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Article

Neurological Outcomes Following Mitoquinone Supplementation in Mice, 3 and 7 Days Post Repeated Mild Traumatic Brain Injury

Mohammad Amine Reslan[1], Maya Jammoul[2], Leila Nasrallah[1,3], Maha Tabet[1,4], Marya El-Kurdi[1], Sarin Mekhjian[1], Judith Nwaiwu[1], Mona Goli[5], Yehia Mechref[5], Kevin K Wang[6], Firas Kobeissy[1,6*] and Riyad El Khoury[7*]

1 Department of Biochemistry and Molecular Genetics, Faculty of Medicine, American University of Beirut, Beirut, Lebanon; mr99@aub.edu.lb
2 Department of Anatomy, Cell Biology, and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut, Lebanon; mwj05@mail.aub.edu.lb
3 Department of Psychiatry and Neuroscience, Faculty of Medicine, Université Laval, Quebec City, QC, Canada; leila.nasrallah@hotmail.com
4 Molecular, Cellular and Developmental Biology Department (MCD), Centre de Biologie Integrative (CBI), University of Toulouse, CNRS, UPS, 31062 Toulouse, France; mahatabet.mt@gmail.com; marya.elkurdi@gmail.com
5 Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX, USA; yehia.mechref@ttu.edu; mona.goli@ttu.edu
6 Program for Neurotrauma, Neuroproteomics & Biomarkers Research, Departments of Emergency Medicine, Psychiatry, Neuroscience and Chemistry, University of Florida, Gainesville, FL, USA; kwang@ufl.edu; firasko@gmail.com
7 Department of Pathology and Laboratory Medicine, Neuromuscular Diagnostic Laboratory, American University of Beirut Medical Center, Beirut 1107 2020, Lebanon; re70@aub.edu.lb
* Correspondence: firasko@gmail.com (FK); re70@aub.edu.lb (REK)

Abstract: Mild traumatic brain injury (mTBI) or concussion accounts for the bulk of all head injuries and represents a major health concern. Although an mTBI event may not manifest in neurobehavioral impairment, repeated injuries, known as repeated mTBI (rmTBI), can result in a cumulative effect that may progress to long-term cognitive and functional deficits. To date, there is no FDA-approved drug for TBI in general and rmTBI in particular. In previous studies, we have demonstrated the neuroprotective role of mitoquinone (MitoQ), a mitochondrial antioxidant, in an open head injury model and a model of repeated mild TBI (rmTBI) at a chronic time point (30 days). In this work, we set out to assess the neuroprotective potential of MitoQ at acute (3 days) and subacute time points (7 days) post-injury in a controlled cortical impact model of rmTBI. C57BL/6 male mice were injected intraperitoneally with MitoQ (5 mg/kg) one hour after the first mTBI, and three days after the first injury in both the 3-day and 7-day MitoQ + rmTBI subgroups, with an additional injection four days after the second injection in the 7-day group. Cognitive function was evaluated using the Morris water maze (MWM) while gross and fine motor functions were evaluated by the pole climbing, grip strength, and ladder rung tests. Dihydroethidium (DHE) staining was performed to evaluate oxidative stress while qRT-PCR was used to measure the gene expression of different antioxidant enzymes. Also, immunofluorescence staining was performed on brain tissue to assess the degree of microgliosis and astrocytosis. Our results showed that MitoQ conferred significant protection on days 3 and 7 post-injury against fine motor function impairment induced by rmTBI. Moreover, MitoQ enhanced cognitive function and reduced astrogliosis, microgliosis, and levels of oxidative stress on day 7 post-injury. However, antioxidant gene expression generally remained unaffected. In light of our results, MitoQ administration may be considered a preventive approach that helps to alleviate the neurological manifestations associated with rmTBI early before symptoms progress to long-term deficits.

Keywords: acute injury; antioxidant; behavior; mitochondria; mitoquinone; neuroinflammation; oxidative stress; repeated mild TBI

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1. Introduction

A mild traumatic brain injury (mTBI) or concussion, despite what its name may suggest, has the potential to cause deleterious complications. Even so, many mTBIs are overlooked and undertreated in most patients as symptoms typically subside within 10 days of injury [1]. Uninformed patients, especially those who neglect medical intervention, may inadvertently be exposed to further mild injuries collectively known as repeated mild traumatic brain injury (rmTBI). In the context of closed head mTBI and rmTBI, a spatial delineation between the primary and secondary injuries that typify moderate-severe TBIs is much less defined [2-5]. As such, the insidious nature of rmTBI leaves its victims vulnerable to developing mild cognitive impairment [6], dementia [7], and other neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and chronic traumatic encephalopathy [8-13]. The pathological mechanisms that underlie the clinical manifestations of rmTBI represent complex interactions involving oxidative stress, excitotoxicity, diffuse axonal injury (DAI), neuroinflammation, mitochondrial dysfunction, and cell death [14]. Different TBI biomarkers have been investigated in the context of mitochondrial dysfunction and protein dysregulation via neuroproteomics and even post-translational modifications [4, 15-18].

Despite its widespread prevalence and serious potential complications, mTBI remains an untreatable disease [19]. The Food and Drug Administration (FDA) has yet to approve a drug aimed at treating or mitigating the deleterious consequences associated with TBIs. Therefore, a prospective drug would ideally target one or more pathomechanisms related to TBI [20, 21]. As such, potential drug candidates may include compounds that prevent oxidation, otherwise known as antioxidants, which have been proposed to be theoretically capable of counteracting oxidative stress and attenuating the chain reaction resulting from stress-induced dysregulation. Recently, a synthetic mitochondria-targeted antioxidant drug known as Mitoquinone (MitoQ) has been shown to be a viable contender for the treatment of TBI (mTBI and rmTBI included). We have recently shown that MitoQ alleviated fine motor function and learning impairments, reduced reactive astrogliosis, microgliosis, dendritic and axonal shearing, and increased the expression of antioxidant enzymes in a mouse model of rmTBI at a chronic time point defined as 30 days post-injury [22]. Moreover, we have shown that MitoQ enhanced neurological and cognitive functions, (2) decreased the activation of astrocytes and microglia, and improved axonal integrity and neuronal cell count in the cortex 30 days post-injury in a mouse model of moderate open head injury [23].

MitoQ contains the antioxidant quinone moiety covalently attached to a lipophilic triphenylphosphonium cation (TPP+). The latter enhances significantly (100–1,000-fold) MitoQ's capacity to accumulate within mitochondria [24], where it is adsorbed to the matrix surface of the inner membrane and continually recycled to the active quinol antioxidant form by complex II of the respiratory chain [24]. MitoQ was also shown to be involved in the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway, through which it may exert some of its neuroprotective effects [25].

Considering the neuroprotective effects of MitoQ that have been established in various experimental models of TBI and neurodegenerative diseases [20], we set out to conduct a thorough investigation of the neurological outcomes of MitoQ supplementation at acute and subacute time points after rmTBI. An efficient treatment capable of slowing down or reversing the earliest TBI-associated anomalies may significantly improve later outcomes. This is of particular importance in the case of rmTBI, a condition that seldom exhibits early cautionary clinical signs. In this study, we demonstrate that MitoQ confers neuroprotection as early as 3- and 7-days days post-injury in a controlled cortical impact (CCI) model of rmTBI.

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2. Materials and Methods

2.1. Animals

Seventy-two male C57BL/6 mice, acquired from the Animal Care Facility at the American University of Beirut (AUB), were group-housed under closely regulated conditions at a room temperature of 22°C ± 1°C, a 12-h/12-h light/dark cycle, under pathogen-free conditions, and with unrestricted access to food and water. The Institutional Animal Care and Use Committees (IACUC) at AUB authorized all experimental procedures performed on the animals under the approval number 17-01-458. Based on previous published studies, we did not observe differences in male/female outcomes; thus, this current work involved male mice [23, 26, 27].

2.2. Sample size calculation and experimental groups

Among the cognitive and motor tests we set out to conduct, the Morris water maze (MWM) behavioral test is the most prone to variability. Therefore, the optimal sample size for our studies was determined based on the typical results of this test [22]. In order to achieve a 90% power to detect a difference with a 95% confidence level, at least 9 mice per experimental group were required. We included 10 to 12 mice per group. Animals were equally and randomly divided into two main groups: the 3-day and 7-day groups, each of which was further divided into three subgroups: sham, rmTBI, and rmTBI + MitoQ.

The ideal total number of mice was 60 with an additional 20% to account for attrition, bringing the total number of animals used to 72 mice. These were divided into three subgroups. Subgroup1: Sham (n = 10x2); subgroup 2: rmTBI (n = 10 x 2); and subgroup 3: rmTBI + MitoQ (n = 10 x 2). Animals were assigned to experimental groups by randomly selecting animals that would receive the treatment from those that underwent CCI surgery. Animals that showed weak health post-surgery and exhibited any disease condition were not included in the study. For the experimental groups, four animals per subgroup (n = 4) were used to perform immunofluorescence and four animals (n = 4) per subgroup for the RT-qPCR. For the MWM and Ladder Rung Test — sham: n = 8; rmTBI: n = 9; rmTBI + MitoQ: n = 10. For the Grip Strength and Pole Climbing Tests — sham: n = 10; rmTBI: n = 11; rmTBI + MitoQ: n = 12 mice. At the time of surgery, the mice were 6 to 8 weeks of age and weighed between 20 and 25 g. The experimenter handled the mice for two weeks prior to any surgical procedure or behavioral test. Microscopic examinations and behavioral test analyses were performed by two independent researchers in a blinded manner. Mice were then sacrificed 3 or 7 days after the third injury.

2.3. Surgical procedures

A combination of Ketamine/Xylazine (50 mg/kg Ketamine, 15 mg/kg Xylazine) was administered by intraperitoneal injection to anesthetize the mice. Subsequently, each mouse had its head firmly fixed with ear bars in a stereotaxic frame and a longitudinal skin incision was carefully made to expose the skull. An ophthalmic ointment (Xailin®, Nicox, France) was applied to prevent eye dryness. Following anesthesia, each animal in the sham subgroups had its skull exposed on three consecutive days, after which the skin incision was sutured without inducing a brain injury. The rmTBI and rmTBI + MitoQ subgroups were subjected to three successive injuries 24 hours apart. Injuries were delivered using the Leica Impact One Angle Controlled Cortical Impact (CCI) machine (Leica Microsystems Inc., Buffalo Grove, IL, USA). To simulate concussive conditions, the head was cushioned, and the impactor tip was modified with a rubber tip 5 mm in diameter. To establish a zero point, the impactor tip was lowered to the point of direct physical contact with the skull on the right parieto-temporal region, midway between the lambda and the bregma. Using the Angle Two™ Software (Leica Microsystems Inc.), the impactor was guided to the site of injury, and its center was positioned above the somatosensory area of the parietal cortex (+1.0 mm AP, +1.5 mm ML, and −2 mm DV). The impact duration was kept constant with a dwell time of 1 second and a velocity of 4 m/sec. The depth of the injury was fixed to 0.3 mm. The incision site was sutured and disinfected at the end of

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the procedure with antiseptic liquid. Animals were removed from the stereotaxic frame and laid on a heating pad before reintroduction into home cages.

2.4. Mitoquinone supplementation

According to the protocol described by Xiao et al. [28], MitoQ was administered via intraperitoneal injection, at a dose of 5 mg/kg. The first injection was performed one hour after the first injury in the rmTBI + MitoQ subgroups of the 3-day and 7-day groups. A second dose of MitoQ was administered 3 days after the first injury in both the 3-day and 7-day rmTBI + MitoQ subgroups. Finally, the third injection of MitoQ was performed four days after the second injection in the 7-day rmTBI + MitoQ subgroup (Figure 1). MitoQ (MW=663.64; cat# 10-1363; Focus Biomolecules, Plymouth Meeting, PA, USA) solution was prepared in 10% Dimethyl Sulfoxide (DMSO) and PBS at a concentration of 25 mg/mL, after which it was diluted in PBS to a working concentration of 1 mg/mL.

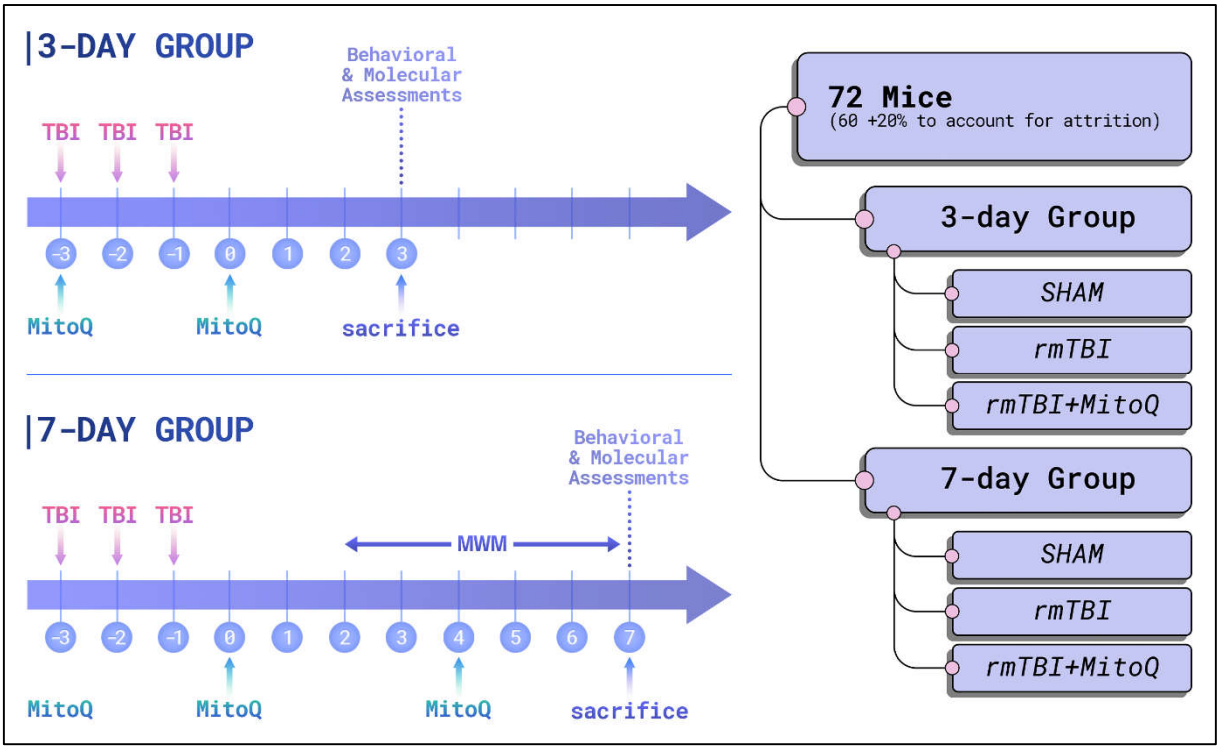

Figure 1. Timeline of the study. Mice were divided into two groups: the 3-day and 7-day groups, each of which was further divided into three subgroups: sham, rmTBI, and rmTBI + MitoQ. Behavioral and molecular studies were performed on day 3 and day 7 after the last injury for the 3-day and 7-day groups respectively except for the Morris water maze, which was only performed in the 7-day group.

2.5. Behavioral assessment of cognitive functions

The Morris water maze (MWM) is a well-validated model to assess spatial learning and memory [29]. The apparatus consists of a large circular pool (diameter: 100 cm diameter; depth: 55 cm) half-filled with water maintained at around 22°C to which a submerged platform of 10 cm in diameter is added. Monochromatic visual cues distributed around the testing area help the animal to navigate from a defined start location toward the escape platform. The three visual cues (in the form of pictures of simple shapes) and the experimenter's location were kept constant throughout the experiment. To prevent water reflection and ensure accurate camera tracking, non-toxic Tempera® white paint was added to render the water opaque. All data were recorded by a video camera suspended above the pool and connected to the automated tracking software ANY-maze 5.2 (Stoelting Co., Wood Dale, Illinois, USA). Each mouse was tested for 5 consecutive days, 3 trials per day, and on day 6, only 1 probe trial was performed; the platform was removed from the pool,

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and the time that the animals spent searching for the platform location was recorded. Typically, the normal animal will spend more time searching in the quadrant where the platform was located, indicating spatial memory. On the first testing day, a one-minute trial (trial 0) was performed before the learning trials. A visible flag is attached to the visible platform to guide the animal to it and ensure that the animals do not have any visual deficits. Following trial 0, three trials were carried out per animal provided that the position of the platform and the starting point of the animal were altered among trials. The pool was virtually divided into four equal quadrants designated as North East (NE), South East (SE), North West (NW), and South West (SW). On day two through day five (the acquisition days) the flag was removed, and the platform was fixed in the NE quadrant. The starting position of the animal, however, varied among the three independent trials. The maximum time set per trial was 60 seconds with an inter-trial interval of 60 seconds. If the animal failed to find the platform during this time, it was guided by the experimenter and allowed to rest on the platform for 30 seconds. If the animal reached the platform before this time, the test was considered fulfilled, and the animal was kept on the platform for 30 seconds. On day six, the probe trial day, the platform was removed, and one trial was performed per animal, during which each mouse was allowed to swim for 60 seconds. Throughout the experiment, mice were dried with a towel after each trial and allowed to rest in a warm cage. This test was solely performed in the 7-day group because it requires at least 6 days to complete.

2.6. Behavioral assessment of motor functions

2.6.1. The pole climbing test

The pole climbing test was conducted to study balance and motor coordination. This test was performed according to the protocol described by Ogawa et al. [30]. Briefly, mice were placed on the top of a vertical metal pole (length: 60 cm; diameter: 1 cm) face facing upwards and were left to descend freely from the topmost point. The time to climb down to the base of the pole (t-total) was measured in 3 consecutive trials for each mouse.

2.6.2. The grip strength test

The grip strength test was performed using a 47200 grip strength meter (UGO BASILE, Gemonio, Italy) in order to evaluate the skeletal muscle force. The grip strength meter harbors a trapeze-shaped metal handle that the mouse was allowed to grasp with both paws while held up by its tail. The instrument automatically measures the force (gram force; gf) exhibited by the mouse to release its grip. The average gf, of three independent trials, was normalized against the mouse's weight in grams.

2.6.3. The ladder rung test

A horizontal ladder rung walking test, developed by Metz et al. [31], was used to assess walking ability and to measure both forelimb and hind limb placement, stepping, and inter-limb coordination. The apparatus used for this experiment consists of removable rungs enclosed by two transparent Plexiglass side walls (length: 70 cm; width: 15 cm). All animals were habituated and trained to cross the ladder for one week before the surgery, and a baseline score was obtained one day before the first injury. On training days, the rungs were spaced equally apart at 1 cm intervals. This pattern was altered for baseline and testing days. All tests were video recorded at a tilted angle to aid in later analysis. The video recording was analyzed using frame-by-frame analysis. On all days, mice were allowed to cross the rungs at least four times. Each forelimb was evaluated according to a foot fault scoring system as described by Metz *et al.* [32]. A minimum of 20 steps was analyzed for each forelimb. The motor score was calculated as the percentage (%) of the baseline.

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2.7. Molecular assessment

2.7.1. Tissue processing

Brains were snap-frozen and stored at -80°C for later analysis *via* qRT-PCR. Immunofluorescence staining was performed on tissue sections of brains harvested from mice perfused transcardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4). Before sectioning on the microtome, brains were post-fixed overnight in 4% PFA at 4°C, cryoprotected in 30% sucrose solution for 24h at 4°C, and frozen in liquid isopentane at -45°C.

2.7.2. Dihydroethidium staining

Dihydroethidium (DHE) staining was performed as previously described [33]. After fixation in 4% PFA, free-floating sections (40 µm thick) were mounted on microscope slides and incubated with 50 µM DHE (D11347, Invitrogen, Carlsbad, CA, USA) for 30 minutes at 37ºC in a humidified light-protected chamber. Sections were then washed three times with PBS. Photographs were obtained by laser scanning confocal microscopy (LSM 710, Carl Zeiss, Oberkochen, Germany) that is operated by the Zen 2011 Software. The mean fluorescence intensity (MFI) of DHE from six independent images per condition was measured using NIH ImageJ software. Images were obtained with consistent acquisition settings for comparison purposes. To determine the mean grey value, images were converted to 8-bit grayscale.

2.7.3. Immunofluorescence (IF) staining

Free-floating brain sections, 40-micron thick, were collected and processed as previously described [34]. Briefly, sections were first washed with PBS then PBS-T (0.1% Triton in PBS) and incubated in a blocking solution of 10% heat-inactivated fetal bovine serum (FBS) in PBS-T for 1.5 hours. Tissue sections were then incubated with primary antibodies diluted in 1% FBS overnight at 4 °C. Anti-GFAP antibody (MCA-5C10, EnCor Biotechnology, Gainesville, Florida, USA) was used to detect astrocytes, while anti-Iba-1 antibody (019-19471, Fujifilm Wako Chemicals, Richmond, Virginia, USA) was used to detect microglia. Both primary antibodies were diluted 500-fold (1:500).

After an overnight incubation with primary antibodies, tissue sections were rinsed with PBS-T and incubated with the appropriate fluorochrome-conjugated secondary antibody at a dilution of 1:1000 for 1 hour at room temperature, followed by three washes in PBS-T. Sections were further counterstained with 1 µg/ml of Hoechst (Sigma Aldrich), diluted in PBS, and mounted on microscope slides using Fluoromount (F4680-25ML, Sigma Aldrich). For each subgroup (sham, rmTBI, or rmTBI+MitoQ), tissue sections from three mice (three sections per mouse) were stained. Images were quantified using ImageJ. To quantify the expression of Iba-1, individual cells were counted, and the resultant number was divided by the area of the selected region. The expression of GFAP was measured similarly to that of DHE.

2.7.4. qRT-PCR

Total RNAs were extracted using Trizol (T9424-100ML, Sigma Aldrich, St. Louis, Missouri, USA). Contaminating genomic DNA was removed with the TURBO DNA-free™ Kit (AM1907, Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. Reverse transcription (1.5 µgs of total RNA) was performed using the iScript™ cDNA Synthesis Kit (1708890, Bio-Rad, Hercules, California, USA). Quantitative real-time PCR was conducted using 0.5 µL of cDNA, 10µM of each of the reverse and forward primers, and the Quantifast® SYBR® Green PCR Master Mix (204054, Qiagen, Hilden, Germany). PCR cycling conditions consisted of a three-step procedure: 1) 10 minutes at 95 °C for one cycle, 2) 10 seconds at 95 °C followed by 30 seconds at 60 °C and 30 seconds at 72 °C for 40 cycles, and 3) 5 minutes at 72 °C for one cycle. Primers used in qRT-PCR experiments are listed in Table 1.

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Table 1. The sequence of primers used in qRT-PCR.

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

2.8. Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Shapiro-Wilk test confirmed that the data did not follow a normal distribution. Considering that, non-parametric tests, namely the Kruskal-Wallis test followed by Dunn's test, were performed to compare the three subgroups. Data were plotted as mean ± SEM. P-values were calculated, and $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ (signified as *, **, ***; respectively) were regarded as significant.

3. Results

3.1. MitoQ improves fine motor function impairment induced by rmTBI at acute and subacute time points

In the current study, we evaluated gross motor functions at subacute time points following rmTBI using the grip strength and the pole climbing tests. Obtained data showed that balance and motor coordination, as assessed by the pole climbing test, were similar in the three groups at both days 3 and 7 post-injury. Muscle strength, however, was significantly ameliorated by MitoQ treatment compared to the rmTBI subgroup at both day 3 and day 7 post-injury as shown by the grip strength test. Muscle strength was even significantly higher in the MitoQ treated subgroup compared to that of the sham subgroup at day 3 post-injury. It should be noted that rmTBI did not result in a significant decrease in muscle strength at both day 3 and day 7 post-injury when compared to the sham subgroup. While gross motor functions are often the subject of evaluation, fine motor functions are seldom assessed post-TBI. Our previous study has shown that fine motor functions were mildly but significantly altered by rmTBI at a chronic time point, and that MitoQ alleviated such impairment. We therefore set out to assess fine motor functions at acute and subacute time points after rmTBI using the ladder rung test [22].

Our results showed that rmTBI caused a significant alteration in fine motor function on days 1, 3 and 7 post-injury ($p \leq 0.01$, $p \leq 0.05$, and $p \leq 0.05$ versus sham mice respectively), with a noticeable spontaneous improvement between day 1 and day 7. MitoQ treatment entirely prevented motor function impairment on days 1, 3, and 7 post-injury ($p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.01$ versus untreated rmTBI mice; respectively) (**Figure 2**). It should be noted that the observed protection provided by MitoQ in treated mice was only evident in the left forelimb.

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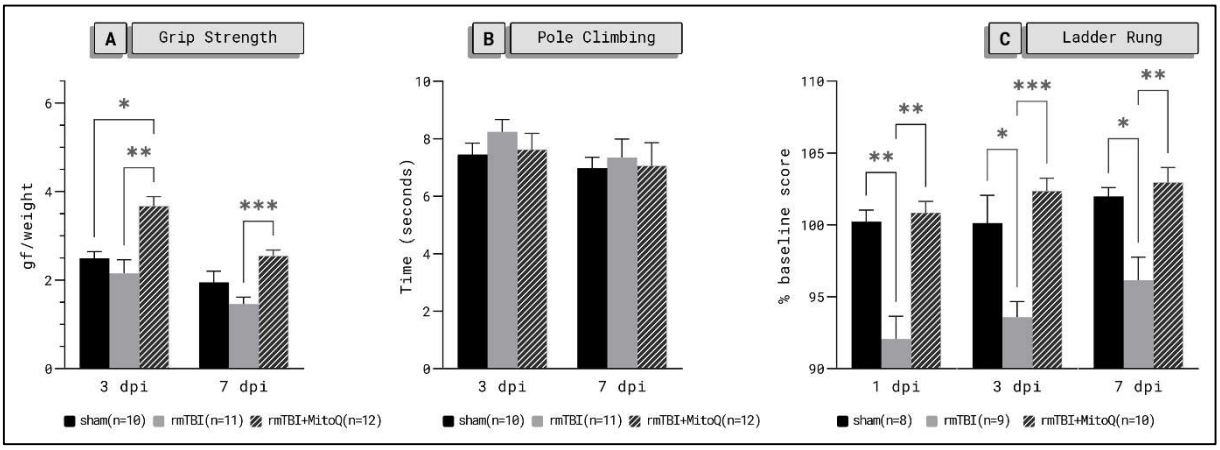

Figure 2. Assessment of gross and fine motor functions. A) Grip strength test, expressed in gf/g (gram force per gram of mouse), was used to assess muscle strength. Although MitoQ treatment showed significant increase in muscle strength, rmTBI did not induce a significant loss in muscle strength when compared to the sham subgroup at both day 3 and day 7 post-injury. B) Pole climbing test, expressed in seconds (total time needed to reach the base of the pole), was used to assess coordination and balance. No differences were noted among the three subgroups at both day 3 and day 7 post-injury. C) The ladder rung test expressed as % baseline score (calculated by dividing the score on days 1, 3, and 7 by the baseline score) was used to assess fine motor function. A significant fine motor impairment was induced by rmTBI and entirely prevented by Mito Q treatment on days 1, 3, and 7 post-injury. Only results of left forelimb assessment are shown in the figure. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.2. MitoQ mitigates rmTBI-associated learning deficits at a subacute time point

MitoQ's potential to prevent cognitive deficits induced by rmTBI was then assessed using the Morris water maze (MWM) test. The latter was only applied to mice in the 7-day group. No differences in latency to the platform were noted among the different groups on day one of the data acquisition conducted 4 days after the last TBI (Figure 3). On acquisition days 2, 3, and 4, untreated rmTBI mice required additional time to reach the hidden platform compared to the sham and MitoQ-treated mice. This delay was significant on day 2 ($p \leq 0.05$; rmTBI versus sham) and day 3 ($p \leq 0.05$; rmTBI versus rmTBI + MitoQ) of data acquisition (**Figure 2B**). Our findings are in accordance with other studies that showed an increased latency in brain-injured mice to reach the hidden platform [35-37]. Our findings suggest that rmTBI results in a cognitive learning deficit that is at least partially reduced by MitoQ. It should be noted that no differences in speed were observed among the three subgroups. Hence, observed deficits do not reflect symptoms of vision impairment or a motor deficit but rather a substantial cognitive aberration. Moreover, no significant differences in the latency to reach the target quadrant or in the time spent in that quadrant were recorded on the probe trial day, which indicates that our mTBI model did not result in memory retention deficits (**Figure 3C-D**).

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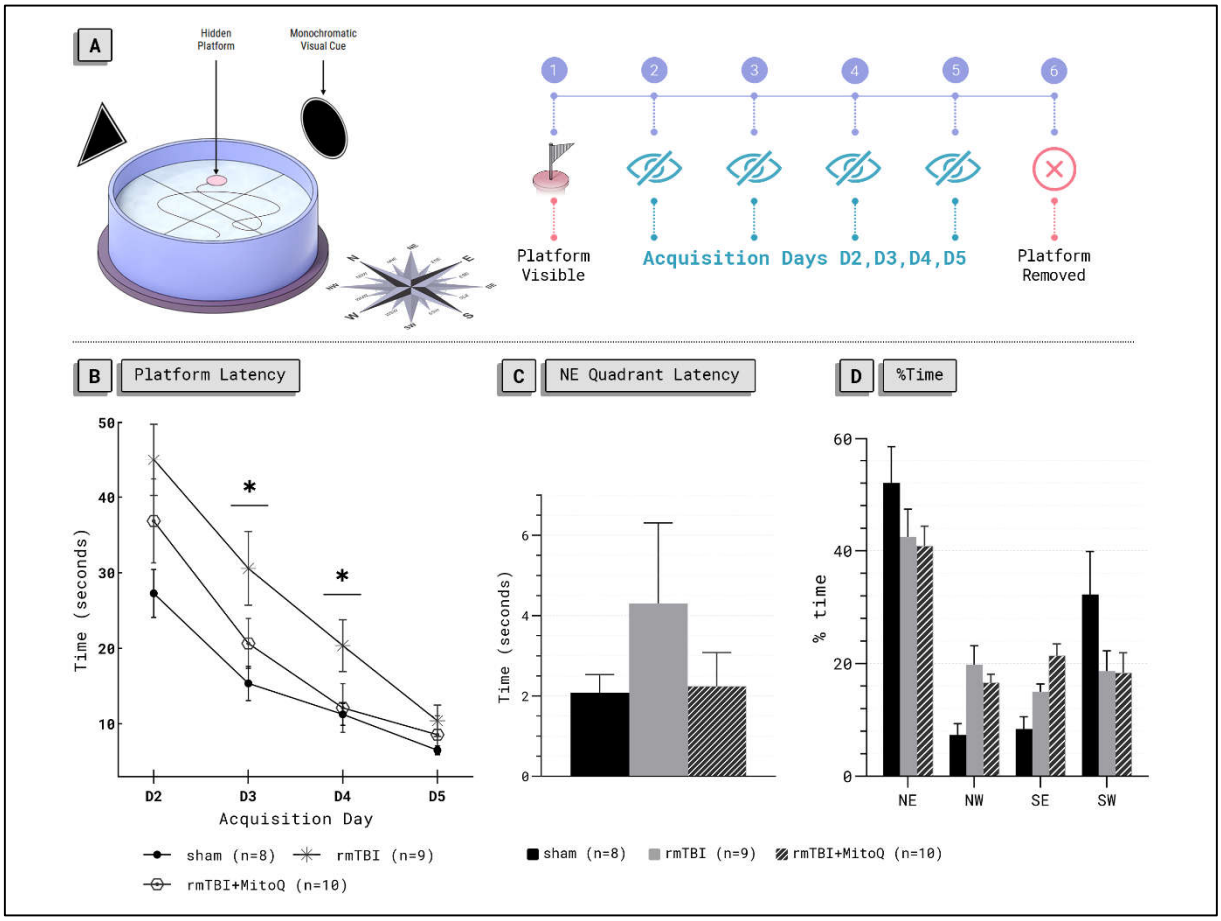

Figure 3. Assessment of spatial learning and memory in mice using the Morris water maze (MWM). A) The MWM test is performed in a circular water pool visually divided into four quadrants and a hidden platform. Acquisition of data was performed on days 2 (D2), 3 (D3), 4 (D4), and 5 (D5), during which the platform was concealed underwater. Days 1 and 6 represent cue day and probe trial day, respectively. B) Represents the time needed by mice to reach the platform recorded on acquisition days D2, D3, D4, and D5. rmTBI mice showed the worst performance, particularly on D3 and D4. MitoQ treatment significantly alleviated the cognitive function impairment. C) Represents the time needed to reach the target quadrant (NE) on the probe trial day. D) Represents the percentage of time spent in every quadrant. The MWM test was conducted on mice from the 7-day group only. MWM test was initiated 4 days after the last TBI. Data represent the mean ± SEM. * $p \leq 0.05$ sham group versus experimental groups; NE: north-east quadrant; NW: north-west quadrant; SE: south-east quadrant; SW: south-west quadrant.

3.3. MitoQ reduces rmTBI-induced reactive astrogliosis in the hippocampus at a subacute time point

Astrocytes are pivotal early responders to brain trauma. The intermediate filament protein GFAP (Glial Fibrillary Associated Protein) represents a biomarker of astrocyte activation that is known to be profoundly upregulated after TBI [38]. In order to assess the degree of astrocyte activation in our rmTBI model and the potential of MitoQ to mitigate astrogliosis, we evaluated GFAP expression by immunofluorescence using brain sections obtained from the three subgroups. In the cortical parenchyma, astrocytes failed to express sufficiently detectable levels of GFAP (data not shown). It has previously been demonstrated that astrocytes in different cerebral regions may express unequal immunoreactivities to GFAP and that astrocytes in the dorsal cortex and thalamus react weakly to GFAP labeling [39]. Accordingly, analysis of GFAP expression in the cortex was excluded from this study. GFAP expression was significantly induced, however, in the hippocampus of rmTBI mice (both 3-day and 7-day groups) compared to the sham subgroups, delineating astrocytic activation following rmTBI. MitoQ administration significantly

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decreased GFAP expression and therefore the number of reactive astrocytes compared to that noted in the rmTBI mice of the 7-day group ($p \leq 0.01$; rmTBI versus rmTBI). This same effect was not observed in the 3-day group. The morphology of hippocampal astrocytes was also studied in the three subgroups. An increase in the thickness of cell bodies and cellular processes was observed in rmTBI mice while hippocampal astrocytes in sham and rmTBI + MitoQ mice assumed a ramified morphology that is more closely associated with a resting state (**Figure 4B**).

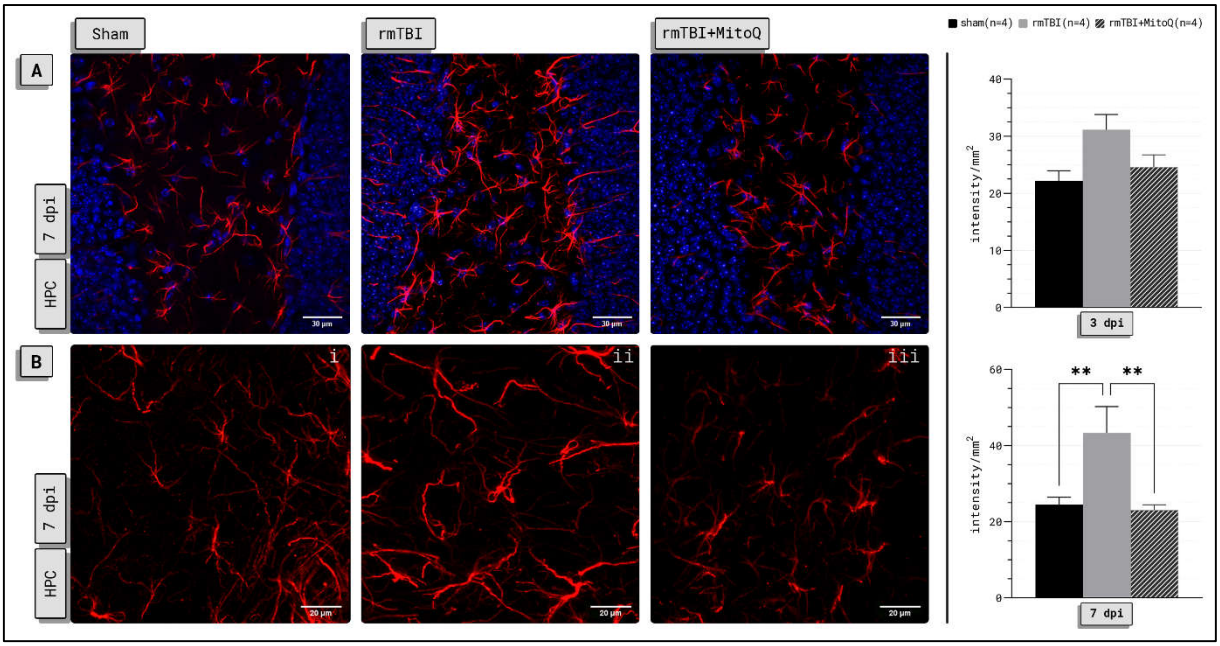

Figure 4. GFAP expression assessed by immunofluorescence staining. A) Reactive astrocytosis, measured in terms of intensity/mm², was more prominent in the hippocampus of rmTBI mice. MitoQ was shown to significantly decrease this overactivation of astrocytes in the 7-day group but not in the 3-day group. B) i) Quiescent astrocytes in the hippocampus of sham mice. ii) Activated astrocytes showing increased thickening of the cell body and processes. iii) MitoQ administration reverted hippocampal astrocytes to a more "resting state", indicating decreased reactive astrogliosis. The mean fluorescence intensity (MFI) associated with GFAP was estimated using the NIH ImageJ software in nine independent fields. Bar graphs display averages ± SEM of GFAP MFI/ mm². dpi: days post-injury; CTX: cortex; HPC: hippocampus; **: $p \leq 0.01$.

3.4. MitoQ reduces rmTBI-induced cortical microgliosis at a subacute time point

Microglia, the resident macrophages of the CNS, respond to damage by proliferating and migrating towards the injury site [40]. Accordingly, microglia were quantified in the cortex and the hippocampus, with the assumption that a higher or lower density of microglia in regions affected by the rmTBI would correlate with more or less damage in the tissue, respectively. In the cortex, an increase in microglia count was noted in rmTBI mice as early as 3 days post-injury ($p \leq 0.05$; rmTBI versus sham) (Figure 5). MitoQ administration significantly attenuated microgliosis only 7 days post-injury ($p \leq 0.01$; rmTBI + MitoQ versus rmTBI). In the hippocampus, although a tendency for MitoQ to alleviate microgliosis was observed, the effect was not statistically significant (Figure 5). It should also be noted that changes in microglial cell morphology were not discernible enough to allow for the analysis of the "reactive state", suggesting only mild microgliosis. Such results may imply that MitoQ plays a prominent role in preventing tissue damage induced by rmTBI, which translates into a lesser number of microglia infiltrating the injury site.

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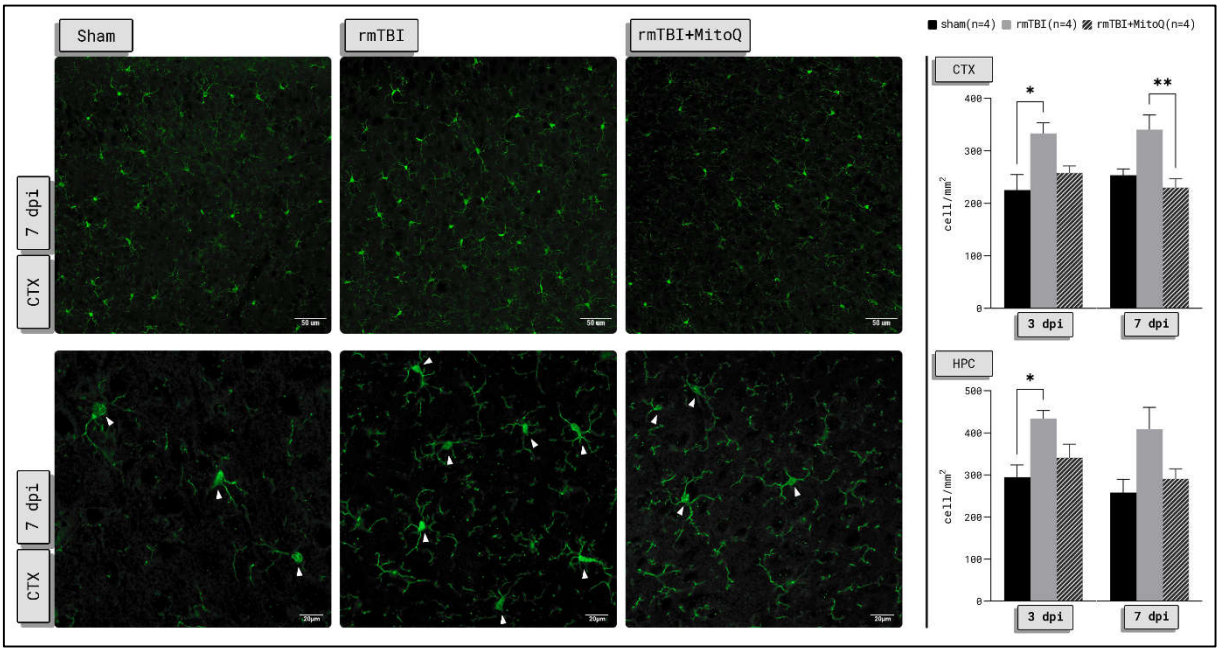

Figure 5. Immunofluorescence staining against Iba-1 revealed a significant increase in microglia in injured tissue following rmTBI, both in the cortex and hippocampus, of mice belonging to the 3-day and 7-day groups. MitoQ administration resulted in a significant alleviation of microgliosis in the cortex of mice of the 7-day group only (upper histogram). No significant impact of MitoQ was observed in the hippocampus (lower histogram). Histograms represent Iba-1 positive cells per mm². The number of Iba-1 positive cells was quantified in nine independent fields. *: $p \leq 0.05$; **: $p \leq 0.01$; dpi: days post-injury; CTX: cortex; HPC: hippocampus.

3.5. No statistically significant increase in antioxidant genes expression following MitoQ administration is observed 3 or 7 days post-rmTBI

MitoQ has previously been shown to enhance the gene expression levels of endogenous antioxidant enzymes such as superoxide dismutase 2 (SOD2) and catalase (CAT) via the Nrf2 pathway [27, 41]. Our data show an induction—albeit not statistically significant—in the expression of the transcription factor Nrf2 at day 3 post-injury in the ipsilateral cortex of the rmTBI and rmTBI + MitoQ subgroups compared to the sham subgroup. Expression levels of antioxidant enzymes SOD2 and CAT were, however, similar in the ipsilateral cortex of the three subgroups. At day 7 post injury, expression levels of the Nrf2, SOD2 and CAT were similar in the ipsilateral cortex of the three subgroups.

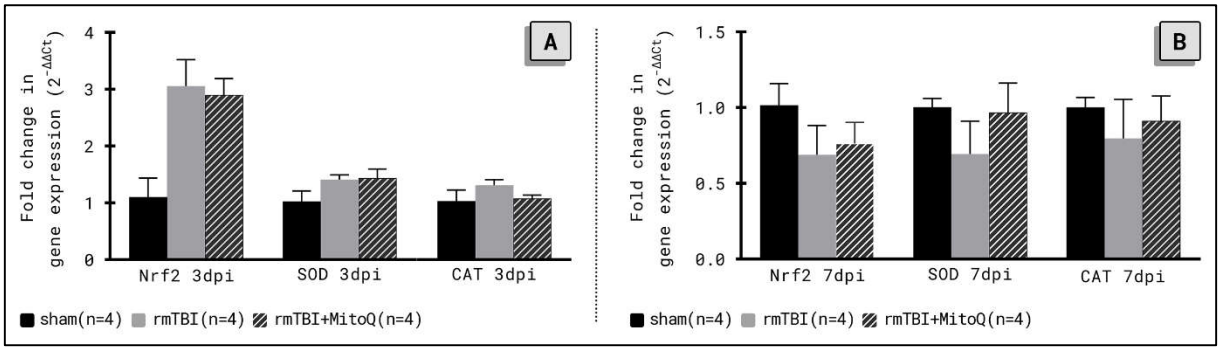

Figure 6. Nrf2 transcription factor and antioxidant enzymes SOD2 and CAT expression levels were assessed in the ipsilateral cortex by qRT-PCR in the three subgroups at day 3 (A) or day 7 (B) post-injury. Expression levels were normalized against those of ß-actin. dpi: days post-injury.

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3.6. MitoQ reduces levels of reactive oxygen species (ROS) overproduction in the cortex and hippocampus of rmTBI mice

We further assessed whether the protective effects of MitoQ could be mediated, at least partially, by its antioxidant potential. For that, we evaluated by dihydroethidium (DHE) staining the level of reactive oxygen species (ROS) in the cortex and the hippocampus of mice belonging to the three study subgroups. While DHE is primarily used to measure ROS, particularly superoxide anions ($O_2^{\cdot-}$), in live cells, it has also been shown to be effective in quantifying ROS levels in fixed tissue sections [42]. Quantification of DHE-stained sections of the cortex and the hippocampus revealed a significant increase in superoxide levels in rmTBI brains 3 and 7 days post-injury compared to the sham brains ($p \leq 0.01$; rmTBI versus sham in the 3-day group — and $p \leq 0.05$; rmTBI versus sham in the 7-day group, only in the cortex) (**Figure 7**). MitoQ treatment did not result in a significant reduction in superoxide levels 3 days after injury. However, MitoQ significantly reversed the rmTBI-induced increase in superoxides in the 7-day group in both the cortex and hippocampus ($p \leq 0.05$) compared to rmTBI mice (**Figure 7**).

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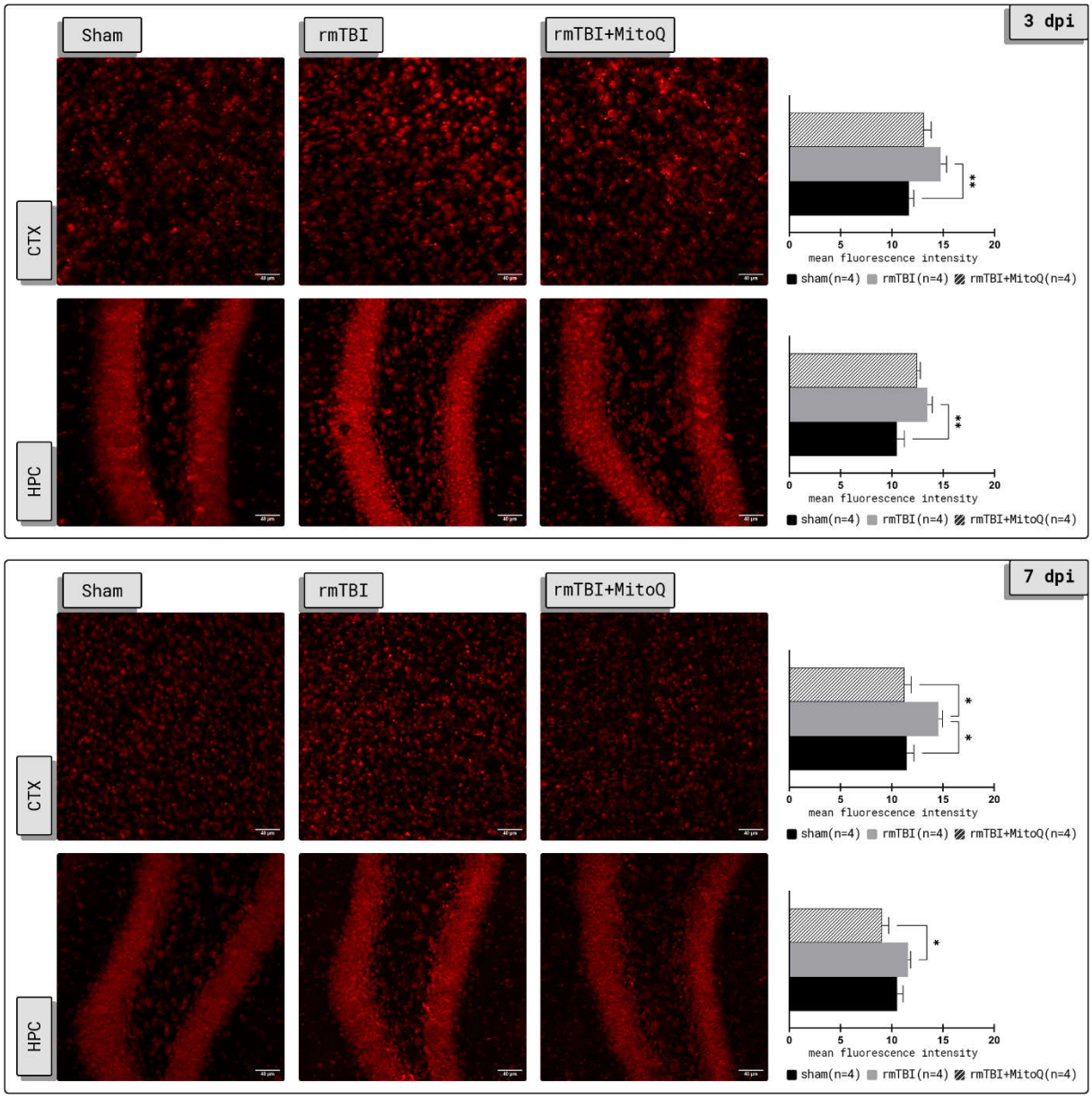

Figure 7. Quantification of ROS levels by dihydroethidium (DHE) staining. Tissue sections from the cortex (CTX) and the hippocampus (HPC) of mice brains from the sham, rmTBI, and rmTBI + MitoQ subgroups of both 3-day and 7-day groups were stained with DHE and analyzed by fluorescence microscopy. Histograms represent the quantification of superoxide ions level expressed as mean fluorescence intensity (MFI). The mean fluorescence intensity (MFI) corresponding to DHE was estimated using the NIH ImageJ software in nine independent fields. Bar graphs display averages ± SEM of DHE MFI/ mm^2. dpi: days post-injury; CTX: cortex; HPC: hippocampus; *: $p \leq 0.05$.

3. Discussion

Epidemiological studies indicate that the bulk of all TBIs (>75 %) are mild (mTBI) [43]. The incidence is likely to be even more pronounced since mTBI results in non-life-threatening events whose symptoms often subside spontaneously in a short period of time. As such, the majority of the patients do not seek medical attention or are discharged immediately after clinical examination. Post concussive symptoms can however persist in 10-40 % of the patients who may develop long-term cognitive deficits, white matter changes, and neuropsychological disorders and increase their risk of developing neurodegenerative diseases, more so in patients who are likely to incur further repetitive mild injuries (rmTBI) [2]. Untangling the neuropathological consequences induced by rmTBI is key in the endeavor to develop adapted treatments. In this study, we evaluated the

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neuroprotective potential of mitochondrial-targeted antioxidant MitoQ at acute and subacute time points post-injury in a CCI model of rmTBI. In a first series of studies, we analyzed various motor and cognitive parameters. Using the ladder rung test we evaluated fine motor functions associated with forebrain control. We have shown that our injury model resulted in a fine motor impairment at days 1, 3 and 7 post-injury that was entirely prevented by MitoQ treatment. Our rmTBI model did not however result in gross motor function deficits at subacute time points following injury as seen by the grip strength and the pole climbing tests. The treated subgroup showed even a slight but significant improvement of muscle strength compared to that of the sham subgroup. This is most likely related to the variability of the injury outcomes, which occurs occasionally, resulting in non-significant gross motor dysfunction. Moreover, we showed that our CCI model of rmTBI resulted in a cognitive learning deficit characterized by an increased latency of brain-injured mice to reach the hidden platform in the MWM. Notably, MitoQ treatment improved significantly both learning deficits and memory retention. Such executive functions are controlled particularly by medial temporal regions and dorsolateral prefrontal cortex [44], which reflects the diffuse nature of the injury that was significantly alleviated by MitoQ. As a corollary, MitoQ is able to reach those brain regions. Mitochondrial impairment as well as oxidative damage represent integral parts of the pathophysiological process associated with rmTBI therein. The observed neuroprotective effects of MitoQ are also in line with previous studies that demonstrated the safe profile of MitoQ and its efficacy in alleviating cognitive impairments and motor deficiencies [23, 27, 45].

As an antioxidant that efficiently targets the mitochondria, MitoQ may exert its neuroprotective effect through the detoxification of ROS either directly or indirectly by inducing the expression of a set of antioxidant genes. MitoQ antioxidant function may further alleviate oxidative stress and hence prevent mitochondrial impairment, axonal injury, neuroinflammation, *etc*. [46]. In that regard, we showed that MitoQ treatment resulted in a significant decrease in rmTBI-associated ROS overproduction within the cortex and the hippocampus 7 days post-injury. However, contrarily to what was previously shown in several studies [41, 47-49], we didn't observe an induction in the assessed antioxidant enzymes CAT and SOD2, neither on day 3 nor day 7 post-injury.

Similarly, the transcription factor Nrf2 gene expression did not change in the three subgroups on day 7 post-injury. However, on day 3 post-injury we observed a significant increase in Nrf2 expression that was similar in both the rmTBI and rmTBI + MitoQ subgroups. We speculate that such an induction may be related to the direct reaction to the primary phase from the acute injury. The intrinsic antioxidant nature of MitoQ as well as its bioavailability that may vary across experiments, may preclude under specific circumstances an induction of antioxidant enzymes or other specific signaling pathways involved in ROS detoxification. Our results are in favor of such a scenario as our injury model resulted in ROS overproduction that was mitigated by MitoQ probably through direct effect. Oxidative stress and ROS play critical roles in the onset and progression of several diseases including neurodegenerative disorders [50-54]. In this context, cell death observed in Parkinson's disease (PD) was attributed to an increase in oxidative stress induced by a malfunctioning mitochondrial complex-I and impaired oxidative phosphorylation or to the loss of mitochondrial membrane potential [55]. While MitoQ was shown not to slow down PD progression in untreated Parkinson's patients, an *in vitro* study on a PD model demonstrated that it could prevent mitochondrial fragmentation induced by oxidative stress [56, 57]. In preclinical studies in primary neurons from a mouse model (Tg2576 line) of AD and in the *Caenorhabditis elegans* model of AD overexpressing human Aβ, MitoQ showed a therapeutic potential by preventing amyloid beta-induced cell toxicity and restoring mitochondrial oxidative capacities [58, 59]. Similarly, MitoQ treatment showed neuroprotection in a mouse model of ALS ($SOD1^{G93A}$) and highlighted the roles of mitochondria and nitro oxidative stress in the pathogenesis of ALS. Moreover, MitoQ treatment ameliorated fine motor control, and reduced oxidative damage and ROS-induced autophagy particularly in skeletal muscle in a mouse model of Huntington's disease [60, 61]. The significant neuroprotection conferred by MitoQ in various

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neurodegenerative models, in addition to the mechanistic evidence that it provided for the role of mitochondria, oxidative damage, and other cellular processes in such diseases, warrant further basic and clinical studies on neurodegenerative disorders and particularly on mTBI using MitoQ.

The clinical outcomes of mTBI can be divided into acute phase and secondary late-phase symptoms [62]. In the acute phase, patients may exhibit dizziness, fatigue, impaired cognition, sleep troubles, and headaches, while in the late phase, the symptoms progress and are mainly affected by psychological factors [62]. Similar to neurodegenerative diseases, rmTBI can impair mitochondrial function, increase oxidative stress, among other cellular processes, and cause long-lasting symptoms attributed to epigenetic changes and oxidative damage to mitochondrial DNA [63, 64]. The acute phase after rmTBI presents an important therapeutic window to counteract these symptoms that may be exacerbated by subsequent mTBI, and ultimately lead to permanent deficits [65]. mTBI not only causes mitochondrial impairment but also leads to apoptosis, axonal injury, and a breakdown in the blood-brain-barrier (BBB) [66, 67]. Axonal injury and BBB breakdown are worsened following rmTBI compared to a single injury [68]. Moreover, increased levels of reactive oxygen species can contribute to BBB breakdown by activating metalloproteinases capable of degrading the basement membrane and tight junctions surrounding the BBB [65]. Interestingly, due to its hydrophobicity, MitoQ can cross the BBB and cellular membranes to reach the mitochondria [69]. This mechanism may explain MitoQ's ability to protect against symptoms of rmTBI by acting as an antioxidant.

In addition to its impact on ROS production, we have shown that MitoQ treatment reduced rmTBI-induced astrogliosis in the hippocampus and rmTBI-induced microgliosis in the cortex 7 days post-injury. The antioxidant potential of MitoQ may have resulted in the observed mitigation of the neuroinflammatory profile by alleviating rmTBI-induced oxidative stress [70, 71].

MitoQ was also shown to reduce neuroinflammation by decreasing the level of pro-inflammatory cytokines and increasing the levels of anti-inflammatory cytokines in microglial cells [72]. Neuroinflammation is yet another mechanism that may involve a mitochondrial dysfunction and ROS overproduction that may be controlled by MitoQ treatment. In that regard, a growing number of evidence suggest a role of mitochondria and ROS in the assembly and activation of the NLRP3 (NOD-like receptor family pyrin domain-containing 3) inflammasome. The latter is an essential component of the innate immune system that fine-tunes the inflammatory process mediated particularly by microglia and macrophages. Hence, mitochondria-induced damages in rmTBI may induce a dysregulation in the NLRP3 inflammasome and therefore contribute partly to the neuroinflammation associated with rmTBI [73].

4. Conclusion

The neurological sequelae of rmTBI are multifold and can greatly impact the life quality of patients as time progresses. This study showed that MitoQ administration at acute and subacute time points protects from cognitive and motor impairment following rmTBI and reduces neuroinflammation and oxidative stress. These findings provide evidence for the potential role of mitochondria, among other mechanisms, in the pathophysiology of rmTBI and highlight the potential to prevent or mitigate acute and subacute rmTBI symptoms before they worsen and lead to permanent deficits. The use of MitoQ can also be extended to treat motor and cognitive impairment observed in patients with neurodegenerative diseases involving mitochondrial dysfunction and oxidative stress. The efficacy of MitoQ highlights the need to investigate further how mitochondrial dysfunction contributes to the initiation and/or progression of the symptoms following injury. In that context, studying the impact of rmTBI on mitochondrial oxidative capacities, mitochondrial network, and mitochondrial membrane potential is needed. Even more importantly, investigating its effect on mitochondrial inner membrane lipid composition, integrity of glial and neuronal cells, and the capacity of MitoQ to overcome potential mitochondrial

impairments is also highly warranted. Finally, future studies are needed to identify the potential interaction between mitochondrial dysfunction, increased oxidative stress, neuroinflammation, and apoptosis in mediating the neurological manifestations of rmTBI in the early phase.

Author Contributions: REK, KW, YM and FK were responsible for the conceptualization of the research idea. MAR, MK MG, and MT performed the experiments. MAR, LN, MG, JN, SM and MJ analyzed the data. MAR, MJ, REK, YM, FK and MG wrote the manuscript. All authors read and approved the final manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committees (IACUC) at the American University of Beirut (approval number 17-01-458).

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare that they have no competing interests.

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