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NEUROLOGICAL, COGNITIVE, AND MOLECULAR EFFECTS OF MITOQUINONE IN OPEN HEAD TRAUMATIC BRAIN INJURY MOUSE MODEL AT CHRONIC TIME-POINT

by
MUHAMMAD ALI HUSSEIN HAIDAR)

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MUHAMMAD ALI HUSSEIN HAIDAR

Approved by:

Firas Kobeissy

Dr. Firas Kobeissy, Assistant Professor — Advisor
Department of Biochemistry and Molecular Genetics

Dr. Aida Habib, Professor — Member of Committee
Department of Biochemistry and Molecular Genetics

Nadine Darwiche

Dr. Nadine Darwiche, Professor — Member of Committee
Department of Biochemistry and Molecular Genetic

Dr. Riyad El-Khoury, Assistant Professor — Member of Committee
Pathology and Laboratory Medicine, AUBMC

Date of thesis defense: [8, 2021]

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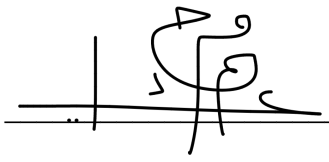

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ABSTRACT
OF THE THESIS OF

Muhammad Ali Hussein Haidar for Master of Science
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Title: Neurological, Cognitive, and Molecular Effects of Mitoquinone in Open Head Traumatic Brain Injury Mouse Model at Chronic Time-Point

Traumatic brain injury (TBI) is a heterogeneous disease in terms of its origin, pathology, and prognosis. Globally, it is one of the leading causes of death and long-lasting disability. TBI is classified according to the mechanism of injury and its severity. TBI can be either an open head injury where the skull is fractured, also known as penetrating brain injury (PBI), or a closed head injury where the skull remains intact. TBI exerts its effects through two major, dynamic, and overlapping events denoted as the primary and the secondary injuries. The primary injury is the result of the mechanical force exerted, leading to the disruption and necrosis of neural tissue, hemorrhage, and axonal damage. Pathological changes trigger then the secondary injury, initiating a cascade of metabolic events that include further excitotoxicity, neuroinflammation, disruption of the blood-brain barrier (BBB), and cell death. Notably, mitochondria play an important role in the pathology of TBI via reactive oxygen species (ROS) overproduction creating a state of oxidative stress and via apoptosis induction. Finding therapies for TBI, and PBI specifically, remains one major challenge since there are currently no FDA-approved drugs. Some suggested treatments target oxidative stress since antioxidants have been shown to ameliorate the pathology of TBI. An example of this, is the mitochondria-targeted drug called Mitoquinone (MitoQ). It is synthesized by conjugating a ubiquinone moiety to a triphenylphosphonium cation (TPP+). Previous studies from our lab have shown that MitoQ improves behavioral and cognitive impairments, oxidative stress, and neuro-inflammation in a model of repetitive mild TBI at acute, subacute and chronic time-points. So, this study aimed at investigating the effect of MitoQ supplementation on neurological, behavioral, and molecular functions 30 days post-open head TBI mouse model. It found that MitoQ reduces long-term effects of open head TBI by decreasing the state of oxidative stress and enhancing neurological and behavioral sequences. This is along with dampening the chronic activation of astrocytes and microglia, leading to a reduced inflammatory state.

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ABBREVIATIONS

AD: Alzheimer’s disease

ADP: Adenosine diphosphate

AIF: Apoptosis inducing factor

ANT: Adenine nucleotide translocase

ARE: Antioxidant response elements

ATLS: Advanced trauma life support

ATP: Adenosine triphosphate

BBB: Blood-brain barrier

CAT: Catalase

CCI: Controlled compact impactor

CoQ: Coenzyme Q

CSF: Cerebrospinal fluid

CTE: Chronic traumatic encephalopathy

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ETC: Electron transport chain

FDA: Food and drug administration

FST: Forced swim test

GCS: Glasgow coma scale

GFAP: Glial fibrillary acidic protein

HO: Heme-oxygenase

Iba-1: Ionized calcium-binding adaptor protein 1

IMM: Inner mitochondrial membrane

IMS: Intermembrane space

IP: Intraperitoneal

LOC: Loss of consciousness

MBP: Myelin basic protein

MitoQ: Mitoquinone

MMP: Mitochondrial membrane permeabilization

MTP: Membrane transition pore

MWM: Morris water maze

NADH: Nicotinamide adenine dinucleotide

NMDA: N-methyl-D-aspartate

NQO-1: NAD(P)H dehydrogenase

Nrf2: Nuclear factor erythroid 2 (NFE2)-related factor 2

NSE: Neuron specific enolase

NF: Neurofilament

OCD: Obsessive compulsive disorder

OMM: Outer mitochondrial membrane

PBI: Penetrating brain injury

PBS: Phosphate-buffered saline

PFA: Paraformaldehyde

PTA: Post-traumatic amnesia

PTPC: Permeability transition pore complex

PTSD: Post-traumatic stress disorder

QPCR: Quantitative polymerase chain reaction

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

RT-PCR: Real time polymerase chain reaction

Smac: Second mitochondria-derived activator of caspase

SOD: Superoxide dismutase

TBI: Traumatic brain injury

TOM: Translocase of outer membrane

TPP+: Triphenylphosphonium cation

VDAC: Voltage-dependent anion channel

$\Delta\Psi m$: Mitochondrial transmembrane potential

CHAPTER I

INTRODUCTION

Traumatic brain injury (TBI) is a heterogenous neurological disease in terms of its origin, pathology, and prognosis. Globally, TBI is one of the leading causes of death and long-lasting disability. Survivors suffering from disabilities are often forced to endure major socioeconomic burdens as well. In 2010, the economic impact of TBI in the United States was estimated to be $76.5 billion in direct and indirect costs [1]. TBI is classified according to two categories: a) the mechanism of injury and b) its severity. According to the former, TBI can either be an open head injury where the skull is fractured, also known as penetrating brain injury (PBI), or a closed head injury where the skull remains intact [2]. As for the latter category, TBI can be severe, moderate, or mild, usually assessed using the Glasgow Coma Scale [3]. PBI, which represents 12% of all TBI cases, is the most lethal form of traumatic brain injury, with gunshot wounds being the most common cause of mortality [4]. Only 10% of PBI patients survive to reach the emergency department, half of which ultimately cannot manage to stay alive while the other half often lives with major long-term neurological sequelae [5].

TBI leads to neurological complications through two major events, dynamic and overlapping, denoted as the primary and the secondary injuries. The primary injury is the result of the mechanical force which leads to the disruption and necrosis of neural tissue, hemorrhage, and axonal damage [6]. The mechanical damage herein causes an ionic imbalance where calcium (Ca^{2+}) and glutamate increase inside the cell [7]. The resultant excitotoxicity leads to an energetic crisis and oxidative stress through increasing the production of reactive oxygen species (ROS) [8]. Oxidative stress can directly stimulate the release of cytokines and pro-inflammatory factors contributing to

an increased inflammatory state [9, 10]. Following this, the secondary injury is triggered, initiating a cascade of metabolic events that include further excitotoxicity, neuroinflammation, disruption of the blood-brain barrier (BBB), and cell death [11]. Notably, mitochondrial dysfunction plays an important role in the pathology of TBI via the excessive production of ROS, leading to apoptotic cell death. This comes secondary to the disruption in their membrane along with a disturbance between mitochondrial fission and fusion balance [12].

Finding therapies for TBI remains one major challenge as there are currently no FDA-approved drugs for TBI therapy. Present treatments mainly target the symptoms of the injury. Some suggested treatments target oxidative stress since antioxidants have been shown to ameliorate the pathology of TBI [13, 14]. An example of such drugs is the mitochondria-targeted drug called Mitoquinone (MitoQ). It is synthesized by conjugating a ubiquinone moiety to a triphenylphosphonium cation (TPP+) [15]. MitoQ can easily cross the BBB [16] and acts by activating the nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) pathway [17]. Previous studies at our lab have shown that MitoQ improves behavioral and cognitive impairments, oxidative stress, and neuro-inflammation in a model of repetitive mild TBI at acute, subacute, and chronic time-points. We hypothesize that MitoQ is effective in ameliorating the long-term behavioral, cognitive, and molecular outcomes of PBI.

CHAPTER II

LITERATURE REVIEW

A. Definition and Epidemiology

TBI is a common and devastating health concern that inflicts hundreds of individuals each second [18]. TBI occurs when the brain is subjected to a direct mechanical insult like stretching, compression, and tearing of the brain parenchyma leading to primary cell loss and biochemical changes [6]. The incidence of TBI is estimated to be 939 in 10,000 worldwide with major causes being falls, vehicle accidents, wars, and sports [19]. The mortality rates worldwide are assessed to be between 7% and 23%, 90% of which are in developing countries [20]. TBI imposes a massive financial load with an annual global cost of 400 billion dollars [21]. Studies in the Middle East concerning TBI are limited; however, it was predicted that the incidence in the region is 45 per 100,000 [20]. A recent systematic review showed that the TBI mortality rate in the MENA region was at 12.9% with leading causes of death being motor vehicle accidents (MVA) and military injuries [22]. TBI studies in Lebanon are not well established. However, 682 cases of TBI were reported in the past 30 years with a mortality rate of 1.6% [23].

B. Penetrating Brain Injury (PBI)

PBI is caused by a foreign object piercing the skull and extending to the underlying brain's parenchyma resulting in focal damage [24]. Penetrating brain injury can be classified into two subcategories according to the velocity of the object. This results in either high-velocity injuries like in the case of bullets and shockwaves or low-velocity injuries like in the case of knife stabs [25]. Penetrating head injury is less

prevalent than a closed head injury, but it results in the worst outcomes [26, 27]. Symptoms associated with this injury are physical, cognitive, and psychiatric. Physical symptoms include loss of consciousness, headache, nausea, vomiting, convulsions or seizures, weakness or numbness in toes and fingers, and loss of coordination. Cognitive symptoms include disturbed attention and confusion, agitation, combativeness, and coma [28]. As for psychiatric complications, they include major depression, panic disorder, generalized anxiety disorder, phobia disorders, sleep disorder, aggression, poor impulse control, irritability, anhedonia, and apathy [29].

The development of neuropsychological and cognitive sequelae post-brain injury is a multifactorial process. The behavioral changes after TBI may also be manifested as personality changes like aggression, impulsivity, irritability, emotional lability, or apathy [30-32]. Major depression is the most reported neuropsychiatric consequence of TBI with a prevalence of 25%-50% [33-35]. A prospective multicenter study done by Seel *et al*. showed that fatigue, irritability, rumination, and distractibility are the most common depressive symptoms [36]. Notably, however, the link between the severity of depression and TBI is still not clear [37]. In a prospective cohort study of Group Health Cooperative of Puget Sound members, computerized records of 939 health plan members who were diagnosed with TBI showed that the risk of developing psychosis after moderate to severe TBI increases progressively one to three years post-injury [38]. In addition, TBI is a risk factor for many psychiatric disorders like post-traumatic stress disorder (PTSD), obsessive-compulsive disorder (OCD), and panic disorders [39]. On the long run, TBI is linked to neurodegenerative diseases such as Alzheimer's Disease (AD), cytoskeletal changes, and persistent neuro-inflammation [40-42]. Studies performed on animal models revealed that memory impairments,

assessed via Morris Water Maze (MWM) test, are evidently starting at a month or even up to a year post-injury [43-45].

C. Neuropathology of PBI

1. Excitotoxicity

The primary mechanical injury disrupts the BBB leading to an ionic imbalance via the influx of calcium (Ca^{2+}) and sodium (Na^{+}) ions [7]. This is accompanied by the secretion of excitatory amino acids and neurotransmitters like glutamate, acetylcholine, norepinephrine, and serotonin to the extracellular space. Upon this release, post-synaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-D-aspartate) receptors are overexcited leading to further, non-physiological elevation in Ca^{2+} [46]. Excessive amounts of Ca^{2+} activate an array of catalytic enzymes that damage the mitochondrial membrane, i.e. phosphatases and phospholipases, and fragment the DNA, i.e. endonucleases, leading to necrosis and apoptosis [47]. One important protein affected by this Ca^{2+} influx is the membrane transition pore (MTP), a mitochondrial Ca^{2+} dependent pore, that when activated for a long time, it leads to an elevation in mitochondrial permeability. The increased permeability, in turn, causes the uncoupling of the electron transport chain (ETC), resulting in dampened ATP production [48]. These metabolic changes in the mitochondria lead to an excessive release of ROS and initiate a state of oxidative stress that contributes to the secondary mechanisms post-injury.

2. Oxidative Stress

Oxidative stress represents a state where oxygen-derived free radicals—superoxide anions ($O2^{-}$), hydroxyl (OH^{-}), and peroxyl (ROO^{-}) radicals—overwhelm

the scavenging antioxidant system, playing a major role in the secondary injury post-TBI [49]. Superoxide (O^{2-}) is the most abundant ROS and is mainly produced by the mitochondrial complexes I and III [50]. During the first few minutes and hours after injury, several mechanisms contribute to the production of superoxides like the auto-oxidation of biogenic amine neurotransmitters, mitochondrial leak facilitated by MTP, xanthine oxidase activity, and the oxidation of extravasated hemoglobin [51]. At later time points, activated microglia, astrocytes, infiltrating neutrophils, and macrophages provide additional sources of superoxide.

In the presence of H^{+}, superoxide is reduced to H_2O_2 by the enzyme superoxide dismutase (SOD). SOD exists in three forms: SOD1 in the cytosol, SOD2 in the mitochondria, and SOD3 in the extracellular space [52]. H_2O_2 is more stable than O^{2-} and can diffuse through the cellular membranes to be deactivated by catalase (CAT) that converts it to water (H_2O) and O_2. In addition, glutathione peroxidases are considered important antioxidative enzymes that facilitate the reduction of H_2O_2 to yield glutathione and H_2O [53].

When the antioxidative system is overwhelmed, ROS elevation leads to several consequences that induce inflammatory processes and early or late apoptotic programs. Brain tissue is specifically vulnerable to oxidative damage due to its high oxidative metabolic activity and relatively low antioxidant capacity[54]. Studies have shown that superoxide and nitric oxide are able to form peroxynitrite which is a potent oxidant involved in the impairment of cerebral vascular function after injury [55-57]. The consequentially altered circulation and ischemia result in perturbed oxygenation in brain tissue. Also, the production of these radicals impairs energy metabolism in cells resulting in further excitotoxicity [55]. This state of oxidative stress is usually followed

by lipid peroxidation characterized by the breakdown of polyunsaturated fatty acids in lipid membranes resulting in disturbed ionic gradients and leading to membrane lysis. To counteract this ionic imbalance, membrane ionic pumps are activated leading to increased uptake of glucose, depletion of energy stores, and further influx of Ca^{2+} into the mitochondria. The resultant disrupted metabolism leads to lactate production, acidosis, and edema. Eventually, DNA oxidation and fragmentation and protein nitration take place leading to cell death via apoptosis or necrosis [6].

3. Neuroinflammation

Mechanical injury leads to the secretion of neuropeptides like substance P which leads to the activation of resident microglia and astrocytes [58]. These cells start secreting interleukin and cytokines that in turn activate the complement system in an attempt to protect brain tissue. However, this activation increases the recruitment of neutrophils, monocytes, and leukocytes across the disrupted blood-brain barrier (BBB) leading to the secretion of prostaglandins, chemokines, and cell adhesion molecules [59, 60]. Among these cytokines are the tumor necrosis factor-alpha (TNF- α) and the interleukins IL-1β, IL-6, and IL-12 [61]. Although several cell types can produce pro-inflammatory cytokines, microglia are the major contributors to the production of molecules involved in the initiation and sustenance of the neuroinflammatory cascade [62]. Notedly, it is plausible that peripheral macrophages, especially upon the disruption of BBB during PBI, can infiltrate brain tissue and differentiate into microglia to reinforce inflammatory pathways [62]. Despite this fact, microglia in the injured brain also produce anti-inflammatory mediators and scavenger cellular debris and control neuroprotective processes to promote neurological recovery [63]. However, the excess

production of pro-inflammatory molecules versus anti-inflammatory molecules, especially in the chronic activation of microglia, is what tips the balance towards exacerbating brain damage. For example, it has been shown that levels of the proinflammatory cytokines interleukin 1 beta (IL-1β), IL-6, IL-17, tumor necrosis factor-a (TNF-a), interferon-γ (IFN-γ), and as well as the chemokines macrophage chemotactic protein-1 (MCP-1)/CCL2, macrophage inflammatory protein 2 (MIP-2)/CCL4, chemokine (C-C motif) ligand 5 (CCL5) are significantly increased while levels of the anti-inflammatory cytokines IL-4, IL-10, IL-13, and transforming growth factor-β1 (TGF-β1) are reduced in the injured brain of TBI rats [62].

These dual roles of microglia may be accounted for by their polarization state and functional responses after injury. It has been suggested by recent reports that microglia not only differentiate into M1 (a classical pro-inflammatory state) or M2 (an alternative anti-inflammatory state) phenotype but also mixed phenotype over time after TBI[64]. Specifically, early after CCI, microglia express both M1-and M2-type phenotypic markers in mice[64]. But the transient upregulation of the M2-type phenotype is followed by a predominant M1-type or mixed (Mtran) phenotype that expresses high levels of reactive oxygen species-producing nicotinamide adenine dinucleotide phosphate oxidase (NOX2) at the site of injury[65]. This has been shown to be accompanied by ongoing cortical and hippocampal neurodegeneration [63, 65].

On the other hand, another important cell type that plays a role in neuroinflammation is astrocytes. There is increasing evidence that astrocytes exhibit structural, molecular, and functional diversity in healthy CNS and responses to CNS insults[66]. Their activation is firstly initiated by the mechanical injury that takes place during PBI. This is because astrocytes express mechanotransducing ion channels and

non-traditional, stretch-sensitive cation channels such as NMDA receptors and big potassium (BK) 5channels, which may all contribute to the rapid influx of extracellular Ca^{2+} and Na^{+} observed in physically stressed astrocytes[67]. *In vitro* studies have shown that astroglial cells respond diversly to physical strain upon their plasma membrane stretching, which includes initiation of mitogen-activated protein kinase and protein kinase B (AKT) signaling, elevations in intracellular calcium, and adenosine triphosphate (ATP) release [68-70].

Astrocytes can both respond to and produce many immunomodulatory molecules, including cytokines, chemokines, and inflammatory mediators such as danger-associate molecular patterns (DAMPs) and alarmins [67]. It is also noteworthy that astrocytes contain dense networks of intermediate filaments such as glial fibrillary acidic protein (GFAP). TBI-associated mechanical strain transduced by these flexible intermediate filament networks could serve to encode the severity of astrocyte deformation[67]. GFAP and other intermediate filaments like vimentin and nestin are markedly upregulated following brain trauma and stroke [71].

Persistent neuroinflammation in TBI patients may contribute to progressive and long-lasting impairments in their physical, cognitive, behavioral, and social performance signal pathway [7]. Neuroinflammation can become maladaptive over time, especially when macrophages, microglia, and astrocytes remain in an inflammatory state in the brain for months or years post-injury and acquire abnormal functions [62]. Chronic traumatic brain inflammation manifested by extensive microglial and astroglia activation may be the most important cause of post-traumatic neurodegeneration including chronic traumatic encephalopathy (CTE) [62].

4. Cell Death

Cell death can either result from necrosis or apoptosis [72]. Apoptosis is defined as programmed cell death mediated by the mitochondria and result in internucleosomal DNA fragmentation. It can be mediated via intrinsic or extrinsic pathways [73]. The extrinsic pathway is mediated by transmembrane receptors including the tumor necrosis factor (TNF) superfamily [74]. The most studied receptors of which are FASR and TNFR1 receptors [75, 76]. It can also be either caspase-dependent, where caspase 3 and caspase 12 are involved [77], or caspase-independent, where apoptosis-inducing factor (AIF) initiates the signal without the involvement of enzymes[78]. These apoptotic events are controlled by the Bcl-2 family that contains pro-apoptotic members such as Bax, Bak, Bid, and Bim, and anti-apoptotic members such as Bcl-2 and Bcl-XL [77]. These modulators are subjected to disruption post-TBI leading to neuronal death [77].

Accumulated preclinical studies have revealed that neuronal cell death was observed in the pericontusional region and the hippocampus [79, 80]. Interestingly, using the moderate lateral fluid-percussion brain injury model, apoptotic cells in the injured cortex were observed at as early as 24 h, whereas in the hippocampus and the thalamus, the apoptotic response was delayed, peaking at 48 h and 2 weeks after injury, respectively [81]. In contrast, using the CCI model, neuronal apoptotic cells were the most apparent in the contusional region and hippocampus at between 24 and 48 h after injury[81, 82]. Therefore, the type, extent, and spatiotemporal distribution of neuronal apoptosis could be related to injury type and severity.

The excitotoxicity resulting from the injury contributes to the initiation of cell death pathways. Intracellular Ca2+ concentration increases and initiates cytoplasmic and nuclear events, involving the intrinsic apoptotic pathway[83]. An immediate

response to increased intracellular Ca^{2+} is the activation of neutral proteases, calpain, which have been implicated in the cleaving of Bcl-2 interaction domain (bid) and its truncated active form (tBID) in ischemic stroke [84]. Elevated intracellular Ca2+ is also associated with activation of the caspase gene family, leading to the induction of apoptosis [83]. Ca2+-dependent endonucleases are also activated following TBI, causing DNA damage in the form of the orderly chromatin cleavage patterns which are typical of apoptosis.

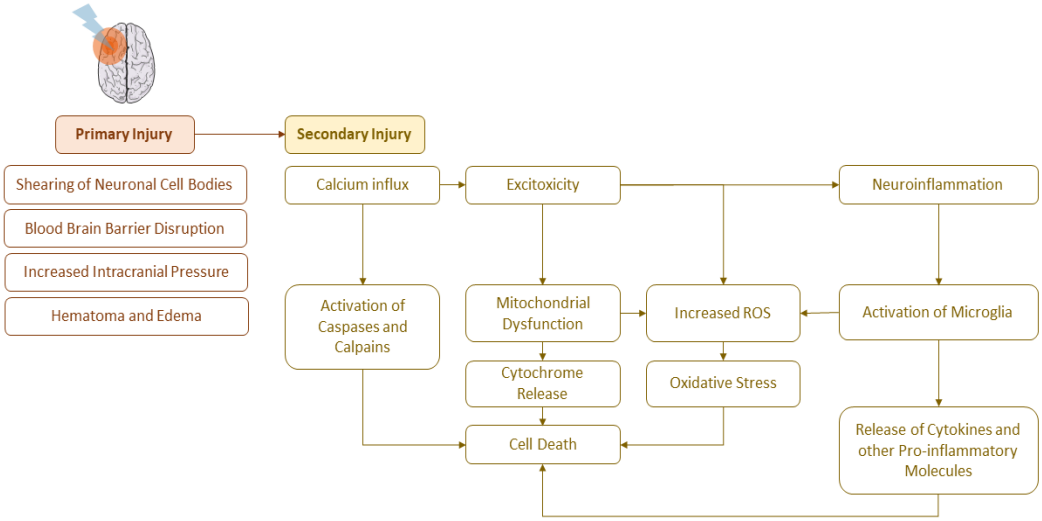

Figure 1 Neuropathology of PBI

D. The Mitochondrial Structure

The mitochondria are organelles found in all cells and possess two membranes: the outer mitochondrial membrane (OMM), an inner mitochondrial membrane (IMM), and three distinctive structures: intermembrane space (IMS), intra-Cristal space, and the matrix [85]. The OMM is highly permeable to molecules of less than 5kDa that can

diffuse freely across it through integral proteins called porins [86]. The most abundant OMM porin is the voltage-dependent anion channel (VDAC) which facilitates the import of several molecules including ATP, Ca^{2+}, and other ions [3]. The transportation of larger proteins occurs through the translocase of the outer membrane (TOM), which is a multi-subunit protein [87]. As for the IMM, it forms many folds known as cristae that extend to the matrix of the mitochondria and is highly impermeable [88]. Notably, most molecules need specific membrane transporters to reach the matrix [89]. The impermeability of the IMM and its electrochemical gradient is generated from the basis of the mitochondrial transmembrane potential ($\Delta\Psi m$) used to drive ATP synthesis by ATP synthase or Complex V of the electron transport chain (ETC) [90].

The electron transport chain (ETC) is a component of IMM and consists of five complexes. Electrons from nicotinamide adenine dinucleotide (NADH) enter the ETC at complex I and are then transferred through the membrane via coenzyme Q (CoQ) or ubiquinone to complex III. From complex III, electrons are carried by cytochrome B (CytB) and CytC to complex IV. At complex IV, electrons are eventually transferred to oxygen (O_2). Complex II receives electrons from succinate, a component of the citric acid cycle, and transfers them to Flavin adenine dinucleotide ($FADH_2$) then to CoQ. The energy resulting from this transport creates a proton (H^+) gradient across IMM which is utilized by complex V for ATP synthesis by the phosphorylation of adenosine diphosphate (ADP) [50, 91].

IMM contains the mitochondrial fission and fusion proteins that are indispensable for the proper functioning of cells in response to local changes [88]. A channel known as permeability transition pore complex (PTPC) is formed at the junction between IMM and OMM. It is composed of several elements including the

ADP/ATP carrier adenine nucleotide translocase (ANT) which belongs to IMM, VDAC, and cyclophilin D (Cyp D) [89, 92, 93].

Furthermore, IMS contains several proteins, some of which play integral roles in cell death such as CytC, AIF, the second mitochondria-derived activator of caspase (Smac), and its homolog DIABLO, and the mitochondrial serine protease OMI/HtrA2 [93]. CytC can also be bound to mitochondrial lipids, mainly to cardiolipin, and it plays an integral role in ETC [94]. AIF is a mitochondrial redo-oxidative enzyme responsible for detoxification of ROS and maintenance of complex I of the ETC [95]. The matrix contains most proteins in the mitochondria, including enzymes of metabolic pathways, and mitochondrial DNA, which encodes ribosomal subunits, CytC subunits, and ATPase subunits [96].

E. Antioxidant Therapy: Mitoquinone

The optimal treatment of penetrating brain injury demands adequate comprehension of the pathology of the injury. To manage the damaging outcomes of the injury guidelines were set according to *Guidelines for the Management of Penetrating Brain Injury in 2001 that attempted to standardize the management of PBI* [97]. Before the patient reaches the hospital, the penetrating object shall be stabilized and not removed until they reach where they can get definitive care. In the emergency unit, revitalization and maintenance should be provided. Airway, Breathing, Circulation, Disability, Exposure (ABCDE's) shall be managed using Advanced Trauma Life Support (ATLS) guidelines [98, 99].

Antioxidants have been targets for ongoing research to look for neurotherapeutics that can help with the outcomes of TBI. One of those is Mitoquinone.

Mitoquinone (MitoQ) is a mitochondria-targeted ubiquinone molecule that was developed in American radiolabeled chemicals in the United States of America in the 1990s to freely cross the BBB and neuronal membranes. It is synthesized by the covalent conjugation of a ubiquinone or coenzyme Q moiety to a triphenylphosphonium cation (TPP+) (Figure 2) [15]. TPP^{+} is a lipophilic cation that directs the ubiquinone moiety to the inner mitochondrial membrane due to the high electrochemical potential [100]. Ubiquinone plays the role of electron carrier in the IMM due to its redox property and will be reduced to ubiquinol after receiving two electrons and two protons. The active antioxidant ubiquinol scavenges excess ROS. After reducing ROS, ubiquinol will get oxidized back to ubiquinone after protecting the cell against lipid peroxidation [101]. Ubiquinone if supplemented will reach the brain in small amounts and will not be effective in boosting the mitochondrial content due to its hydrophobic properties [102].

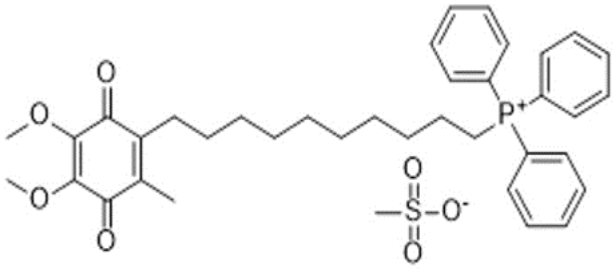

Figure 2 The Chemical Structure of MitoQ

MitoQ can be easily taken up by the brain's mitochondria by the action of ΔΨm where it is reduced to Mitoquinol by complex II of the ETC. Nonetheless, its reduced form cannot be oxidized by complex III, and thus it cannot restore mitochondrial respiration [101, 103]. MitoQ increases the production of SOD in the forward electron transport which goes from complex I to MitoQ [104-107]. This elevation of SOD due to the action of MitoQ consequently leads to the activation of the antioxidant transcription factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2). Naturally, the Nrf2 is

sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) [108, 109]. However, MitoQ modifies the stability of the bond between Keap1 and Nrf2 making it weaker and thus increasing the availability of Nrf2 [110]. Upon this action, Nrf2 translocates to the nucleus where it acts with Maf proteins to bind to antioxidant responsive elements (ARE). These are regulatory sequences upstream of antioxidant enzyme genes such as glutathione S-transferase, NAD(P)H (quinone) dehydrogenase (NQO-1), heme-oxygenase (HO), SOD, and CAT. This binding enhances the expression of the antioxidant genes and thus reduces oxidative stress [108, 111].

The first study to demonstrate the effects of MitoQ in TBI was recently completed. The treatment was shown to significantly improve neurological deficits, alleviate brain edema, and inhibit cortical neuronal apoptosis in a TBI mouse model[17]. Mice treated with 4 mg/kg of MitoQ showed significantly improved neurobehavioral functions, coupled with increased activity of different antioxidant enzymes, including SOD and GPx [17]. Remarkably, MitoQ accelerated the nuclear translocation of Nrf2 and subsequently upregulated the expression of downstream proteins, including HO-1 [17]. Therefore, such findings demonstrate that MitoQ mediates its neuroprotective effects via activating the Nrf2/ARE pathway.

Interestingly, MitoQ administration does not seem to have adverse consequences or side effects. There are clinical trials that have already used MitoQ. Snow *et al.* found that patients can be given MitoQ doses without any side effects for up to a year (Snow et al., 2010). Gane *et al.* showed that MitoQ helped decrease liver damage in hepatitis C patients with no adverse side effects over 28 days [16]. More recently, Rossmann *et al.* studied the effect of MitoQ on vascular function in adults. The study done over six weeks showed no significant adverse side effects following the administration [112].

Nevertheless, most studies utilizing MitoQ were done on animal models. Rodriguez-Cuenca *et al.* showed that the long-term administration of MitoQ on wild-type mice for up to 28 weeks was safe and did not act as a pro-oxidant or cause damage over this period [113].

CHAPTER III

AIMS AND OBJECTIVES

In the absence of an FDA-approved drug that targets the consequences of TBI, identifying a therapeutic target represents an unmet need for the field of neurotrauma. As the mitochondria are important for initiating the cascades involved in oxidative stress, they represent a major target for therapy in PBI. In consequence, we suggested that targeting the mitochondria with MitoQ would be a valuable approach to ameliorate the secondary injury and subsequently improve the long-term cognitive, behavioral, and molecular consequences of PBI. To properly assess this hypothesis, we set up three specific aims:

- *Specific Aim 1 is* to investigate the effects of MitoQ on neurological, cognitive, and behavioral functions using a battery of tests between mice treated with MitoQ for 30 days after being subjected to PBI, mice subjected to PBI without treatment, and sham mice.
- *Specific Aim 2 is* to investigate whether MitoQ can lower oxidative stress by assessing the expression profiles of key enzymes like SOD2, CAT, and the transcription factor Nrf-2 involved in the production and elimination of ROS.
- *Specific Aim 3 is* to explore the inflammatory state in the cortex and the hippocampus after MitQ injections by assessing activation markers Iba-1 and GFAP of microglia and astrocytes respectively. This is along with quantifying neuronal cells in both regions with the NeuN cell marker via immunofluorescence.

CHAPTER IV
MATERIAL AND METHODS

A. Animals

The study was carried out at the Animal Care Facility of the American University of Beirut (AUB) and all animal experiments were performed in compliance with the AUB Institutional Animal Care and Use Committee (IACUC) guidelines with the reference number: (17-01-458). C57BL/6 male mice were housed in a controlled environment (12 h light/dark cycles, 22 ± 2 °C). All animals were handled under pathogen-free conditions and fed chow diet ad libitum. Male mice 7–8 weeks were divided into three groups: Sham (n=12), CCI (n=12), and CCI+MitoQ (n=12). The mice in the CCI and CCI+MitoQ groups were subjected to TBI using a controlled cortical impact machine (CCI). 30 minutes after TBI, mice in the CCI+MitoQ were injected intraperitoneally with MitoQ with a dosage of 8 mg/kg given every 3 days for a period of 30 days (until sacrificed). The dose was adopted from the previous study on MitoQ and TBI[17]. Animals were randomly attributed to experimental groups in a blinded manner by randomly selecting which animal received the treatment from those that underwent the injury. Animals that showed weak health post-surgery and exhibited any disease condition were not included in the study as per the IACUC regulations. We had around 2 to 3 animals eliminated from each group with approximately 14% mortality rate. We have used 10 animals per group for each behavioral testing.

B. Controlled Cortical Impact

Each mouse was first anesthetized by Ketamine/Xylazine mixture (50 mg/kg and 15 mg/kg respectively) administered intraperitoneally. Isoflurane (Forane®) was used to maintain anesthesia until the animal lost its reflexes. Each mouse was then fixed on a stereotaxic frame and a longitudinal skin incision was made in the middle of the mouse's head by a surgical scalpel to expose the skull. An ointment (Xailin®) was applied to the eyes to protect vision during surgery. Using a drill and forceps, the part of the skull above the somatosensory area of the parietal lobe was removed to expose the brain. The injury was done using the Leica Impact One Angle Controlled Cortical Impact (CCI) machine. The center of the impactor was placed above the somatosensory area of the parietal cortex of the brain using these coordinates (+1.0 mm AP, +1.5 mm ML, and −2 mm DV). The duration of impact was kept constant with a dwell time of 1 second and at a velocity of 4 m/s. The depth of the injury was set to 2 mm. The wound was closed using silk sutures (MERSILK™-W587H). Each mouse was placed on a heating pad to maintain the body's temperature. The CCI+MitoQ group received the first MitoQ injection 30 minutes following injury. For the animals in the Sham group, drilling was performed to remove confounding factors. Figure 4 shows how the injury was delivered using the Leica Impact One Angle Controlled Cortical Impact (CCI) machine

and Figure 3 shows the Bregma and Lambda pointed out to the software (Angle Two system ®) and the target area (parietal cortex).

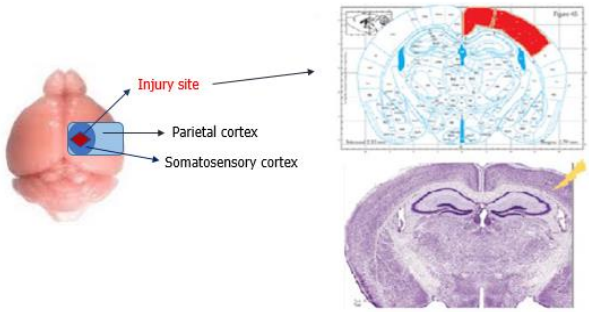

Figure 3 Stereotaxic Coordinates of the Injury Site

Figure 4 Controlled Cortical Impact Machine

C. Mitoquinone Supplementation

Mitoquinone (Focus Biomolecules, 10-1363, MW=663.64) was prepared by dissolving 25mg in 100 µl of 10% dimethylsulfoxide (DMSO). 900 µl of phosphate-buffered saline (PBS) was then added to obtain a stock solution of 25 mg/mL. Further dilutions using PBS were carried out to obtain working solutions of 1 mg/mL. MitoQ was intraperitoneally (IP) supplemented at a dose of 8 mg/kg, 3 times per week over 30 days starting at 30 minutes post-injury.

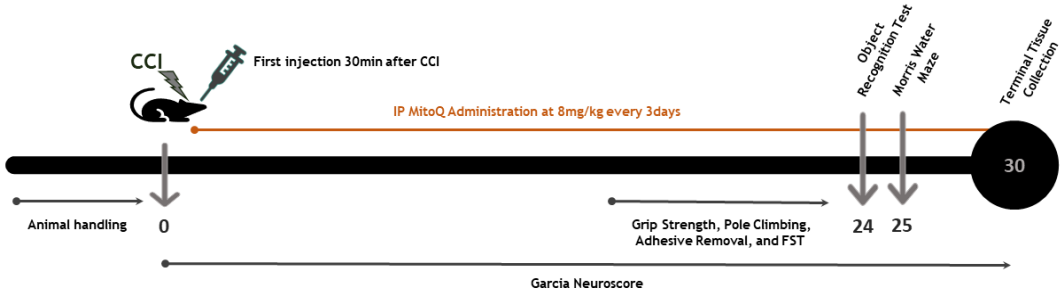

Figure 5 Experimental Timeline

D. Garcia Neuroscore

Neuroscore was adopted from Garcia *et al.* to assess the integrity of neurological function via different criteria. Every group was tested on day -3 before CCI and then the experiment was repeated on 3, 7, and 30 days after the surgery. The animals underwent 6 different evaluations on every test day, each being scored from 0 to 3 (Table 1).

Table 1 Evaluation Criteria for Garcia Neuroscore

Criteria	Evaluation
Spontaneous Activity	Ability to approach all four walls of the cage
Limb Symmetry	Limb symmetry when held by the tail

Forepaw Outstretching	Outstretching symmetry of both forelimbs while the hindlimbs are kept in the air
Climbing	Ability to climb and hang into the cage
Body Perception	Reaction to stimulus while the mouse is touched on each side of the body with a stick
Vibrissae Touch	Reaction to stimulus while whiskers of the mouse are touched with a stick without entering the visual field

E. Grip Strength Test

This forced motility test was used to assess endurance of motor skills and muscular dysfunction. The 4700 grip strength meter (UGO BASILE-Italy®) was used to assess muscle strength. Each animal was held by its tail and was allowed to catch a trapeze-shaped metal with both of its paws. A total of three trials were done for each animal. Muscle strength in gram force (gf) was recorded along with the total time of grip and the time the force was applied by the mouse. For analysis, the average of muscle strength, in gf, in the three trials, is normalized to the weight of the mouse [114]. The apparatus used for the grip strength test is shown in Figure 6.

Figure 6 Grip Strength Test Meter

F. Pole Climbing Test

The pole climbing test was done to evaluate motor coordination. The apparatus consists of a metal pole with a length of 60 cm and a diameter of 1 cm, set perpendicularly in a big cage. The pole is wrapped with tape to facilitate the animal's grip on the apparatus. Animals were habituated on the testing days to descend on the pole for three trials before the recorded testing. The mice were placed on top of the pole with their heads directed upwards and were then allowed to descend freely [115]. The time needed for each mouse to reach the bottom of the pole (total time) was recorded. For each animal, a total of three testing trials were done on each testing day. Figure 7 shows the pole climbing apparatus.

Figure 7 Pole Climbing Apparatus

G. Adhesive Removal Test

This test was performed by placing a rectangular adhesive tape strip on each mouse's nostril to assess sensorimotor function. Then the animal is placed back to its cage. Two different values are recorded using two stopwatches: (1) the time needed to make the first contact with the tape which represents nose sensitivity and (2) the time needed to remove the tape which represents dexterity.

H. Forced Swim Test

This test was used to assess despair and depression-like behavior. Animals were placed in a glass cylinder [20 cm (height) × 11 cm (diameter)] filled with 25°C water to a depth of 15 cm for 6 minutes. The 6 minutes included a one-minute acclimation period at the beginning of the test followed immediately by five minutes test. Depression is reflected by the time mice spend floating on the surface of the water without moving. Animals were then dried with a towel and returned to their heated home cage. The behavior of the mice was recorded using a camera and immobility time was analyzed later during the last five-minute period of the test. Immobility time is defined as the time a mouse spends floating making only necessary movements to keep its body balanced and head above water.

I. Morris Water Maze Test

The Morris water maze (MWM) is used to study defects in spatial learning and memory. The apparatus consists of a circular pool (110 cm diameter, 55 cm depth) half-filled with water and maintained at 22 °C. A 10 cm diameter platform is placed in the water. Three extra-maze cues are placed around the pool to spatially guide the mice. Black and white cues were used to omit any variance in color discrimination among mice. Non-toxic white paint was added to the water surface to make it opaque to ensures accurate tracking. The ANY-maze 5.2 software (Stoelting Co., Wood Dale, Illinois, USA) was used to track the movement of mice and record the different parameters to be evaluated. On the first day of testing, the platform was made visible by mounting a flag on it for cued trials. Four trials were carried out for each animal where

the position of the platform and the starting position of animals changed among trials. On days two through five, which are the acquisition days, the flag was removed, and the platform was fixed in the North East (NE) quadrant submerged in water. However, the starting position of the animals varied among trials where three trials were carried out. The maximum time of a trial was set to one minute. If the animal failed to find the platform during this time, it was guided to the platform by the experimenter where the animals were allowed to sit on the platform for 20 seconds for memory consolidation. If the animals found the platform before this time, the test was considered completed, and the animals were kept on the platform for five seconds. On day six, or the probe trial day, the platform was removed, and the mice were allowed to swim for 1 minute for one trial only. After each trial on all days, the mice were dried with a towel and allowed to rest in a heated cage.

J. Novel Object Recognition Test

Novel object recognition depends on the innate capacity of rodents to discriminate a novel object from a familiar object (previously encountered). Mice were placed in the center of an open field at the beginning of each trial and freely explored the open field and objects (Figure 8). At the end of each trial (5 min), mice were removed from the open fields and placed in their home cages next to each testing area for the inter-trial interval (ITI- 5 min). The open fields and objects were cleaned with 70% ethanol during the ITI. During Trial 1 (learning trial), the animal explored an open field with two identical objects. During trial 2 (testing trial), one object was kept in the testing field while the other was replaced by a novel object for exploration. All data were recorded by a video camera suspended above the 4 open fields and connected to

image analyzer software (Any Maze-IL-USA®). The zones were located 5 cm around each object on the software, and the software recorded the time the animal spent in each zone with its head directed toward the object.

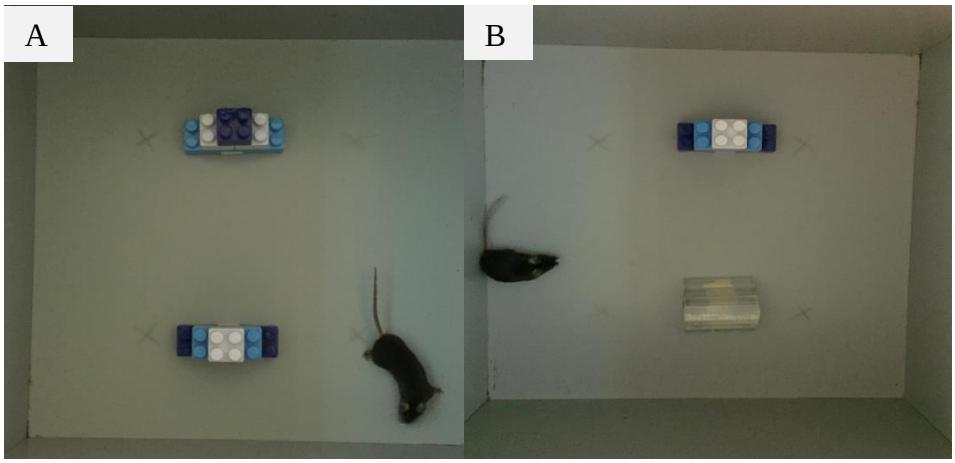

Figure 8 Object Recognition Test
(A): A mouse in the learning session of the object recognition test
(B): A mouse during the testing session of the object recognition test

K. RT-qPCR

RNA was extracted using Trizol (T9424-100ML, Sigma Aldrich, St. Louis, Missouri, USA), then any contaminating genomic DNA was removed using TURBO DNA-free™ Kit (AM1907, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. 1.5µgs of total RNA were reverse transcribed using the iScript™ cDNA Synthesis Kit (1708890, Bio-Rad, Hercules, California, USA). Quantitative real-time PCR was applied to the obtained cDNA using the Quantifast® SYBR® Green PCR Master Mix (204054, Qiagen, Hilden, Germany) and 10µM of each of the reverse and forward primers and 1 µL of cDNA. Cycling conditions were as follows: 95°C for 10 minutes for one cycle, then 95°C for 10 seconds followed by 60°C for 30 seconds and 72°C for 30 seconds for 40 cycles, and finally 72°C for 5 minutes for one cycle. The primers used are listed in Table 2.

Table 2 Primer Sequences for RT-qPCR

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
mSOD2	GGCCAAGGGAGATGTTACAA	GAACCTTGGACTCCCACA
mCAT	TGAGAAGCCTAAGAACGCAAT TC	CCCTTCGCAGCCATGTG
mNrf2	CGAGATATACGCAGGAGAGGTAAGA	GCTCGACAATGTTCTCCAGCTT
mβ-actin	CAGCTGAGAGGGAAATCGTG	CGTTGCCAATAGTGATGA CC

L. Immunofluorescence Staining

Free-floating brain sections (40 μm thick) were washed with PBS followed by PBST (0.1% Triton in PBS), then incubated for 1.5 h in a blocking solution of 10% heat-inactivated fetal bovine serum (FBS) in PBST, as previously described [116]. Tissues were then incubated overnight at 4 °C with primary antibodies, diluted in 1% FBS solution. The primary antibodies used were: anti-GFAP (1:1000 dilution; MCA-5C10, Encor Biotechnology, Gainesville, Florida, USA; https://encorbio.com/) as an astrocyte marker; anti-NeuN (1:1000 dilution; RPCA-FOX3-AP, Encor Biotechnology) as a marker of mature neurons; anti-Iba-1 (1:1000 dilution; 019-19471, Fujifilm Wako Chemicals, Richmond, Virginia, USA) as microglia marker. Then sections were rinsed in PBST and incubated with the appropriate fluorochrome-conjugated secondary antibody (1:1000 dilution) for 1 hour at room temperature, followed by three washes in PBST. The secondaries used are: Donkey Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150105) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 568) preadsorbed (ab175696). Finally, all sections were counterstained in 1μg/ml of Hoechst (Sigma

Aldrich), diluted in PBS, and mounted using Fluoromount (F4680-25ML, Sigma Aldrich).

M. Statistical Analysis

Statistical analysis was done using GraphPad Prism 9. Non-parametric tests were carried out for variables with a non-normal distribution where the one-way ANOVA test was done to compare the data sets that contained more than two groups and then Kruskal-Wallis test to compare the three groups simultaneously. All results were considered significant for p-value <0.05: ***($p<0.001$), **($p<0.01$), * ($p< 0.05$).

CHAPTER V

RESULTS

A. MitoQ enhances the expression of Nrf2, SOD2, and CAT at 30 days post-CCI

MitoQ has been previously shown to exerts its antioxidant effects via the activation of the transcription factor Nrf2 which, in turn, upregulates the expression of antioxidant enzymes including SOD and CAT[17]. Therefore, the expression level of Nrf2 and downstream antioxidant enzymes, particularly CAT and SOD2, was quantified by RT-qPCR to draw similarities between previous research and make sure MitoQ acted on the same axis in this experiment.

The experiments were performed on total mRNA obtained from the cortex of mice in all three groups (n=4 for every group). In the case of Nrf2, MitoQ treatment resulted in a significant improvement in expression as compared to CCI ($p<0.05$) in the cortex (**Figure 8**). This improvement resulted in a basal expression level a little less than that of Sham. As for SOD2 and CAT, both genes were significantly increased as compared to CCI ($p<0.05$) and there was an apparent increase over the basal level expressed by the Sham (Figure 9). This seems to comply with the data since MitoQ's mode of action focuses on the localization of Nrf2 rather than expression, which is sequentially followed by the increase in oxidative enzyme expression.

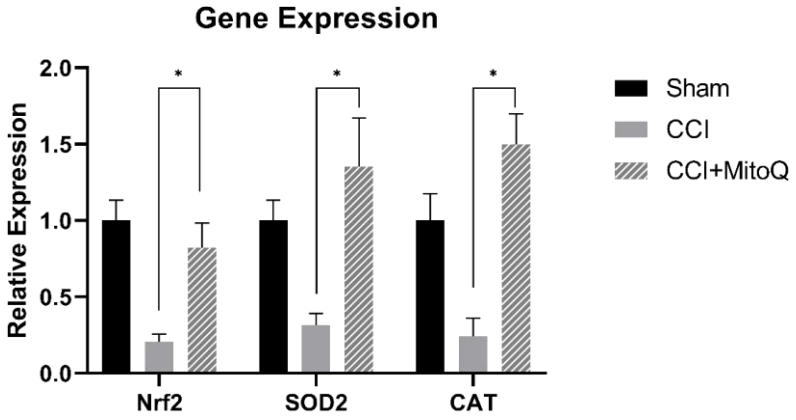

Figure 9 MitoQ Improves Expression of Nrf2, SOD2, and CAT in the Cortex post-CCI.

The mRNA levels of Nrf2, CAT, and SOD2 were determined using RT-qPCR in sham (n=4), CCI (n=4), and CCI+MitoQ (n=4). Data were normalized to β-actin. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

B. MitoQ ameliorates neurological performance post-CCI by improving sensorimotor deficits and gross motor function

TBI has been shown to induce a battery of neurological dysfunctions that hinder affected individuals from carrying on their daily tasks[6, 55]. The pathophysiology of TBI has been usually linked to sensorimotor deficits due to cellular death and other neurodegenerative mechanisms that arise from a state of oxidative stress [51, 117]. Since MitoQ has been previously shown to decrease CNS complications due to oxidative stress, and now that it was shown to successfully exert its effects via the previous RT-qPCR analysis, it was hypothesized that it would help in decreasing neurological deficits post-CCI[17, 54]. This is especially interesting because, after extensive review in the literature, there is a lack of evidence that investigated the link between MitoQ and open head TBI. For this reason, the first test that was conducted to

look at the overall neurological function of mice was the Garcia Neurological Test. The experiment was conducted three days before the CCI to make sure that all animals (n=10 for every group) had a similar basal score (Figure 10A). The test was then repeated on 3, 7, and 30 days after CCI. During all test runs, CCI+MitoQ performed significantly better on all test parameters than the CCI group ($p<0.05$). This showed that MitoQ improved overall neurological performance in mice. However, to be able to further assess this, additional testing was performed to evaluate specific sensorimotor deficits and look at overall motor coordination.

Firstly, the adhesive removal test was performed to assess sensorimotor function by looking at sensitivity and dexterity. CCI+MitoQ group performed significantly better than the CCI group by establishing the first contact faster ($p<0.05$) (Figure 10B and C). Time to remove the adhesive was also recorded, showing that CCI+MitoQ group required less time to remove the adhesive ($p<0.05$). This shows that MitoQ specifically enhances sensorimotor function. Secondly, motor function was investigated by exploring motor coordination via pole climbing test and muscle strength via grip strength test. MitoQ group was able to perform better by taking less time to descend the pole ($p<0.05$) (Figure 10E) and showed increased muscle strength ($p<0.001$) (Figure 10D) than the CCI group. Therefore, MitoQ administration helped improve gross motor function.

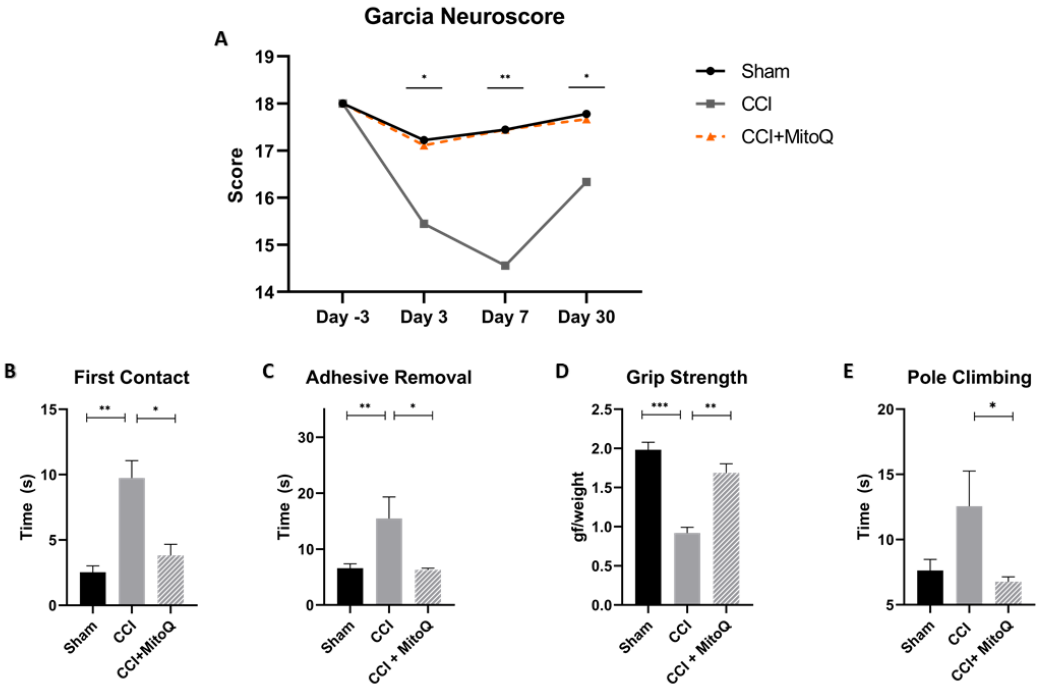

Figure 10 MitoQ Ameliorates Neurological Deficits post-CCI

(A) Garcia Neuroscore allowed testing for neurological function in mice and showed that MitoQ improved overall neurological performance 3-, 7- and 30-days post CCI. (B) Further testing was conducted to assess sensorimotor function via adhesive remove test. Mice that received MitoQ established contact and were able to remove the adhesive faster than CCI group. Additionally, gross motor function was investigated by two tests. (D) Grip strength allowed showing that MitoQ improved overall muscle strength and pole climbing test (E) showed that CCI+MitoQ had better motor coordination. All tests were executed on sham (n=10), CCI (n=10), and CCI+MitoQ (n=10). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

C. MitoQ alleviates learning deficits and recognition memory dysfunction at 30 days post-CCI

MitoQ's ability to potentially alleviate cognitive deficits that result from CCI was subsequently evaluated. For this purpose, we used the Morris Water Maze test. No

differences in latency to the platform were found among groups on day one of the tests, which was performed 24 days following CCI. Moreover, there were no differences in speed among the three study groups on all days. This indicates that any potential deficit that may be found on the following days of the test will not be the result of an eyesight impairment or due to a motor deficit, but the result of a genuine cognitive impairment. During the acquisition days of the test, mice from the CCI group took more time to reach the hidden platform as compared to the Sham and CCI+MitoQ. This delay was significant on days 2 ($p<0.05$), 3 ($p<0.001$), and 4 ($p<0.05$) of the acquisition days (Figure 11A). The data indicate that CCI results in a cognitive learning deficit that is reversed upon MitoQ administration. These findings were in line with other studies that showed increased latency to the hidden platform in brain-injured mice [45, 81, 82]. On the probe trial day of the test, there were differences in the latency to reach the target quadrant by the CCI group indicating that retention deficits are caused by CCI in our model (Figure 11B and C), however, this was shown to be attenuated by MitoQ since CC+MitoQ group required less time to reach the target quadrant ($p<0.05$) and spent more time in it ($p<0.05$) (Figure 11B and C).

For further learning assessment and recognition memory testing, the “novel object recognition” test was conducted. On the testing day, after carrying habituation and training for the mice in the platform, the total exploration time for all three groups was recorded. There was no significant difference between them showing that all animals explored the area equally and therefore they wouldn’t be excluded from analysis (Figure 11F). The results showed that the CCI group didn’t exhibit differentiation between familiar and novel objects due to dysfunction in recognition memory and learning, which was not the case in the CCI+MitoQ group where they

spent more time exploring the novel object, resulting in a positive differentiation index ($p<0.05$) (Figure 11G). This reveals that MitoQ improved cognitive function by enhancing recognition memory and learning.

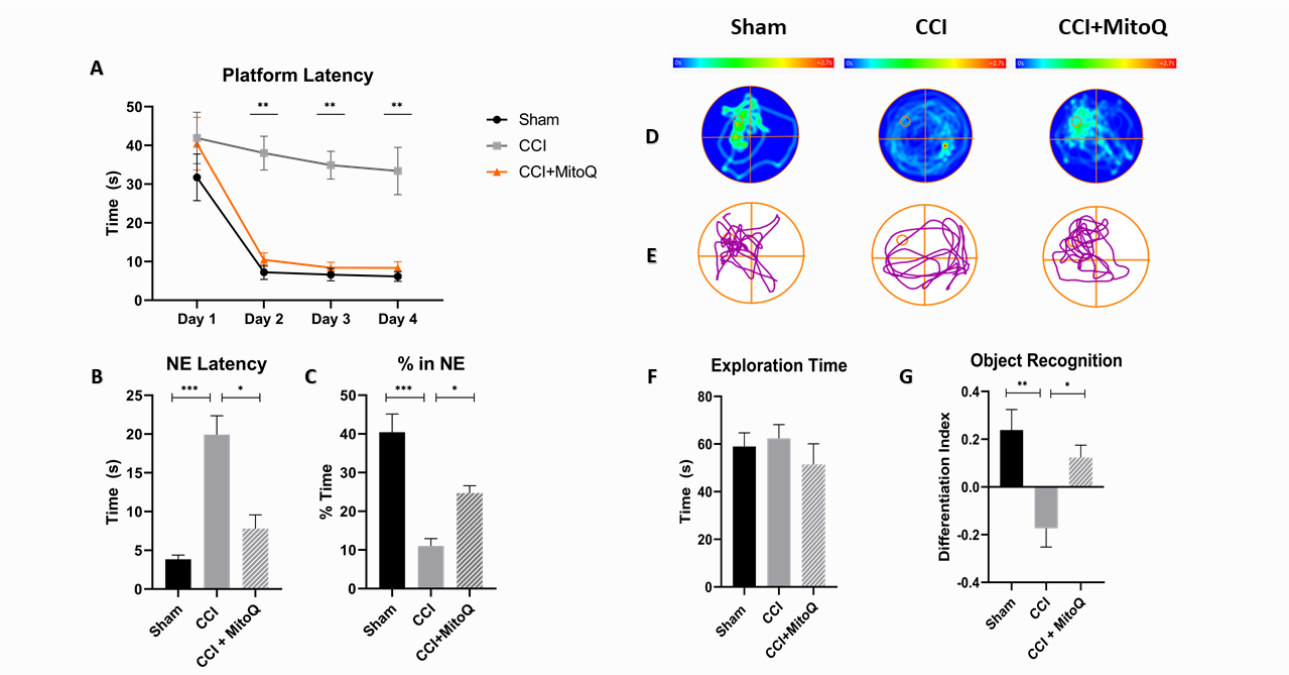

Figure 11 MitoQ Reverses Learning Deficits and Recognition Memory Caused by CCI

Spatial learning and memory in mice from the three groups were evaluated using the Morris water maze. (A) shows the time taken for the first arrival at the platform by mice from the three study groups on the acquisition days where the mice learn how to reach the platform based on visual cues. (B) shows the time taken to reach the target quadrant (NE) on the probe trial day. (C) shows the percentage of time spent in the target quadrant. (D) and (E) show the representative heat maps and trace plots on the probe trial day, respectively. For further investigation of memory acquisition, novel object recognition test was performed. (F) Exploration time for all three groups was recorded and (G) discrimination index was calculated, showing that recognition memory was enhanced in the CCI+MitoQ group. Data represent the mean ± SEM (n = 10 per group). *p <0.05, **p<0.01, ***p <0.001

D. MitoQ decreases depressive-like behavior

It has been widely shown that TBI, in all its forms, is associated with neuropsychiatric consequences, especially in the long run [29]. The most common complications that arise are depression, post-traumatic stress disorder, and generalized anxiety disorder[33]. For this reason, mice that underwent CCI were tested for depressive-like behavior using the forced swim test (FST). When compared to the Sham, the CCI group showed increased immobility time during the test, which is indicative of hopelessness ($p<0.001$). However, this was improved in the CCI+MitoQ as compared to the CCI group ($p<0.05$). Therefore, MitoQ administration helped in reducing depressive-like behavior in mice.

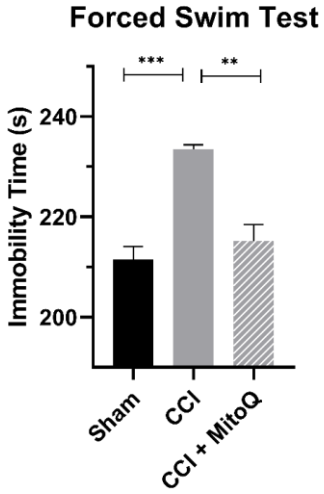

Figure 12 Depressive-like behavior was reduced by MitoQ
Animals were placed in a water container during the forced swim test to measure immobility time as a sign of hopelessness. CCI+MitoQ group performed better than CCI group with decreased immobility time. Data represent the mean ± SEM (n = 10 per group). *p <0.05, **p<0.01, ***p <0.001

E. MitoQ reduces astrogliosis, microgliosis, and neuronal cell loss post-CCI

The increased mRNA levels of antioxidant genes and the improved cognitive and neurological performance of mice once MitoQ was administered required further molecular investigation. Therefore, we tried to assess if the decreased state of oxidative stress could be translated into ameliorated cellular recovery post-CCI. For this reason, immunofluorescence on brain sections for the three groups (n=4 for every group) was performed to examine the activation of immune cells and neuronal loss. Ionized calcium-Binding Adaptor protein-1 (Iba-1), an actin-binding protein, was used to stain activated microglia using immunofluorescence. The results showed that MitoQ administration significantly attenuated the number of activated microglia both in the cortex ($p<0.05$) and hippocampus ($p<0.05$) compared to that of the CCI group (Figure 13A and B). The morphology of the microglial cells is also important to note as the CCI group showed an amoeboid shape with major extensions indicating their activated state (Figure 13C). Taken together, these data show that MitoQ reduces microgliosis following CCI which is indicative of decreased inflammation. Further investigation was done by assessing at the astrocytic activation marker GFAP.

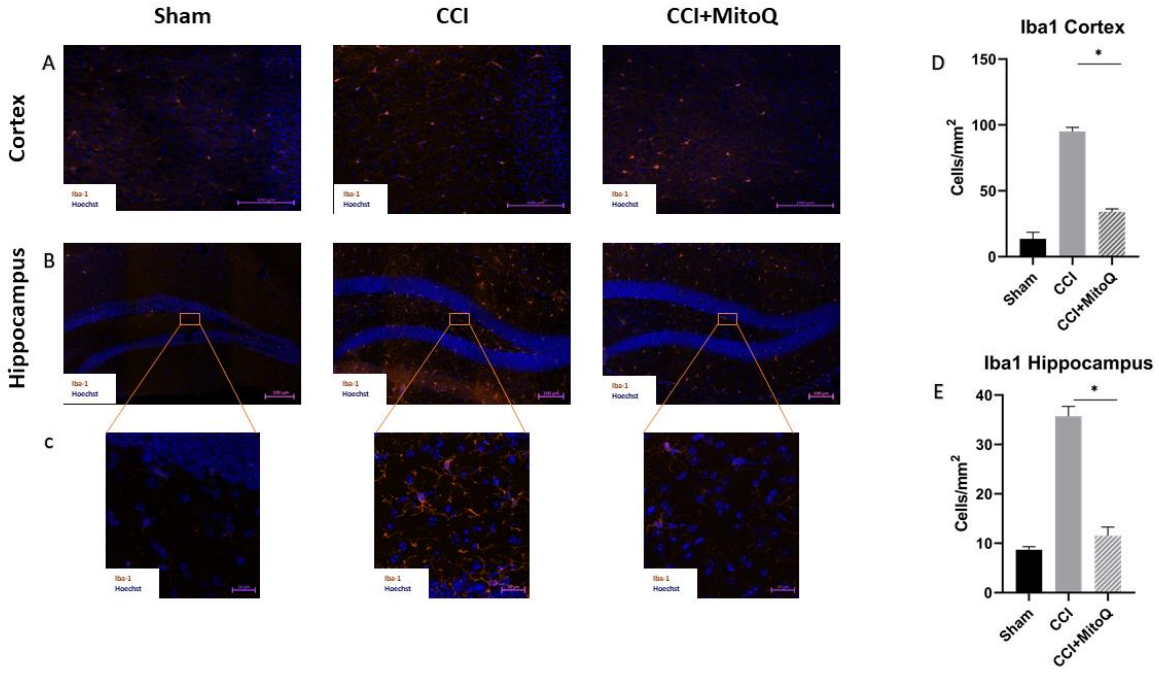

Figure 13 MitoQ reduces microgliosis following CCI

Microgliosis was assessed by immunofluorescence staining for Iba 1. (A) shows iba-1 positive cells in the cortex and (B) in the hippocampus. Hoechst was used as a nuclear counter stain. Iba-1 positive cells were counted and then the obtained number was divided by the area of the selected field in an average of nine fields. (C) Higher magnification images were taken to show unique morphology of microglia when they are activated as opposed to when they are not. (D) and (E) show the mean ± SEM (n = 4 per group). *p <0.05, **p<0.01, ***p <0.001

Glial Fibrillary Associated Protein (GFAP) is an intermediate filament protein used as a marker of reactive astrocytes [52]. Astrocytic activation plays an important role in the progression of the inflammatory response post-injury and has numerous neurotoxic effects[67]. GFAP expression was significantly higher in the hippocampus and cortex of the CCI group compared to that of the sham group($p<0.05$), outlining the

increased astrogliosis that follows TBI (**Figure 13**). This state of increased GFAP expression was significantly improved upon MitoQ administration in the CCI+MitoQ group as opposed to the CCI group in the cortex ($p<0.05$) and the hippocampus ($p<0.05$). What is also noticeable is the morphology of the astrocytes that were activated in the CCI group. Z-stack imaging showed that there is significant arborization of astrocytes upon activation post-CCI, which is absent in the case of Sham and when MitoQ is administered (Figure 14C).

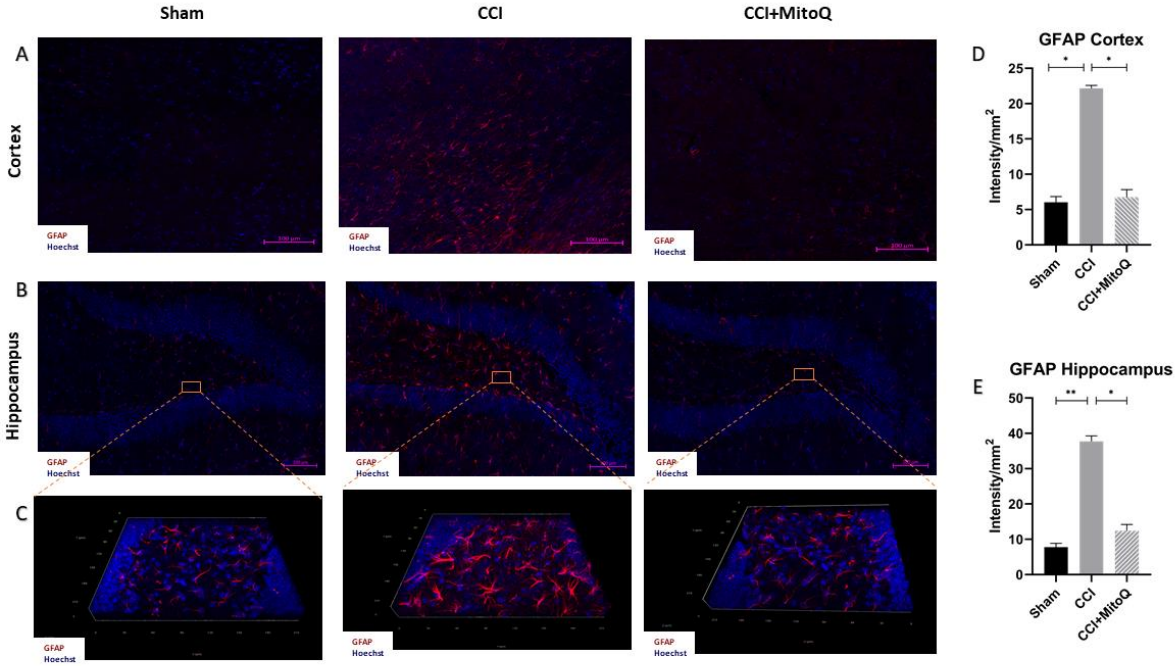

Figure 14 MitoQ Mitigates Astrocytosis post-CCI.

Astrogliosis was assessed by immunofluorescent staining of GFAP. Representative fluorescent images of GFAP staining of the cortex (A) and hippocampus (B) from different groups were obtained. Hoechst was used as a nuclear counterstain. (C) 3D images were obtained using Z-stacking to showcase unique morphology of activated astrocytes. (D) and (E) show the mean intensity ± SEM (n = 4 per group). *$p<0.05$, **$p<0.01$, ***$p<0.001$

Finally, immunofluorescence staining for NeuN, a nuclear protein found in the nuclei and perinuclear cytoplasm of neurons, was used to assess the number of mature neurons among the three study groups in a semi-quantitative manner [118]. This is especially important to assess based on the literature that links neuronal death to oxidative stress, something that MitoQ helps in ameliorating as suggested by previous results. There was a significant decrease in neuronal cell count in CCI as compared to sham in the cortex and the hippocampus ($p<0.05$) (Figure 15). This seemed to be improved in the cortex significantly ($p<0.05$) in the CCI+MitoQ group. However, in the hippocampus, the increased number of cells albeit apparent, was not significant enough.

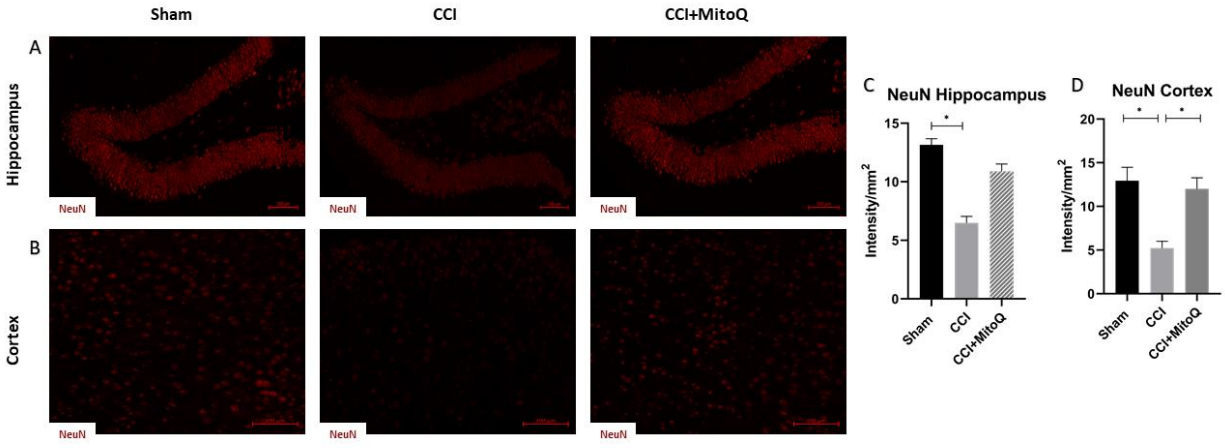

Figure 15 MitoQ reduced neuronal cell loss in cortex post CCI.
NeuN staining was performed to assess the number of mature neurons. (A) shows representative fluorescent images of NeuN staining in the cortex from the different study groups. Hoechst was used as a nuclear counterstain. NeuN positive cells were counted then the number was divided by the area of the field. (B) same as in (A), but in the hippocampus. (C) and (D) show the mean ± SEM (n = 4 per group). *p <0.05, ***p <0.001

CHAPTER VI

DISCUSSION

Due to the lack of FDA-approved drugs that help in treating the pathology of TBI, especially PBI, there is a need to investigate potential neurotherapeutics that help ameliorating post-injury consequences. For that purpose, this study was conducted to assess the potential effect of MitoQ as an antioxidative approach to PBI. We were able to show that MitoQ enhanced the expression of genes involved in the antioxidative axis like Nrf2, SOD2, and CAT. MitoQ administration was also able to improve sensorimotor and gross motor function, enhance learning and recognition memory, and help with depression-like behavior. On a molecular level, MitoQ decreased the activation of microglia and astrocytes in the hippocampus and the cortex, indicating that it has effects in alleviating inflammation. This is along with improving neuronal cell count in the cortex, owning to decreased neuronal loss due to the injury. These results are in line with previous research that investigated MitoQ effects in TBI, adding more evidence to the efficacy of this antioxidant[17].

As a first step to consider the importance of MitoQ, it is crucial to note the safety of taking these supplements as assessed by previous research. Rodriguez-Cuenca *et al.* demonstrated that long-term administration of MitoQ in wild-type mice for up to 28 weeks is safe and did not act as a pro-oxidant or cause any damage over the study period [113]. In addition, it has been found that MitoQ can be safely administered to patients at a dose of 80 mg for up to a year [119]. Studies on MitoQ and TBI are extremely limited. In one study by *Zhou et al.* MitoQ exhibited a neuroprotective effect by upregulating the expression of antioxidant enzymes through the Nrf2-ARE pathway

in a weight-drop model of TBI [120]. Other aspects related to TBI pathophysiology were not however investigated in that study. This stresses the importance of conducting further research in other models of TBI, like the current CCI model. The mechanism of action of MitoQ was confirmed in this study as an expression of the three genes, Nrf2, SOD, and CAT, was assessed. The results showed that Nrf2 was improved when MitoQ is administered but it was still below the levels exhibited by the Sham. This is true since MitoQ specifically works on the translocation rather than the expression of Nr2. However, downstream oxidative enzymes were significantly increased showing that MitoQ helps in creating advantageous conditions in the cortex to decrease oxidative stress. However, further investigation in the hippocampus specifically is still necessary.

On a macroscale, it was important to investigate if MitoQ's beneficial effects in reducing oxidative stress translated to neurological and behavioral levels. This is especially true since previous studies have clearly stated the link between heightened oxidative stress and neurological dysfunction[54, 55]. To start with, general neurological testing was carried via the Garcia neurological score test. This allowed scoring the animals on a scale from 0-18 to establish the general sensorimotor function, motor skill, and coordination-based functions[121]. The test was firstly formulated in an ischemic stroke model but was later adapted to TBI studies[122, 123]. In this study, MitoQ was shown to improve overall neurological integrity by resulting in better scores on the three days of testing. Further assessments to look for sensorimotor deficit and gross motor dysfunction were carried out. Adhesive removal test showed that our CCI model induced sensorimotor deficits in mice that took longer to establish contact with and remove a tape that was placed on their noses. However, MitoQ was able to shorten this time showing that it improved sensorimotor function at a chronic timepoint. During

the pole climbing test and grip strength test, the current CCI model displayed decreased muscle strength and adversely affected motor coordination, both of which were improved by MitoQ. In addition to this, MitoQ demonstrated a positive impact on depressive-like behavior during the forced swim test, revealing that it helped with the notion of hopelessness. This is especially important as TBI has been linked to many neuropsychiatric sequalae on the long run[29, 33].

Moreover, CCI was shown to result in learning deficits and impairment of recognition memory as seen in the Morris Water Maze test and Novel Object Recognition test. Firstly, in the former, the CCI group displayed latency to reach the platform on the learning days of the MWM test and a tendency towards a worsened memory retention as seen by the latency to the target quadrant and the time spent in it on the probe trial day. During the latter, the CCI group showed no differentiation between the familiar and the novel objects, revealing recognition memory dysfunction. These findings are in line with other studies that showed increased latency to the hidden platform in mice subjected to TBI [43, 44, 124]. The observed alterations in cognitive functions can be correlated to damage to the neuronal structures in the cortex and hippocampus because of neuronal loss and other secondary injury mechanisms. Furthermore, circuit alterations in the hippocampus due to synapse disruption and other pathological effects have been linked to memory impairment in TBI [125]. These circuit alterations have been suggested to occur because of disruptive cellular pathologies like oxidative stress and glutamate altercations[126]. Interestingly, MitoQ treatment following CCI resulted in an enhanced learning ability and recognition memory. Such an improvement may be related to MitoQ's capacity to alleviate the pathophysiological alterations following CCI including oxidative stress, astrogliosis, microgliosis, and

neuronal damage. After examining the effect of MitoQ on antioxidative enzymes, it was important to investigate other parameters that might be related to improved cognition and neurological function. This was done via immunofluorescence.

Previous studies have shown that TBI was followed by microglia and astrocyte activation which is correlated with a heightened inflammatory state in the brain[60, 64, 67, 70]. This is accompanied by neuronal cell loss via activation of cell death machinery[77, 79, 80]. Our CCI model exhibited results that were in line with the previous studies. There was an increased expression of GFAP, a marker of astrocyte activation, and Iba-1, a marker of microglia activation, showing that there is chronic activation of both cell types, an indication of an active inflammatory state [63, 67]. The injury also led to decreased neuronal count as examined using neuronal marker NeuN. This pathology in the cortex and the hippocampus is reflected in the depressive-like state exhibited by the mice and by disrupted neurological function and cognition. MitoQ was able to help decrease these effects where astrocytes and microglia showed dampened activation and there was reduced neuronal cell loss in the cortex. The mitigated neuroinflammatory profile may be related to the antioxidant activity of MitoQ that enhances oxidative enzyme production and therefore prevents excessive production of ROS. Enhanced ROS production is known to directly stimulate the expression of the pro-inflammatory interleukins IL-1α and IL-1β within hours after injury [9, 10]. Secreted pro-inflammatory cytokines contribute subsequently to the activation of Ca^{2+}-dependent proteolytic enzymes that activate cell death machinery and induce a pro-inflammatory state [127].

The advantages of mouse models to study genetic and cellular components are balanced by the limitations of the small size and lack of complexity of the mouse brain.

Mouse models are most useful as tools to screen for pathology that may be otherwise hard to distinguish. These findings in mice pave for further development of techniques to be applied in higher mammals and humans. However, a limitation of this study is the lack of evaluation of the genes and signaling pathways involved in inflammation and other phenotypes mentioned above which are not exclusive to the oxidative stress axis. This is in addition to the fact that only male mice were used in the animal model considering that sex differences have been demonstrated in PBI when it comes to gene expression, development of pathological proteins, and behavior.

Overall, MitoQ administration appears to be efficient in reducing the deleterious consequences associated with PBI on both the molecular and behavioral levels. This study paves the way towards identifying a potential preventive therapy for PBI that may also improve the quality of life for those affected by it. Moreover, obtained data provide an additional line of evidence to the potential role of oxidative stress and mitochondria in the pathology of brain disorders by mechanisms that need to be unraveled. This is along with opening up more questions on the effects of this supplement on the mitochondrial complexes and neuronal regeneration.

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