Methylene Blue Is Neuroprotective against Mild Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. Methylene blue (MB) has known energy-enhancing and antioxidant properties. This study tested the hypothesis that MB treatment reduces lesion volume and behavioral deficits in a rat model of mild TBI. In a randomized double-blinded design, animals received either MB (n = 5) or vehicle (n = 6) after TBI. Studies were performed on 0, 1, 2, 7, and 14 days following an impact to the primary forelimb somatosensory cortex. MRI lesion was not apparent 1 h after TBI, became apparent 3 h after TBI, and peaked at 2 days for both groups. The MB-treated animals showed significantly smaller MRI lesion volume than the vehicle-treated animals at all time points studied. The MB-treated animals exhibited significantly improved scores on forelimb placement asymmetry and foot fault tests than did the vehicle-treated animals at all time points studied. Smaller numbers of dark-stained Nissl cells and Fluoro-Jade/C210 positive cells were observed in the MB-treated group than in vehicle-treated animals 14 days post-TBI. In conclusion, MB treatment minimized lesion volume, behavioral deficits, and neuronal degeneration following mild TBI. MB is already approved by the United States Food and Drug Administration (FDA) to treat a number of indications, likely expediting future clinical trials in TBI.

Key words: antioxidant; mitochondria; MRI; oxidative stress; vasogenic edema

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability.1 Common causes of TBI include falls, violence, injuries from wars, and vehicle and sport accidents. The initial direct mechanical damage in TBI is followed by secondary damage that includes impaired cerebral blood flow (CBF) and oxygen delivery, cerebrovascular autoregulation, and metabolic function. Ischemia-like events (such as membrane depolarization, ion dysregulation, oxidative stress, excitotoxicity, and inflammation, among others) subsequently lead to apoptotic and necrotic cell death. The multidimensional cascades of secondary brain injury in TBI offer many potential targets for therapeutic interventions.

Mitochondria are the cells’ power plants that generate adenosine triphosphate (ATP), necessary for sustaining normal cellular function and repair. As the brain is dependent upon a continuous supply of energy to maintain cellular integrity, damage to the mitochondria can be detrimental. Mitochondrial dysfunction may occur within minutes of TBI, and persist for days following the injury. In addition, reactive oxygen species (ROS) – natural by-products of oxygen metabolism – increase rapidly following TBI, and overwhelm the cells’ natural antioxidant capabilities, resulting in oxidative stress. Treatments that can restore mitochondrial function have the potential to improve outcomes following TBI injury. As such, mitochondria have become an important target for neuroprotection in TBI.2,3

Methylene blue (MB) is used clinically to treat methemoglobinemia, malaria, carbon monoxide poisoning, and cyanide poisoning.4 At low doses, MB forms a reversible reduction-oxidation (redox) system in the cell with auto-oxidizing capacity.5 Low-dose MB has redox recycling properties in that it acts as an electron cyclers and facilitates electron transfer in the mitochondrial electron transport chain by accepting electrons from nicotinamide adenine dinucleotide (NADH) and transferring them to cytochrome c, bypassing complex I-III.6 MB thus enhances or sustains ATP production in cells7–9 and brain oxygen consumption in vitro.10 In bypassing complex I-III to generate ATP, MB also minimizes free radical production in the mitochondrial electron transport chain, which could have positive effects under metabolic stressed conditions (i.e., brain injury) in which excessive production of free radicals may lead to cellular damage and cell death. Moreover, MB can induce long-lasting effects by modifying mitochondrial pathways.11 In vivo studies also
show that MB significantly affects cerebral metabolism, hemodynamics, and evoked responses in normal rats; namely, that, MB enhances global glucose uptake, oxygen consumption, CBF and evoked responses. Recently, MB treatment has been shown to reduce behavioral impairments in animal models of Parkinson’s disease and Alzheimer’s disease, and to reduce cerebral infarct volume by histology. The goal of this study was to evaluate the therapeutic efficacy of MB in a rat model of mild TBI. We tested the hypothesis that MB treatment reduces lesion volume, neurologic deficit, and neuronal degeneration following mild TBI in rats. In a randomized, double-blinded design, animals received either MB or vehicle after TBI. MRI lesion progression, forelimb placement asymmetry, and foot fault behavioral tests were longitudinally evaluated up to 14 days post-TBI. Immunohistology (Nissl and Fluoro-Jade B) was used to quantify neuronal degeneration.

Methods

Animal preparations

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center, San Antonio. Male Sprague–Dawley rats (250–350 g, n = 12) were anesthetized initially with 5% isoflurane mixed with room air and maintained at 1.5% isoflurane throughout all surgical and imaging procedures. The animal was secured in a MRI-compatible stereotaxic frame and a 5 mm craniotomy was created over the surgical and imaging procedures. The animal was secured in a MRI-compatible stereotaxic frame and a 5 mm craniotomy was created over the lesioned area. For each slice imaged, 10 regions located under the impact zone were selected for imaging by sampling beginning 3.5 mm from the median plane and directly under the impact zone 3.5 mm from the median plane and left (both) forelimb placements onto the wall of the cylinder, exposing the dura matter. The intact dura matter was impacted using a pneumatic controlled cortical impactor (Precision Systems and Instrumentation, LLC, Fairfax Station, VA) at 1 mg/kg 1 h post-TBI and 0.5 mg/kg 3 h post-TBI received saline vehicle. A daily dose of 0.5–5 mg/kg MB is considered safe for humans and animals.4,11,19,20 MRI was performed on day 14 post-TBI. Brains were removed and fixed for 2–5 h at 4°C, and subsequently cryopreserved in 30% sucrose for 48 h. Coronal sections (25 μm thick) were cut on a cryostat and affixed to gelatin-coated slides and dried overnight at 37°C. Slides were hydrated through a series of graded alcohols to distilled water followed by 0.1% cresyl violet acetate for 7 min. Brain sections were then dehydrated, cleared in xylene and cover-slipped with mounting medium.

Images were acquired on an Olympus BX60F microscope equipped with an Olympus DP70 camera using a 100× oil objective for morphological analysis. A 10× objective was utilized to acquire images for mosaic full brain images assimilated using Microsoft ICE software. Intact neurons were defined as nonbasophilic neurons with pale nuclei and discrete nucleoli, and having an intact neuronal body. Dark stained neurons were defined as neurons with abnormal morphologies such as hyperbasophilic neurons and those with shrunken morphology. Four slices from each brain were selected for analysis by determining corresponding slices to the images acquired from T2 MRI using stereotaxic coordinates. The slices were 1 mm apart to ensure adequate sampling throughout the lesioned area. For each slice imaged, 10 regions located under the impact zone were selected for imaging by sampling beginning directly under the impact zone 3.5 mm from the median plane and 1.5 mm from the edge of the cortex. Additional images were acquired on either side of and below this area in two rows spaced 250 μm apart. Ten corresponding regions on the contralateral side were selected and imaged at 60× (175 μm x 175 μm) from each brain collected. The numbers of dark stained cells were counted and averaged. Results were expressed as the number of dark stained cells per field.
Fluoro-Jade B staining

Fluoro-Jade B is an anionic fluorescein derivative used to stain degenerating neurons.\textsuperscript{27,28} Sections were incubated in a solution of 1\% NaOH in 80\% ethanol for 5 min, followed by hydration in graded ethanols (75, 50, and 25\%) to distilled water for 5 min each. The sections were then incubated for 10 min in 0.06\% potassium permanganate, rinsed with distilled water, and incubated in 0.004\% Fluoro-Jade B (Hist-Chem Inc, Jefferson, AR) for 20 min. Sections were rinsed with distilled water three times for 2 min each. The slides were then dried on a slide warmer for 10 min, cleared with Histo-Clear and cover-slipped with DPX (Fluka). Images were acquired on a Nikon C1si microscope using a 60\textsuperscript{·} water immersion objective. Image analysis was performed similar to that done for Nissl staining.

Statistical analysis

Unpaired \textit{t} tests were used to compare T2 lesion volumes between vehicle- and MB-treated groups. Differences in the number of dark-stained Nissl neurons and Fluoro-Jade B positive cells between vehicle- and MB-treated animals were assessed using a two tailed \textit{t} test. Mann–Whitney \textit{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, foot fault scores, and Nissl and Fluoro-Jade B staining values.

Results

MB reduced lesion volume

Multislice T2 maps show the temporal progression of TBI lesions in a vehicle-treated and an MB-treated animal (Fig. 1A). The hyperintense area in the S1 cortex (arrows) indicates the lesion, which has been enlarged in the panels to the right of each representative group in Figure 1A. Within the lesions, there were also hypointense spots, likely indicating signs of hemorrhage. T2 MRI at 1 h after TBI showed no apparent lesion in either the vehicle- or MB-treated group. MRI lesions were apparent 3 h after TBI in both treatment groups. In the vehicle-treated group, the lesion volume peaked at 2 days, and decreased by 14 days post-TBI (Fig. 1B). In the MB-treated group, by contrast, the lesion volumes did not change substantially with time, and were significantly smaller than those of the vehicle-treated group at all time points studied. These results indicate that MB treatment markedly reduces TBI lesion volume and vasogenic edema.

MB improved behavioral outcomes

Sensorimotor function was assessed using the forelimb asymmetry (cylinder) and foot fault tests. Before TBI, the mean forelimb asymmetry scores were not significantly different between the vehicle- and MB-treated groups (49±2\% vs. 52±2\%, \(p>0.05\); Fig. 2A) indicating symmetrical use of the two forelimbs. In the vehicle-treated group, by contrast, the lesion volumes did not change substantially with time, and were significantly smaller than those of the vehicle-treated group at all time points studied. These results indicate that MB treatment markedly reduces TBI lesion volume and vasogenic edema.

FIG. 1. T2 MRI. (A) Representative T2 maps are shown for vehicle- and methylene blue (MB)-treated animals at 3 h, and 1, 2, 7, and 14 days post-traumatic brain injury (TBI). The arrows indicate hyperintense lesions in the ipsilesional cortex. The white boxes in the second panel indicate the enlarged image of the lesion area demonstrated to the right of each panel to further demonstrate the evolution of the lesion with time. (B) Bar graph shows the progression of lesion volumes in MB-treated animals compared with vehicle-treated animals (mean±SEM, \(n=6\) per group,\(^*p<0.05\)).

The percentages of foot faults were not statistically different between the vehicle- and MB-treated groups prior to TBI induction (5.13±0.64 vs. 6.57±1.04\%, \(p>0.05\); Fig. 2B). In the vehicle-treated group, foot fault scores worsened in the right forelimb dramatically on day 1, persisted on days 2 and 7 after TBI, and improved slightly on day 14. In the MB-treated group, by contrast, right foot faults were only slightly elevated on day 1 post-TBI but did not reach the severity observed in the vehicle group. Moreover,
by day 2, the right foot fault scores returned close to pre-TBI values, and persisted through days 7 and 14. There were significantly lower numbers of foot faults in the MB-treated group than in the vehicle-treated group on days 1, 2, and 7 post TBI (\( p = 0.043, 0.018, \) and 0.0058, respectively) but not on day 14. Together, these data indicated that MB markedly reduced sensorimotor deficits following TBI. These data also suggest that there were functional compensations in both groups, and that the extent of functional compensation differed between groups.

Correlation analysis between T2 MRI lesion volume and forelimb asymmetry scores for all time points yielded an \( R^2 \) value of 0.32 (\( p = 0.002 \)) (Fig. 3A). Correlation analysis between MRI-determined lesion volume and foot fault scores for all time points yielded an \( R^2 \) value of 0.43 (\( p = 0.0002 \)) (Fig. 3B). Vasogenic edema and T2 MRI lesion volume were in general agreement with behavioral scores.

**MB reduced Nissl-positive cells**

Nissl staining of degenerating neurons was analyzed in the primary somatosensory cortex below the impact zone for both experimental groups on day 14 after TBI. Mosaic images (Fig. 4A) and magnified images (Fig. 4B) demonstrated the extent of the injury and altered morphological characteristics in the ipsilesional cortex. In the vehicle-treated group, the number of Nissl-positive cells in the ipsilesional S1 cortex was 73 ± 17 cells/field and that in the contralateral S1 cortex was 26 ± 9 cells/field (\( n = 6, p < 0.05 \)). In the MB-treated group, the number of Nissl-positive cells in the ipsilesional S1 cortex was 41 ± 7 cells/field and that in the ipsilesional S1 cortex was 21 ± 6 cells/field (\( n = 6, p < 0.05 \)). The numbers of positive Nissl cells in the contralateral S1 cortex were not significantly different between the vehicle- and MB-treated groups (\( p > 0.05 \)).

**MB reduced Fluoro-Jade B positive cells**

Fluoro-Jade B staining for degenerating neurons was performed 14 days after TBI. Figure 5A demonstrates representative images from the contralateral and ipsilesional cortex from vehicle- and MB-treated groups.

**FIG. 2.** Line graphs of the (A) cylinder and (B) foot fault tests for the vehicle- and methylene blue (MB)-treated animals at pre-traumatic brain injury (TBI), and at 1, 2, 7, and 14 days post-TBI (mean ± SEM, \( n = 6 \) each group, *\( p < 0.05 \), **\( p < 0.01 \) between vehicle- and MB-treated groups).

**FIG. 3.** Multiple regression correlation plots are demonstrated for T2 MRI lesion volume versus (A) asymmetry and (B) foot fault, with different symbols indicating data from different days post TBI.
MB-treated animals. In the vehicle-treated group, the number of Fluoro-Jade B positive cells (white arrows) in the ipsilesional S1 cortex was 368 ± 23 cells/field, and that in the contralesional S1 cortex was 158 ± 4.8 cells/field \((n = 6, \ p < 0.05)\) (Fig. 5B). In the MB-treated group, the number of Fluoro-Jade B positive cells in the ipsilesional S1 cortex was 270 ± 18 cells/field, and that in the contralesional S1 cortex was 149 ± 15 cells/field \((n = 6, \ p < 0.05)\). The numbers of Fluoro-Jade B positive cells in the ipsilesional S1 cortex were significantly different between the vehicle- and MB-treated groups \((p < 0.05)\). The numbers of Fluoro-Jade B positive cells in the contralateral S1 cortex were not significantly different between the vehicle- and MB-treated groups \((p > 0.05)\). The results from the Nissl and Fluoro-Jade B experiments indicated that MB reduced neurodegeneration following TBI.
Correlation analysis between T2 MRI lesion volume and Nissl values at 14 days revealed significant correlation with an $R^2$ value of 0.978 ($p = 0.000005$) (Fig. 6A). Correlation analysis between T2 MRI lesion volume and Fluoro-Jade B values at 14 days revealed significant correlation, with an $R^2$ value of 0.963 ($p = 0.008$) (Fig. 6B). Vasogenic edema and T2 MRI lesion volume were in general agreement with markers of neurodegeneration.

**Discussion**

This study demonstrated a neuroprotective effect of MB in a rat model of mild TBI using a double-blinded randomized design. MB minimized lesion volume and decreased functional deficits compared with vehicle-treated animals. Immunohistological staining for neuronal degeneration corroborated MRI lesion volume and behavioral data. MB neuroprotection against mild TBI in our study is consistent with previous MB studies that reported reduced behavioral deficits in animal models of Parkinson’s disease, Alzheimer’s disease, and cerebral infarct volume, supporting the notion that MB’s energy-enhancing and antioxidant properties have therapeutic effects in a number of neurological injuries and disorders.

TBI-induced edema peaked at day 2 and resolved substantially by days 7 and 14. The reduction in edema by day 14 is in general agreement with improvement in the behavioral scores. Lesion volume and behavioral scores also generally correlated well with the improvement by MB treatment. However, despite the presence of lesions at day 7 and 14, forepaw asymmetry scores returned to normal for both groups. These results suggest that there is likely functional compensation (or reorganization). By contrast, the
forelimb foot fault scores remained significantly abnormal on day 7 and returned closer to normal on day 14. These differences in our studies suggest that the foot fault test is more sensitive to milder injury, especially in the initial days following injury. The two behavioral tests showed different but complementary sensitivities to injury in this animal TBI model.

In vivo studies have shown that MB readily crosses the blood–brain barrier. MB concentration in the brain is 10–20 times higher than that in the circulation after intravenous injection in rats. No negative side effects of MB in animals or humans at low doses of MB (0.5–5 mg/kg) have been reported, but adverse effects (e.g., methemoglobinemia) have been reported at high MB doses (>10 mg/kg) because MB interferes with electron transfer function in the mitochondria. The dosage utilized in this study was based on published results that daily low-dose MB is safe in humans and rats.

A single MB dose of 1 mg/kg affects cerebral metabolism, hemodynamics, and evoked responses in normal rats. MB enhances global glucose uptake, oxygen consumption, and CBF in normal rats. In addition, MB markedly potentiates forelimb evoked blood oxygenation level-dependent (BOLD), CBF and cerebral metabolic rate of oxygen (CMRO2) changes focally in the forelimb somatosensory cortices, suggesting that MB has effects localized to regions with enhanced activity or metabolic stress. Moreover, during mild hypoxia, MB-treated rats are better able to sustain global glucose uptake, oxygen consumption, and CBF than are vehicle-treated rats. MB-treated animals have also shown larger functional MRI (fMRI) responses and O2 consumption changes than have vehicle-treated rats. These findings further support the notion that MB is an energy enhancer in vivo under metabolically stressful conditions. The ability to sustain some level of ATP production after TBI likely accounted, in part, for the observed reduction in vasogenic edema, lesion volume, neuronal cell death, and behavioral deficits.

A likely mechanism of neuroprotection is that MB acts as an energy enhancer, sustaining some level of ATP production after TBI. Other studies have targeted the mitochondria to sustain energy production and mitochondrial integrity following TBI. For example, 2-methylthioadenosine diphosphate trisodium salt, which targets the mitochondria through the P2Y1 receptor, stimulates energy production through an IP3 mediated pathway, was recently reported to decrease edema formation and neuronal loss in a closed-skull model of TBI in mice. In addition to stimulation of ATP production through the mitochondrial electron transport chain, the preservation of mitochondrial integrity has also been targeted using hyperoxia treatment. In a fluid percussion injury model, hyperoxia treatment significantly improved mitochondrial metabolic activity, and contributed to increased B-cell lymphoma 2 (Bcl-2) expression and increased oxygen availability. Loss of mitochondrial integrity can also affect the mitochondrial permeability transition pore, leading to increased release of pro-apoptotic factors following injury. Cyclosporine A binds to cyclophilin D, and results in stabilization of the mitochondrial permeability transition pore, reduced axonal damage, and lesion volume following TBI. Phase II clinical trials have demonstrated that cyclosporin A improves cerebral perfusion pressure and cerebral metabolism, and cyclosporin A has moved into Phase III clinical trials.

Taken together, these data provide evidence that improving mitochondrial integrity and production of ATP results in improved outcome following TBI.

In addition to being an energy enhancer, MB also has antioxidant properties. Therefore, 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 and neuron cell death induced by oxidative stress. MB inhibits rotenone-induced lipid peroxidation, and decreases oxidative damage following ischemic reperfusion injury. Many studies have targeted reduction in the production of ROS from the mitochondria following brain injury. Superoxide dismutases (SOD), which are considered one of the primary antioxidant defenses in cells, have been shown to maintain cytosolic calcium levels and reduce superoxide. The free radical scavenger, α-tocopherol, has also been shown to exert neuroprotective effects in experimental models of TBI by decreasing lipid peroxidation, neuronal necrosis, and reactive gliosis, and by reducing edema formation. MB’s antioxidant property likely contributed to the observed reduction in vasogenic edema, lesion volume, neuronal cell death, and behavioral deficit in our study.

Conclusions

The present study provides evidence that MB – a unique energy-enhancer and antioxidant – is neuroprotective against mild TBI as measured by the reduction in lesion volume, behavioral deficit, and...
neurodegeneration. These findings suggest that targeting mitochondrial function via sustaining energy production and reducing free oxygen radicals could be a promising treatment strategy for mild TBI. Future studies will optimize multiple MB dosing and timing regimens to improve efficacy, impact other brain regions such as those involved in memory and cognition, explore milder TBI along with more sensitive behavioral tests, determine the mechanistic temporal profiles, and use additional MRI measures such as CBF, diffusion tensor, neurovascular coupling, evoked responses, and blood–brain permeability. Combination therapy targeting multiple mechanisms will also be explored to further minimize damage and enhance recovery. Because MB is already a drug approved by the United States Food and Drug Administration (FDA), with an excellent safety profile at low doses, TBI clinical trials in humans utilizing MB can be readily explored.

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Author Disclosure Statement

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