Nitroxides affect neurological deficits and lesion size induced by a rat model of traumatic brain injury

Razia Zakarya[1,2], Arjun Sapkota[1], Yik Lung Chan[1], Jadvi Shah[1], Sonia Saad[3], Steven E. Bottle[4], Brian G. Oliver[1,2], *Catherine A. Gorrie[1], *Hui Chen[1]

1 School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW, Australia

2 RCMB, Woolcock Institute of Medical Research, The University of Sydney, NSW, Australia

3 Kolling Institute of Medical Research, Royal North Shore Hospital, The University of Sydney, NSW, Australia

4 Science and Engineering Faculty, Queensland University of Technology, QLD, Australia.

*** Correspondence:**

Assoc Prof Catherine A. Gorrie. School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW, Australia. Tel: + 61 2 9514 8298, E-mail: Catherine.gorrie@uts.edu.au

Assoc Prof Hui Chen. School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, NSW, Australia. Tel: + 61 2 9514 1328, E-mail: hui.chen-1@uts.edu.au

Abstract

Research has attributed tissue damage post-traumatic brain injury (TBI) to two-pronged effects, increased reactive oxygen species (ROS) and impairment of endogenous antioxidant defence systems, underpinned by manganese superoxide dismutase (MnSOD). Novel antioxidant nitroxides have been shown to mimic MnSOD to ameliorate oxidative stress related disorders. This project aims to investigate the effects of two nitroxides, CTMIO and DCTEIO, on the neurological outcomes following moderate TBI in rats induced by a weight drop device. The rats were immediately treated with CTMIO and DCTEIO (40mM in drinking water) post-injury for up to 2 weeks. The brains were histologically examined at 24 hours and 6 weeks post injury. DCTEIO reduced the lesion size at both 24h and 6 weeks, with normalised performance in sensory, motor and cognitive tests at 24h post-injury. Astrogliosis was heightened by DCTEIO at 24h and still elevated at 6 weeks in this group. In TBI brians, cellular damage was evident as reflected by changes in markers of mitophagy and autophagy (increased fission marker dynamin-related protein (Drp)-1, and autophagy marker light chain 3 (LC3)A/B and reduced fusion marker optic atrophy (Opa)-1). These were normalised by DCTEIO treatment. CTMIO, on the other hand, seems to be toxic to the injured brains, by increasing injury size at 6 weeks. In conclusion, DCTEIO significantly improved tissue repair and preserved neurological function in rats with TBI possibly via a mitophagy mechanism. This study provides evidence for DCTEIO as a promising new option to alleviate lesion severity after moderate TBI, which is not actively treated.

Keywords: TBI, neurological function, Nitroxide, antioxidant, mitophagy.

1 INTRODUCTION

Traumatic brain injury (TBI) is caused by sudden mechanical damage to the brain, by either rapid acceleration or deceleration, or by an impact to the upper body including the head. Globally, more than 10 million people affected by TBI are hospitalized [1; 2]. The incidence of TBI worldwide is rising, primarily due to the increasing frequency of motor vehicle accidents [1; 2].

The initial mechanical force on the brain tissue results in haemorrhage and immediate cell death [3; 4], followed by cascades of biochemical reactions, which can ultimately result in neurological defects [5]. Tissue energy demands elevate post-injury to facilitate tissue repair, thereby adding to the mitochondrial burden and subsequent buildup of reactive oxygen species (ROS) as a by-product. We have previously shown increased inflammation and oxidative stress at the site of injury in a rat model of moderate TBI induced by cortical contusion. This led to overconsumption of the endogenous antioxidant manganese superoxide dismutase (MnSOD) [6]. As the major site of ROS production, mitochondria are particularly vulnerable to oxidative damage. Indeed, increased oxidative stress and related mitochondrial damage have been implicated in tissue damage and neurological functional decline post-TBI by several studies [7; 8; 9].

Mitochondria are dynamic organelles with integrity maintained by fission and fusion processes [10]. Fission separates damaged mitochondrial fragments from the healthy parts and is facilitated by dynamin-related protein (Drp)-1, whilst fusion merges two healthy fragments via optic atrophy (Opa)-1 to form new mitochondrion. However, increased cellular stress – similar to that induced by TBI – leads to a skew towards fission over fusion, thereby impeding energy production. We posit that reducing oxidative stress to maintain mitochondrial integrity and function may prevent excessive tissue damage and neurological functional decline after TBI.

Antioxidants such as resveratrol have been shown to benefit tissue repair and neurological function in animal models of TBI [11; 12; 13], yet poor bioavailability has impeded its efficacy in humans. As a consequence, there remains a clear need for discovery of a novel neuroprotective antioxidant treatment for mild to moderate TBI. The novel nitroxide antioxidants 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) and 5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl (DCTEIO) are possible superoxide dismutase mimetics that have been reported to be neuroprotective in ataxia-telangiectasiathrough scavenging ROS and reducing oxidative stress [14]. However, CTMIO and DCTEIO, have never been tested in the context of oxidative stress in TBI. Importantly, we have

recently shown that these moieties, incorporated within a fluorescent probe, are strongly responsive to the redox environment of the mitochondria [15] and protective against ROS derived retinal injury *in vitro* (661W photoreceptor cells) and in a rat model [16]. The study described herein aimed to investigate the effects of CTMIO and DCTEIO in promoting tissue repair and improving neurological functional outcomes in a rat model of mild TBI. Tissue damage, neurocognitive functions, neuro-inflammatory response, and markers of endogenous antioxidant, autophagy, and mitophagy, were measured.

2 METHODS AND MATERIALS

2.1 Antioxidants

The nitroxide antioxidant CTMIO and DCTEIO (structure in Figure 1) were prepared as previously described [17; 18] and stored as solids under an inert atmosphere at reduced temperature until use. The antioxidant solution was prepared by dissolving powdered CTMIO and DCTEIO in drinking water to make 40mM aqueous solutions.

2.2 Modelling Traumatic Brain injury

Animal experiments were approved by the University of Technology Animal Care and Ethics Committee (ACEC#2014-478). All protocols were performed according to the code for the care and use of animals for scientific purposes of the Australian National Health & Medical Research Council.

Female Sprague-Dawley rats (~250g, Animal Resources Centre, Perth, Australia) were housed at 20°C and kept at a 12-h light and 12-h dark cycle with *ad libitum* access to food and water. After acclimatisation, rats were stochastically divided into four groups: sham (n=12), TBI only (n=12), TBI-CTMIO (n=12) and TBI-DCTEIO (n=12). After anaesthesia with isoflurane (2.5%), an incision was made to expose the skull and a burr hole (5 mm in diameter) was drilled at 2.5 mm posterior and 3.0 mm lateral to the bregma as we have previously published [6]. A weight (5 cm height, 2.5 mm in diameter) was dropped onto the brain surface to induce a cortical contusion using New York University Impactor for the TBI rats. This results in a mild focal injury to the right side of the brain that produces small functional deficits in the contralateral limbs. The sham rats went through the same surgical procedure as the TBI groups, sans the weight drop. Sham rats did not receive any nitroxide treatment and are included as surgical controls. Two subgroups of rats with TBI were treated with CTMIO or DCTEIO added to their drinking water (40mM) immediately after the surgery for either 24 hours (acute injury) or for 2 weeks, as most neurological deficits had recovered by this stage in our previous studies

[6]. For the longer term study, the drinking water for each cage was discarded after 2 days and replaced with fresh batches containing a fresh CTMIO or DCTEIO solution. Water intake for individual rats was measured daily with no difference was seen between treatment groups. All animals received analgesics (Buprenorphine 0.03mg/kg) and antibiotics (Cephazolin sodium, 0.08ml/kg) twice daily for 3 days post-surgery. Rats were euthanised by lethal injection (pentobarbitone sodium, 10ml/kg, i.p.) at 24 hours or 6 weeks post-surgery (n=6 per group). and their brains were examined. In order to minimize the number of animals used, brains were bisected through the centre of the lesion site (or equivalent level in sham) in the coronal plane. A slice containing half the lesion from the rostral (anterior) part was snap-frozen in liquid nitrogen and kept at -80°C for western blotting. The caudal (posterior) half was fixed in 10% formalin and processed through graded alcohol and paraffin embedded. As a result, we were unable to measure the full volumetric extent of the lesion, but employed a semi-quantitative scale to compare the severity of the lesion between groups as previously published (Chen et al, 2016a).

2.3 Behaviour tests

Sticky tape test. The sticky tape test is used as a measure of sensory function [19]. A circular sticker (20 mm in diameter) was attached to the lower region of the phalanges and hairless part of the forepaw (the metacarpal pads, thenar, and hypothenar). Shaking of the limb, looking at, or touching the adhesive with the other paw or nose/whiskers was considered acknowledgement of the sticker. The time required for the rat to acknowledge the sticker was recorded. The result is expressed as the ratio of the time of the left over right paw to acknowledge the sticker [19]. A normal uninjured rat typically has a ratio of 1. If there is a sensory deficit in the left (affected) paw the ratio will be greater than 1.

Error ladder test. The error ladder test is used as a measure of motor function. The rat walked on a horizontal ladder with uneven runs for 2 minutes. Stepping error was expressed as a percentage. calculated from the number of left forepaw stepping errors compared to the number of total steps within the 2 minutes. A normal uninjured rat typically has a low baseline percentage of stepping errors (< 10%). If there is a motor deficit of the left (affected) paw the percentage will be higher than baseline.

Novel objective recognition (NOR) test. This test evaluates short term memory retention. The test was set up as we have previously published [6]. All rats were habituated to the testing apparatus (dark-wall chamber with floor space floor 40 x 29cm2) for 1 week prior to the surgery. During the NOR test, each rat underwent a familiarisation phase (5 mins in the chamber exposed to two identical objects (green square blocks). They were then returned to the home cage for 1 hour before being returned to the chamber for the test phase (5 mins exposed to one old (green square block) and one novel (orange

triangular block) object. Both sessions were digitally captured for later analysis and the time spent investigating each block was recorded. The results are presented as the percentage of the total time spent on the novel object divided by the total time spent exploring both objects in the test phase. A normal uninjured rat typically spends more time exploring the novel object in the test phase (~70%). If there is a memory deficit then the rat is likely to spend equal time (~50%) exploring both objects in the test phase.

2.4 Histology and immunohistochemistry

A series of coronal sections (5µm) were cut at the level of the lesion from paraffin embedded blocks using a rotary microtome. One set of slides was stained with Mayer's haematoxylin and eosin in order to examine the maximum severity of the lesion. The right (affected) side of the brain was imaged and the lesion graded using a semi-quantitative scoring system (Grade 0: No observable injury in the brain regions; Grade 1: Slight haemorrhage or disruption of tissue seen in at a very low level; Grade 2: Tissue loss with haemorrhage, or tissue disruption; Grade 3: Considerable tissue loss, extensive haemorrhage and/or tissue disruption) as previously described [6; 20]. The percentage of injury area, based on the area of the visible lesion divided by the area of the brain hemisphere, was also determined using ImageJ software (National Institute of Health, Bethesda, Maryland, USA).

Immunohistochemical staining was undertaken on adjacent slides. Astrocytes were stained using glial fibrillary acid protein (Rabbit anti-GFAP, 1:1000, Dako, CA, USA). The slides were incubated with primary antibody overnight at 4°C, followed by secondary antibody (Alexa fluor 488 anti-rabbit IgG, 1:200, or Alexa fluor 568 anti-mouse IgG, 1:200, Invitrogen, CA, USA) at room temperature for two hours. The slides were then washed and counterstained with a Hoechst counterstain (1:5000, Invitrogen, CA, USA) for 10 minutes, and coverslipped using fluoromount (DAKO, CA, USA). Images were taken using an Olympus BX51 Upright Fluorescence Microscope with Olympus U-RFL-T fluorescent burner (Nikon Instrument Inc, USA). The staining intensity (GFAP) was analysed using mean greyscale per ROI in the cortex (adjacent to the lesion), hippocampus and thalamus using ImageJ software (National Institute of Health, Bethesda, Maryland, USA) by an observer (AJ) who was blinded as to treatment group.

2.5 Western blotting

The protein levels of endogenous antioxidant MnSOD, Drp-1, Opa-1 and mitochondrial light chain 3(LC)3A/B were analysed by western blotting. The brain was homogenised using cell lysis buffer to

extract the whole protein, and mitochondrial protein. Protein samples (15 µg) were first separated on NuPage®Novex®4-12% Bis-Tris gels (Life Technologies, CA, USA) and then transferred to nitrocellulose membrane (Thermo Fisher Scientific, Massachusetts, USA). The membrane was then blocked with 5% skim milk in TBST and incubated with primary antibodies β-Actin (1:5000, Santa Cruz Biotechnology), MnSOD (1:2000, Sigma-Aldrich Co), Drp 1 (1:2000, Novus biologicals), Opa1 (1:2000, Novus biologicals) and LC3A/B (1:2000, Cell signalling), followed by secondary antibody conjugated horseradish peroxidase (HRP) anti-Mouse (Thermo Fisher Scientific, MA, USA). Protein expression was then detected by SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific Massachusetts, USA) by exposing the membrane in Amersham Imager 600 (GE Healthcare Australia).

2.6 Statistical methods

Results are expressed as mean ± standard error of the mean (SEM). The data were analysed by one way ANOVA followed by Fisher's LSD Difference post hoc tests (Statistica 10, Statsoft Inc. OK, USA). A conditional t test was used if the difference from the Sham group is more than 1 fold. $P<0.05$ was considered statistically significant.

3 RESULTS

3.1 Behavioural tests

Sensory function, as shown by the sticky tape test (Figure 2A), was markedly impaired in the TBI rats at 24hrs ($P<0.01$ vs sham by conditional t test), was still impaired at one week ($P<0.05$ vs sham) and had returned to normal by six weeks. TBI+CTMIO group also showed deficits in sensory function ($P<0.01$ vs sham) at 24hrs, but was normalised by one week. On the other hand, there were no sensory deficits observed for the TBI+DCTEIO group at any time point.

Motor function, as shown by the error ladder test (Figure 2B), was significantly impaired in TBI rats at 24h and one week post-injury ($P<0.005$ vs sham) and had returned to normal by six weeks. TBI+CTMIO group showed similar time course of deficits in motor function as the TBI group ($P<0.005$ vs sham). At 24hrs post-injury TBI+DCTEIO group showed no difference in motor function compared to the sham group ($P<0.05$). At one-week post-injury motor function in TBI+DCTEIO group was reduced ($P<0.005$) which was normalised by six weeks of motor function in all groups had.

Short term memory function, as shown by the novel object recognition test (Figure 2C), was significantly reduced in the TBI ($P<0.01$) and TBI+CTMIO ($P<0.05$) groups compared to the sham at 24h post-injury. At 24hrs post-injury the TBI+DCTEIO group showed no difference in short term memory function compared to the sham group and it was improved compared to the TBI group ($P<0.05$). By one week post-injury, short term memory function in all groups had normalized.

3.2 Tissue injury

Sham brains were graded 0 as no visible injuries were caused by sham surgery. Significant tissue injury was observed in the cortex, hippocampus and thalamus of the TBI rats at 24h post-injury (both $P<0.01$ vs sham, Figure 3A) and this was sustained at 6 weeks post-injury ($P<0.01$ and $P<0.05$ vs sham in the cortex and hippocampus respectively; Figure 3B). CTMIO had no measurable impact on injury grade throughout 6 weeks. DCTEIO administration significantly reduced injury in the cortex and hippocampus at 24h post-injury (both $P<0.05$ vs TBI, Figure 3A). There was also a trend of reduction in tissue injury in cortex and hippocampus regions at 6 weeks although this did not reach statistical significance, while no injury was visible in the thalamus of TBI+DCTEIO rats (Figure 3B).

Since grading analysis was based on the severity of haemorrhage, tissue disruptions, and cavity formation, we also measured the maximal extent of the lesion as a percentage of total hemispheric surface area. At 24h post-injury, TBI rats had $15.48 \pm 1.26\%$ of area affected by the injury ($P<0.001$ vs sham). The CTMIO groups had less area affected ($10.96 \pm 3.84\%$) than the TBI rats, although it was not statistically significant. The injury size was further reduced in the TBI+DCTEIO groups ($9.98 \pm 0.28\%$, $P=0.09$ vs TBI). At 6 weeks, the TBI group had $4.74 \pm 0.95\%$ of area injured ($P< 0.001$ vs sham). The TBI+CTMIO group had a bigger injury size than the TBI rats of area injured ($7.22 \pm 0.23\%$, $P<0.001$ vs TBI), consistent with the grading results in Figure 2B. The TBI+DCTEIO group had only $1.39 \pm 0.68\%$ area affected, which was less than 1/3 of the injury size in the TBI group ($P<0.01$ vs TBI).

3.3 Astrogliosis

Astrogliosis was measured using GFAP staining intensity as shown in Figure 4. At 24h, GFAP staining was significantly increased in the cortex of all rats with TBI regardless of the treatment ($P<0.01$ all vs sham, Figure 5A). An increase in GFAP staining intensity was only significant in the hippocampus of TBI+CTMIO rats ($P<0.05$ vs sham, Figure 5A); whereas in the thalamus, GFAP staining was only significantly increased in the TBI+DCTEIO rats ($P<0.05$ vs TBI, Figure 5A). At 6 weeks post-injury,

GFAP staining was much lower in the cortex and thalamus compared with 24h, whereas its levels were still high in the hippocampus (Figure 5B). In the cortex, TBI and TBI+DCTEIO rats still have significantly higher level of GFAP staining than the sham rats ($P<0.05$); in the hippocampus, GFAP were significantly increased in all rats with TBI ($P<0.01$, TBI, TBI+CTMIO and TBI+DCTEIO vs sham). There was no difference in GFAP levels in the thalamus between groups.

3.4 Markers of endogenous antioxidant and mitochondrial integrity

At 24h, MnSOD protein levels were significantly reduced in the TBI group ($P<0.05$ vs sham), and more so in the TBI+CTMIO and TBI+DCTEIO groups (both $P<0.01$ vs sham, Figure 6A). MnSOD levels also negtively correlated with cortex and hippocampus tissue damage gradings (both $R^2 = 0.640$, $F = 17.79$, $P<0.01$), and the performance in error ladder test ($R^2 = 0.367$, $F = 5.792$, $P<0.05$). The fission marker Drp-1 was non-significantly reduced by 20% in the TBI group, while its level was significantly increased by both CTMIO and DCTEIO treatments (both $P<0.01$ vs TBI, Figure 6A). The fusion marker Opa-1 was significantly reduced in the TBI group by half ($P<0.05$ vs sham), which was not significantly reversed by either treatment. Opal levels also positively correlates with the performance perservation in sticky tape test ($R2 = 0.579$, $F = 22.01$, $P < 0.01$), error ladder test ($R2 = 0.671$, $F = 20.38$, $P < 0.01$) and novel objective recognition test ($R2 = 0.317$, $F = 7.423$, $P < 0.05$). The autophagy marker LC3A/B was only significantly upregulated in the TBI+CTMIO group ($P<0.05$, vs sham, Figure 6A), and negatively correlates with the performance perservation in sticky tape test ($R^2 = 0.234$, $F = 4.592$, $P < 0.05$), and novel objective recognition test ($R^2 = 0.232$, $F = 5.121$, $P < 0.05$).

At 6 weeks, the fission marker Drp-1 was significantly increased by 60% in the TBI group ($P<0.01$ vs sham), which was only normalised by DCTEIO treatment ($P<0.01$, TBI+DCTEIO vs TBI, Figure 6B). The autophagy marker LC3A/B was also significantly increased in both TBI and TBI+CTMIO groups (both $P<0.01$ vs sham), while the level in TBI+DCTEIO group was almost the same as the sham group ($P<0.01$ vs TBI, Figure 6B). There was no difference in MnSOD and Opa-1 levels among the 3 groups (Figure 6B).

4 DISCUSSION

Oxidative stress and inflammation are two major events contributing to tissue damage and subsequent neural defects after TBI [21; 22], whereas the treatment against oxidative stress after mild to moderate TBI has not been well established. This study examined the impact of the nitroxide-based antioxidant, CTMIO and DCTEIO on tissue and functional recovery after moderate TBI in rats. The major finding

is that DCTEIO treatment immediately post-injury ameliorated functional deficits in rats with TBI and reduced tissue damage, with enhanced gliosis, mitochondrial fission and autophagy response compared with non-treated TBI rats at 24h post-injury. However, CTMIO treated rats with TBI showed enlarged tissue injury size in the long term although motor function seemed to be only affected at 24 hrs. Based on these findings, we posit that DCTEIO represents a potential therapeutic option for early treatment of moderate TBI, particularly in an acute setting, whereas CTMIO may be toxic.

The rats with TBI showed marked haemorrhage, tissue disruption and cavity formation in the cortex which is consistent with our previous studies, and the pathophysiological changes in humans with ischemic brain injury [23; 24]. Although the epicentre of the injury was in the sensory and motor cortex region of the right hemisphere, the force from the weight drop was extended to the deep areas including the hippocampus and thalamus regions. This aligns with our previous observations using the same model [6]. As a result, sensory, motor, and cognitive functions were significantly impaired 24h post injury. However, those deficits were fully recovered by 6 weeks, even in the non-treated rats, most likely due to the resilience and neuroplasticity of the uninjured side to compensate for the impaired function, as cortical neurons are believed to have no capacity to regenerate after the injury [25]. Further, new neurons formation has been shown in the hippocampus of adult rats, especially after injury [26; 27]. It should be noted that we used female rats in this study due to the high motility and mortality rate in humans [28]; even with the formation of a cavity on the injury site by 6 weeks [6], the injury in our model is relatively mild in terms of the impact size which may explain the rapid recovery in neurological functions in the TBI rats.

TBI significantly increased astrogliosis as reflected by GFAP staining in the early stages of TBI. During TBI, studies have found that there is either proliferation or activation of new astrocytes [29]. In this study, at both time points, GFAP staining intensity was higher in the TBI brains from 24h lasting until 6 weeks, consistent with previous studies [30; 31]. As the most abundant supporting cells in the central nervous system, astrocytes are critical to provide energy support and maintain the iron and neurotransmitter balance in the neurons [32]. After injury, astrocytes form the scar to restrict the inflammatory response and neurotransmitter leakage [33; 34], and promote angiogenesis for restoration of blood supply [35]. In this study, astrogliosis was still prominent at 6 weeks in the cortex adjacent to the lesion suggesting that the tissue repair has not been completed, when neurological functions have returned to the control level. In fact, such a response may linger for years after TBI and leads to the development of other neurological disorders [36]. Thus, these results highlight the need for longer term

follow up studies to examine long term neurological functional changes.

Upon injury, inflammatory responses can induce oxidative stress. As such, in the TBI rats, the antioxidant MnSOD was reduced, consistent with our previous observation using the same model [6]. MnSOD knockout not only leads to increased oxidative stress but also impairs mitochondrial dynamics such as fission, fusion, and autophagy [37]. Drp1 is a guanosine triphosphatase, found in the cytoplasm that catalyses mitochondrial fission activity [38; 39]. During cellular stress such as TBI, Drp1 induce constriction of the mitochondrial outer membrane, ultimately causing binary fission of the mitochondrion [40]. The end products are a healthy fragment and a damaged fragment. The healthy part binds to a healthy fragment from another mitochondrion to form new mitochondrion via a fusion process mediated by Opa-1. The damaged fragment undergoes autophagy after being engulfed by autophagosomes formed by LC3A/B protein. In this study, impairment in mitochondrial fusion seems to occur earlier than fission, suggesting less healthy mitochondrial fragments recycle in the acute phases of TBI. The fission marker Drp-1 was increased at 6 weeks with high levels of autophagosome forming protein LC3A/B, suggesting prolonged mitochondrial and cellular damage after TBI. It needs to be noted that overall, MnSOD level seems to correlates with primary tissue injury, while fusion marker and autophagy markers related to secondary behavioural performance. The lack of correlation for fission mark in this study could be due to its lack of acute response to TBI.

Nitroxides are cell permeable and stable free radical scavengers that have been shown to deliver potent antioxidant action by ameliorating ROS production in vivo and in vitro models of oxidative stress [16]. Several mechanisms have been proposed, such as superoxide dismutase (SOD) activity, catalase mimic activity, radical–radical interactions and detoxification of secondary organic radicals [16]. Pre-treatment with DCTEIO has been shown to prevent ischemic-reperfusion caused mitochondrial function loss in retina, whereas CTMIO did not have much effect [16]. Resveratrol is a well-known antioxidant to protect cardiovascular system. In the abovementioned model, DCTEIO showed a more potent protective effect on mitochondria than Resveratrol [16]. Interestingly, DCTEIO treatment immediately after TBI seems to accelerate the neurological recovery process. The lesion size of the TBI rats with DCTEIO treatment was 30% smaller than the non-treated TBI rats, with a decreased injury severity score, leading to the smallest lesion size at 6 weeks. This supports the original hypothesis that treatment improves the cellular outcomes for the brain. Although the tissue was not affected at 24h, the rats treated with DCTEIO performed similarly to the sham rats in all neurological functions tested at this time point. On the other hand, CTMIO promoted fission at 24hr resulting in a

larger cavity size at 6 weeks. Whether this leads to later neurocognitive functional decline requires further investigation. The lower efficacy of CTMIO compared to DCTEIO could arise from the enhanced bio-availability of ethyl-analogue nitroxides compared to the methyl versions [16; 41].

However, it is unknown whether this rapid recovery was due to an increase in new neuron formation around the epicentre or synaptogenesis to form better connections between the uninjured and injured sides to compensate for the lost function [26]. Further study will need to focus on the possible impact of DCTEIO on accelerating neurogenesis in response to injury stimuli, especially in the cortex and hippocampal areas. Physiologically, synaptogenesis seems to be supported by robust astrogenesis, as the early astrocytes can release synapse-forming factors [26]. In the rats treated with DCTEIO, there was heightened astrogliosis at 24h in both cortex and thalamus, which was still prominent in the cortex hippocampus at 6 weeks. Astrogliosis can be considered both beneficial or detrimental in the CNS [42] and appears in this study that early astrogenesis is critical for rapid functional recovery. As DCTEIO has been shown to mimic the action of endogenous SOD [14], it may directly scavenge the free radicals in the cells. Therefore, there is no need for the cells to generate additional MnSOD in the acute phase; however, in the long term MnSOD level recovered in the DCTEIO treated rats. We don't exclude the possibility that other mitochondrial redox markers are affected by DCTEIO, which requires further investigation. This study is the first step to prove the beneficial effect of DCTEIO on TBI recovery. Fission function seems to be increased in TBI+DCTEIO, which may help to eliminate damaged mitochondria to maintain cell homeostasis.

However, a dose-response analysis must be completed first to confirm the above findings. It would also be interesting to compare DCTEIO to other stable antioxidants such as Tempol that have been reported to be neuroprotective in rodent stroke and trauma models [43; 44; 45; 46]. Further studies should also consider examining delayed DCTEIO administration, which was not done in the current study. In addition, the efficacy of DCTEIO also needs to be confirmed in the male gender for clinical translation.

CONCLUSION

This study investigated a novel antioxidant as a potential treatment to cellular and neurological outcome in rats with TBI. DCTEIO significantly reduced lesion size and preserved neurological function after acute injury. Further studies are needed to elucidate the exact mechanism of action of DCTEIO in intervening in TBI, to investigate optimal doses and modes of delivery, and to test the effects of sex in this outcome, however the results presented here demonstrate the potential of this

nitroxide as a lead for the development of new therapeutics for moderate TBI.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

HC, RZ, WL, SS, SB, BO & CG all contributed to the study design, planning and implementation, data analysis and manuscript preparation. HC, CG, AS, JS and YLC undertook the experimental work, analysed data and prepared results for publication. SB provided the nitroxides used in this study.

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Figures

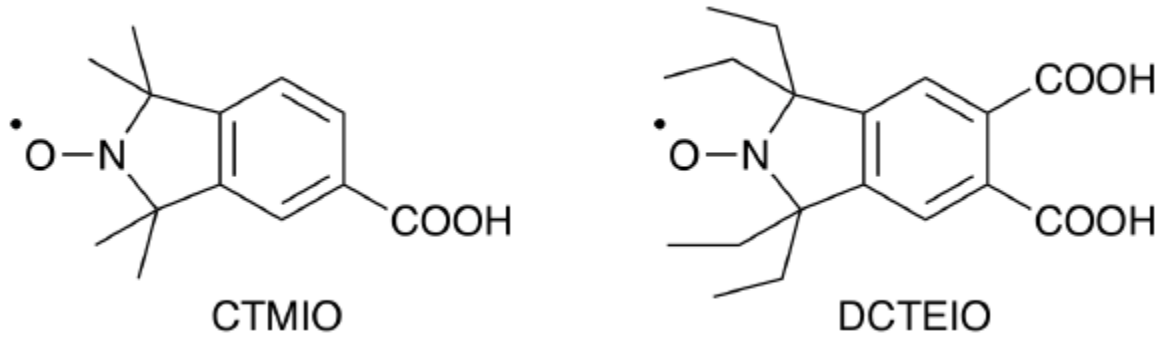

Figure 1. The chemical structures of the two nitroxides CTMIO and DCTEIO employed in the study. CTMIO: 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl; DCTEIO: 5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl.

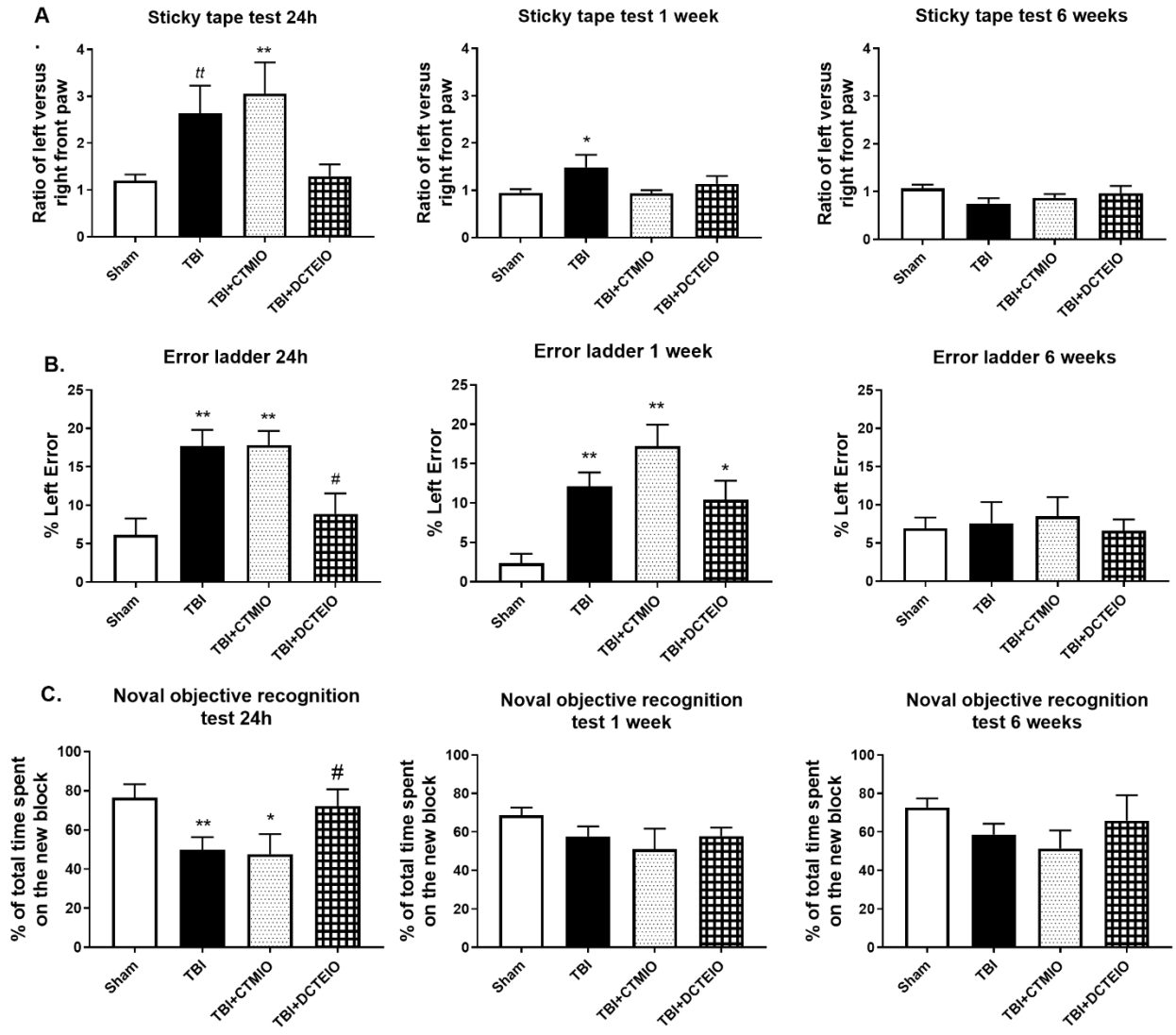

Figure 2. The outcome in sticky tape test (A), error ladder (B), and novel objective recognition test (C) at 24 hours, 1 week, and 6 weeks post-surgery. Results are expressed as mean ± SEM, n=6-12. * P<0.05, ** P<0.01, tt P<0.01 (conditional t test) vs Sham; # P<0.05, vs TBI.

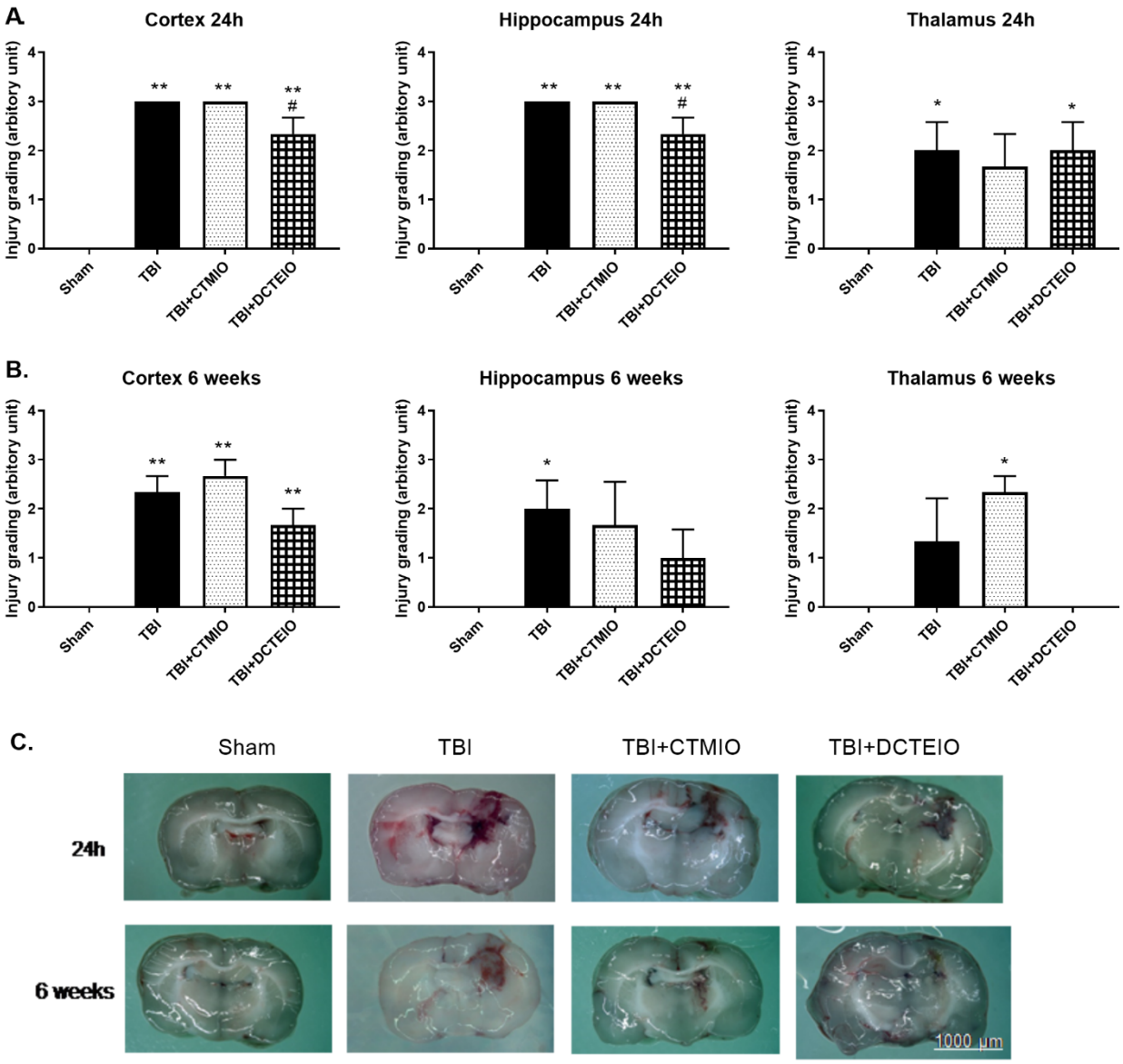

Figure 3. Tissue damage grading in the cortex, hippocampus, and thalamus at 24h (A) and 6 weeks post-surgery (B), and representative images showing brain slices through the right hemisphere cortical lesion site at 24h and 6 weeks post-surgery (C). Results are expressed as mean ± SEM, n=6. * P<0.05, ** P<0.01 vs Sham; # P<0.05 vs TBI.

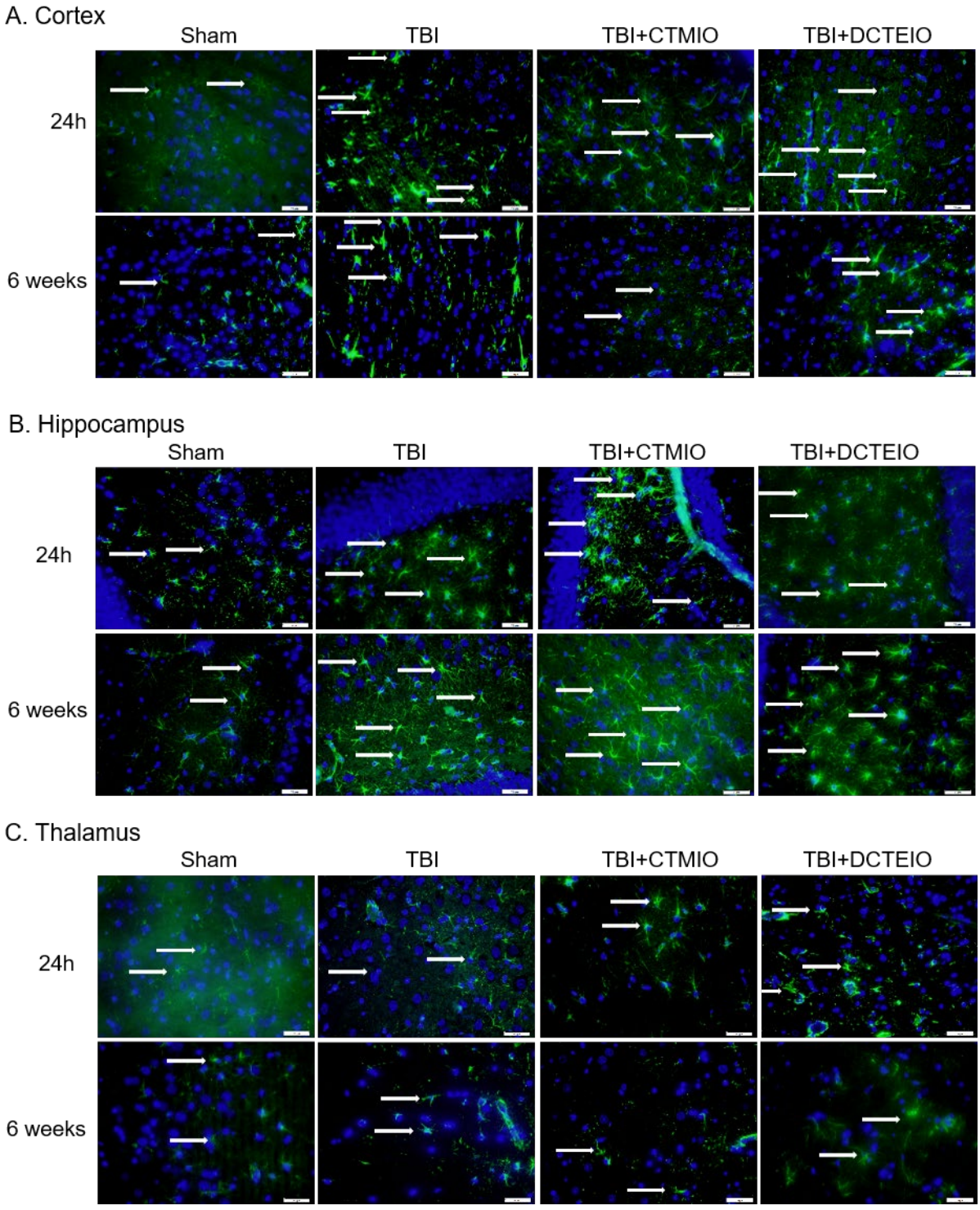

Figure 4. Representative images showing $GFAP^+$ astrocytes (green, arrows) in the cortex (A), hippocampus (B), and thalamus (C) at 24 hours and 6 weeks post-surgery. Scale bar = 20μm

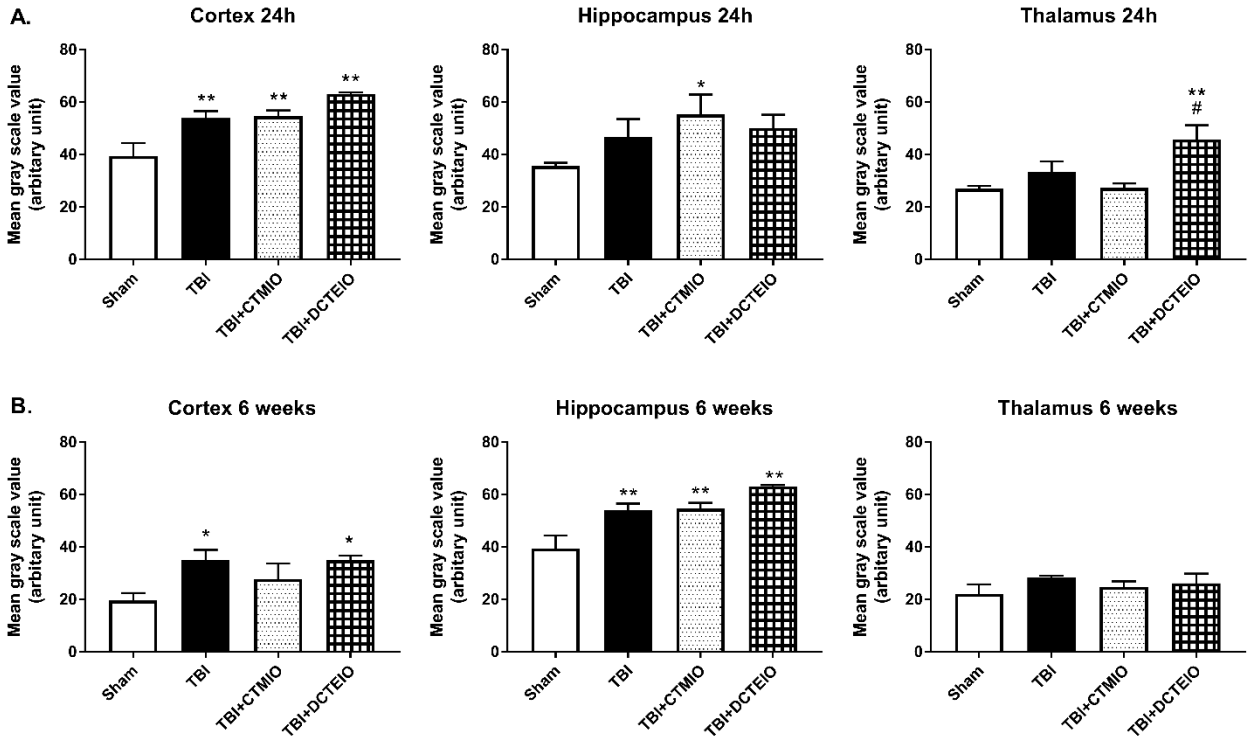

Figure 5. GFAP staining intensity in the cortex, hippocampus, and thalamus **a**t 24 hours (A) and 6 weeks (B) post-surgery. Results are shown as mean ± SEM, n=6. * P<0.05, ** P<0.01 vs Sham; # P<0.05 vs TBI.

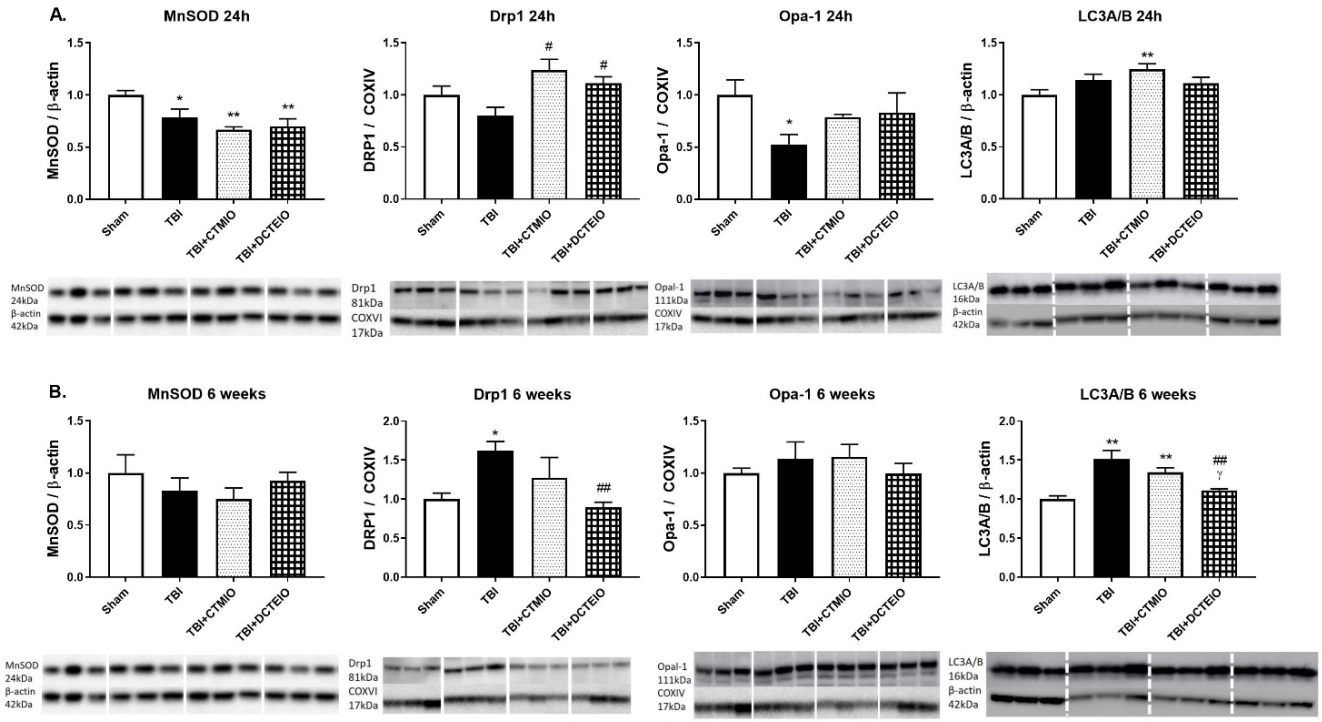

Figure 6. The protein level of manganese superoxide dismutase (MnSOD, 24kDa), dynamin-related protein 1 (Drp1, 81kDa), mitochondrial optic atrophy 1 (Opa-1, 111kDa), and mitochondrial light chain 3 (LC3A/B, 16kDA) at 24 hours (A) and 6 weeks (B) post-surgery. Results are shown as mean ± S.E.M, n=6. *P<0.05, **P<0.1 vs Sham; # P<0.05, ##P<0.01 vs TBI, γ P<0.05 vs CTMIO.