Original Article

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Methylene Blue Treatment Enhances Mitochondrial Function and Locomotor Activity in a C57BL/6 Mouse Model of Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a neurodegenerative disease. Although multiple factors are involved in the pathogenesis of MS, there are several lines of evidence that oxidative stress and mitochondrial dysfunction are involved in neuronal demyelination and deterioration of MS symptoms. Hence, compounds that could modulate mitochondrial function and decrease mitochondria-mediated ROS formation might be able to decrease MS symptoms. Methylene blue (MB) is a compound widely used in the treatment of central nervous system disease (e.g., Alzheimer's disease). It has been found that MB could robustly suppress mitochondria-mediated ROS formation at low concentrations. The current study was designed to evaluate the effect of MB on neuronal demyelination, mitochondrial function, and ROS formation in an animal model of MS. C57BL/6 male mice received cuprizone (0.1% w: w in chow diet for 42 consecutive days). MB (0.5 and 1 mg/kg, oral) was simultaneously administered. Significant demyelination was detected in CPZ-treated animals, which confirm the induction of MS in the mice model. Decreased animals' locomotor activity, including significant suppression of open field movement, stride length, and decreased time on the rotarod, was evident in CPZ-treated mice. Mitochondrial indices, including significantly elevated lipid peroxidation, mitochondrial depolarization, significant mitochondrial permeabilization, and decreased ATP levels, were also detected in the CPZ group. It was found that MB administration significantly improved animals' locomotor activity and mitochondrial indices in the current animal model of MS. The effects of MB on mitochondria and mitochondria-mediated ROS formation might play a fundamental role in the protective effects of this compound.

Keywords: ATP, Mitochondrial impairment, Neurodegeneration, Neurotoxicity, Oxidative stress.

1. Introduction

Methylene blue (MB; 3, 7-Bis (dimethylamino) phenothiazine-5-ium, Figure 1) is a phenothiazine compound tested for the treatment of a variety of ailments in the past 100 years (1). The positive effects of MB on different experimental models of human diseases have been reported (2-9). It has been found that MB significantly alleviated inflammatory disorders, ischemia-reperfusion-induced organ injury, as well as xenobiotics-induced damage toward biological systems (9-14). On the other hand, the effects of MB on

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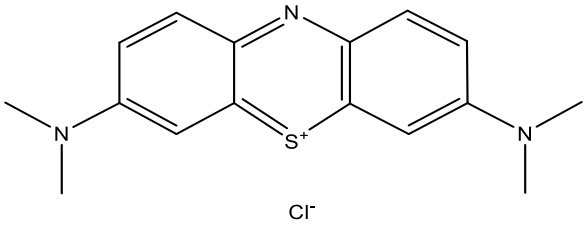

Figure 1. Methylene blue (3, 7-Bis (dimethyl amino) phenothiazine-5-ium) chloride.

central nervous system diseases are an interesting feature of this compound (15-19). The effects of MB on the central nervous system (CNS) diseases such as Alzheimer's are the most impressive therapeutic properties of this compound (20-22). Interestingly, some formulations of MB even underwent clinical trials (e.g., Rember™) in humans (1, 23, 24). MB is an FDA-approved drug for the treatment of methemoglobinemia with different etiologies (25).

Multiple sclerosis (MS) is a debilitating disease characterized by neuron demyelination and several locomotor dysfunctions in patients (26, 27). Multiple neurological deficits have been associated with MS (27). The role of the immune response in the pathogenesis of MS has been well-documented (26, 28). However, several other factors have also been identified in the pathobiology of MS disease (28, 29). Reactive oxygen species (ROS) and oxidative stress is a primary mechanism involved in the progression of MS (30-32).

Although there is no precise mechanism for the source of ROS in the brain of MS patients, the activity of inflammatory cells, the accumulation of redox-active metals such as iron in the brain tissue, as well as mitochondrial impairment has been identified as a source of ROS in the brain of MS patients (33-35). Cellular mitochondria are the primary source of ROS formation (36, 37). Therefore, in the current study, the role of MB administration on the brain mitochondrial function has been investigated.

Various investigations mentioned the effects of MB on mitochondrial function and regulation of mitochondria-mediated reactive oxygen (ROS) formation (38). It has been found that MB significantly decreased mitochondria-mediated ROS formation (38). The effects of MB on cellular mitochondria is concentration-dependent (38). Low concentrations of MB decrease mitochondria ROS formation (38). Higher concentrations of this compound (>5 μM) deteriorate mitochondrial function and could cause cell death (38).

The current investigation aimed to evaluate the effects of MB supplementation on the locomotor activity and mitochondrial function in an animal model of MS.

2. Material and methods

2.1. Reagents

Methylene blue (3, 7-Bis (dimethylamino) phenothiazine-5-ium), trichloroacetic acid (TCA), thiobarbituric acid (TBA), sodium citrate, ethylene diamine tetra-acetic acid (EDTA), meta-phosphoric acid, methanol HPLC grade, 2 amino 2-hydroxymethyl-propane-1,3-diol-Hydrochloride (Tris-HCl), and all salts used for making buffer solutions were obtained from Merck (Darmstadt, Germany). Cuprizone (bis-cyclohexanone oxaldihydrazone) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

2.2. Animals

C57BL6 male mice weighing 20-25 g were purchased from the Center of Comparative and Experimental Medicine (Shiraz, Iran). The use of animals complied with the guidelines established by the Animal Care Committee of this institute (94-01-36-11043). Animals were housed in a standard environment (temperature of 23±1 ºC and 45 ± 5% humidity). Animals had access to a rodents chow diet (RoyanFeed®, Isfahan, Iran), and tap water ad libitum.

2.3. Animal model of multiple sclerosis

Cuprizone (Oxalic bis (cyclohexylidene-hydrazide; CPZ)-fed C57BL6 mice were used as an animal model of MS (n=8) (39). Mice were treated with a rodent chow diet containing 0.2% (w: w) CPZ ad libitum (39, 40). The control group

consisted C57BL/6 mice (n=8) that were not fed CPZ (n=8). Two other groups were simultaneously treated with CPZ and MB (0.5 mg/kg and 1 mg/kg, oral) for 42 consecutive days (n=8/group). At day 43, animals underwent a series of behavioral investigations. Afterward, animals were sacrificed (thiopental 80 mg/kg, i.p), and their brain was excised and used for further experiments.

2.4. Behavioral studies

2.4.1. Rotarod test

Following a reported procedure, each rat underwent five sessions of rotarod performance on a 'Techno' (Lucknow, India) made a rotarod apparatus. The speed of the rotarod was fixed with the increments of 5, 10, and 15 rpm. Each session had three trials for each rat with 10 min interval, and the time that rats stayed on the rotating rod was recorded automatically (41-43).

2.4.2. Gait stride

Using a runway procedure, the rats, with their hind paws wetted with the ink, were allowed to walk down on a paper strip (60 cm long, 10 cm wide) from the brightly lit corridor toward a dark side. The distance between the prints of left and right paws was measured and recorded (41-44).

2.4.3. Open field test

Open-field behavior is used as an index of animals' locomotor activity in the animal models of hyperammonemia and hepatic encephalopathy (45, 46). The open-field test was conducted for each group five hours before animal anesthesia and blood and liver sample collection. The apparatus was made of the white wood box (100 cm L × 100 cm W × 30 cm H, box floor was divided into 25 squares of 20×20 cm). The open-field arena was equipped with a webcam (2.0-Megapixel, Gigaware, UK), and all activities were monitored and recorded from a separate room. Animals' behavior was recorded for fifteen minutes, and the total number of crossed squares were counted (Total locomotion) (47, 48).

2.5. Histopathological evaluation of the brain tissue

Brain tissue sections were preserved in a formalin buffer solution (0.4% sodium phosphate monobasic, 0.64% sodium phosphate dibasic, and 10% formaldehyde in double-distilled water). Luxol fast blue (LFB) staining was applied to detect neuronal demyelination in the corpus callosum region of the brain of different experimental groups (49, 50).

2.6. Isolation of the liver mitochondria

Mice liver mitochondria were isolated as previously described (51). Briefly, animals were anesthetized (ketamine/xylazine, 50/10 mg/kg, i.p), and their liver was excised and washed with ice-cold saline (sodium chloride 0.9%) (51-53). The organ was homogenized with an Ultra-Turrax® Tube (IKA) homogenizer (8000 rpm, 10 sec), in a buffer containing 220 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 2 mM HEPES, 0.1% mostly fatty acid-free bovine serum albumin (pH=7.4) at a 10:1 buffer to liver (v/w) ratio (51, 54). Afterward, the liver homogenate was centrifuged at 1,000 g for 20 minutes at 4oC to remove intact cells and nuclei. The supernatants were further centrifuged (15,000 g, 4 °C, 20 minutes) to precipitate the heavy membrane fractions (mitochondria) (55, 56). This step was repeated three times using a fresh buffer medium. As mentioned, all manipulations for liver mitochondria isolation were performed at 4 °C or on ice to minimize mitochondrial injury (51).

2.7. Mitochondrial ATP levels

Based on a previously reported protocol, mitochondrial ATP level was assessed by HPLC (57). Briefly, isolated mitochondria (1 mg protein/mL) were treated with 100 µL ice-cooled phosphoric acid (50 % w: v, 4 ºC) and centrifuged (10 min, 15,000 g, 4 ºC). Afterward, the supernatant (100 µL) was treated with its equivalent volume of ice-cooled 1 M KOH solution. Samples (25 µL) were injected into an HPLC system consisted of an LC-18 column (µ-Bondapak, 25 cm) (58). The mobile phase was composed of 100 mM KH2PO4 (pH = 7 adjusted with KOH), tetrabutylammonium hydroxide (1 mM), and acetonitrile (2.5 % v: v). The flow rate was 1 mL/min, and the UV detector was set at λ=254 nm (57, 58).

2.8. Lipid peroxidation in isolated mitochondria

Thiobarbituric acid-reactive substances (TBARS) were measured in isolated kidney mitochondria (59, 60). Previous studies mentioned that sucrose interferes with the lipid peroxidation test in isolated mitochondria preparations (51). Therefore, mitochondria preparations were washed once (to remove sucrose) in ice-cooled MOPS-KCl buffer (50 mM MOPS, 100 mM KCl, 4 ºC, pH = 7.4). For this purpose, isolated kidney mitochondria were suspended in 5 mL of MOPS-KCl buffer and centrifuged (17,000 g, 15 min). The pellet was re-suspended in MOPS-KCl buffer and used for TBARs assay. The mitochondrial suspension (1 mg protein/mL) was mixed with 1 mL of a solution containing trichloroacetic acid (15 % w: v), HCl (0.24 N), thiobarbituric acid (0.375 % w: v), and Trolox (500 µM) (61-63). Samples were heated for 15 min at 100 ºC (51). Then 1 mL of n-butanol was added and vortexed (5 min). Samples were centrifuged (17,000 g, 10 min), and the absorbance of the upper phase (n-butanol phase) was measured (EPOCH plate reader, BioTek® Instruments, Highland Park, USA, λ=532 nm) (51).

2.9. Mitochondrial depolarization

Mitochondrial uptake of rhodamine 123 was applied for the evaluation of mitochondrial depolarization (64-66). Rhodamine 123 accumulates in the mitochondrial matrix by facilitated diffusion. When the mitochondrion is depolarized, there is no facilitated diffusion. Therefore, the amount of rhodamine 123 in the supernatant will be increased (67-69). In the current study, the mitochondrial fractions (0.5 mg protein/mL; in the depolarization assay buffer) were incubated with rhodamine 123 (10 µM, 30 min, 37 °C, in the dark) (70-73). Afterward, samples were centrifuged (17,000 g, 2 min, 4 °C), and the fluorescence intensity of the supernatant was monitored (FLUOstar Omega®; BMG Labtech, Germany; $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm) (64, 74).

2.10. Mitochondrial permeabilization and swelling

Mitochondrial swelling was estimated by analyzing the changes in optical density at λ=540 nm (51, 75). Briefly, isolated mitochondria (0.5 mg protein/ml) were suspended in swelling buffer (125 mM Sucrose, 65 mM KCl, 10 mM HEPES, pH=7.2) (76). The absorbance was monitored (λ=540 nm, 30°C, during 30 min) (77, 78), using an EPOCH plate reader (Bio-Tek® Instruments, Highland Park, USA). A decrease in absorbance is connected with an increase in mitochondrial swelling. The results are reported as maximal mitochondrial swelling amplitude (ΔOD 540 nm) (51, 75).

2.11. Mitochondrial dehydrogenases activity

The 3-(4, 5-dimethylthiazol-2-yl)-2, the 5-diphenyltetrazolium bromide (MTT) assay was applied as a colorimetric method for the estimation of mitochondrial dehydrogenases activity in isolated mice liver mitochondria (79-81). Mitochondrial suspension in a buffer containing 0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4, was incubated with 0.4% of MTT at 37 °C for 30 minutes. The product of purple formazan crystals was dissolved in 1 ml dimethyl sulfoxide (DMSO) (61, 82-84). Then, 100 µL of dissolved formazan was added to a 96-well plate, and the optical density at λ=570 nm was measured with an EPOCH plate reader (BioTek® Instruments, Highland Park, USA). Samples protein concentrations were determined by the Bradford method (61, 85).

2.12. Statistical methods

Data are given as Mean±SD. The comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as a post hoc test. Values of $P<0.05$ were considered statistically significant.

3. Results

A significant sign of demyelination was evident in CPZ-treated animals, as revealed by LFB staining of the brain tissue (Figure 2). On the other hand, it was found that MB administration (0.5 and 1 mg/kg) dose-dependently decreased the number of demyelinated neurons in the corpus callosum (CC) region of CPZ-treated mice (Figure 2).

Animals locomotor activities were impaired in the mice model of MS (Figure 3). A significant decrease in the open field activity, hind paw stride length, and time on the rotarod were detected in CPZ-treated mice (Figure 3). MB treat-

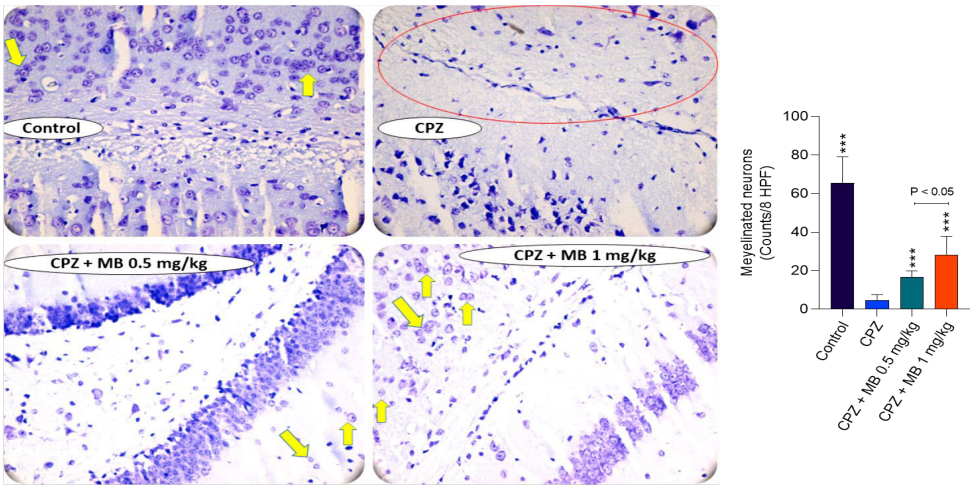

Figure 2. Luxol fast blue (LFB) stain revealed myelinated neurons with dark blue (Yellow arrow) in the corpus callosum (CC) of the control group. Demyelination in the CC of C57BL/6 mice treated with cuprizone (Red circle). Significant changes in neurons demyelination was detected in MB-treated animals. CPZ: Cuprizone; MB: Methylene blue.

ment (0.5 and 1 mg/kg) significantly improved the animals' locomotor function in the CPZ group (Figure 3). The effects of MB on animals' locomotor activity was not dose-dependent (Figure 3).

Impaired mitochondrial function, including decreased mitochondrial dehydrogenases activity, mitochondrial permeabilization, depleted mitochondrial ATP, and mitochondrial depolarization, were evident in CPZ-treated mice (Figure 4). It was found that MB treatment significantly

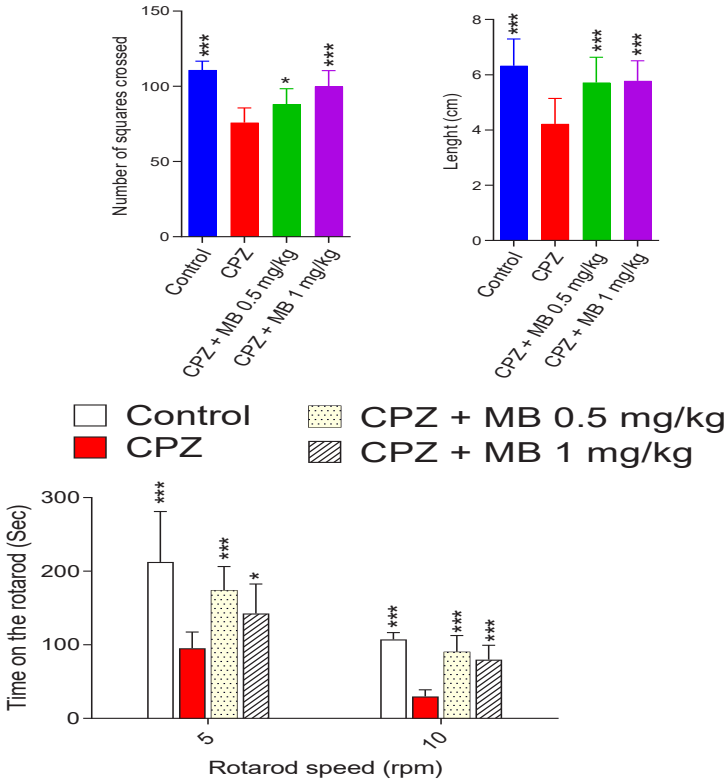

Figure 3. Effect of methylene blue (MB) supplementation on the locomotor activity in the cuprizone (CPZ) model of multiple sclerosis. Data are represented as mean±SD (n=8). Asterisks indicate significantly different as compared with the CPZ-treated group (* P<0.05 and ** P<0.01).

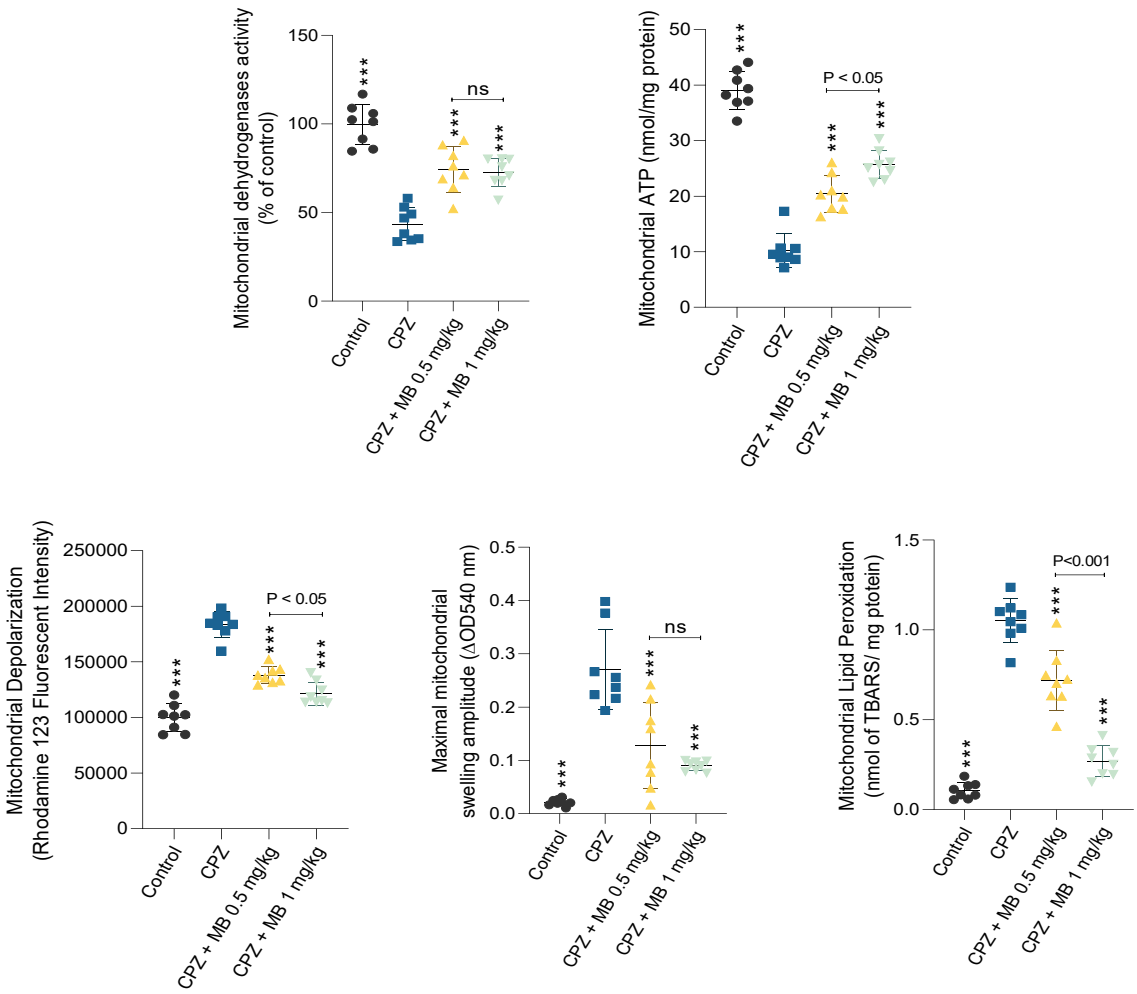

Figure 4. Mitochondrial indices in the whole brain mitochondria of multiple sclerosis (MS) mouse model. CPZ: Cuprizone; MB: Methylene blue. Data are represented as mean±SD (n=8). ***Indicate significantly different as compared with the CPZ-treated group (P<0.001).

prevented brain mitochondrial impairment in the current animal model of MS (Figure 4). The effects of MB on mitochondrial indices were not dose-dependent in the current study (Figure 4).

4. Discussion

MS is a neurodegenerative disease that severely affects the patients' quality of life. Hence, finding therapeutic options against this disease is of great clinical value. Several pharmacological targets, including the immune system and cellular mitochondria, have been identified for the treatment of MS. In the current investigation, the effects of MB, a mitochondrion regulating compound, have been evaluated in an animal model of MS. It was found that MB treatment (0.5 and 1 mg/kg, oral, for 42 consecutive days) significantly impaired mitochondrial indices and enhanced animal locomotor activity in the MS mouse model.

The role of mitochondrial impairment in the pathogenesis of other neurodegenerative diseases such as Alzheimer's disease and Parkinsonism has been widely investigated (86, 87). There is no precise mechanism for mitochondria-mediated ROS formation in MS (36, 88, 89). However, inflammatory response and mitochondrial impairment might play a pivotal role in brain oxidative stress in MS patients. There is also a large body of evidence that mitochondrial impairment could play a pivotal role in the pathophysiology of the MS disease (90-92). It has been found that mitochondrial dysfunction plays a significant role in

the release of cell death mediators and neuronal demyelination (88, 93-95). Mitochondria-facilitated ROS formation could damage several targets, including proteins, lipids, and mitochondrial DNA (mtDNA) (89, 93). A high level of mitochondrial ROS could lead to mutated mtDNA (89, 93). The enzymes for the repair of mitochondrial DNA are not as efficient as those for nuclear DNA. Hence, mtDNA is more prone to ROS damage. When the mutant mtDNA increase to a pivotal threshold, the symptoms of the mitochondria-related disease could occur (36, 88, 89). Damaged mtDNA could lead to deterioration in mitochondrial electron transport chain proteins synthesis, more ROS formation, and decreased ATP production.

Several studies also reported the impairment in mitochondrial electron transport chain (ETC) components in the MS disease (92, 93, 96-100). Decreased activity of complex I, III, and IV from the neurons in the different brain regions of MS models (92, 93, 96-100). Decreased activity of ETC could be directly related to mtDNA damage induced by oxidative stress. The final result of this process is decreased ATP production in mitochondria. ATP plays a fundamental role in many physiological processes, such as neurotransmitter release and ion channel activity (22, 88). Hence, the mitochondrial energy crisis in neurons of MS patients could lead to severe complications. MB could affect the function of mitochondrial ETC components a which finally leads to a higher ATP level (38).

There are several lines of evidence that mention the interaction of MB with cellular mitochondria in different experimental models (2, 38, 101-104). It has been found that MB (at low concentrations) significantly mitigated mitochondria-mediated ROS formation (38). Cellular mitochondria are the major sources of ROS (105, 106). A large amount of ROS is produced during the oxidative phosphorylation process, which is counteracted by mitochondrial antioxidant systems (107, 108). However, when the mitochondrial function is impaired or mitochondrial antioxidant capacity is overwhelmed (e.g., in MS disease), mitochondria-facilitated ROS formation could damage this organelle and finally lead to cellular injury.

Interestingly, it has been mentioned that the antioxidant activity of MB is mediated through the interaction of this compound with the ETC (38). The cycling between the oxidized (MB) and reduced (leucomethylene blue; MBH2) forms of MB plays a vital role in its effects on mitigating mitochondria-mediated ROS formation (38). The complex I of the ETC use NADH to reduce oxidized MB to reduced MBH2 (38). On the other hand, cytochrome c and complex IV of ETC accept an electron from MBH2 and re-oxidized it to MB (38). Therefore, some ETC complexes (II and III), which are involved in ROS production (e.g., superoxide anion formation) are bypassed. Therefore, this could be one of the most important mechanisms for the positive effects of low concentrations of MB on mitochondria-mediated ROS formation. The effects of MB on mitochondrial biogenesis is another important mechanism that could enhance cellular energy level and decrease cell injury in tissues fronted energy crisis (e.g., neuronal ATP deficit in MS disease) (109-111). As mentioned, some ETC components might have defected in MS disease (92, 93, 96-100). Hence, MB might help neurons and glia to produce more energy (ATP) and prevent an energy crisis in these cells. Some other studies revealed that MB could indirectly enhance cellular antioxidant defense mechanisms (109). This effect of MB could be mediated through the activation of the antioxidant enzymes gene expression (109). All these data mention the positive effects of MB on cellular mitochondria and its important therapeutic potential in mitochondria-linked disorders.

MB is widely investigated in central nervous system (CNS) disorders as well as xenobiotics-induced CNS injury (112-116). The effects of MB on Alzheimer's disease is one of the most investigated aspects of this compound on CNS diseases (22, 117-119). A formulation of MB even went under clinical trial (Rember™) for Alzheimer's disease (1, 23). MB is also an FDA-approved drug for the treatment of methemoglobinemia (25). Hence, MB could be readily administered to patients.

Although MS is a multifaceted neurodegenerative disease that several mechanisms are involved in its pathogenesis, mitochondrial impairment seems to play a significant role in the

pathobiology of this disease. Hence, mitochondria targeting could be a promising therapy for MS patients. In the current study, we found that MB administration prevented neuronal demyelination, mitochondria-mediated ROS formation, and improved locomotor activity. More investigations are warranted for the determination of the precise effects of MB on MS disease progression and the use of this phenothiazine compound in the management of neurological deficits in patients.

Acknowledgments

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Conflict of Interest

None declared.

5. References

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