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Targeting Mitochondrial Dysfunction in CNS Injury Using Methylene Blue; Still a Magic Bullet?

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Targeting mitochondrial dysfunction in CNS injury using Methylene Blue; still a magic bullet?

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Abstract

Complex, multi-factorial secondary injury cascades are initiated following traumatic brain injury, which makes this a difficult disease to treat. The secondary injury cascades following the primary mechanical tissue damage, are likely where effective therapeutic interventions may be targeted. One promising therapeutic target following brain injury are mitochondria. Mitochondria are complex organelles found within the cell, which act as powerhouses within all cells by supplying ATP. These organelles are also necessary for calcium cycling, redox signaling and play a major role in the initiation of cell death pathways. When mitochondria become dysfunctional, there is a tendency for the cell to loose cellular homeostasis and can lead to eventual cell death. Targeting of mitochondrial dysfunction in various diseases has proven a successful approach, lending support to mitochondria as a pivotal player in TBI cell death and loss of behavioral function.

Within this mixed mini review/research article there will be a general discussion of mitochondrial bioenergetics, followed by a brief discussion of traumatic brain injury and how mitochondria play an integral role in the neuropathological sequelae following an injury. We will also give an overview of one relatively new TBI therapeutic approach, Methylene Blue, currently being studied to ameliorate mitochondrial dysfunction following brain injury. We will also present novel experimental findings, that for the first time, characterize the *ex vivo* effect of Methylene Blue on mitochondrial function in synaptic and non-synaptic populations of mitochondria.

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1. Introduction

1.1 Mitochondria, Powerhouse and Death Switch of Cells

Oxygen mediated respiration is vital for the survival of eukaryotes, and the functional role of respiration is assigned to a specialized organelle, the mitochondria. The role of mitochondria is not only that of the powerhouse of the cell, but is also a dynamic center for cellular homeostasis [1]. Newer information about mitochondria has shifted focus onto their crucial role in cellular plasticity and regulation. Owing to the complexity of the mammalian brain and its high dependency on oxygen, mitochondria play a very important role in ATP production and maintaining brain homeostasis.

The primary role of mitochondria is to generate ATP through the TCA cycle and oxidative phosphorylation, which is essential for driving neuronal functions including maintenance of the membrane potential and neurotransmission. Mitochondria contain a double membrane structure. The outer membrane is permeable allowing small molecules and ions to pass through via pore forming protein complexes like voltage dependent anion channels (VDAC) [2]. The inner membrane is almost impermeable and only selectively allows controlled transport of specific ions like K^+, Na^+ and Ca^{2+} and various metabolites like ADP, amino acids and pyruvate. The inner membrane is highly invaginated allowing it to maximize its surface area and house the electron transport chain (ETC), the FoF1 ATPase, and various other specific transporters [3]. Complex I (NADH/ubiquinone oxidoreductase) accepts electrons from NADH generated through the breakdown of the metabolic substrates like carbohydrates, lipids and proteins and transfer them to complex III via Coenzyme Q. The transport of electrons continue through complex III (cytochrome c reductase) through complex IV (cytochrome c oxidase) and ultimately reduces and splits molecular oxygen to generate two molecules of water (H_2O). Each electron transfer step pumps two protons from matrix into the intermembrane space. This imbalance/gradient of protons across the inner membrane leads to the generation of electromotive force. This voltage difference drives back the movement of protons through FoF1 ATPase, which utilizes this electrochemical energy to drive the enzymatic phosphorylation of ADP to generate ATP [3].

In the past few decades mitochondria have gained special attention for their role in apoptosis, cell signaling, cell cycle regulation, Ca^{2+} homeostasis, and ROS production. Mitochondria play a vital role as a biological switch that determines the fate of the cell under stress [4]. They trigger apoptosis by releasing important mediators like Cytochrome C and Apoptosis inducing factor (AIF) [5]. Mitochondria have a strong capacity to sequester cytosolic calcium mainly due to electrochemical gradient and Ca^{2+} uniporter present in its inner membrane. Under normal physiological conditions, mitochondria regulate Ca^{2+} homeostasis by acting as a cytosolic Ca^{2+} buffer [6–8]. Mitochondrial uptake of Ca^{2+} beyond a critical level signals opening of the mitochondrial permeability transition pore (mPTP) leading to a series of events including breached mitochondrial permeability, lowered ATP, increased ROS, eventually causing cell death via apoptosis or necrosis [6, 9]. As ETC involves transfer of electrons through numerous electron carriers, mitochondria are the major contributor of reactive oxygen species (ROS) [10]. Leakage of single electrons during the electron transfer process causes a reduction of molecular oxygen (O_2) leading to the

generation of a primary ROS entity called superoxide radicals ($O_2^{\cdot -}$). The primary sites within the ETC that contribute to generation of ROS are Complex I and Complex III [10, 11]. $O_2^{\cdot -}$ is highly reactive, but under physiological conditions it is typically rapidly reduced further to H_2O_2 by the enzyme Superoxide dismutase (SOD) [12].

1.2 Introduction to Traumatic Brain Injury

Traumatic brain injury [13] is defined as an insult to the brain caused by an external physical force, leading to an alteration in brain functioning [14]. The Centers for Disease Control and Prevention estimates that at least 1.7 million cases of TBI occur in the United States annually [15], and is steadily on the rise. Currently, approximately 3.2 and 5.3 million people live with long-term disabilities as a result of TBI. Furthermore, it has been estimated that TBIs contribute to approximately 30.5% of all injury-related deaths [15]. TBI is considered a leading cause of death and long-term disability worldwide and affects all age groups and genders; however, children and the elderly are often considered at a higher risk of experiencing a TBI [16]. Military personnel are also considered to be at a higher risk for sustaining TBI, with the Department of Defense reporting over 235,000 service members between 2000 and 2011 diagnosed with a TBI [17].

The initial insult can be the result of blunt trauma, penetrating injury (bullet wound), acceleration-deceleration, or blast waves injuries [18]. The leading cause of TBI-related death occurs from motor vehicle accidents, suicide or falls [19]. The result of these impacts varies highly from patient to patient, and is dependent on location of injury, and has been correlated with injury severity. The severity of a TBI is classified from mild to severe based on the Glascow Coma scale. While the intracranial location and severity of injury contribute to the extent of functional deficits, there are also likely contributions from physiological changes that occur during hours to months after injury. The progression of molecular changes following TBI leads to multiple physiological changes including: mitochondrial dysfunction, neuronal degeneration, inflammation, blood-brain barrier (BBB) dysfunction, and edema formation [20, 21]. These changes in the cellular microenvironment contribute to the secondary injury phase when neuronal loss either permanently disables victims, decreasing their quality of life, or results in death.

Despite the tremendous effort invested in TBI research, the ability to minimize chronic neurological deficits in TBI patients has remained extremely limited. This is likely a result of the vast complexity of the pathophysiology of a TBI. As such, it is necessary to continue to explore and understand the underlying mechanisms of a TBI, and how these mechanisms relate to functional outcomes.

1.3 Mitochondria & TBI

It has become increasingly clear that TBI, as well as other neurological disorders, are the cause or effect of mitochondrial dysfunction [6, 22–31]. Secondary injury following TBI is initiated by a massive depolarization of the plasma membrane by voltage- dependent Na^+ channels. Along with glutamate release, this depolarization causes a massive Ca^{2+} influx into the cell which can activate many damaging cellular enzymes within the cytosol, and as such must be sequestered by intracellular organelles, mainly the mitochondria [25, 32, 33].

After Ca^{2+} is sequestered into mitochondria via membrane potential- driven transporters, it is stored as a Ca^{2+} phosphate compound within the matrix, causing the matrix to have an almost gel-like consistency [25].

The Ca^{2+} buffering capacity of mitochondria is finite and eventually the Ca^{2+} influx becomes too great, resulting in mitochondrial dysfunction and subsequent initiation of cell death pathways [6, 24, 34, 35]. This causes the mitochondria and the cell to swell and eventually burst, which are characteristic signs of necrotic cell death [6, 25]. It is also important to note that inhibition of mitochondrial Ca^{2+} uptake by reducing membrane potential ($\Delta\Psi$) (chemical uncoupling) following TBI is neuroprotective, emphasizing the pivotal role of mitochondrial Ca^{2+} uptake in neuronal cell death following TBI [36–38].

The formation of the mPTP results in mitochondrial dysfunction and has been shown to occur after acute TBI and excitotoxic insult [6, 25, 39]. It has been repeatedly shown that cyclosporine A [40] (and CsA analogs) inhibits opening of the mPTP and reduce neuronal cell death following TBI homeostasis [39, 41–45]. Specifically, NIM811, a non-immunosuppressive CsA analog, at 10 mg/kg improves tissue sparing, mitochondrial functionality, and reduces 4-HNE levels following TBI [44]. Recently, it has also been shown that CsA improves respiration by targeting the more damaged synaptic mitochondria, relative to non-synaptic mitochondria [46]. In clinical studies, CsA has been demonstrated to improve outcome in dose-escalation studies with treatment initiated within 8 hrs post-injury but in a second phase II study, which included a broader 12 hr post-injury therapeutic window, no evidence of efficacy was demonstrated [47, 48]. These lines of evidence point to mitochondria as a valid, therapeutic target following TBI.

1.4 Methylene blue

Methylene Blue (MB), also known as methylthioninium chloride, is a cationic thiazine that contains a tri-heterocyclic thiazine ring structure (S(C6H4)2NH4). It was discovered and synthesized in the late 1880 by Heinrich Caro, and has been applied widely as a dye, redox indicator, and medication [49–51]. The pharmacological use of MB began in 1890s when Nobel laureate Paul Ehrlich discovered its usefulness in treating malaria [52]. He noted that MB was selectively taken up by the nervous tissue, and the term “magic bullet” was coined by this phenomenon. Since then, MB has been used in humans safely for over 120 years to treat variety of diseases, including methemoglobinemia, urinary tract infection, nitric oxide-induced hypotensive complications from septic shock, cardiopulmonary bypass, and in diagnostic procedures and Vasoplegic syndrome [53–55] [56–59]. It is also an antidote to cyanide and carbon monoxide poisoning [60], and shows neuroprotective property for stroke, Parkinson’s disease, Alzheimer’s disease, and optic neuropathy [61–64].

In recent studies, MB was found to be able to improve mitochondrial functions [65]. Specifically, MB can function as an alternative electron carrier that efficiently shuttles electrons between NADH (mitochondrial complex I) and cytochrome *c* (complex IV). This process reroutes electron transfer upon complex I/III inhibition, reduces electron leakage, increase mitochondrial oxidative phosphorylation, and attenuates reactive oxygen species (ROS) overproduction under pathological conditions. Through this shunt, MB causes an increase in cellular oxygen consumption and a corresponding decrease in anaerobic

glycolysis *in vitro* and *in vivo* [66–68]. In addition, chronic exposure to MB results in increased activity and expression of mitochondria complex IV [65, 69]. It is therefore has been proposed as a metabolic enhancer that attenuates neurodegeneration induced by metabolic challenge [64, 70].

1.5 Methylene Blue in CNS injury models

Recently there has been resurgence in the study of MB in diseases related to the central nervous system. This is in part due to MBs rapid ability to cross the blood brain barrier following intravenous injection, where the concentration in the brain rapidly (less than 1 hour) reaches 20 times that of plasma[71]. Further, as it is an FDA approved medication, its safety profile has already been determined, as well as the optimal clinical dose to use in humans. Preclinical research has suggested a potential neuroprotective effect following the administration of MB in various CNS injury models including Parkinson's Disease, Alzheimer's Disease, amyotrophic lateral sclerosis, ischemic stroke, and more recently in traumatic brain injury [64]. The common theme in the use of MB in these conditions is their link to deficits in mitochondrial function.

Mitochondria are sensitive to changes in the physiological state of a cell, and their dysfunction has been implicated in numerous neuropathological diseases, including TBI. Increasing evidence suggests that TBI leads to both direct mechanical damage and functional disturbances in mitochondria and plays a key role in contributing to apoptotic and necrotic cell death. As the brain is a highly aerobic, energy-demanding tissue, an imbalance in energy demand for repair of cellular damage and concomitant decrease in energy generation caused by mitochondrial dysfunction leads to increased cellular damage. Excitotoxicity, production of reactive oxygen species (ROS), Ca^{2+} overload, apoptosis-inducing factor, and caspases are some of the primary candidates that induce mitochondrial damage following TBI [72–74]. During injury, the surge in ROS facilitates a vicious cycle that accelerates mitochondrial damage, excitotoxicity, lipid peroxidation, and inflammation [75]. Mitochondrial membrane potential is a key indicator of damage to the mitochondria and may be linked to early-stage apoptosis. Thus, mitochondrial targeting strategies in stroke, ischemia and TBI have been increasingly studied, as their maintenance could potentially preserve brain function [76, 77].

In normal rats a single *low-dose* of MB has significant effects on CBF, metabolism, and fMRI responses [70]. MB also increases global glucose uptake, oxygen consumption, CBF [70], and fMRI responses [78] in normal rats, but has no significant effects on heart rate, respiration rate, or blood pressure. MB has been demonstrated to be effective at reducing lesion volume, vasogenic edema formation, function deficits and neurodegeneration in a cortical impact model of TBI in rats [79, 80]. In these studies MB was administered 1 and 3 hours[25] or 24 hours[24] after TBI. In the vehicle group, the lesion volume peaked at 2 days and decreased at 14 days post TBI. In the MB group, by contrast, the lesion volume did not change substantially with time and was significantly smaller than the vehicle group at all time points studied.

The objective of this paper was to determine the *in vitro* effects of MB on mouse cortex mitochondrial bioenergetics and reactive oxygen species production. We hypothesized that

MB treatment would improve mitochondrial bioenergetics, and decrease the production of reactive oxygen species. To test this hypothesis mitochondria were isolated from mouse cortex and further purified and separated into synaptic and nonsynaptic fractions. The fractions were then treated with a range of doses of MB and mitochondrial bioenergetics and production of H_2O_2 were measured.

2. Materials and Methods

2.1 Isolation of mitochondria from the mouse cortex

All animal experimental protocols were approved by the University of Kentucky Animal Use and Care Committee and complied with National Institutes of Health guide for the care and use of Laboratory animals. Mitochondrial isolation protocol was adapted from the previously described protocols with modifications [81–83]. All the steps were carried out at 4°C or on ice. Briefly 6–8 week old male C57BL/6 mice (n=10; 20–22 g) were euthanized with CO_2 and the cortex was dissected out and homogenized using Teflon-glass dounce homogenizer containing isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, Adjusted pH 7.2 with KOH). The homogenate was transferred to 2ml micro centrifuge tube and spun at 1300 × g for 3 minutes. The supernatant was further transferred in a fresh 2ml micro centrifuge tube and spun at 13,000 × g for 10 minutes. The supernatant was discarded and the crude mitochondrial pellet was resuspended in 500μL of isolation buffer. For all experiments biological n=4 to 6 and technical replicates n≥3.

2.2 Separation and purification of Synaptic and Non-synaptic mitochondria

The mitochondrial fraction was further purified and separated into synaptic and nonsynaptic fractions using double layered discontinuous Ficoll density gradient centrifugation [84]. Ficoll stock (20% Ficoll, 0.3M Sucrose, 10mM Tris and 0.2mM EGTA) was diluted using isolation buffer to make 7.5% and 10% Ficoll solution. The double layer Ficoll gradient was prepared using 2ml each of 7.5% Ficoll layered over 10% Ficoll solution. The mitochondrial suspension was layered on top of the gradient and was centrifuged at 100,000 ×g for 30 min using ultracentrifuge to get neurosynaptosomes and non-synaptic mitochondria. Based on density difference, the neurosynaptosomes were collected from the interface of the two Ficoll layers and non-synaptic mitochondria were collected from the pellet. The neurosynaptosomal fraction was transferred into a 2 ml centrifuge tube and 4X diluted with isolation buffer and centrifuged at 13,000 ×g for 10 min. The neurosynaptosomal pellet was resuspended in 400 μl of isolation buffer and the synaptic mitochondria were released using a pressurized nitrogen cell disruptor at 1200 psi for 10 min at 4 °C. The synaptic mitochondria were again purified using Ficoll purification steps as done before. This time synaptic mitochondria released from synaptosomes were collected from the pellet. Both non-synaptic and synaptic mitochondrial pellets from the two ultracentrifugation steps were resuspended in 600 μl of EGTA-free isolation buffer and were pelleted at 10,000 × g for 10 min at 4 °C. The supernatants were discarded and the synaptic and non-synaptic mitochondrial pellets were resuspended in EGTA-free isolation buffer to get ~10ug/ul approximate concentration of mitochondria. The absolute protein concentration was determined using BCA protein assay kit (Pierce, Cat # 23227) by recording absorbance at 560 nm on Biotek Synergy HT plate reader (Winooski, Vermont).

2.3 Mitochondrial bioenergetics measurements

The mitochondrial bioenergetic measurements in the presence of varying concentrations of Methylene Blue (0.0001 μg/ml to 10 μg/ml were carried out using a Seahorse XF^{e}24 Extracellular Flux Analyzer (Agilent Technologies, USA). It determines the bioenergetics of mitochondria by measuring the Oxygen Consumption Rates (OCR) during various states of respiration. The OCR were measured in the presence of different substrates, inhibitors and uncouplers of Electron Transport Chain (ETC) using previous methods with slight modifications [84–88]. The stocks used for the assays are 500 mM pyruvate, 250 mM malate, and 30 mM adenosine diphosphate (ADP), and 1 M succinate (pH for all were adjusted to 7.2) plus assay solutions of 1 mg/mL (1.26 mM) oligomycin A, 1 mM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 1 mM rotenone were prepared in ethanol. As per the instructions from XF^{e}24 Extracellular Flux kit, the sensor cartridge was hydrated and kept at 37 °C overnight before the experiment. The injection ports A to D of the sensor cartridge were loaded with 75ul of different combinations of the above substrates/inhibitors/uncouplers as follows. Before loading, the stocks were diluted appropriately in the respiration buffer (RB) (215 mM mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES, 2 - M $MgCl_2$, and 2.5 mM KH_2PO_4, Adjusted pH 7.2) to get the final concentrations in the respiration chamber of 5 mM pyruvate, 2.5 mM malate and 1 mM ADP (via Port A), 1 μM oligomycin A (via Port B). 4 μM FCCP (via Port C) and 0.1 μM rotenone and 10 mM of succinate (via Port D) starting with the initial volume of 525ul RB in the chamber and diluting it to 9X, 10X, 11X and 12X with every injection through ports A to D respectively. Once loaded, the sensor cartridge was placed into the Seahorse XF^{e}24 Flux Analyzer for automated calibration.

Seahorse Standard XF24 assay plates were used separately for loading mitochondria. Initially the purified synaptic and non-synaptic mitochondria were diluted to 2.5 μg/50ul in RB and 50 μl was loaded in each well resulting in 2.5 μg mitochondria/well. The assay plates were centrifuged at 3000 rpm for 4 minutes at 4°C to adhere the mitochondria at the bottom of the wells. After centrifugation, 475 μL RB (pre-incubated to 37°C) was added without disturbing the mitochondrial layer to obtain a final volume of 525 μL per well. After the instrument calibration with the sensor cartridge was complete, the utility plate was replaced by the plate loaded with mitochondria for bioenergetics analysis.

The assays were carried out under previously optimized protocol. [88]. Briefly, it involved cyclic steps of mixing, sequential injections of substrates/inhibitors via Ports A thru D, mixing, equilibration, and measurement of the OCR and pH through fluorimetric optical probes. The data output gives **State III** respiration in the presence of pyruvate, malate and ADP (Port A) followed by **State IV** rate in presence of oligomycin A (Port B). Sequentially, it gives uncoupled Respiration **State V_{PM}** (State V1) and **State V_{Succ}** (State V2) in the presence of FCCP (Port C) and rotenone plus succinate (Port D) respectively.

2.4 Mitochondrial H_2O_2 production

The H_2O_2 generated by actively respiring mitochondria was measured using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (molecular probes, Cat#A22188) using a 96-well plate format. In a 130μl assay system, a series of MB concentrations from 0.0001 μg/ml to 1

μg/ml were prepared along with the combinations of mitochondrial substrates (Final concentrations 5 mM pyruvate, 2.5 mM malate and 150 μM ADP, 1 μM oligomycin A. 4 μM FCCP and 0.1 μM rotenone and 10 mM of succinate) prepared in Respiration buffer (RB mentioned in the OCR measurement protocol). The plate was placed in the 37°C pre-incubated plate reader (Biotek Synergy HT plate reader, Winooski, Vermont) and 25μl (5X) of Amplex solution (125μM Amplex + 0.5U/ml HRP) prepared in RB was added to each well using automated injectors. The fluorescence was measured at one-minute intervals at the wavelengths λ^{EX} 571 and λ^{EM} 585. The relative H_2O_2 generated was calculated from the difference in the fluorescence measurements.

2.5 Statistical Analysis

For all statistical comparisons, significance was set at $p \leq 0.05$. OCR and ROS data were analyzed using a one-factor analysis of variance (ANOVA) followed by post-hoc analysis (Dunnett test) when warranted.

3 Results and Discussion

3.1 Effect of MB on brain mitochondrial bioenergetics

The *in vitro* effects of MB on mouse cortex mitochondrial bioenergetics were assessed by exposing them to different concentrations of MB ranging from 0.0001μg/ml to 10μg/ml in respiration buffer just before initiating OCR measurements on seahorse XF^e24 (Figures 1 and 2). The dose range of MB was chosen based on previous studies *in vitro* and *in vivo* [66, 67, 89, 90]. Dosages above 7mg/kg are considered cytotoxic [63, 91]. The synaptic and non-synaptic mitochondria showed varied sensitivity to MB. In both, the state III levels showed a nonsignificant surge at 0.0001μg/ml. With increasing concentrations non-synaptic mitochondria were more sensitive to MB compared to synaptic with a significant drop in state III at 0.01μg/ml of MB, whereas the synaptic mitochondria failed to show a significant decrease in state III respiration even at 1μg/ml. There was a sharp drop in the OCR at 10μg/ml of MB in both synaptic and non-synaptic mitochondria. In both, there were no significant increases in state IV respiration at the lower concentrations of MB till 0.1μg/ml, but both the mitochondrial populations demonstrated a significant increase in State IV at 1μg/ml which declined at the highest concentration (10μg/ml), indicating a complete shutdown of mitochondrial respiration. Respiration Control Ratio [92] which is an important indicator of mitochondrial uncoupling remained unchanged in non-synaptic mitochondria until 0.1μg/ml whereas the synaptic mitochondria showed a significant decrease in the uncoupling activity even at 0.001μg/ml of MB (Figure 1 and 2). State V_{PM}, which indicates Complex I mediated uncoupled respiration showed a response similar to that of State III in both mitochondrial populations but was significant only at the higher dose of 10μg/ml of MB. There was no major change in State V_{succ}, a complex II mediated uncoupled respiration in either population of mitochondria, indicating that MB is interrupting mitochondrial respiration mainly through Complex I and uncoupling mechanisms. The overall results suggest that MB has a varied response to both synaptic and non-synaptic mitochondrial populations. In case of non-synaptic, MB is targeting respiratory complex I directly causing a suppression of respiration, but has less effect on the RCR. In comparison, synaptic mitochondrial complex I is less sensitive to MB but there was significant uncoupling as

indicated by the drop in RCR. This seems to indicate basic differences exist between the two mitochondrial populations and their response to external redox stimuli like MB. It should be noted that the lack of effect on complex-II driven OCR at concentrations that completely shut down complex-I driven respiration in either population of mitochondria is in contrast to the proposed site of action for MB which is hypothesized to be upstream at complex III. These data seem to indicate that at higher concentrations, MB is altering mitochondrial respiration at some target that has yet to be elucidated.

3.2 ROS production in respiring mitochondria

Mitochondrial ROS generation was measured in terms of H_2O_2 production using Amplex assay. As discussed earlier, mitochondria are a major source of ROS and H_2O_2 generation was measured in actively respiring mitochondria using specific substrates and inhibitors. This was done to induce maximum ROS production (in presence of oligomycin) and minimal ROS (presence of FCCP) under control conditions. Looking at the control data of synaptic and non-synaptic mitochondria (Figure), compared to starved mitochondria (Baseline), under respiring conditions in the presence of PM (pyruvate/malate) and ADP (state II and III respiration respectively), there was a sharp increase in the H_2O_2 generation due to activation of ETC (Figure 3 and 4). State IV respiration, in the presence of Oligomycin, an inhibitor of ATPase, generates a high electric potential difference across the inner mitochondrial membrane resulting in more slippage of electrons and results in increased ROS production. Under state V respiration in the presence of FCCP due to uncoupling of mitochondria the H_2O_2 production returns to the baseline levels. In the presence of rotenone (state V) there is an increase in the ROS generation even in the relaxed state as rotenone is known to inhibit complex I, and release free electrons from complex I. This H_2O_2 production remained unaffected even in the presence of succinate because in the uncoupled state there is a minor contribution of complex II mediated respiration to the ROS generation.

3.3 Effect of MB on mitochondrial ROS production

MB showed similar effects on synaptic and non-synaptic mitochondrial ROS production measured in the form of H_2O_2 production (Figure 3 and 4). Although both mitochondrial populations showed similar responses, overall the synaptic mitochondria showed slightly higher H_2O_2 production compared to non-synaptic in the presence of MB (given the effects of 10μg/ml MB on respiration, we did not assess its effect on ROS production). In both populations, there was no significant change observed in ROS production until 0.01μg/ml of MB under most of the conditions. However, there was a significant increase in H_2O_2 at 0.1 and 1μg/ml of MB. In this range MB affects mitochondrial respiration irrespective of the coupled or uncoupled state, but to varied extents. At the high concentration of 1μg/ml, MB showed a maximum rise in H_2O_2 generation which is consistent with the state III and state IV alterations in OCR measurements. Considering the fold change in the production of ROS for each individual state, State III (ADP) and State V (FCCP), where there is maximum flow of electrons thru the ETS, showed the maximum fold change in H_2O_2 production compared to their respective controls with no MB. This suggests that at high concentrations, MB may be directly releasing/donating free electrons irrespective of the mitochondrial membrane potential.

4. Discussion and Conclusions

Following a TBI, the body seems to enter a state of metabolic crisis, mitochondrial dysfunction become apparent, oxidative stress increases and a dysregulation of excitatory amino acids occurs rapidly. Mitochondrial dysfunction is a well-documented hallmark of traumatic CNS injury and strategies that target this dysfunction have been experimentally successful. This indicates that targeting mitochondrial dysfunction following a brain injury is not only a well hypothesized target but a valid target both in the laboratory and the clinic. One such compound is Methylene Blue, which has been studied for over 100 years and can function as an alternative electron carrier to shuttle electrons between mitochondrial complex I and cytochrome *c* and has shown promise in experimental models of TBI. Our current results demonstrate that Methylene Blue alters mitochondrial bioenergetics differently depending on the compartmental source of the mitochondria (synaptic vs non-synaptic) by inhibiting respiration at complex I or upstream of complex I in non-synaptic mitochondria. However, Methylene Blue was demonstrated to increase mitochondrial ROS production regardless of the mitochondrial source in a dose dependent manner. Granted, our studies employed well-coupled mitochondria isolated from experimental naïve animals and that future work should examine the effect of this compound on mitochondria isolated from TBI animals or using *ex vivo* bioenergetics stressors. At face value, these data would seem to indicate that a fine line exists in the utilization of Methylene Blue to promote mitochondrial function and that care should be taken to generate rigorous dose-response curves for this compound. Alternatively, these data could point to other neuroprotective mechanisms being utilized by Methylene Blue, including activation of endogenous antioxidant systems such as Nrf2 by increasing mitochondrial ROS production, or perhaps by increasing mitochondrial biogenesis by reducing mitochondrial respiration [93]. Regardless, Methylene Blue offers a valid approach to reduce the effects of bioenergetics failure following TBI and may still prove to be a “magic bullet”.

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Highlights

- Mitochondrial play a pivotal role in determining neuronal cell survival and outcome following traumatic brain injury (TBI).
- Strategies that target mitochondrial dysfunction following TBI have proven to be neuroprotective.
- Methylene Blue (MB) is one relatively new TBI therapeutic approach that may be targeting mitochondrial dysfunction.
- MB alters mitochondrial function differentially in synaptic vs non-synaptic brain mitochondria including shutting down mitochondrial respiration and increasing ROS production.

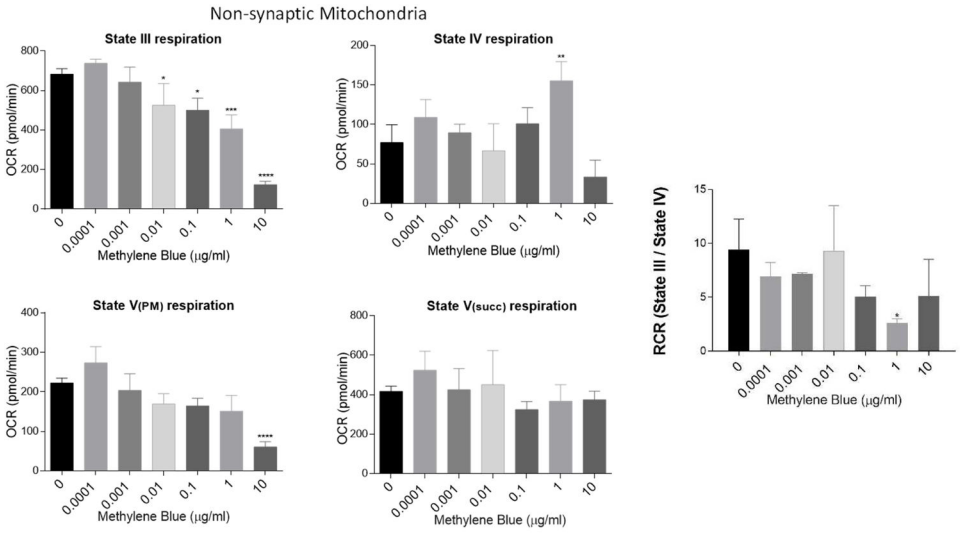

Figure 1. Effect of Methylene Blue on cortical non-synaptic mitochondrial respiration

Mitochondrial bioenergetics were assessed in the presence of varying concentrations of Methylene Blue (0.0001 μg/ml to 10 μg/ml) during classic states of respiration (III – V) and RCRs calculated. (PM=pyruvate/malate; SUCC=succinate in presence of rotenone) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control. Bars are mean ± SD.

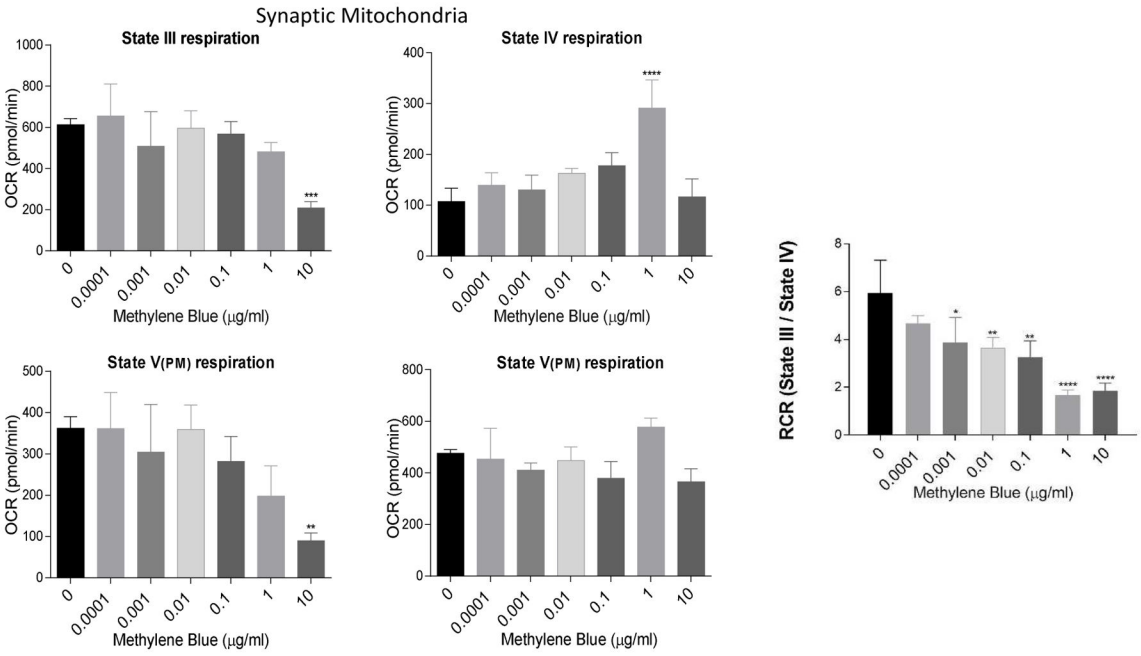

Figure 2. Effect of Methylene Blue on cortical synaptic mitochondrial respiration

Mitochondrial bioenergetics were assessed in the presence of varying concentrations of Methylene Blue (0.0001 μg/ml to 10 μg/ml) during classic states of respiration (III – V) and RCRs calculated. (PM=pyruvate/malate; SUCC=succinate in presence of rotenone) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control OCR. Bars are mean ± SD.

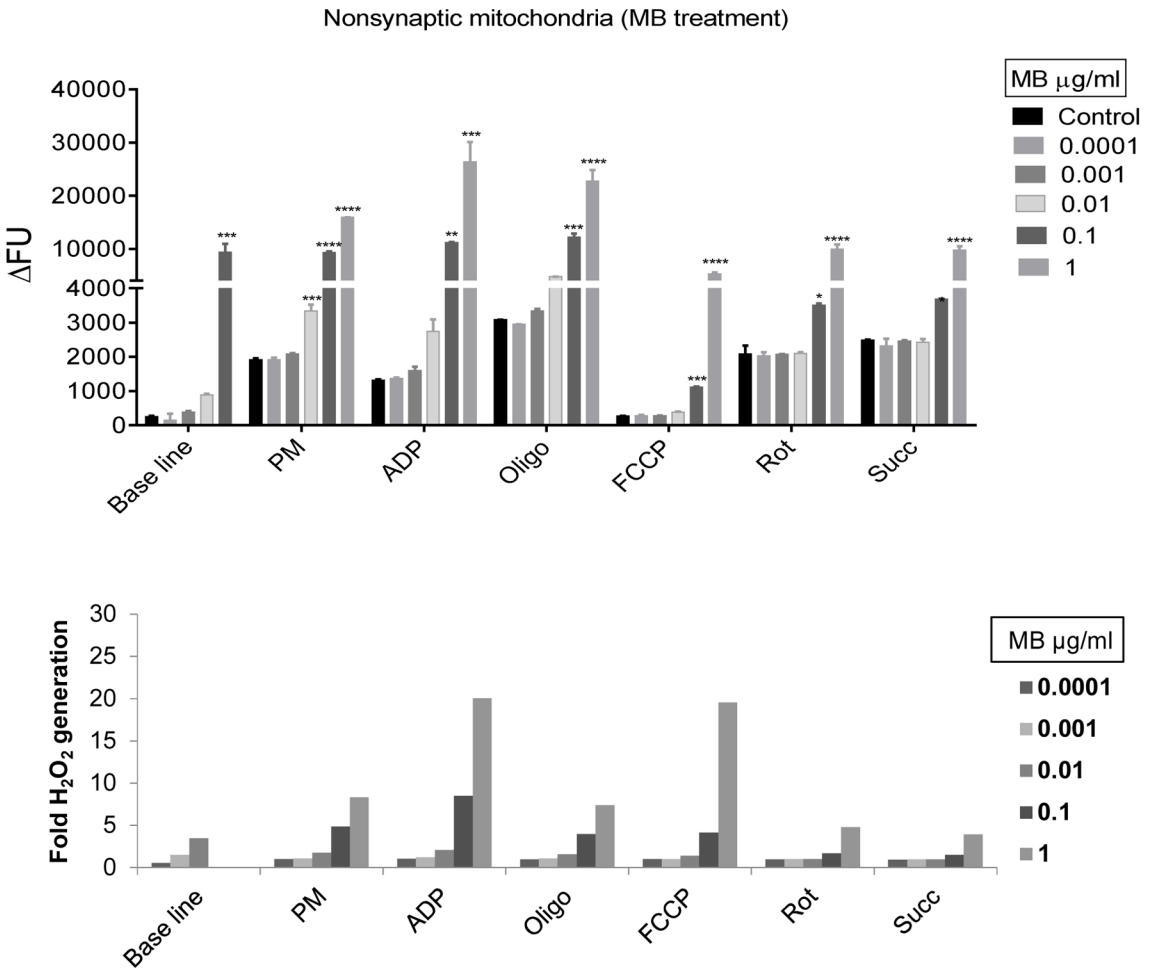

Figure 3. Effect of Methylene Blue on cortical non-synaptic mitochondrial ROS production

Mitochondrial ROS production was measured using the H_2O_2 indicator Amplex Red under various states of respiration (PM-pyruvate/malate, ADP, Oligo-Oligomycin, FCCP, Rot-Rotenone, Succ-Succinate) and with and without MB (0.0001 µg/ml to 1 µg/ml). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control fluorescence (FU). Fold change is plotted in the lower panel. Bars are mean ± SD.

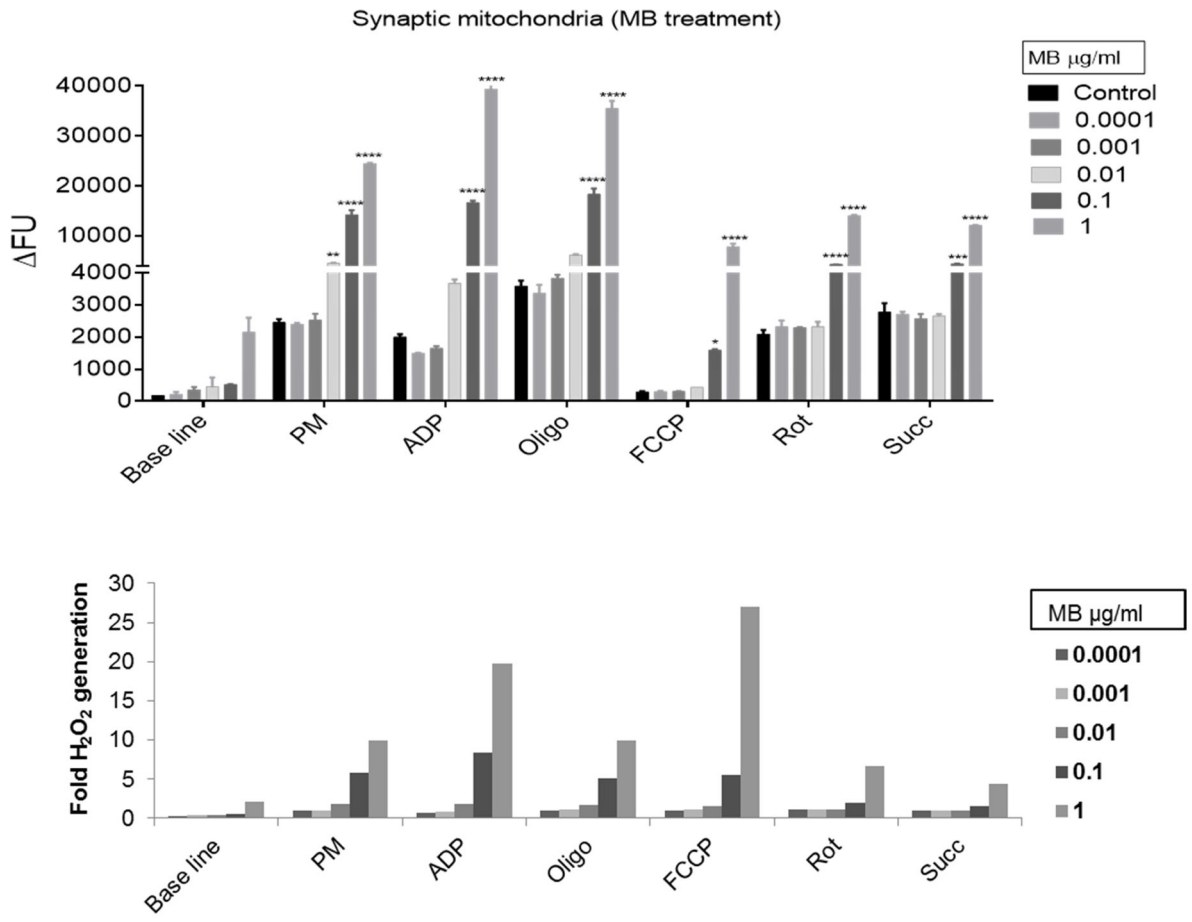

Figure 4. Effect of Methylene Blue on cortical synaptic mitochondrial ROS production

Mitochondrial ROS production was measured using the H_2O_2 indicator Amplex Red under various states of respiration (PM-pyruvate/malate, ADP, Oligo-Oligomycin, FCCP, Rot-Rotenone, Succ-Succinate) and with and without MB (0.0001 µg/ml to 1 µg/ml). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to control fluorescence (FU). Fold change is plotted in the lower panel. Bars are mean ± SD.