Methylene blue improves lesion volume, multi-parametric quantitative MRI measurements, and behavioral outcome following TBI

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Abstract

Traumatic brain injury (TBI) remains a primary cause of death and disability in both civilian and military populations worldwide. There is a critical need for the development of neuroprotective agents that can circumvent the damage and provide functional recovery. We previously showed that Methylene Blue (MB), a FDA grandfathered drug with energy-enhancing and antioxidant properties, given one and three hours post-TBI had neuroprotective effects in rats. This study aimed to further investigate the neuroprotection of delayed MB treatment (24 hours post-injury) following TBI as measured by lesion volume and functional outcomes. Comparisons were made with vehicle and acute MB treatment. Multimodal MRI and behavioral studies were performed at 1 and 3 hours, 2, 7, and 14 days following an impact to the primary forelimb somatosensory cortex. We found that delaying MB treatment 24 hours post-injury still minimized lesion volume and functional deficits compared to vehicle-treated animals. The data further supports the potential for MB as a neuroprotective treatment especially when medical teatment is not readily available. MB has an excellent safety profile and is clinically approved for other indications. MB clinical trials on TBI can thus be readily explored.

Key words: TBI, methylene blue, mitochondria, CCI, behavioral outcomes, MRI

Background

Traumatic brain injury (TBI) is characterized as a sudden physical impact to the head and is considered a leading cause of death and disability in civilian and military populations.[1] In the hours and days following TBI, there is a progression of molecular changes that lead to mitochondrial dysfunction, edema formation, inflammation, blood brain barrier dysfunction, and neuronal degeneration.[2, 3] Importantly there are no neuroprotective agents clinically available to counteract the progressive nature of a TBI.

Mitochondrial oxidative damage is well known to precede the onset of neuronal damage and loss, and is therefore considered an important mediator of secondary brain injury. During injury, a surge in reactive oxygen species facilitates a vicious cycle that accelerates mitochondrial damage, excitotoxicity, lipid peroxidation, and inflammation.[4] Thus, mitochondrial targeting strategies in TBI have been increasingly studied as their maintenance could preserve brain function.[5, 6] However, the majority of studies using agents that target mitochondria demonstrating neuroprotection have utilized a therapeutic treatment window of less than one hour post TBI. The administration of cyclosporine A [7] was demonstrated to significantly reduce mitochondrial dysfunction (given 15 min post-injury)[8], cortical damage (given 5 min before to immediately after injury) [9,10, 11], and cytoskeleton and axonal dysfunction (given as a pretreatment prior to injury).[12, 13] Furthermore, dietary supplementation with creatine was also shown to be effective in ameliorating neuronal cell death likley by reducing mitochondrial ROS production and maintaining ATP production after TBI.[11] While these studies provide increasing evidence of the importance of mitochondrial dysfunction and the effects of its failure in secondary brain injury, the potential therapeutic value of pharmacological mitochondrial protection, the time course of intervention needed, and the causes of mitochondrial dysfunction have not yet been fully defined.

Methylene Blue (MB), a FDA-grandfathered drug currently used to treat methemoglobinemia, carbon monoxide poisoning and cyanide poisoning in humans[14, 15], is well established to act on the mitochondria by improving energy

production and reducing oxidative damage. MB has also recently been shown to reduce neurobehavioral impairment in animal models of optic neuropathy[16, 17], Parkinson's disease[15, 18], Alzheimer disease[19-21], and ischemic stroke.[22, 23]_ MB has redox recycling properties whereby it acts as an electron cycler and facilitates electron transfer in the mitochondrial electron transport chain by accepting electrons from NADH and transfering them to cytochrome c, bypassing complex I-III.[24] We previously showed that low-dose MB (1mg/kg) adminstered acutely at 1 and 3 hours after TBI in rats reduces lesion volume, neurologic deficits and neuronal degeneration.[25]

The goals of this study were to: 1) extend our previous findings to test whether delayed (24 hrs) MB treatment would show efficacy in reducing lesion and behavioral deficits following an open-skull TBI, and 2) to determine the effects of MB on MRI parameters (T_2, apparent diffusion coefficient (ADC), fractional anisotropy (FA), and cerebral blood flow (CBF)) in vehicle, acute and delayed MB treatment groups. A controlled cortical impact was applied over the left primary forelimb somatosensory cortex (S1FL) in rats. Multi-parametric quantitative MRI measurements (T_2, ADC, FA, and CBF) and longitudinal behavioral assessments (foot fault and forelimb asymmetry tests)[26-28] were made from 3 hours to 14 days post-TBI. Comparisons were made with our previously published vehicle and acute MB treatment groups in terms of lesion volume and behavioral measures.[25] Moreover, analysis was also performed on how MB affects T_2, ADC, FA and CBF of the vehicle, acute and delayed MB treatment groups.

Methods

Animal preparation

All animal procedures follow the ARRIVE guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center San Antonio. The induction of TBI was done as previously described.[29] Three groups of animals were studied: 1) Vehicle treated (N=6), 2) acute MB treated (N=5), where MB was given at one and three hours post-TBI,

and 3) delayed MB treated group (N=10), where MB was given 24 hours post-TBI. Note that the lesion volume and behavioral measures data from vehicle and acute MB treatment groups have been previously published.[25] The T_2, ADC, FA and CBF in the acute MB treatment group have not been previously published.

Male Sprague Dawley rats (250-350g) were anesthetized initially with 5% isoflurane mixed with room air and maintained at 1.2% isoflurane throughout all surgical and imaging procedures. The animal was secured in a stereotaxic frame and an incision was made as posterior (at the level of the cerebellum) from the impact site as possible to prevent artifacts during MRI acquisition and the periosteum was removed over the impact site. A Ø5mm craniotomy was created over the left forelimb primary somatosensory cortex (S1FL: +0.25mm anterior and 3.5mm lateral to bregma), exposing the dura matter. The intact dura matter was impacted using a pneumatic controlled cortical impactor (Precision Systems and Instrumentation, LLC, Fairfax Station, Virginia) fitted with a Ø3mm tip (5.0m/s, 250μs dwell time, 1mm depth) to mimic a mild focal TBI. Following the impact the cranial opening was sealed with bone wax, the scalp sutured closed and antibiotic ointment applied. Saline was placed under the skin to facilitate the removal of air pockets between the scalp and the skull to minimize artifacts during MRI acquisition. Buprenex (0.05mg/kg) was given subcutaneously every 12 hours for three days for pain.

Magnetic Resonance Imaging

Magnetic resonance imaging (Bruker 7-Tesla Biospec) was acquired on the day of the TBI procedure (1-3 hours post-TBI), and again on days 2, 7 and 14 after TBI onset to longitudinally monitor lesion volume. The animal was anesthetized with 1.5% isoflurane and secured in a MRI-compatible stereotaxic holder with ear and tooth bars. A transceiver surface coil of 2-cm in diameter was placed on top of the rat's head. T_2-weighted images were acquired using a fast spin-echo sequence with repetition time (TR) = 3s (90^o flip angle), effective echo time (TE) = 18, 54, 90 and 126 ms, 8 echo train length, seven 1.0-mm thick coronal images, field of view = 2.56 x 2.56 cm, matrix 96 x 96 and reconstructed

to 128 x 128, and 4 transients for signal averaging. Images were co-registered across time points using QuickVol and MRIAnalysisPak software. The lesion volumes were determined as pixels that had T_2 values higher than the mean plus 2 standard deviations of the value in the homologous contralesional region.[30]

Functional assessment

Behavioral assessments were made 1-3 days prior to TBI and again 1, 2, 7, and 14 days post-TBI prior to the MRI experiments in the same animals. Behavioral tests were not performed on the day of TBI induction due to incomplete recovery from anesthetic. Sensorimotor function was assessed using the asymmetry forelimb placement (cylinder) test and foot-fault test.[29] Previous behavioral studies have demonstrated that these functional tests have the appropriate sensitivity for this injury model.[26-28] Testing was conducted 1-3 days prior to TBI and again 1, 2, 7, and 14 days post-TBI.

Foot fault test: The foot fault test is utilized as a measure of neurologcal deficits associated with motor impairment and has been used widely to measure deficits following stroke. In this test the rat is placed on a grid like surface and allowed to move freely upon it for five minutes. When an animal places the limbs inaccurately on the grid the limb falls through and is counted as a foot fault.

Cylinder test: The cylinder test provides a way to evaluate a rodent's spontaneous forelimb use and is commonly used to evaluate motor/sensory behavior. A rat is placed in a Plexiglas cylinder and the rat will actively explore the vertical surfaces by rearing. The number of individual limb placements and simultaneous placements are observed for each limb. Unilateral brain damage will result in an asymmetry in forelimb use.

Statistical Analysis: Unpaired t-tests were used to compare T_2 lesion volumes between vehicle- and MB-treated groups. Mann-Whitney U tests were used to compare differences in asymmetrical limb use and the percentage of foot faults between vehicle-and MB-treated animals. Values are presented as mean ± SEM.

Statistical significance was set at $p<0.05$. Multiple regression analysis was performed to determine the correlation between MRI determined lesion volume and asymmetry scores and foot fault scores.

Results

MB improves lesion volumes: Figure 1A displays T_2 lesion volumes for vehicle, acute MB, and delayed MB treated animals at 3 hrs and 2, 7 and 14 days post-TBI. The delayed MB group had similar lesion volumes at the three-hour time point (as MB has not yet been delivered in the delayed MB treatment group) compared to vehicle treated group as expected, while the acute MB group (after MB) had reduced lesion volume at this time point. By day 2 post-TBI the delayed treatment group demonstrated a similar reduction in lesion volume as the acutely treated animals suggesting that delayed treatment is as effective as acute treatment. The delayed group continued to show significant reductions in lesion volume surpasing those of the acute treatment group on days 7 and 14. Figure 1B demonstrates representative T_2 maps for vehicle, acute MB, and delayed MB treated animals at 2 days post-TBI with white arrows indicating the lesion. In the vehicle treated animals the lesion spread over four slices indicated by the hyperintense areas of the cortex, while the acute and delayed MB treatment groups only spanned two slices. The data suggests that delayed treatment is more effective at reducing lesion volume than the acute treatment.

MB effects on MRI measured parameters: T_2, ADC, FA and CBF percent differences from the contralesional cortex were analyzed using ROI analysis for vehicle, acute MB, and delayed MB treated groups at 3 hrs and 2, 7 and 14 days post-TBI.

Vehicle treated animals had increased T_2 values in the cortex by 3 hours and remained elevated on day 2 post-injury. The T_2 values decreased by day 7 and continued to decrease through day 14. Similarly, 3 hrs after injury, the delayed MB group did not show significant differences from the vehicle group in

T_2, as expected. The acute MB group showed a significant decrease in T_2 from both the vehicle and delayed MB groups at both the 3 hour and 2 day time points. By day 7 and all groups had decrased T_2 values which continued to decrease through day 14 with no significant differences detected among the groups (**Figure 2A).**

ADC was significantly elevated at 3 hrs, peaked on day 2, and returned toward normal values on day 14 in vehicle treated animals (**Figure 2B**). In a similar fashion, acute and delayed MB treated animals had significant elevations in ADC on day 2, however they did not reach the severity of the vehicle treated animals. On day 7 post-injury the MB treated groups had significantly lower ADC values compared to vehicle treated animals. All treatment groups had returned towards normal on day 14 with no significant differences between the groups.

In vehicle treated animals abnormal FA in the cortex was apparent within 3 hrs of the impact. FA was significantly reduced at 3 hrs, remained reduced on days 2 and 7 but returned toward normal values on day 14 (**Figure 2B**). In contrast the acute MB treated animals had significantly reduced FA compared to vehicle treated animals at 3 hours, and on days 2 and 7. The delayed MB treated group showed significantly reduced FA, similar to vehicle treated animals. By day 2 MB treatment had minimized the FA decrease similar to the acute MB treated group. By day 14 all treatment groups had returned toward normal.

Cortical CBF peaked on day 2 in all groups, reduced on day 7 and increased again on day 14. There were no significant differences in the CBF amongst vehicle, acute and delayed MB groups until day 14 post-TBI (**Figure 2D**). On day 14 acute and delayed MB treatment increased CBF values compared to vehicle treated animals.

MB improves behavioral outcomes:

Sensorimotor function was assessed using the forelimb asymmetry (cylinder) and foot fault tests. Prior to TBI induction, mean forelimb asymmetry scores were not significantly different between the vehicle-, acute MB-treated, and delayed MB-treated groups (49±2%, 52±2%, and 53%±3% respectively;

P>0.05; Figure 3A) indicating symmetrical use of the two forelimbs. Data from our previous study demonstrated that in vehicle treated animals forelimb asymmetry scores worsened on days 1 and 2 after TBI and that acute MB treatment significantly reduced the asymetrical use of the forelimbs. We extended functional assessment in this study using delayed treatment of MB twenty-four hours post-TBI. In the delayed MB group forelimb asymmetry scores peaked on day 1. By day 2 the asymmetry scores returned to baseline levels in the delayed MB group. By day 7 and 14 the scores rose slightly although they were not significantly different than vehicle or acute-MB treated animals **(Figure 3A).**

The percentages of foot faults were not statistically different among the vehicle and acute or delayed-MB treated groups prior to TBI induction **(Figure 3B)**. Vehicle treated animals had significant increases in the number of right forepaw foot faults by day 1 that persisted through day 7, and improved on day 14. In the acute MB group, by contrast, we previously demonstated that right foot faults were only slightly elevated post-TBI, did not reach the severity observed in the vehicle group and were significantly lower compared to the vehicle-treated group on days 1, 2 and 7 post-TBI (P=0.043, 0.018 and 0.0058, respectively). In the delayed MB group, foot faults followed the same pattern as the vehicle treated animals but were lower at each time point studied. **(Figure 3B)**. The vehicle and delayed MB treated groups showed no difference in the number of foot faults on day 1 as expected as the animals were tested prior to the administration of MB. On days 2, 7, and 14 there was a trend of decreasing foot faults compared to vehicle treated animals but did not reach significance. Together, these data indicate that acute and delayed MB treatment reduces sensorimotor deficits following TBI.

Correlation analysis between T_2 MRI lesion volume and forelimb asymmetry scores for all time points is plotted in Figure 4A for vehicle, 1-3 hour MB and 24 hour MB treated animals. Regression analysis revealed an R^2 value of 0.1202 (p=0.0022) for lesion volume and % asymmetry scores in all groups.

Individual regression analysis for each group was also performed between lesion volume and % asymmetry where in vehicle treated animals yielded a significant correlation with an R^2 value of 0.32 (p=0.002) (data not shown). Methylene Blue treated animals yielded non-significant correlation with an R^2 value of 0.009 (p=0.67) in acutely treated animals and an R^2 value of 0.0007 (p=0.89) in treatment delayed animals when comparisons were made between lesion volume to asymmetry scores. Correlation analysis between MRI determined lesion volume and foot fault scores for vehicle treated animals for all time points is plotted in Figure 4B for vehicle, 1-3 hour MB and 24 hour MB treated animals. Regression analysis revealed an R^2 value of 0.2870 (p<0.0001) for lesion volume and % asymmetry scores in all groups. Individual regression analysis for each group was also performed between lesion volume and % foot fault where in vehicle treated animals yielded a significant correlation with an R^2 value of 0.43 (p=0.0002) (data not shown). Methylene Blue treated animals yielded a non-significant correlation with an R^2 value of 0.009 (p=0.88) in acutely treated animals, while a significant correlation was found in delayed MB treated animals with an R^2 value of 0.286 (p=0.004) when comparisons were made between lesion volume to foot fault scores. Vasogenic edema and T_2 MRI lesion volume were in general agreement with behavioral scores.

Discussion

This study demonstrates delayed methylene blue treatment is also neuroprotective following TBI using an open-skull, controlled cortical impact (CCI) model in rats. The major findings are: 1) Delayed MB treatment was effective in reducing lesion volumes and behavioral deficits on days 2, 7 and 14

compared to vehicle treatment, 2) T_2 determined lesion volume peaked on day 2 in vehicle treated animals and acute MB treated animals while delayed MB lesion volume peaked at three hours, 3) increased T_2 was apparent 3hrs after TBI, and was significantly decreased by 3 hours in acutely treated MB animals and by day 2 in delayed MB animals, with all groups gradually returning toward normal by day 14, 4) the increase in ADC was significantly reduced by acute and delayed MB treatment on days 2 and 7 and gradually returned toward normal at day 14; 5) CBF measures indicated severe hypoperfusion at three hours, marked hyperperfusion peaking on day 2, hypoperfusion again on day 7, and a return towards normal on day 14 in all groups with only a significant increase with acute and delayed MB found on day 14; and 6) acute and delayed MB treatment reduce sensorimotor deficits following TBI. In sum, we provide further evidence that MB acts as a neuroprotective agent that decreases lesion volume, reduces cytotoxic edema formation, improved MRI defined parameters, and improves functional outcome.

Methylene Blue reduces lesion volume

Delayed MB treatment was effective in reducing lesion volumes and behavioral deficits on days 2, 7 and 14 compared to vehicle treatment. The delayed MB group had similar lesion volumes at the three-hour time point (before MB) compared to the vehicle group as expected, whereas the acute MB group at the three-hour time point (after MB) had reduced lesion volume compared to the vehicle group. These findings suggest that MB has immediate effects. A likely mechanism of neuroprotection is that MB acts as an energy enhancer, sustaining some level of ATP production after TBI. MB also has antioxidant properties. Thus, MB also likely reduces oxidative damage from excessive oxygen free radicals following TBI. MB has been shown to decrease ROS production in ischemia/reperfusion injury[31] and neuron cell death induced by oxidative stress.[32] In addition, MB inhibits rotenone-induced lipid peroxidation[17] and decreases oxidative damage following ischemic-reperfusion injury.[33, 34] The ability of delayed MB to exert improved lesion volumes compared to acutely treated

animals may be due to the time course of oxidative damage that occurs post TBI. Ansari et al., demonstrated using the same CCI model utilized in this study that the level of lipid peroxidation peaks around 24-48 hours post injury with a concomitant peak decrease in the primary antioxidant glutathione at the same time.[35] In addition the activity of several antioxidant enzymes are also significantly reduced 24 hours post-TBI including glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase. This suggests that antioxidant defenses are unable to counteract increased production of reactive oxygen species. Therefore, MB's antioxidant property likely contributes to the observed reduction in vasogenic edema, lesion volume, neuronal cell death and behavioral deficits in our study when oxidative stress levels are at their highest.

MRI characterization of TBI

T_2: We previously demonstrated that T_2 and diffusion MRI parameters are sensitive to both hyperacute and subacute changes in a rat model of mild TBI without treatment.[36] This study compared the quantitative analysis of multimodal MRI parameters of both the acute and delayed MB treatments (which have not been previously published) to those from our previous multimodal MRI study of vehicle-treated animals.[36] Increased T_2 values were apparent 3hrs after TBI in both vehicle and delayed MB treated animals as expected and suggest increased water that likely indicates edema formation within the impacted area. There are a number of cellular mediators released following TBI that likely contribute to increased brain edema including glutamate, lactate, nitric oxide, calcium, potassium and hydrogen among others. TBI induced disruption of the BBB is also an important mediator of increased edema formation, especially during the acute phase after injury. However, significantly decreased T_2 was detected by 3 hours in acutely treated MB animals and by 48 hours in the delayed MB treatment group suggesting that MB alleviates edema formation. The mechanisms underlying MB effects on edema formation are not well understood. However, our findings have been corroborated by Fenn et al., who recently

demonstrated that an acute single dose of methylene blue caused a reduction in cerebral edema within twenty four hours after injury using the fluid percussion model of TBI.[37] The authors also found a reduction in neuroinflammation which could also contribute to the decrease in edema formation.

Diffusion: While cytotoxic edema has been described as the predominant form of edema post-injury, vasogenic edema is known to cause increased intracranial pressure and secondary ischemic events.[38, 39] In our model of TBI we detect heterogeneous ADC decreases and increases around the impacted area during the hyperacute phase (3 hrs), indicating a combination of cytotoxic and vasogenic edema, respectively. Furthermore, the time course of edema formation within the ipsilesional impact area began as early as 1-3 hours post-injury, peaked at 48 hours and begin to disipate by day 7 correlating with behavioral recovery.[36] Both acute and delayed MB treatment resulted in significant elevations of ADC on day 2, however they did not reach the severity of the vehicle treated animals. Subsequently, MB treated animals had significantly reduced ADC values compared to vehicle treated animals. These data suggest that MB has important effects on reducing vasogenic edema formation even when given in a delayed manner (24 hours post-injury). MB has previously been shown to have hemodynamic effects in models of pulmonary hypertenstion by reducing edema formation likely through inhibiting cyclooxygenase products of arachidonic acid [40] and suggests that MB has important *in vivo* effects on edema formation. The data for acute MB treatment are supported by a study by Fenn et al., 2014 who demonstrated that immediate injection of MB reduces cerebral edema using the wet-dry weight method following fluid percussion injury in mice.[41] However, there are no other studies that have assessed this phenonmenon following delayed treatment of MB in TBI.

Diffusion tensor imaging parameters such as fractional anisotropy have been increasingly used to investigate axonal injury in mild to severe TBI.[42,43] Following TBI there is a reduction in brain white matter FA that may be associated with changes in parenchymal structure [44,45,46], however it is not fully understood. The potential parenchymal changes associated with TBI include

edema formation effects, axonal degeneration and fiber disruption. The present study is in line with previous studies that have demonstrated a reduction in FA post-TBI, which peaked two days after the injury and steadily returned towards normal. Acute and delayed MB administration reduced the abnormalities in FA compared to vehicle treated animals suggesting that MB reduces axonal injury. The mechanisms of this protection may be due to the reduction in oxidative stress however there is no direct evidence to support this theory in the literature. One study has shown that treating with Edaravone, a free radical scavenger, resulted in suppressed oxidative stress and axonal injury.[47]The authors suggest that the protective function of Edaravone may be mediated in part by down regulation of nNOS and iNOS with a concomitant increase in iNOS and its free radical scavenging ability. Due to the similarity in the potential mechanisms of MB as a free radical scavenger, MB could be limiting axonal injury by reducing the level of free radicals however additional studies are needed.

CBF: The effects of TBI on CBF measurements have been reported using multiple methods in various TBI models.[48-49] However, the results have been variable with hypoperfusion, hyperperfusion or a combination of the two found following TBI in animal models. In our model CBF was markedly reduced acutely (at 3 hrs), significantly increased on day 2, and returned toward near-normal values by days 7 and 14. The initial reductions in CBF could be due to local increases in intracranial pressure and/or damaged to blood vessels due to mechanical injury during the impact. The subsequent hyperperfusion observed at 24 and 48 hours post injury could be related to decreased vasoconstriction or impaired vasoreactivity. Hyperperfusion elevates cerebral blood volume causing increased intracranial pressure altering autoregulation. Dore-Duffy et al., suggest that following TBI hyperperfusion could be caused by altered regulation of blood flow within the microvessels by endothelin -1-induced pericytes.[50] These finding are also supported by Thomale et al. who found severe hypoperfusion using laser Doppler flowmetry in a similar rat model of moderate CCI in the area of the impact at 0.5-6.0 hrs and hyperperfusion at 24 and 48 hours.[51] Other studies found CBF reduction only on day 0 post-TBI but did not observe subsequent

hyperperfusion.[52,53] The observed hyperperfusion could lead to detrimental changes by causing increased edema and potentially increasing the rate of hemorrhage.[54]

Interestingly, MB had minimal effects on CBF when compared to vehicle treated animals. Previous studies have reported that MB does not affect CBF [55] or increased CBF [56] only slightly in normal animals. In this study acute and delayed methylene blue increased CBF values only on day 14 compared to vehicle treated animals.

Behavioral improvement with Methylene Blue

We utilized the foot fault and asymmetry test to determine functional outcomes following TBI to the S1FL cortex and the effects of MB on functional recovery. Despite the presence of a significant lesion in vehicle treated animals at day 7 and 14, forepaw asymmetry scores returned to normal for all groups while the forelimb foot fault scores remained significantly abnormal on day 7 returning to normal on day 14. Delayed MB treatment was as effective as acute MB treatment in terms of percent asymmetry measures despite the fact that lesion volumes were significantly lower in the delayed administration group. Interestingly the foot fault test revealed a slower recovery in functional deficits compared to the asymmetry test. This suggests that the foot fault test may be a more sensitive measure of forelimb functional deficits in mild to moderate brain injuries. Regression analysis also confirmed increased correlation between lesion volume and % foot faults scores when compared to lesion volume and % asymmetry scores correlation analysis further supporting that the foot fault test is a more sensitive measure of motor impairment in TBI rats. In all groups, for both functional tests, the animals recovered by day 14. This suggests that there may be functional compensation or reorganization occurring with moderate traumatic brain injury. Previous studies have revealed a similar phenomenon and have suggested that recovery of brain function may occur due to diaschisis, behavioral substitution or that another area of the brain may take over the function.[57] For diaschisis to occur areas outside of the injury that may be depressed in terms of

function initially recover over time and begin to function again. The rats may also learn new strategies over time to compensate for the functional deficits. However, the tests utilized in this study may also not be sensitive enough to detect more subtle injuries. Therefore, the utilization of enhanced multimodal imaging methods may provide increased identification of potential outcome measures that are not readily detectable by standard behavioral tests.

Limitations of the current study

There are a few limitations to the current study. 1) Differences in initial lesion volume could confound the experimental data as each individual animal may start with differing lesion volume. The CCI model utilized in this study minimizes the variability of starting lesion volume and the results demonstrate non-significant differences in initial lesion volume between animals further validating the findings. 2) The functional outcome measures may not be sensitive enough to detect chronic abnormalities in motor function. Therefore, future directions will include behavioral tests with increased sensitivity for motor deficits. We also intend on including assessments for sensory deficits as this is a common pathology following TBI. We will assess fine motor impairments using the Vermicili handling test and sensory deficits using the Morris Water maze in future studies. 3) Histological data was not included in the current study as we have previously reported such data and found no significant difference in lesion volume detected by MRI and Nissl or Fluro Jade immunohistology[25]. Future studies will include assessment of neurodegeneration and markers of inflammation.

Conclusion

The present study provides additional evidence that delayed methylene blue treatment is neuroprotecitve against mild TBI and further substantiates that targeting the mitochondria provides a mechanism of neuroprotection post-injury. Importantly, delayed administration of MB was also found to attenuate secondary

brain injury effects. This has important implications for patients that do not have medical treatment readily available following an acute injury to the brain.

Acknowledgement

This work was supported in part by NIH/NINDS R01 NS45879 (TQD), a TL1 grant (JAL) and KL2 (LTW) via the Clinical Translational Science Awards (CTSA, parent grant 8UL1TR000149, TL1TR001119, and TR001118) and Mike Hogg Fund (LTW).

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Figure legends:

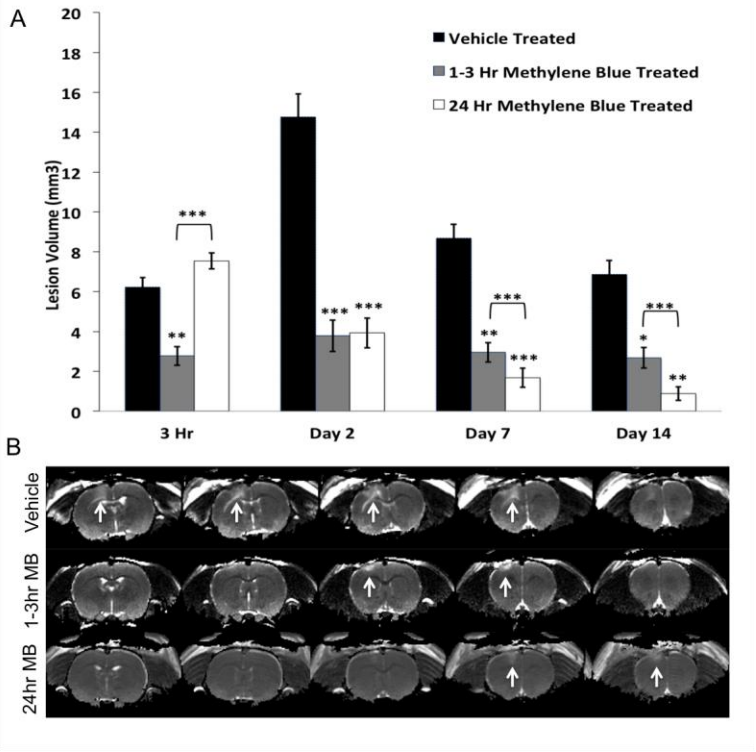

Figure 1: A. Lesion volumes for MB and vehicle treated animals are shown. MB treated animals were either treated acutely (1-3 hour) or subacutely (24 hour). (n = 10 per group, ±SEM). B. Representative T_2 maps for vehicle, 1-3 hr MB, and 24 hr MB treated animals on day 2 post TBI. White arrows indicate areas of abnormal T_2 indicating the lesion.

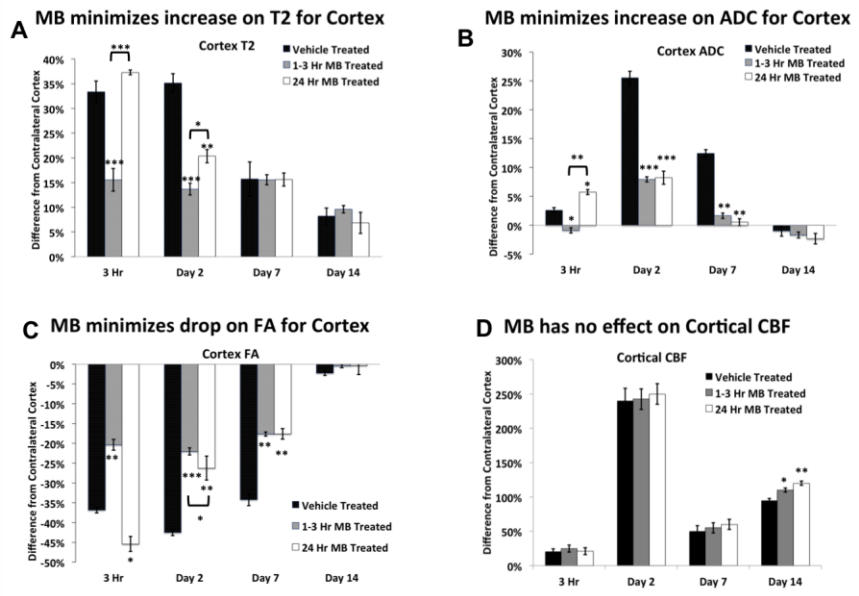

Figure 2: Percent differences from the contralesional cortex were caluclated for T_2, ADC, FA and cortical CBF in vehicle, acute (1-3 hr) and delayed (24 hr) methylene blue treated groups after TBI (n = 10 per group, ±SEM).

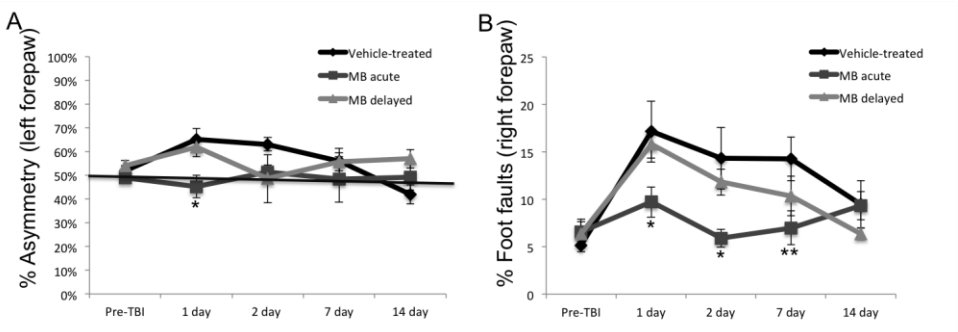

Figure 3: A. Line graph demonstrates the averaged % asymmetry of the left forepaw for vehicle, acute (at 1 and 3 hours) and delayed (at 24 hours) MB treated animals prior to injury and post-injury on days 1, 2, 7 and 14. *p<0.05 compared to vehicle treated animals; n=10 per group; average±SEM. B. Line graph demonstrating the % foot faults of the right forepaw in vehicle, acute (at 1 and 3 hours) and delayed (at 24 hours) MB treated animals prior to injury and 1, 2, 7, and 14 days post-injury. *p<0.05 and **p<0.01 compared to vehicle treated animals; n=10 per group; average±SEM.

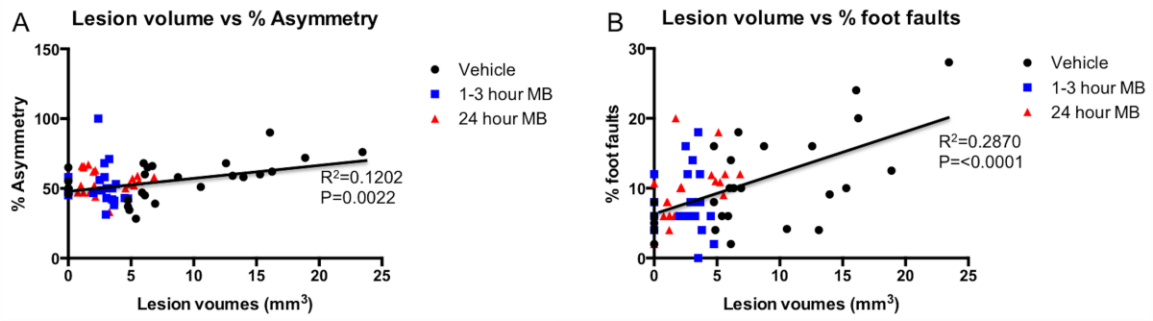

Figure 4: A. Multiple regression correlation plots are demonstrated for T_2 MRI lesion volume versus % asymmetry scores in vehicle, 1-3 hour MB treated animals and 24 hour MB treated animals with different symbols indicating data from the different treatment groups. B. Multiple regression correlation plots are also demonstrated for T_2 MRI lesion volume versus % foot fault scores in vehicle, 1-3 hour MB treated animals and 24 hour MB treated animals with different symbols indicating data from the different treatment groups.