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Methylene Blue Improves Brain Mitochondrial ABAD Functions and Decreases Aβ in a Neuroinflammatory Alzheimer's Disease Mouse Model

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Abstract Methylene blue (MB) phase II clinical trials reported improvements in cognitive functions of Alzheimer's disease (AD) patients. Regarding MB mechanism of action, its antioxidant and mitochondrial protective effects have been previously described. In relation to AD, it has been recently reported that MB reduced amyloid beta (Aβ) levels in AD models. The mitochondrial enzyme amyloid-binding alcohol dehydrogenase (ABAD) has been shown to bind Aβ inducing mitochondrial dysfunction, providing a direct relation between Aβ toxicity and mitochondrial dysfunction occurring in AD. Since it has been reported that inhibiting ABAD protects mitochondrial functions and prevents Aβ-induced toxicity, the aim of the current study was to investigate if the protective effects of MB could be associated with an effect on ABAD levels and functions. The current study shows that MB is able to enhance cell viability, reduce both reactive oxygen species levels and importantly Aβ oligomers in a lipopolysaccharide (LPS) mouse model. Interestingly, ABAD levels were increased in the brains of the LPS mouse model and MB treatment was able to reduce its levels. Given that regulation of the estradiol level is a well-established function of ABAD, brain estradiol level was compared in LPS mouse model and in MB-treated mice. The results of the current study show that MB treatment is able to improve significantly the LPS-induced decrease of estradiol levels in mice brains, indicating its ability to modulate both levels and function of ABAD. These results give a new insight to possible mechanisms of MB in AD.

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Introduction

Methylene blue (MB) is an FDA-approved drug used to treat methemoglobinemia and cyanide poisoning [1]. Recently, MB became a drug of interest for treatment of neurodegenerative diseases mainly Alzheimer's disease (AD). Phase II clinical trials of MB under the name Rember® showed that 321 patients with mild to moderate AD had an 81 % decrease in rate of mental decline compared with those taking placebo after 1 year [2]. Consequently, with such encouraging results, Rember® is currently in phase III clinical trials.

However to date, the mechanism of action of MB in AD is still unidentified. Several studies reported that MB has an effect on protein aggregation especially tau protein [3, 4]. Lately, the effect of MB on amyloid beta (Aβ) aggregation and clearance has been described. In vitro studies showed that MB remodels toxic mature soluble oligomers into benign conformers [5] This effect of MB on Aβ aggregation may explain its neuroprotective effects since the toxicity of Aβ is lowest with its monomeric form followed by the fibrillar form then the oligomers which are the most toxic. However, the exact mechanism of structure remodeling by MB is still unknown. Another recent study indicated that MB treatment of fibrils led to the loss of beta sheet structure and formation of disordered, amorphous aggregates, therefore destabilizing and remodeling the Aβ peptide [6]. An in vivo study examined the possible mechanism of action of MB in an AD transgenic mouse model

which was treated with MB for 16 weeks. Soluble Aβ levels in the brain of these mice decreased significantly, and this effect was related to the ability of MB to increase Aβ degradation by increasing the activity of trypsin and chemotrypsin. In addition, learning and memory improved in this mouse model upon MB treatment [7]. After the findings of Medina et al., the effect of MB in vivo on memory enhancement and its ability to decrease Aβ peptide was further confirmed in transgenic PSAPP mouse and (APP/PS1) transgenic mice models [8, 9].

Moreover, mitochondrial dysfunction and disruption of the electron transport chain complexes has been widely reported in AD [10]. Since mitochondria are the power house of the cell, its inadequate energy supply to the neurons in AD is the main reason for neuronal cell death and synaptic loss [11]. Multiple in vitro and in vivo studies have pointed out the role of MB in protecting the mitochondria and enhancing its functions. These studies showed that MB enhances mitochondrial respiration and mitochondrial complex I-III activity [12, 13]. They demonstrated that MB is able to bypass complex I/III blockage, by accepting electrons from NADH (complex I) and transferring it to cytochrome C (complex IV) [12]. This alternative electron route helps in reduction of electron leakage and therefore inhibiting mitochondrial induced reactive oxygen species (ROS) overproduction. Furthermore, in vivo treatment with MB was capable of attenuating the neurotoxic effect of an inhibitor of cytochrome c oxidase, improving memory and learning functions [14, 15].

The presence of a link between mitochondrial dysfunction and Aβ accumulation has been suggested in many recent reports [16–18] Several lines of evidence suggest that accumulation of Aβ alters mitochondrial enzymes activity and induces high oxidative stress level [17, 19] One of these important mitochondrial enzymes is amyloid beta-binding alcohol dehydrogenase (ABAD) which belongs to the 17 β hydroxysteroid dehydrogenase family [20]. ABAD is the only mitochondrial enzyme responsible for converting estradiol to estrone and vice versa [21], which has been demonstrated to be crucial for neuronal survival [22]. Estradiol has been shown to have several protective effects on mitochondria. First, it increases the ETC. activity and increases glucose utilization, ultimately leading to a significant increase in ATP production [23]. Moreover, estradiol stabilizes the mitochondrial membrane potential, decreases the ROS production, and controls the calcium influx-induced excitotoxicity [24]. ABAD is also responsible for catalyzing the reduction of aldehydes and ketones and the oxidation of alcohols [25, 26].

Aβ was shown to bind to ABAD enzyme leading to modulation of its functions, and the overexpression of its inactive form as reported in transgenic mice models and AD affected brain regions in humans [20, 27, 28]. Aβ binding to ABAD induces changes in ABAD structure, forming Aβ-ABAD complex which prevents the binding of the coenzyme NAD+ [29]. It was reported that Aβ-ABAD complex potentiates cellular stress by changing mitochondrial membrane permeability and decreasing the activity of respiratory enzymes, thereby increasing ROS levels [30]. Moreover, Aβ-ABAD complex was shown to activate apoptosis cascade which leads to neuronal death, as well as impairment of learning and memory seen in transgenic mice [29, 31, 32]. In view of these findings, the link between mitochondrial dysfunction and Aβ-ABAD-associated toxicity may be an effective target for therapeutic intervention in AD. As shown in other studies, inhibiting the interaction between Aβ and ABAD can attenuate the complex toxicity [22, 27]. Accordingly, the aim of the current study was to investigate whether in vivo MB treatment affects ABAD expression and functions in association with its mitochondrial protective properties against Aβ toxicity.

Materials and Methods

Animals and Treatment

Swiss male albino mice 20–30 g of body weight were used. Mice were obtained from the animal house colony of the National Research Centre (Cairo, Egypt). Mice were kept at room temperature and constant humidity at a 12 h dark/night cycle. Standard laboratory food and water were provided ad libitum. Animal procedures were performed in accordance to both the GUC and the National Research Centre ethics committee's guidelines following the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were divided in four groups: control, LPS 055: B5 (Sigma-Aldrich, Germany), LPS + MB, and MB (Sigma-Aldrich, Germany).

LPS mouse model was developed according to Lee et al. [33]. The LPS group received 250 μg/kg of LPS i.p for 7 consecutive days. The LPS + MB group received in the last 4 days 4 mg/kg of MB i.p 2 h after LPS injection. The control group received vehicle only. The MB group was treated with MB only in the last 4 days. Each group contained seven male mice. Animals were sacrificed and brains were extracted. One of two hemispheres was used to obtain dissociated brain cells, while the other hemisphere was stored in –80 °C.

Preparation of Dissociated Brain Cells (DBC)

DBC were prepared as previously described, by modification of the method of Stoll et al.[34, 35]. Briefly, mice were sacrificed by cervical dislocation, and brains were extracted

on ice. The cerebellum was removed and one hemisphere was washed using ice-cold medium (I) (138 NaCl, 5.4 KCl, 0.17 Na_2HPO_4, 0.22 K_2PO4, 5.5 glucose, and 58.4 sucrose, all in mmol/liter, pH 7.35). The cerebrum was dissected, and tissue was dissociated using a Pasteur pipette. The suspension was passed through a coarse sieve of nylon mesh size 210 μm, then through a fine sieve size 100 μm. Cell suspension was then centrifuged for 3 min at 2000 rpm at 4 °C. The pellet was washed twice with Medium (II) (110 NaCl, 5.3 KCl, 1.8 $CaCl_2{\cdot}H_2O$, 1 $MgCl_2{\cdot}6H_2O$, 25 glucose, 70 sucrose, and 20 HEPES, all in mmol/l, pH 7.4). Protein content was determined using the BCA protein assay kit (Thermo Scientific, USA).

Cell Viability Assay

Cell viability was measured using WST-1 MTT assay (Clontech, USA) according to the manufacturer's instructions. Briefly, DBCs were seeded in 96-well tissue culture plates (100 μl/well). Plates were incubated for 4 h. Two hours before the end of the incubation period, 10 μl WST-1 MTT was added. The absorbance was measured by Perkin Elmer Victor^3 multilabel counter 1420 (Massachusetts, USA) at 450 nm. Protein content was determined using BCA kit, and values were normalized to the amount of protein.

Mitochondrial ROS Determination

Mitochondrial ROS was measured using dihydrorhodamine 123 (DHR) (Invitrogen, USA) as previously described [34]. DHR is an uncharged and non fluorescent probe. Upon oxidation of DHR by ROS cationic fluorescent rhodamine 123 is formed, which localizes in the mitochondria and exhibits green fluorescence. 250 μl of DBC suspension was seeded per well into a 48-well plate. After incubating the cells for 2 h, 5 μM of DHR was added per well for another 30 min. Plates were washed twice with PBS. Cells were resuspended in PBS and measured by Perkin Elmer Victor^3 multilabel counter 1420 (excitation at 488 and emission at 515 nm). Protein content was determined using BCA kit and values were normalized to the amount of protein.

Immunohistochemistry

Brain hemispheres were fixed in 4 % formaldehyde, then embedded in paraffin, and sectioned (5 to 8-μm thick). Paraffin-embedded sections were deparaffinized in xylene and rehydrated in serial concentrations of ethanol. Sections were incubated in 3 % hydrogen peroxide for 10 min to block endogenous peroxidase. Each section was blocked with a blocking buffer for 1 h at room temperature. The amyloid beta $_{[1–42]}$ monoclonal antibody (1:5000, Invitrogen, USA) was added onto the slides for 15 to 30 min. EconoTek Biontinylated anti-polyvalent was added onto slides and left to incubate for 30 min. The slides were then washed with TBS buffer. Immunoreactive structures were visualized in brown color using a standard DAB reaction (Scytek Laboratories, USA).

Protein Extraction and Western Blot Analysis

Brain hemispheres were homogenized in RIPA lysis buffer (NaCl, 1 M Tris (pH 7.2), 10 % SDS, Triton x- 100, Na deoxycholate, and 0.5 M EDTA) supplemented with 1 mM PMSF (Sigma, St. Louis, MO). Samples were centrifuged (14,000 rpm) for 20 min at 4 °C, and then the supernatant was collected and used to determine protein levels. For all samples, protein level was quantified using BCA Protein Assay Kit (Pierce Biotechnology, USA).

One hundred microgram of protein was separated on a 12 % SDS gel, and then transferred to a nitrocellulose membrane (Cayman, USA). Blots were blocked for 1 h at room temperature with 5 % (*w*/*v*) nonfat dry milk in TBST. The membrane was incubated at room temperature with specific antibodies for 90 min. The primary antibodies used were 6E10 (1:1000, Covance, USA), anti-ABAD antibody (1:500, Abcam, USA), and B-actin (1:2000, Abcam, USA) as a loading control protein. The blot was washed, and incubated with the corresponding secondary anti-mouse or anti- rabbit immunoglobulin G-horseradish peroxidase antibodies (1:5000, Abcam, USA) for 1 h. Immunoreactivity was detected using TMB peroxidase kit (KPL, USA). Images were captured and protein band densities were analyzed using the image J 2× software.

Quantification of Estradiol Levels

The estradiol assay was performed according to the manufacturer's guidelines (Estradiol EIA Kit, Cayman, USA). In brief, the plate was loaded with brain homogenate from the different treatment groups, along with the estradiol tracer and the specific antiserum to estradiol and incubated for 1 h at room temperature. After five washing steps, Ellman's reagent was added and the plate was developed for 90 min with gentle shaking at room temperature. The calculated estradiol concentration was normalized to the total protein content of the samples.

Statistical Analysis

Data were analyzed using Prism 5.0 software (GraphPad Software, LaJolla, CA, USA). Statistical significance was assessed by Student's *t* tests. Data are expressed as mean±SEM; $P<0.05$ was considered statistically significant.

Results

LPS Effect on Brain Aβ Load

The LPS mouse model used in the current study is a neuroinflammatory model that was developed according to Lee et al. [33]. Lee et al. fully characterized this model reporting memory impairment determined by passive avoidance and water maze tests in mice. Moreover, they showed an accumulation of Aβ1–42 in the hippocampus and cerebral cortex, as well as increase in both β and γ secretase activity. Mice were injected i.p with 250 μg/kg LPS for 7 consecutive days. For confirmation of the mouse model in our laboratory, $Aβ_{1–42}$ deposition in the cortex of the LPS injected mice was examined using immunohistochemistry. The LPS-injected mice showed an accumulation of $Aβ_{1–42}$ deposition in the cortex compared to the control group (Fig. 1).

Effect of MB Treatment on Mitochondrial ROS Levels in LPS-Induced AD Mouse Model

Aβ induces mitochondrial defects through the decrease in complex IV activity, accumulation of calcium ions in mitochondrial matrix [36], damage of mitochondrial DNA [37], inhibition of mitochondrial enzymes [17], and formation of inactive complexes such as Aβ-ABAD [38]. Consequently, Aβ burdens the mitochondria leading to increase in ROS levels [39] Therefore, one of the parameters examined in this model is ROS levels in mitochondria and the outcome of MB treatment. DHR probe was employed which upon oxidization by ROS is converted to the fluorescent dye rotenone that is localized in mitochondria. DBC obtained from the LPS-induced AD animal model showed significant increase in rotenone fluorescence (26526±1770, n=6, P<0.001) compared to the control group, indicating an increase in the ROS levels. On the other hand, MB treatment decreased the ROS levels (20,380±1770, n=6, P<0.05) significantly in the LPS group, however, not being able to completely return it to the control level as shown in Fig. 2. These results confirm that MB has antioxidant properties by decreasing the LPS-induced increase in ROS level. MB had no effect on ROS levels in the control mice.

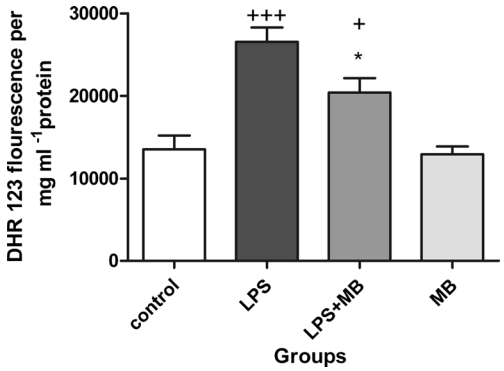

Fig. 2 Effect of MB treatment on mitochondria associated oxidative stress in LPS mouse model. DBC acquired from the four different treatment groups of mice were incubated with 5 μM DHR for 30 min, followed by measuring fluorescence. The LPS mouse model group showed significant increase in fluorescence indicating an increase in mitochondrial ROS compared to the control group. LPS + MB showed a significant difference in ROS compared to both LPS group and control group. Data are represented as mean ± SEM (n=6–7) (+++P<0.001, + P<0.05 vs. control and *P<0.05 vs. LPS group)

Effect of MB Treatment on Cell Viability in LPS-Induced AD Mouse Model

DBC obtained from the different mice groups were incubated with wst-1 reagent for 2 h, followed by measuring absorbance as an indication for cell viability. The DBC acquired from the LPS-treated mice showed significant decrease in cell viability (0.9099±0.08122, n=8, P<0.01) compared to the control mice (Fig. 3). DBC acquired after treatment with LPS + MB showed significant increase in cell viability (1.240±0.06373, n=6, P<0.05) compared to the LPS mice. Both LPS + MB group and MB groups showed no statistical difference when compared to the control group (Fig. 3).

Effect of MB Treatment on LPS-Induced Aβ Overexpression

The effect of MB on Aβ levels in transgenic AD mouse models was recently described. $Aβ_{1-42}$ oligomers are believed to be the most toxic form of $Aβ_{1-42}$ responsible for pathological changes related to AD [40]. Accordingly, the effect of MB treatment on Aβ oligomers was examined by measuring their

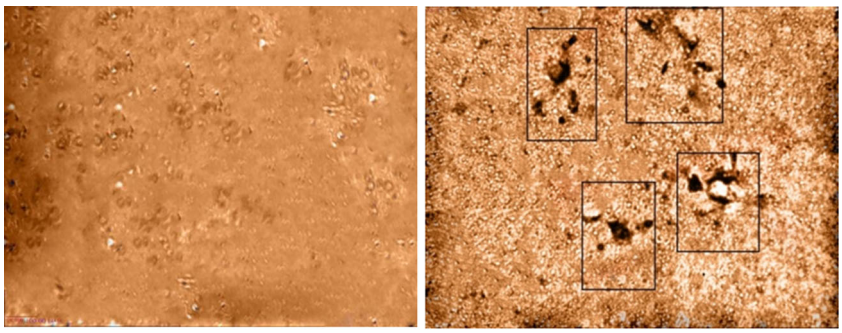

Fig. 1 $Aβ_{1-42}$ plaques formation in cerebral cortex of LPS mouse model. Immunohistochemical staining with amyloid beta [1–42] monoclonal antibody of brain sections shows neuritic plaques with a dense core in the cerebral cortex of LPS-injected mice compared to the control group

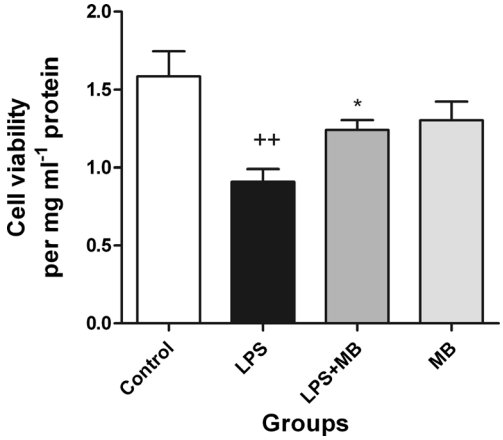

Fig. 3 Effect of MB treatment on cell viability. The LPS model showed a significant decrease in cell viability compared to the control group, while MB treatment significantly increased cell viability compared to the LPS group. No statistical difference was found when comparing LPS + MB group or MB group to the control group, respectively. Data are represented as mean±SEM (n=6–8). ++P<0.01 vs. control group and *P<0.05 vs. LPS group

levels in the mouse brain. In this experiment, Western blot analysis revealed a major Aβ band at ≈27 kDa, which corresponds to Aβ hexamer [41]. The LPS group showed a significant increase in the levels of Aβ protein (3.713±0.1596, n=5, P<0.001) compared to the control group. MB treatment of LPS-induced AD mouse model showed significant decrease in the Aβ band (1.864± 0.3006, n=5, P<0.01) compared to the LPS mouse model (Fig. 4b), and no significant difference compared to the control group. MB alone did not alter Aβ levels in the control group.

MB Impact on the LPS-Induced ABAD Expression and Function

ABAD is a mitochondrial enzyme which has been recently reported to represent a direct link between Aβ toxicity and mitochondrial dysfunction. ABAD levels were found to be elevated in AD human brains as well as in AD mouse models, while its functions were found to be decreased [20, 29]. Therefore, the next step in this study was to measure the levels and functions of ABAD in our mouse model before and after treatment with MB. The level of ABAD protein was measured using western blot analysis (Fig. 4a) Indeed the level of ABAD protein was elevated significantly in the LPS mouse model (1.325±0.09723, n=5, P<0.05) compared to the control group. Interestingly, treatment with MB decreased ABAD level (1.016±0.1168, n=5, P<0.05) compared to the LPS-treated mice (Fig. 4c), and there was no significant difference compared to the control group. MB alone did not alter ABAD levels,

In order to test the function of ABAD enzyme, estradiol levels were measured in the treated mice. The regulation of estradiol levels is a well-known established function of ABAD [20]. Level of estradiol was significantly decreased in the brains of the LPS mouse model (54.65±8.669, n=4, P<0.001) compared to the control group. Moreover, MB-treated mice restored the estradiol level (107.4±14.14, n=4,

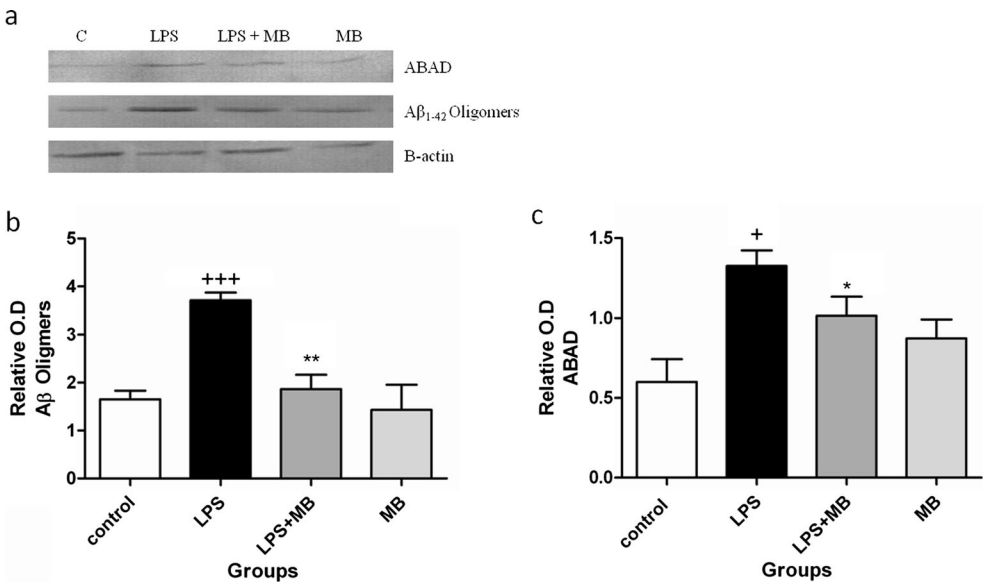

Fig. 4 Effect of MB on Aβ oligomers and ABAD levels in LPS mouse model. Western blots with the 6E10 antibody, anti-ABAD antibody, and B-actin as a loading control using 12 % SDS gel (**a**). Software analysis of band intensity shows a significant increase in brain $A\beta_{1-42}$ oligomers (27 kDa) (**b**) and ABAD levels (**c**) in the LPS model compared to the controls. MB treatment significantly decreased $A\beta_{1-42}$ oligomers (**b**) and ABAD (**c**) compared to the LPS group. No significant difference in $A\beta_{1-42}$ oligomers or ABAD levels was found between the control group and the MB + LPS group. Data is represented as mean±SEM (n=5) (+++ P<0.001, +P<0.05 vs. control group and **P<0.01,*P<0.05 vs. LPS group)

$P<0.01$), indicating an enhancement in ABAD function. Surprisingly, MB treatment in the control mice increased the levels of estradiol levels dramatically (Fig. 5).

Discussion

Methylene blue (MB) phase II clinical trials reported improvements in cognitive functions of AD patients after MB treatment. However, its mechanism of action in AD is debated by many researchers. One of the main mechanisms of action that has been described for MB is inhibition of protein aggregation. Several in vitro studies concentrated on the ability of MB to decrease tau aggregation [42]. The effect of MB on tau aggregation was tested also in vivo, and MB was shown to indeed improve memory and decrease the level of soluble tau, however, it was not able to decrease the levels of aggregated tau [4, 43, 44, 9].

Recently, MB was proposed to decrease soluble Aβ levels which were noticed in vivo in 3×Tg-AD mice [7]. MB action on Aβ was further supported by other studies that suggested different mechanisms of action, most importantly, soluble Aβ degradation [7], Aβ oligomeric structure fibrillization [5] or destabilization of the plaques into disordered aggregates [6].

Nevertheless, compelling data support that MB action on protein aggregates is not the sole mechanism of action of MB. As MB showed improvement in memory in normal rodents, as well as rodents with impaired memory due to metabolic deficits simulating mitochondrial dysfunction in AD [14, 44]. The mechanisms of action proposed was an increase in complex IV activity by increasing heme synthesis [45] as well as acting as an alternative electron transporter [12].

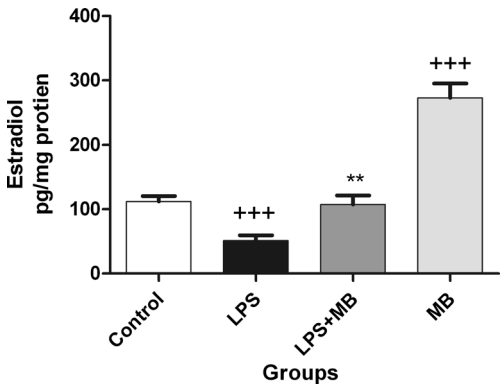

Fig. 5 MB effect on estradiol levels. Brain homogenates obtained from all mice groups were analyzed for the estradiol levels using the EIA kit. LPS mice demonstrate a significant decrease in the level of brain estradiol compared to the control group. MB treatment of LPS group significantly increased the brain estradiol levels back to normal, no significant difference was noted between LPS + MB and control group. Treating normal mice with MB increased the brain estradiol levels significantly compared to control group. Data are represented as mean±SEM (n=4–5) (+++$P<0.001$ vs. untreated control and **$P<0.01$ vs. LPS group)

Recently, the mitochondrial enzyme ABAD has been shown to bind Aβ-inducing mitochondrial dysfunction, providing a direct relation between Aβ toxicity and mitochondrial dysfunction occurring in AD. Previous studies have shown that inhibiting ABAD protects mitochondrial functions and prevents Aβ-induced toxicity [23, 26]. Taking into consideration the previously reported mitochondrial protective effect of MB and the recent data suggesting its ability to reduce Aβ level, the current study aimed at investigating if these protective effects of MB could be associated with an effect on ABAD levels and functions.

The effect of MB was tested in an LPS mouse model that has been previously described to induce memory impairment, with Aβ accumulation in hippocampus and cerebral cortex [33]. In the current study, the immunohistochemistry results have confirmed the deposition of Aβ in the cortex of LPS-induced AD mouse model in comparison to the control group in addition to the significant increase of Aβ oligomers shown by Western blot analysis (Fig. 1).

Aβ has been previously reported to cause deleterious effects on the mitochondria and causes neurotoxicity [19, 46, 47]. This Aβ-mediated mitochondrial dysfunction is either caused directly or through interaction with mitochondrial enzyme ABAD. This interaction causes elevation of nonfunctional ABAD levels, increase in ROS levels, inhibition of complex IV, decrease ATP levels, and finally cell death [29, 30]. To test whether MB overcomes this Aβ-induced mitochondrial toxicity, mitochondrial ROS and cell viability were assessed in DBC obtained from MB-treated mice. In this study, results showed a significant increase in mitochondrial ROS in DBC obtained from the LPS group compared to the control group. There was a decrease in the ROS levels in the MB-treated LPS mice (Fig. 2). In accordance with our results, the antioxidant property of MB, the enhancement of mitochondrial metabolic functions, and the increase in the activity of complex IV have been previously described [45, 48, 49]. Furthermore, Poteet et al. suggested that MB can act as an alternative electron transporter, transporting electrons from complex I to IV through accepting electrons from NADH and is reduced to leuco-MB which can act as free radical scavenger by accepting free electrons to be oxidized back to MB [50]. The protective effect of MB against LPS-induced mitochondrial toxicity was accompanied by an enhancement of DBC cell viability as shown in Fig. 3. MB treatment significantly increased cell viability to a level that did not differ statistically from the control group (Fig. 3). This protective effect of MB on cell viability has been recently reported in different models of mitochondrial toxicity [50, 12].

After noticing that MB overcomes the LPS-induced mitochondrial toxicity, the next step was to find out whether this could be due to a direct effect of MB on Aβ. Brains obtained from LPS mouse model showed an increase in $A\beta_{1–42}$ oligomers of 27 kDa (Fig. 4). This size of $A\beta_{1–42}$ is a highly toxic

form previously described as Aβ derived diffusible ligands (ADDLs) which can diffuse inside the cell, disrupting ion transport and resulting in reduced cell viability [51]. MB treatment of our LPS mouse model showed significant decrease in $A\beta_{1-42}$ oligomers (Fig. 4). Our in vivo results are in consistency with an in vitro study by Ladiwala et al.; where addition of MB to Aβ soluble conformers decreased oligomeric structure significantly [5]. Moreover, the current results are in accordance with the study by Medina et al., where MB treatment of AD transgenic mouse model decreased soluble Aβ levels [7]. More recently, two other studies on transgenic mouse models demonstrated that MB treatment reduced amyloid beta levels in transgenic AD mouse models, again supporting our findings [8, 9].

Finally, we addressed the question; does MB protect the mitochondria by decreasing the levels of Aβ oligomers only as seen in this study, or does it affect the interaction of Aβ with mitochondria by preventing Aβ-ABAD interaction. Previous studies have shown that Aβ-ABAD interaction leads to enzyme conformational change, preventing the NAD^+ from binding to ABAD inducing mitochondrial dysfunction. The NAD^+ is the coenzyme needed for ABAD to act on ketones, steroids and fatty acid chains, which produces acetyl coA, as well as ATP, and NADH [24, 47]. The binding of Aβ to ABAD does not only lead to the loss of function of ABAD and accumulation of toxic aldehydes in the brain, but affects complex IV in ETC. leading to production of ROS [52]. Moreover, it stimulates the release of cytochrome C which activates caspase-3 leading to DNA fragmentation and neuronal cell death [30].

In the brains, obtained from the LPS group, significant increase in ABAD levels was noticed. According to our knowledge, the effect of LPS model on ABAD levels is first to be mentioned in the current study. However, this is in accordance to what is reported—that ABAD is found to be significantly increased in the cerebral cortex and hippocampus of AD-affected brain regions and in mouse models of AD [20, 27, 28, 53].

Interestingly, treatment with MB showed significant decrease in ABAD levels. Here again, the effect of MB on ABAD levels was not previously reported, however, it was previously reported that inhibiting ABAD levels leads to mitochondrial protection and neuroprotective effect. This was demonstrated in a study by Lim et al., showing that inhibiting ABAD by a novel compound (AG18051) reduced accumulation of ROS and neuronal cell death in vitro using SH-SY5Y neuroblastoma cell lines [22]. Furthermore, these observations were confirmed as well in a study by Yao et al., which demonstrated that the inhibition of the Aβ-ABAD interaction using a decoy peptide protected mitochondrial function and enhanced memory in vivo [27]. These studies are in accordance with the current study confirming that decreasing ABAD and Aβ levels in brains of MB-treated mice decreased mitochondrial ROS and prevented cell death. Finally to confirm our hypothesis that MB may exert its mitochondrial effects by affecting Aβ-ABAD interaction and therefore restoring the function of ABAD, we decided to measure the functional activity of ABAD. A well-known function of ABAD is to convert estrone to estradiol [25], and Lim et al. were able to show in vitro that Aβ-mediated decrease in estradiol levels is prevented by ABAD inhibitor [22].

Therefore, after confirming the effect of MB on the levels of ABAD in the brains of the LPS mouse model, the estradiol levels were measured. Our results indeed demonstrated that MB protects the function of ABAD, increasing significantly the estradiol levels that were decreased in the LPS mouse model (Fig. 5). Our results are supported by many recent studies that suggested a protective role of estrogens on cognition and mitochondrial function. Estradiol has been reported to improve brain mitochondrial efficiency and increases respiratory function through promoting the expression and activity of the electron transport chain complex IV [54, 55]. Enhancement of cognition in normal rodents was previously reported in a study by Callway et al. They reported that 1 mg/kg of MB given to normal rats for 5 days showed significant enhancement in spatial memory retention [56]. Moreover, Riha et al. reported improved behavioral habituation and object memory recognition after treatment with 4 mg/Kg MB for 4 consecutive days [57]. While the current study showed consistent effects of MB on enhancing cell viability, decreasing ROS level, decreasing overexpressed ABAD, and increasing estradiol levels in LPS mouse model, the increase seen in estradiol level in normal mice suggests that MB could have other mechanisms of actions on cognition through the estrogen pathway, which requires more investigations.

In conclusion, it has been recently reported that Aβ causes mitochondrial toxicity by binding to ABAD leading to mitochondrial dysfunction. In this study, we hypothesized that MB decreases mitochondrial dysfunction by preventing Aβ-ABAD induced toxicity. It was proven in this study that MB decreases the overexpressed ABAD levels. Moreover, it also decreases the Aβ levels, therefore, decreasing its availability for binding to ABAD; and according to previous studies, MB reduces NADH-forming NAD+ the cofactor for ABAD [12, 50]. This may lead to competition of NAD+ with Aβ for ABAD active site, preserving ABAD functions and preventing mitochondrial dysfunction. The MB mitochondrial protective effect and the enhancement of estradiol levels indicating preservation of ABAD functions in this study are a further confirmation for the proposed hypothesis. Further future binding studies testing the interaction of NAD+ and Aβ with ABAD in the presence of MB need to be carried to confirm this hypothesis.

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