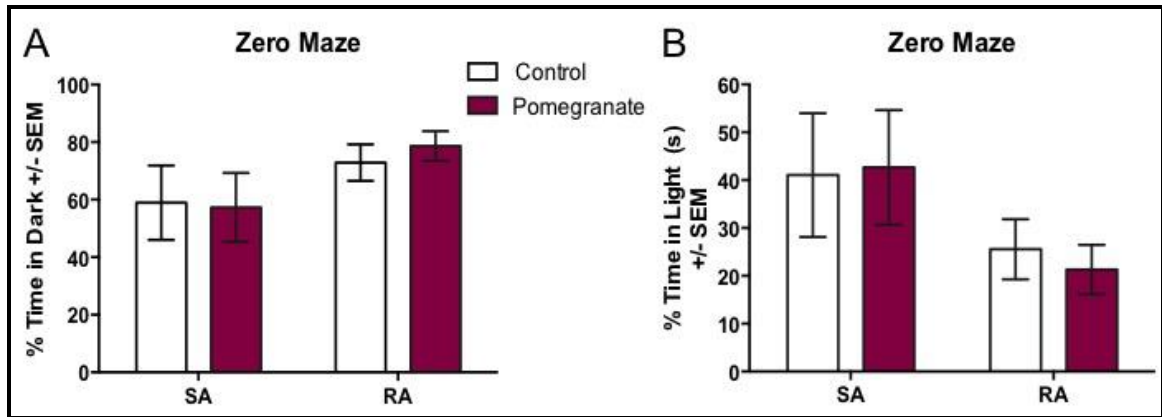
Figure 42. (A) Anesthesia or treatment did not influence the percentage of time mice spent in the darker quadrants in the elevated zero maze test ($p > .05$). (B) SA mice trended towards spending more time in the lit quadrants, regardless of diet, compared to RA mice in the elevated zero maze test.

Depression-like Behaviors

Depression-like behaviors were assessed with the tail-suspension test. SA and RA mice did not differ in latency (time when they first gave up; $p > .05$; Figure 43A), the time spent mobile after latency ($p > .05$; Figure 43B), total time spent immobile ($p > .05$; Figure 43C), or the percentage of time they spent immobile in the test (Figure 43D).
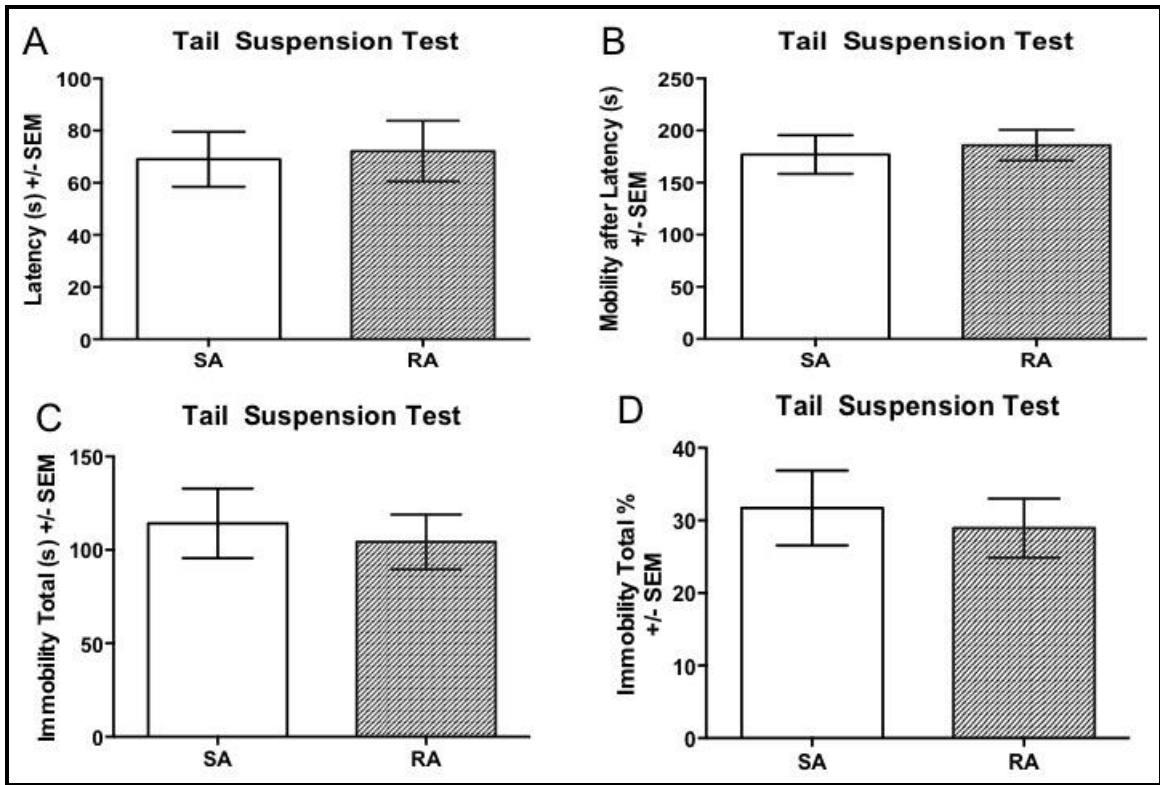
Figure 43. (A, B, C, D) SA and RA mice did not differ in depression-like behavior in the tail suspension test ($p > .05$).

Water treatment did not influence latency ($p > .05$; Figure 44A), mobility after latency ($p > .05$; Figure 44B), total time mice spent immobile ($p > .05$; Figure 44C), or the percentage of time mice spent immobile ($p > .05$; Figure 44D).
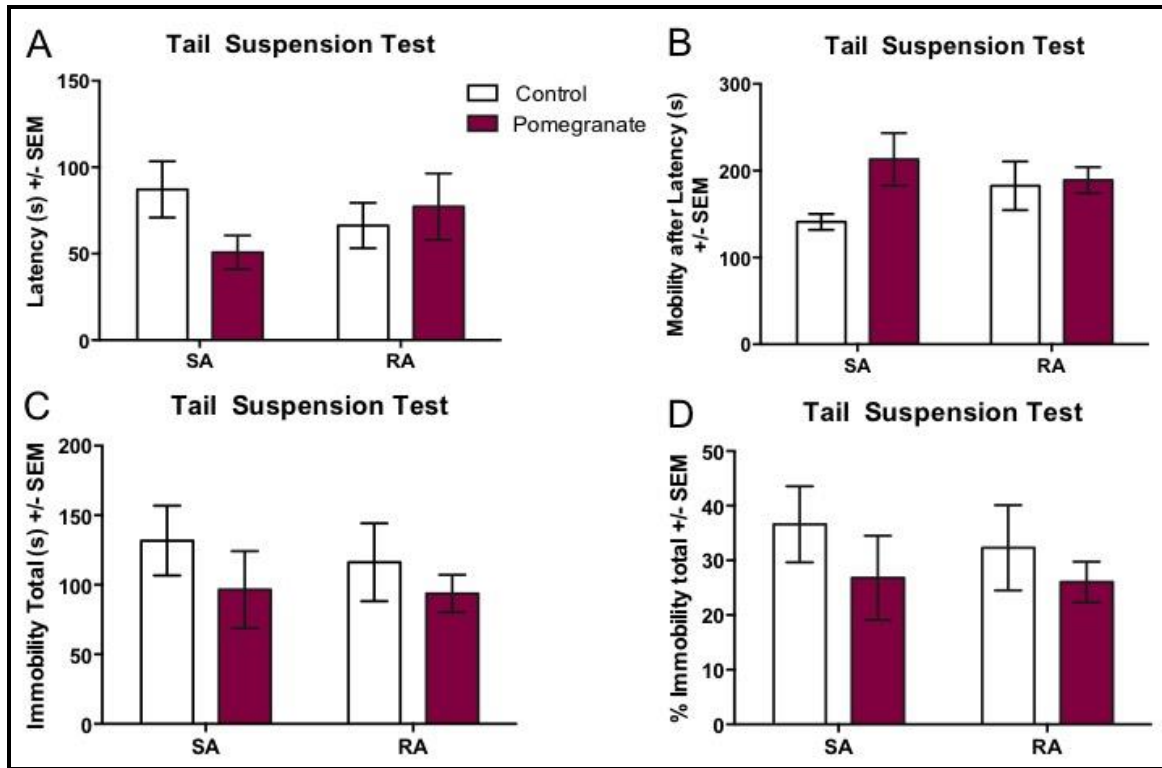
Figure 44. (A, B, C, D) Water treatment did not influence depressive-like behaviors in the tail suspension test ($p > .05$).

Learning/Memory

Learning and memory were assessed with the water maze. A repeated measures ANOVA was conducted to analyze cued water maze performance across blocks. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(9) = 42.92$, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = .51$). Mice showed evidence of learning the locations of the visible platform during the cued training water maze trials by finding the platform quicker across blocks, ($F(2.05, 51.36) = 30.14$, p = .00, partial $\eta^2 = .55$, power = 1.00; Figure 45A). There were no significant main effects or interactions.

In the spatial 1 water maze, mice showed evidence of learning the location of the submerged platform across blocks, ($F(4, 100) = 2.89$, p = .03, partial $\eta^2 = .10$, power = .76; Figure 45B). Additionally, there was a trend towards a significant interaction between anesthesia group and blocks, ($F(4, 100) = 2.28$, p = .07, partial $\eta^2 = .08$, power = .65; Figure 45B). Tukeys's HSD post hoc analysis revealed that RA mice found the location of the platform much faster by block 4 ($p = .10$) and block 5 ($p < .05$), whereas SA mice did not learn the location of the platform at all.

SA and RA mice showed evidence of learning the new location of the submerged platform across blocks on spatial day 2, ($F(4, 100) = 4.50$, p = .00, partial $\eta^2 = .15$, power = .93; Figure 45C). RA mice trended towards taking more time to find the submerged platform on block 1 ($F(1, 25) = 3.35$, p = .08), but these deficits were reversed by block 5, ($F(1, 25) = 3.17$, p = .09).

There were no significant differences in the probe trial between SA and RA mice. Both SA and RA mice swam the maze at random, spending a similar amount of time in each quadrant ($p > .05$).
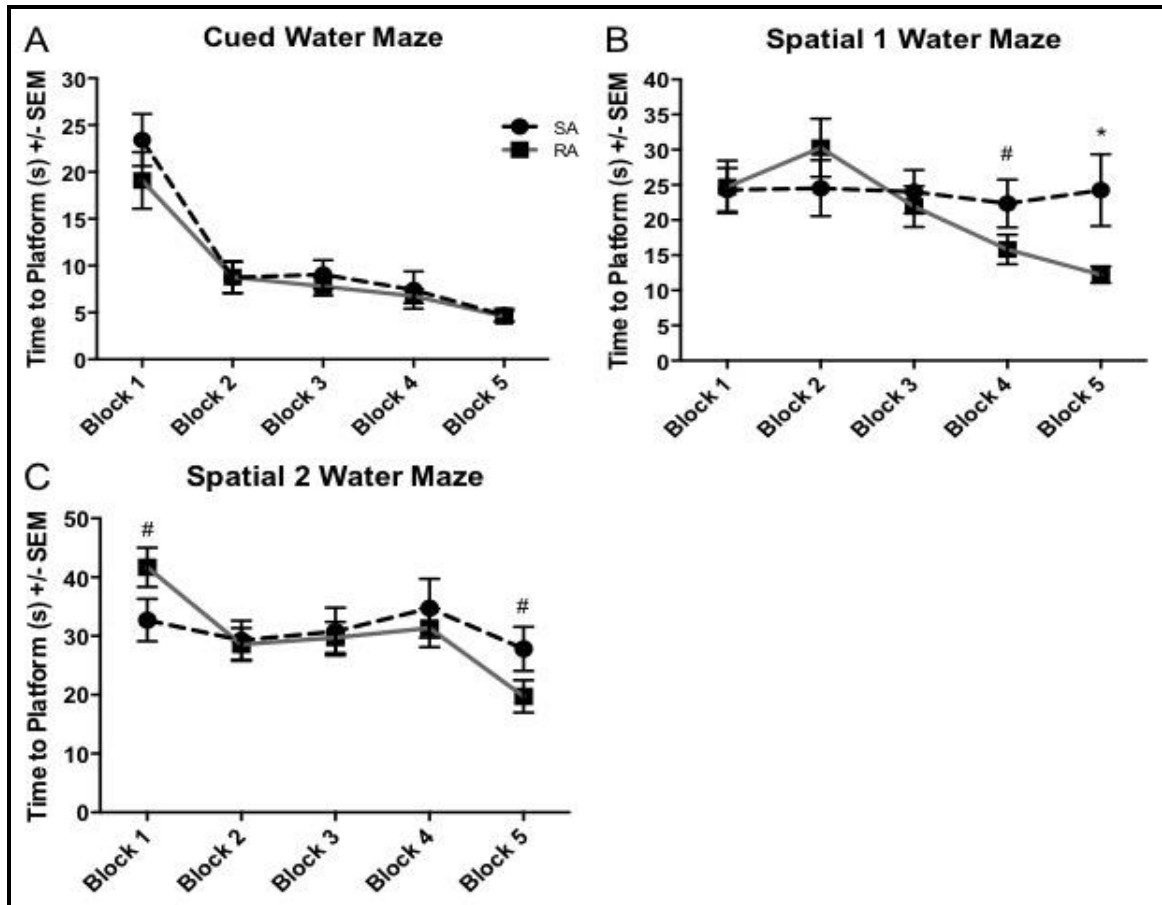
Figure 45. (A) SA and RA mice did not differ in amount of time to locate the visible platform during the cued water maze ($p > .05$); however, all mice learned the location of the platform by block 5 ($p < .05$). (B) All mice appeared to learn the location of the submerged platform in the spatial 1 water maze ($p < .05$); however, there was a strong group effect by block 4. RA mice found the platform faster on block 4 ($p < .10$) and 5 ($p < .05$), compared to SA mice that did not seem to learn the location at all. (C) Performance in the spatial 2 water maze improved across blocks ($p < .05$). Interestingly, RA mice trended towards longer to find the submerged platform on block 1 (#$p = .08$); however, they trended towards finding the platform faster than SA mice by block 5 (#$p = .09$).

There were no differences in swim behavior (turn bias) between SA and RA mice in the water maze, ($p > .05$; Figure 46).
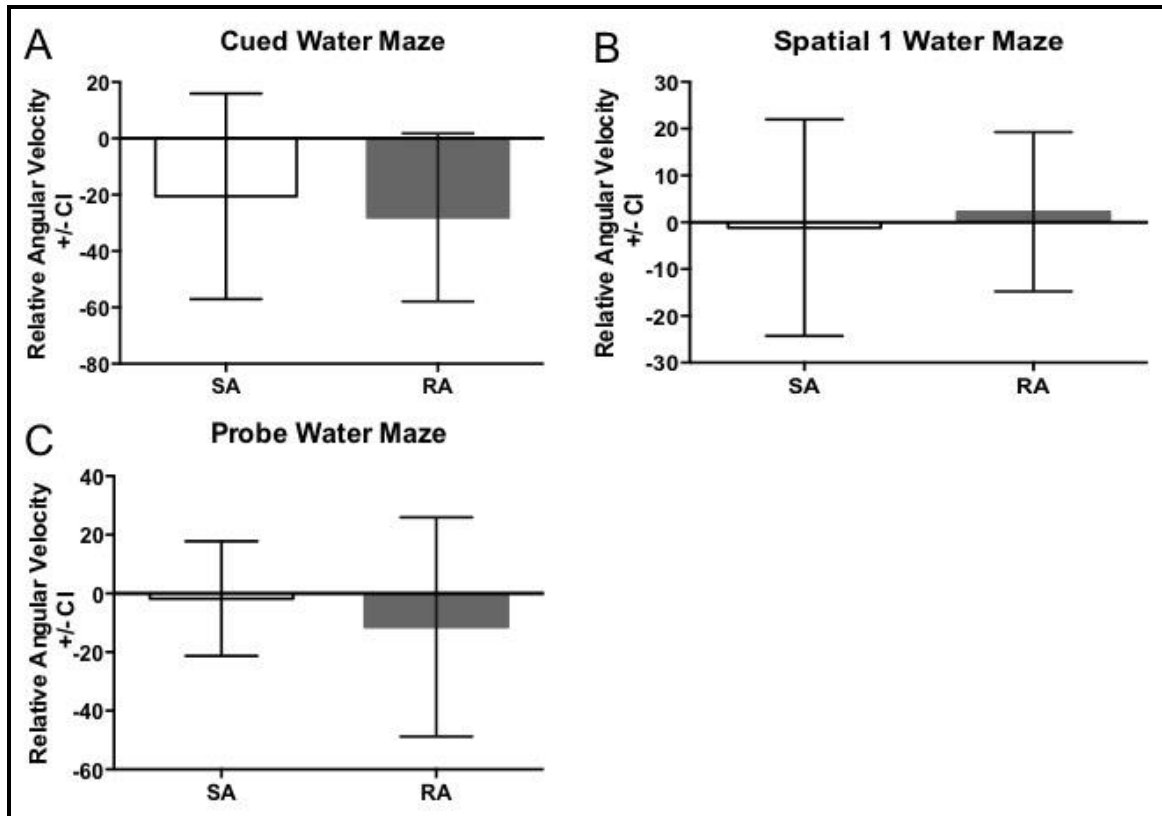
Figure 46. (A, B, C) SA and RA mice did not differ in turn bias in the water maze ($p >$ .05).

Another repeated measures ANOVA was conducted to measure the influence treatment and anesthesia had on cued water maze performance. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2(9) = 38.94$, $p = .00$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon =$ .52). Mice showed evidence of learning the locations of the visible platform during the cued training water maze trials by finding the platform quicker across blocks, ($F(2.06,$ 47.48) = 29.26, p = .00, partial $\eta^2 = .56$, power = 1.00; Figure 47A). There were no significant interactions between anesthesia group and water treatment on performance (Figure 47A).

Another repeated measures ANOVA was conducted to measure the influence treatment and anesthesia group had on spatial 1 water maze performance. There was a main effect of learning the location of the submerged platform across blocks, ($F(4, 92) = 2.99$, p = .02, partial $\eta^2 = .12$, power = .78; Figure 47B), and there were trends towards a significant interactions between anesthesia group and blocks ($F(4, 92) = 2.35$, p = .06, partial $\eta^2 = .09$, power = .66) and treatment and blocks, ($F(4, 92) = 2.28$, p = .06, partial $\eta^2 = .10$, power = .67). Tukey's HSD post hoc analysis revealed that SA mice on the pomegranate treatment took longer to find the platform than SA mice on the control treatment ($p = .08$), RA mice on the control treatment ($p < .05$), and RA mice on the pomegranate treatment ($p < .05$) by block 5 of the spatial 1 water maze. Thus, pomegranate treatment led to learning and memory impairments in SA mice.

Another repeated measures ANOVA was conducted to measure the influence treatment and injury had on spatial 2 water maze performance. There was a main effect of blocks, where mice showed evidence of learning the location of the submerged platform across blocks, ($F(4, 92) = 4.48$, p = .00, partial $\eta^2 = .16$, power = .93; Figure 47C). There was a trend towards significant differences between the groups on block 3, ($F(3, 23) = 2.63$, p = .07). Tukey's HSD post hoc analysis revealed that pomegranate treatment impaired performance of SA mice, taking longer to locate the submerged platform than SA mice on the control treatment ($p = .05$). There were no significant interactions found.
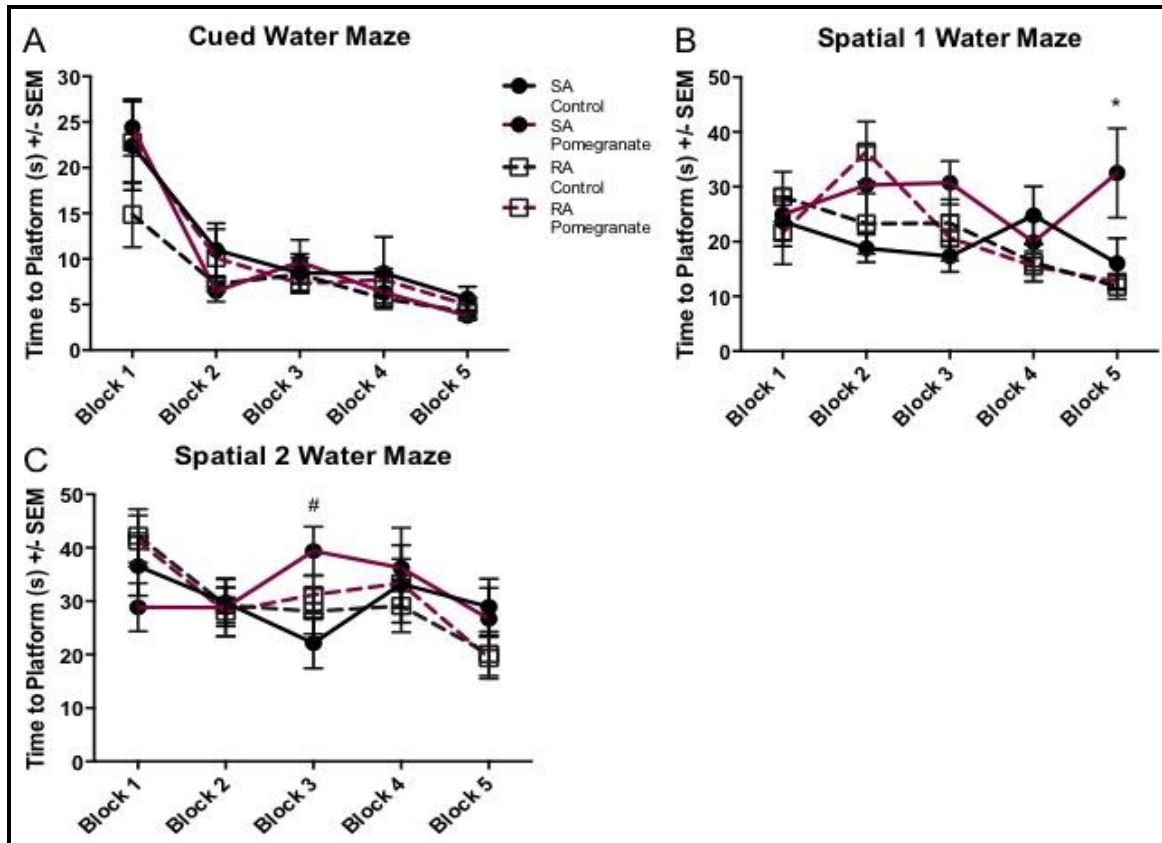
Figure 47. (A) Water treatment did not influence the amount of time SA and RA mice took to locate the visible platform during the cued water maze ($p > .05$); however, all mice learned the location of the platform by block 5 ($p < .05$). (B) Performance in the spatial 1 water maze improved across blocks; however, deficits began to emerge in SA mice with the pomegranate treatment by block 5. SA mice with the pomegranate treatment took longer to find the submerged platform than SA mice on the control treatment (*$p < .10$), RA mice on the control treatment (*$p < .05$), and RA mice on the pomegranate treatment (*$p < .05$). (C) Performance in the spatial 2 water maze improved across blocks ($p < .05$). Interestingly, SA mice on the pomegranate treatment trended towards taking longer to find the submerged platform on block 3 compared to SA mice on the control treatment (#$p = .05$).

A factorial ANOVA was conducted to determine whether treatment and injury influenced probe water maze performance. There was a trend towards a significant interaction between injury and treatment on the distance moved in the probe water maze, ($F(1, 23) = 3.67$, p = .07, power = .45; Figure 48A). Pomegranate treatment may have

impaired performance in SA mice, causing mice to search for the platform longer

compared to SA mice on the control treatment and RA mice on the pomegranate
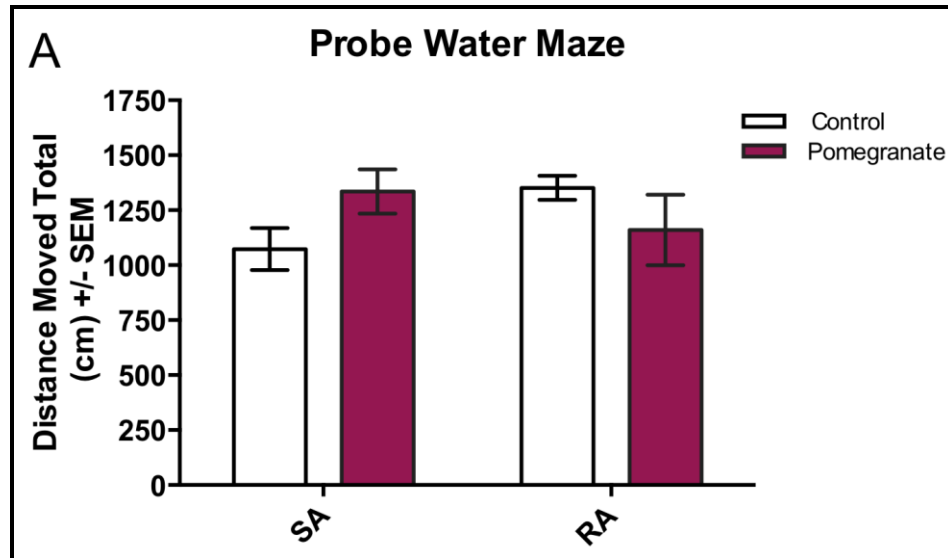
treatment.

Figure 48A. There was a trend towards a significant interaction ($p = .07$) such that SA mice on the pomegranate treatment may have searched for the platform longer compared to SA mice on the control treatment and RA mice on the pomegranate treatment.

Table 3. *Summary of behavioral findings and their associated p-values.*

Test	Summary of Behavioral Findings	p-value
Beam Balance	Overall, RA trended towards more left-sided slips than SA at 5 dpi	< .10
Zero Maze	SA spent more % of time in the lighter quadrants than RA	< .07
Water Maze	SA took longer to find the platform than RA in spatial 1	< .07
	SA with pomegranate treatment took longer to find the platform in spatial 1	< .05
	SA took longer to find the platform in block 5 of spatial 2	< .09
	SA with pomegranate treatment swam farther than RA with pomegranate treatment	< .08

Behavioral Correlations

Surprisingly, the pomegranate treatment seemed to have adverse effects leading to more risk-taking (anxiety-like) and depression-like behaviors in anesthesia mice. SA mice that consumed more of the pomegranate treatment spent a higher percentage of time immobile in the tail suspension test, suggesting increased depression-like symptoms from pomegranates, $r = .91$, $p = .01$ (Figure 49). Also, SA mice that consumed more of the pomegranate treatment spent a higher percentage of time in the lit quadrants of the elevated zero maze ($r = .87$, $p = .02$; Figure 50), suggesting anxiety-like and risk taking behaviors.
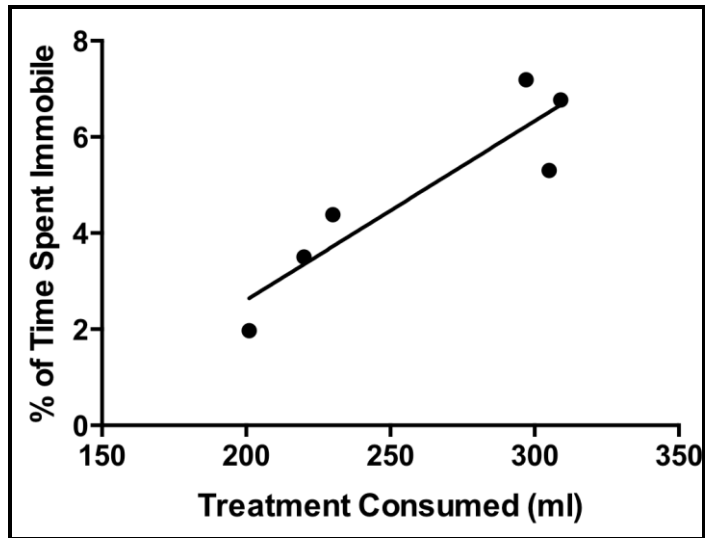
Figure 49. SA mice that consumed more of the pomegranate treatment spent a higher percentage of time immobile in the tail suspension test, $p < .05$.
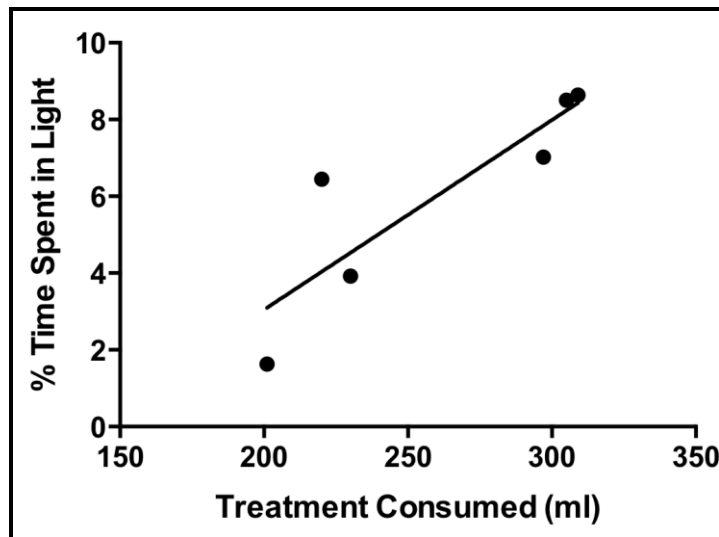
Figure 50. SA mice that consumed more of the pomegranate treatment spent a higher percentage of time in the lit quadrants in the elevated zero maze, $p < .05$.

There was a couple of interesting motor performance related correlations as well. Pomegranate treated SA mice that faulted more on average in the foot fault test, spent less time immobile in the tail suspension test, $r = -.82$, $p = .05$ (Figure 51). In addition, pomegranate treated mice that performed better on average in steady rotarod trials spent more time immobile in the tail suspension test, $r = .84$, $p = .04$ (Figure 52).
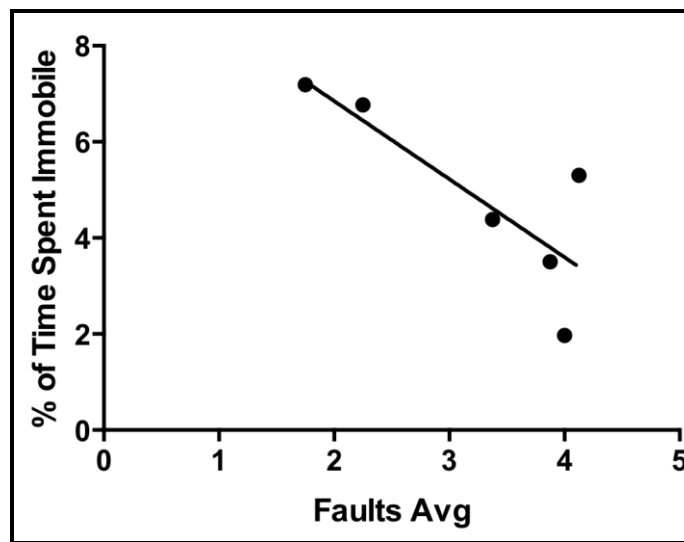
Figure 51. Pomegranate treated SA mice that faulted more in the foot fault spent less time immobile in the tail suspension test, $p < .05$.
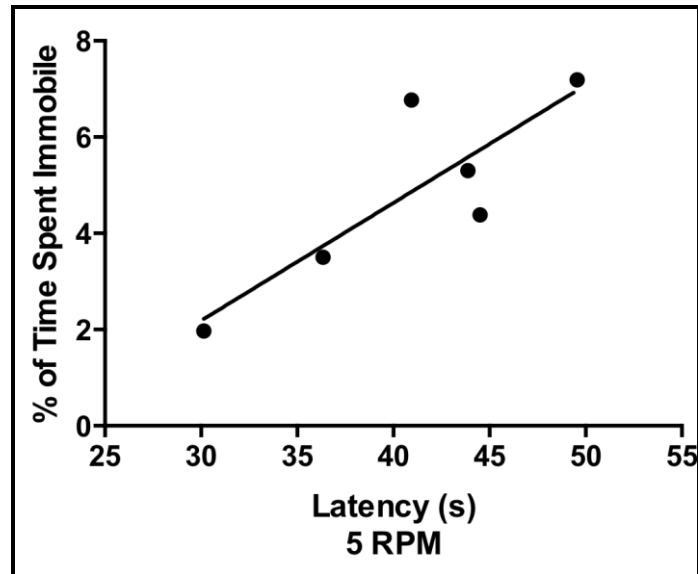
Figure 52. Pomegranate treated SA mice that performed better in the steady rotarod trials spent more time immobile in the tail suspension test, $p < .05$.

Interestingly, the pomegranate treatment seemed to improve motor performance in RA mice across tests. Mice that consumed more pomegranate treatment had a reduction in the number of faults per distance in the foot fault test, $r = -.71$, $p = .05$ (Figure 53). More pomegranate treatment consumption in RA mice also led to better average performance on the rotarod (stayed on the test longer), especially in the most challenging speed of the test, $r = .73$, $p = .04$ (Figure 54). In addition, pomegranate treated RA mice that spent more time in the perimeter of open field had better performance on the rotarod, especially in the most challenging speed of the test, $r = .74$, $p = .04$ (Figure 55).
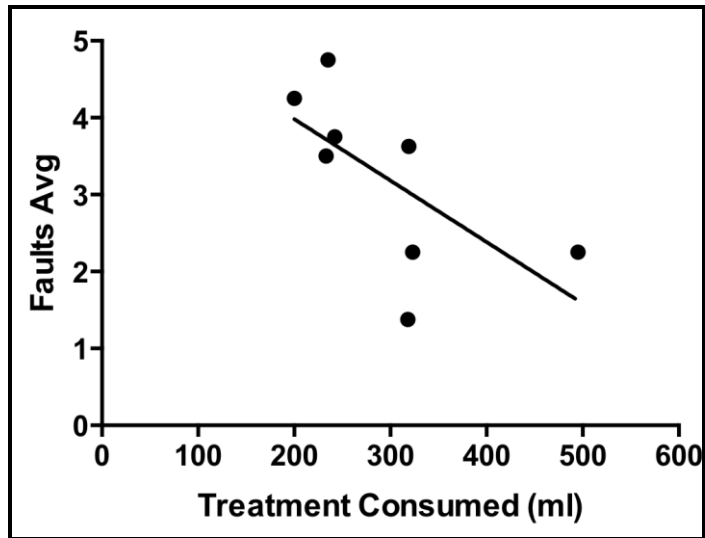
Figure 53. RA mice that consumed more pomegranate treatment made fewer faults in the foot fault test, $p = .05$.
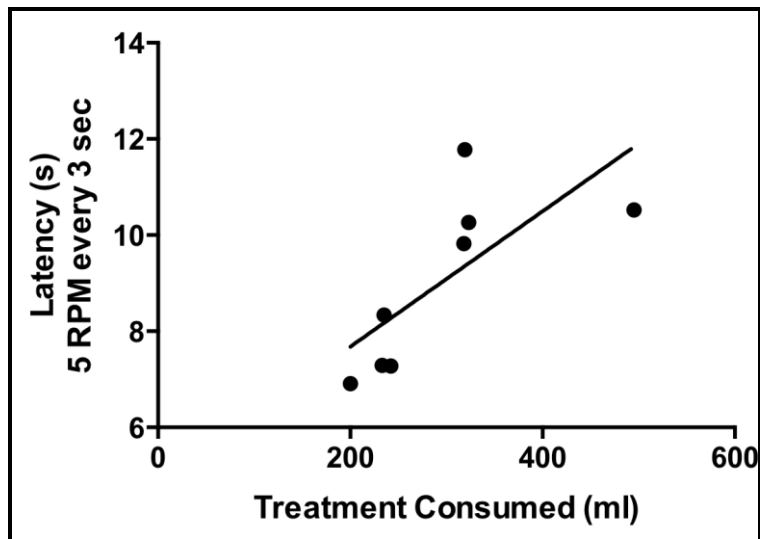
Figure 54. RA mice that consumed more pomegranate treatment had improved performance on average in the most challenging rotarod trials, $p = .05$.
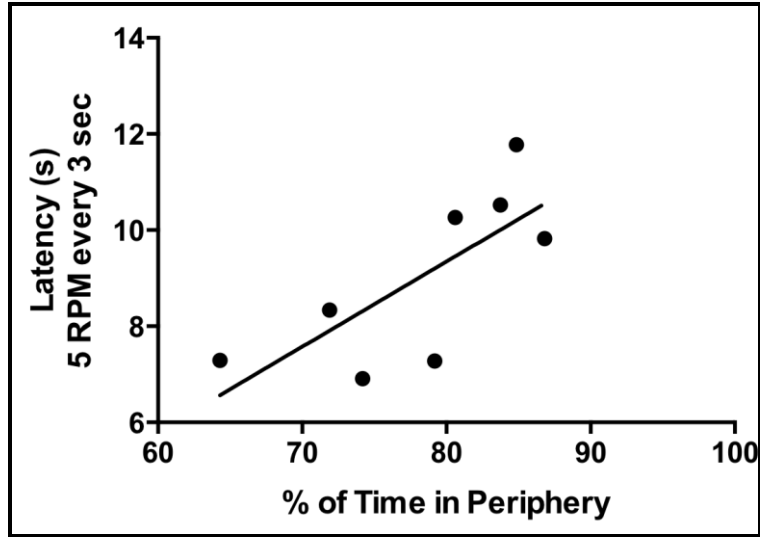
Figure 55. Pomegranate treated RA mice that spent more time in the periphery in open field testing had improved performance on average in the most challenging rotarod trials, $p < .05$.

CHAPTER FOUR

DISCUSSION

Effects of Repeated Concussion

Animal models have been developed to better understand the pathology and consequences of trauma, particularly mild injuries. The purpose of this study was to explore the effects of repeated brain insults on behavior, as well as the protective effects of pomegranate juice on injury-induced deficits. We hypothesized rmCHI would induce behavioral deficits and found that mice that received rmCHI did in fact evidence significant fine motor deficits. Findings from this study are consistent with several studies investigating mild brain injury.

Closed head injury (CHI) is a rodent model of TBI that is more representative of human mTBI/concussive conditions than the more invasive CCI and FPI models. The latter models require craniectomy, which may itself lead to cortical inflammation and behavioral changes (Mouzon et al., 2012). In the CHI model, the pneumatic or electromagnetic impact device drives a motorized to impact the skull directly. This model produces a mild injury that prevents cortical contusions, skull fractures, prolonged apnea, and also keeps mortality to a minimum (Mouzon et al., 2012). In addition, widespread damage to white matter and subsequent connectivity disruption lead to cognitive and neuropsychological impairments (Kim et al., 2014).

The diffuse nature of CHI leads to greater axonal injury over a large brain region, including the motor cortex (Eucker et al., 2014). Alterations in cell function such as excitotoxicity, calcium up-regulation, depolarization, and vulnerability of the blood brain

barrier are components of the secondary response that continue to promote global brain damage (Greve & Zink, 2009; Lakowski, Creed, & Raghupati, 2015). In a midline fluid percussion injury model, diffuse injury leads to the sensitization of microglia and the inflammatory system (Fenn et al., 2013). Our findings demonstrate that motor deficits may have emerged in rmCHI mice because of the brain's increased vulnerability to the initial insult, wherein the subsequent mCHI further exacerbated the brain's sensitivity to injury and contributed to the observation of behavioral deficits.

In a recent study published by our lab, we used a mild closed head injury (mCHI) model to characterize long-term behavioral deficits in young adult mice with a single mCHI and repeated mild CHI (rmCHI). We found that this model (1 mm depression at 40 psi) produced consistent mild rotational injuries that led to the emergence of late-appearing (90 days post-injury) motor and affective deficits (Bajwa, et al., 2016). The injury (2 mm depression at 4 m/s) in rmCHI mice for our study produced mild rotational injuries that led to acute motor and cognitive deficits. These deficits are consistent to several studies investigating repeated TBI.

We found motor deficits in the foot fault task and the beam balance test. However, we did not find hyperactivity or altered rotarod performance as reported in other studies on concussion. This may be due to the differences in our methodology. For example, Kane et al. (2012) used a repeated mild weight-drop TBI mouse model to induce brain injury. Their model may have produced a larger injury than our mild injury. In a study by Mouzon and colleagues (2012), researchers reported impaired rotarod performance after repeated concussions. Yet again, there were variations in the method they followed as compared to ours. In their study, they induced 5 injuries in 48-hour

intervals as opposed to our study where we induced 2 repeated concussions. In addition, their study induced concussion along the midline, whereas we induced bilateral concussions. The rotation of the brain, location of injury, and the pathophysiology in our model may significantly differ and this may explain why they found significant changes in rotarod performance where our study did not.

In our study, repeated CHI led to impaired performance in the water maze. These results are consistent with previous research by Creeley et al. (2004) and Mouzon et al. (2012). In the study by Creeley et al., they used an rmCHI model that included three weight-drop sessions 24 hours apart led. Their results demonstrated impaired water maze spatial learning performance in their subjects. (Creeley et al., 2004). Likewise, Mouzon et al. reported significant spatial learning deficits, where they used a model of repeated concussion where five injuries were given at 48-hour intervals.

We hypothesized that our injury model would result in significant differences in affective symptoms between injured animals and control animals. This hypothesis was based on several studies that demonstrated that brain lesions can contribute to affective outcomes., Frontal lobe and right hemispheric injuries are particularly associated with deviations from normal affective behavior (McDonald, Saykin, & McAllister, 2012). One study that included a repeated mild lateral FPI model revealed that rats subjected to 5 mild concussions demonstrated increased anxiety and depression-like symptoms as compared to comtrol rats. This injury model also produced short (24 hr) and long-term (8 weeks) cognitive impairments (Shultz et al., 2012). Another study that used a midline fluid percussion injury model demonstrated that diffuse injury led to depressive-like behavior at 7 dpi (Fenn et al., 2013). Depressive-like behavior was also manifested at 7

dpi as a result of diffuse injury in another study that used a weight-drop injury model (Milman et al., 2005). However, our model of rmCHI did not lead to increased affective symptoms. This may be attributed to differences in the severity and the pathophysiology of the injury. Repeated CHI mice had motor deficits, but the injury may have not been severe enough to produce ongoing widespread changes that lead to short-term affective symptoms, but may lead to the presentation of long-term depression-like symptoms as we have previously reported (Bajwa et al., 2016).

Effects of Anesthesia

Our results showed that anesthesia (single exposure 25 minutes or 2 exposures separated by 3 days) led to risk taking behaviors and impaired performance in learning tasks. Anesthesia is generally considered a safe intervention and is routinely used in many simple and complex surgical procedures performed in all age groups. Anesthesia acts on the central nervous system by interacting with synaptic transmission and inhibiting neuronal communication between several brain regions (Harrison 1993). Anesthetics are easily controllable and have few long-lasting side effects in clinical and experimental animal research. In research on the topic, it has been shown that single or repeated exposure to general anesthesia resulted in no spatial learning performance changes in young or aged mice when tested less than 24 hours after anesthesia (Butterfield, Graf, Ries, & MacLeod, 2004). In fact, isoflurane has been found to be neuroprotective and may reduce excitotoxicity in rodents (Burchell, Dixon, Tang, & Zhang, 2013; Harada, Kelly, Cole, Drummond, & Patel, 1999; Sakai et al., 2007; Statler et al, 2006).

However, our results are not without precedence: some studies have also reported detrimental effects of anesthesia. Some research findings suggest that despite such widespread use, isoflurane administration can lead to various pathophysiological and cognitive alterations (Jevtovic-Todorovic et al., 2003). Isoflurane reduces the activity of action potentials by causing a reduction in conductance by altering the rate that gap junctions open and close. Too much isoflurane can reduce neuronal signaling, eventually leading to loss of communication and cell death. Some studies show that isoflurane alters metabolic processes such as hypoglycemia (Loepke, McCann, Kurth, & McAuliffe, 2006), cerebral blood flow (Reinstrup et al., 1995; Schlunzen, Cold, Rasmussen, & Vafaee, 2006; Li, Patel, Wang, & Zhang, 2014), induces neurotoxicity leading to both neuronal apoptosis (Kawaguchi et al., 2004; Sinner, Becke, & Engelhard, 2014) and glial apoptosis (Brambrink et al, 2012; Creeley et al., 2014). These physiological changes have been found to impair short-term memory (Ramage et al., 2013), long-term memory, and increase learning deficits (Rothstein, Simkins, & Nunez, 2008; Lee, Chan, Hazarika, Vutskits, & Sall, 2014). Studies have also reported acute spatial learning deficits (< 8 days) in adult rats exposed to a single anesthesia treatment. (Cao et al., 2015; Carr, Torjman, Manu, Dy, & Goldberg, 2011). Another study reported that adult rats tested 4 weeks after 4 hours of isoflurane exposure had poor long-term retention of the platform's location in the water maze, whereas single isoflurane exposure did not affect performance (Callaway, Jones, & Royse, 2012). These findings suggest that behavioral impairments are present several weeks after significant exposure of isoflurane. Our findings suggest that isoflurane may have caused increased cellular apoptosis and associated degeneration that led cognitive deficits (risk-taking behaviors). Thus, an anesthesia control may not be

a suitable control for brain-injured animals. Future studies should also incorporate naïve

animals (without anesthesia) as a control.

Effects of Pomegranate Juice

Mice received pomegranate juice in their drinking water and pomegranate juice is

known to be high in antioxidant and anti-inflammatory properties. We hypothesized that

pomegranate juice supplementation would lessen or even ameliorate the degree of

behavioral deficits induced by rmCHI. Our findings reveal that pomegranate juice led to

fewer motor deficits in rmCHI mice, but not repeated anesthesia mice.

Some studies have reported improved cortical integrity and behavioral outcomes

with pomegranate treatment. Alzheimer's-like transgenic mice that consumed a

pomegranate-enriched water for 6 months found the submerged platform faster in the

water maze (spatial learning tasks) than mice that consumed the control water (Hartman

et al., 2006). In another study from our lab, pomegranate juice treatment for 10 weeks

ameliorated irradiation induced depression-like behavior and improved motor

performance in the rotarod in male mice (Dulcich & Hartman, 2013).

Another study by Loren, et al. showed that maternal supplementation with

pomegranate juice influenced mice pup outcome after brain injury (2005). These pups

had less cortical tissue loss resulting from neonatal stroke when exposed in utero to

pomegranate juice, regardless of dose. Interestingly, these mice were on the treatment for

a total of 15 days and found significant improvements, whereas in our study, we found

only subtle motor improvements from pomegranate juice administration. These

differences may be attributed to the fact that mice were exposed to juice in utero during

critical development periods that may have primed cells against inflammation and injury.

Another reason why we may not have been able to reproduce the beneficial results of other research and those produced by our lab might be because of the duration of treatment in our study. Perhaps, longer treatment duration for adult mice are necessary to see significant improvement in behavior, compared to a shorter duration in young mice.

We also reported that pomegranate treatment worsened performance in the water maze (spatial 1) in rmCHI mice. It is likely that these findings may have been a spontaneous effect. There is also research that suggests the flavonoids found in pomegranate juice are metabolized extensively in the body and, as a result, lower levels of flavonoids may pass through the blood brain barrier to reduce oxidative species (Spencer et al., 2009). The levels of flavonoids that pass through the blood brain barrier may be attributed to individual differences in animals, regardless of the amount consumed.

Additionally, the effectiveness of the pomegranate treatment in our injury model may have been influenced by a hormetic response. Hormesis has been widely researched in the toxicology field; it is defined as "a process in which exposure to a low dose of a chemical agent or environmental factor that is damaging at higher doses induced an adaptive beneficial effect on the cell or organism" (Mattson, 2008). Hormesis is an integral part to the normal physiological function of cells and organisms. The response of a cell or organism to the low dose of the toxin is considered an adaptive compensatory process following an initial disruption in homeostasis (Mattson, 2008). As the disruption occurs, a hormetic stress response is triggered, activating cellular defense mechanisms

that increase antioxidant pathways and proteins associated with cellular survival (i.e. Nrf2 and NFκB) (Luna-Lopez, Gonzalez-Puertos, Lopez-Diazguerrero, & Konigsberg, 2014). Preconditioning ischemia is one of the most common experimental models of hormesis. This occurs when the heart or brain are subjected to a brief period of mild ischemia. During this injury phase, cells become resistant to cellular apoptosis induced by a larger event (i.e. stroke) (Pong, 2004; Yellon & Downey, 2003). Interestingly, ischemia exhibits a dose dependent response, in that brief periods of mild ischemia may be neuroprotective and longer periods result in cellular damage (Mattson, 2008).

As it relates to our research, the first impact in rmCHI may have triggered a neuroprotective response. The second impact was given in a short time frame relative to first impact while the brain had not fully recovered and remained in an inflammatory state. We believe the second impact (3 days later) exacerbated the oxidative and inflammatory response (increased microglia), such that it eliminated the antioxidant and/or anti-inflammatory pathways that were ongoing as a result of pomegranate treatment.

The inflammatory response is triggered as a result of injury or the invasion of pathogens. Inflammation in the central nervous system is characterized by increased blood brain barrier permeability, macrophage activation, glial activation, and the release of cytokines. During injury, microglia are recruited to the site and undergo a series of morphologic and phenotypic changes that attempt to re-establish homeostasis. The increased microglial response may have caused an increase in the release of pro-inflammatory cytokines and proteases. This study has yet to determine whether pomegranate juice influenced the microglia after rmCHI. These results may provide some

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insight on whether pomegranate juice attenuated inflammation in animals and perhaps these effects have yet to be translated to altered cellular changes (behavior). Several studies have reported several ways pomegranates and its derivatives affect inflammatory cascade in the brain. Pomegranate extract has been shown to treat inflammation and attenuate the release of TNFα (pro-inflammatory cytokine) from microglial cells (Jung et al., 2006) and ellagic acid has been shown to reduce oxidative stress, reactive nitrogen species (Priyadarsini et al., 2002), and the release of IL-6 and IL-1β inflammatory cytokines (Farbood et al., 2015).

Future Directions

Determining the specific mechanisms and activation of anti-inflammatory pathways by pomegranate juice in the brain and behavior is beyond the scope of the study. Future projects should determine the levels of oxidative stress, apoptosis, and neurogenesis. In addition, a dose dependent that assess different dilutions of pomegranate juice in our model of repeated concussions should be conducted. These directions may provide critical insight to how pomegranate juice may influence mild brain injuries.

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