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Methylene Blue Improves Mitochondrial Function in The Liver of Cholestatic Rats

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Abstract

Different diseases or xenobiotics could cause cholestasis. The only promising treatment for this disease is the identification of its etiology or liver transplantation in severe cases. Nevertheless, preserving liver function could delay organ injury or help to the treatment of the disease in mild cases. The mechanism of cholestasis-induced liver injury is multifactorial. However, it has been found that hepatocyte mitochondrial function is impaired in this disease. Methylene blue (MB) is a phenothiazine compound. MB is pharmacologically used for a wide range of diseases. It has been found that this compound could significantly improve mitochondrial function and prevent the releases of cell death mediators from this organelle. MB is also well-known for its preventing effect on mitochondria-facilitating reactive oxygen species (ROS) formation. It has been found that mitochondrial function is impaired in the liver tissue in different models of cholestasis. The current study aimed to evaluate the effects of MB administration on mitochondrial indices in cholestatic animals. Rats underwent bile duct ligation (BDL) surgery and treated with MB (0.5 and 1 mg/kg, oral). Significant mitochondrial permeabilization, mitochondrial membrane depolarization, lipid peroxidation, decreased mitochondrial dehydrogenase activity, and depleted ATP content was evident in BDL rats. It was found that mitochondrial indices improved in MB-treated cholestatic animals. Based on the data collected in this study, MB might be useful as a therapeutic agent in cholestasis. The mitochondria protecting properties of this compound could play a major role in its mechanism of action.

Keywords: Bile Acids, Bioenergetics, Cirrhosis, Cholestasis, Liver failure, Mitochondria, Oxidative stress

1. Introduction

Cholestasis could develop with a variety of etiologies. Drugs and xenobiotics, alcoholism, and infectious liver disease could cause cholestasis (1, 2). Un-treated cholestasis could lead to liver fibrosis/cirrhosis and, finally, multiorgan failure (1, 2). When cholestasis occurred, the bile components are accumulated in the liver tissue. Hydrophobic bile acids and bilirubin are major bile constituents identified to be responsible for the liver injury during cholestasis (3-10).

Different mechanisms, including the induction of severe oxidative stress, have been documented in the liver of experimental animal models or human cases of cholestasis (5, 9, 11-13). Disruption of various cellular targets, including biomembrane lipids, DNA, proteins, as well as vital organelles such as endoplasmic reticulum (ER) and mitochondria, could be affected in cholestasis (14-17). Several investigations tried to mitigate cholestasis-induced liver injury by the administration of antioxidants (16, 18-24). The ideal treat-

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ment for cholestasis is the elimination of the cause of cholestasis. However, adjuvant therapies might protect the liver or delay their damage. Although antioxidants revealed significant protective effects against cholestasis, these agents have partial results, perhaps because of the progressive nature of the disease.

Another interesting mechanism of cholestasis-induced liver injury is the involvement of mitochondrial impairment in this complication (5-7, 25-29). It has been reported that hepatocytes ATP levels are depleted in cholestatic animals. Mitochondrial permeabilization decreased mitochondrial dehydrogenases activity, and mitochondrial depolarization is also documented in the liver during cholestasis (5-7, 25-29). Another critical point is the connection between mitochondrial impairment and cellular oxidative stress (30). It is well-known that cellular mitochondria are the primary sources of cellular ROS (31, 32). Hence, mitochondrial impairment in the liver of cholestatic animals could deteriorate the oxidative stress in this organ. It also has been documented that severe oxidative stress additionally damages the cellular mitochondria, and this vicious cycle finally leads to cell death and organ injury.

Methylene blue (MB) is a phenothiazine compound (Figure 1). This chemical was the leading molecule for the discovery of antipsychotic drugs. MB is widely investigated for its pharmacological effects for more than 100 years (33). The effects of MB on the central nervous system (CNS) diseases such as Alzheimer's are the most impressive therapeutic properties of this compound (34-36). MB is widely investigated in CNS disorders as well as xenobiotics-induced neurotoxicity (37-40). However, the effect of this compound on other complications such as liver diseases, renal disorders, lung damage, and cardiovascular diseases also has been investigated (41-48). MB is an FDA-approved compound for methemoglobinemia with different etiologies (33).

Effects of MB on mitochondrial function and regulation of mitochondria-mediated ROS formation is one of the most exciting features of this compound (49). MB (The oxidized form) could accept an electron from the complex I of the electron transport chain (ETC) (49). Reduced MB (MBH) transfers its electron to cytochrome c and complex IV of ETC (49). This process might decrease mitochondria-facilitated ROS formation (49).

As mentioned, mitochondrial impairment is a putative mechanism involved in the pathogenesis of cholestasis-induced liver injury. Therefore, in the current study, the effects of MB on several mitochondrial indices were evaluated in the liver of cholestatic animals. The results might help to reveal the mechanism(s) of hepatoprotective effects of this compound and its potential use in various liver diseases.

2. Material and Methods

2.1. Reagents

N-chloro tosylamide (Chloramine-T), trichloroacetic acid (TCA), sodium acetate, citric acid, n-Propanol, p-Dimethyl amino benzaldehyde, methylene blue (3,7-bis(dimethylamino) phenothiazine-5-ium,) dithiothreitol (DTT), sucrose, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), thiobarbituric acid (TBA), dimethyl sulfoxide, acetonitrile HPLC grade, methanol HPLC grade, sodium citrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethylenediamine tetra-acetic acid (EDTA), phosphoric acid, 2 amino 2-hydroxymethyl-propane-1,3-diol-Hydrochloride (Tris-HCl), were obtained from Merck (Darmstadt, Germany). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ-glutamyl transferase (γ-GT), albumin, and bilirubin kits were obtained from Pars Azmoon® (Tehran, Iran). All salts used for making buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male Sprague-Dawley rats (n=60; 200-250 g weight) were obtained from the Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in a standard environment (temperature of 23±1 ºC and a 12L: 12D photoschedule along with a 40 % of relative humidity). The rats were allowed free access to a regular standard RoyanFeed® (Isfahan, Iran) rodents chow diet and tap water. All the experiments were done in

compliance with the guidelines for care and use of experimental animals approved by an ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (#1396-01-36-14807).

2.3. Surgery

Animals were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p). A midline incision through the linea alba was made. Then, the common bile duct was localized, doubly ligated, and cut between these two ligatures (50). The sham operation consisted of laparotomy and bile duct identification and manipulation without ligation.

2.4. Experimental setup

Animals were equally allotted into five groups (n=8 /group). The treatments were as follows:

1) Sham-operated (Vehicle-treated);
2) BDL;
3) BDL+Methylene blue (0.5 mg/kg, oral, for seven consecutive days);
and 4) BDL+Methylene blue (1 mg/kg, oral, for seven consecutive days). Cholestasis-associated hepatic injury was assessed seven days after BDL surgery (50, 51).

2.5. Serum biochemistry

A Mindray BS-200® auto analyzer and Standard kits (Pars Azmoon®, Tehran, Iran) were used to measure serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ-GT), bilirubin, blood urea nitrogen (BUN), and creatinine (Cr) (52).

2.6. Organ weight index

Animals and their organs (liver, spleen, and kidney) were weighed, and the weight index was measured as Organ weight index=[Wet organ weight (g)/Body weight (g)]×100.

2.7. Isolation of the liver mitochondria

Mice liver mitochondria were isolated as previously described (53). Briefly, animals were anesthetized (Thiopental 80 mg/kg, i.p), and their liver was excised and washed with ice-cold saline (4oC, sodium chloride 0.9%) (53, 54). The liver was homogenized with an Ultra-Turrax® Tube (IKA) homogenizer (8000 rpm, 10 sec), in isolation buffer (220 mM mannitol, 0.5 mM EGTA, 70 mM sucrose, 2 mM HEPES, 0.1% bovine serum albumin, and pH=7.4) at a 10:1 buffer to liver (v: w) ratio (53, 55). 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) (56, 57). The second centrifugation step was repeated at least three times using a fresh buffer medium. All manipulations for liver mitochondria isolation were performed at 4 °C or on ice to minimize mitochondrial injury (53, 58).

2.8. Mitochondrial ATP levels

Based on a previously reported protocol, mitochondrial ATP level was assessed by an HPLC method (55, 59). Briefly, isolated mitochondria (1 mg protein/mL) were treated with 100 µL ice-cooled phosphoric acid (50 % w: v, 4 ºC) and centrifuged (30 min, 17,000 g, 4 ºC). Afterward, the supernatant (100 µL) was treated with 15 µL of ice-cooled 1 M KOH solution. Samples (25 µL) were injected into an HPLC system consisted of an LC-18 column (25 cm, µ-Bondapak). The mobile phase was composed of phosphate buffer (100 mM KH2PO4, pH=7 adjusted with KOH), acetonitrile (2.5 % v: v), and 1 mM tetrabutylammonium hydroxide (55). The flow rate was 1 mL/min, and the UV detector was set at λ=254 nm (55, 59).

2.9. Lipid peroxidation in kidney mitochondria

Thiobarbituric acid-reactive substances (TBARS) were measured in isolated mitochondria (8, 57). Previous studies mentioned that sucrose interferes with the lipid peroxidation test in isolated mitochondria preparations (53). Therefore, mitochondria preparations were washed once (to remove sucrose). For this purpose, isolated mitochondria were suspended in 5 mL of ice-cooled MOPS-KCl buffer (50 mM MOPS, 100 mM KCl, 4 ºC, pH=7.4). Then, isolated mitochondria were centrifuged (17,000 g, 15 min, 4 ºC), and the pellet was re-suspended in MOPS-KCl buffer and used

for TBARs assay (53, 60). 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). Samples were heated for 15 min at 100 ºC (53). 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 was measured (EPOCH plate reader, BioTek® Instruments, Highland Park, USA, λ=532 nm) (53).

2.10. Mitochondrial depolarization

Mitochondrial uptake of the cationic dye rhodamine 123 was applied for the evaluation of mitochondrial depolarization (61-65). Rhodamine 123 accumulates in the mitochondrial matrix by facilitated diffusion (61-63). When the mitochondrion is depolarized, there is no facilitated diffusion. Therefore, the amount of rhodamine 123 in the supernatant will be increased. In the current investigation, the mitochondrial fractions (0.5 mg protein/mL; in the depolarization assay buffer) were incubated with 10 µM of rhodamine 123 (15 min, 37 ºC, in the dark). Afterward, samples were centrifuged (15,000 g, 10 min, 4 ºC), and the fluorescence intensity of the supernatant was monitored (FLUOstar Omega®; multifunctional microplate reader; BMG Labtech, Germany; $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm) (61, 66, 67).

2.11. Mitochondrial permeabilization and swelling

Mitochondrial permeabilization was assessed by monitoring the changes in optical density at λ=540 nm (53, 68). Briefly, isolated mitochondria (0.5 mg protein/ml) were suspended in swelling buffer (65 mM KCl, 10 mM HEPES, 125 mM sucrose, pH=7.2). The absorbance was monitored (25 °C, during 30 min of incubation), using an EPOCH plate reader (Bio-Tek® Instruments, Highland Park, USA) (69, 70). A decrease in absorbance is connected with an increase in mitochondrial permeabilization. The results are reported as maximal mitochondrial swelling amplitude (ΔOD 540 nm) (53, 67, 71).

2.12. Liver 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 (72-76). 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 (77). The product of purple formazan crystals was dissolved in 1 mL of dimethyl sulfoxide. Then, 100 µL of dissolved formazan was added to a 96-well plate, and the optical density at λ=570 nm was measured (EPOCH plate reader; BioTek Instruments, Highland Park, USA). Samples protein concentrations were determined by the Bradford method (78-80).

2.13. Statistical analysis

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

3. Results

Significant elevation in the serum biochemical markers of liver and bile duct injury, including ALT, ALP, γ-GT, bilirubin, AST, and LDH were detected in the rat model of cholestasis (Table 1). On the other hand, signs of hepatomegaly and splenomegaly were evident in the cholestatic animals (Figure 2). These data confirm the cholestasis induction in our model. It was found that the MB administration significantly mitigated splenomegaly and hepatomegaly in BDL rats (Figure 2). The effects of MB on these markers were not dose-dependent. MB treatment also mitigated markers of liver injury in cholestatic animals (Table 1).

It was found that mitochondrial function was interrupted in the liver tissue of cholestatic animals (Figure 3). Severe decrease in mitochondrial dehydrogenases activity, mitochondrial depolarization, decreased mitochondrial ATP content, mitochondrial permeabilization, and increase in mitochondrial lipid peroxidation were detected in mitochondria isolated from the liver of cholestatic animals (Figure 3). It was found that MB administration in both doses (0.5 and 1 mg/kg) significantly improved mitochondrial indices of functionality

Table 1. Serum biochemical measurements in cholestatic rats.

Parameters assessed	Sham	BDL	BDL+MB 0.5 mg/kg	BDL + MB 1 mg/kg
ALT (U/l)	45±7	300±25	148±23[a]	114±11[a]
AST (U/l)	80±14	206±19*	126±6	107±11[a]
LDH (U/l)	587±31	2610±215*	1400±220[a]	1000±90[a]
ALP (U/l)	1183±53	3343±309*	2887±532	2721±339
γ-GT (U/l)	22±7	308±60*	284±47	234±74
Total bilirubin (mg/dl)	0.1±0.05	8.5±2*	11±3	8±3

Data are given as mean±SD (n=8). MB: Methylene blue; BDL: Bile duct ligation.
*Indicates significantly different as compared with the sham group (P<0.001).
[a]Indicates significantly different as compared with the BDL group (P<0.05).

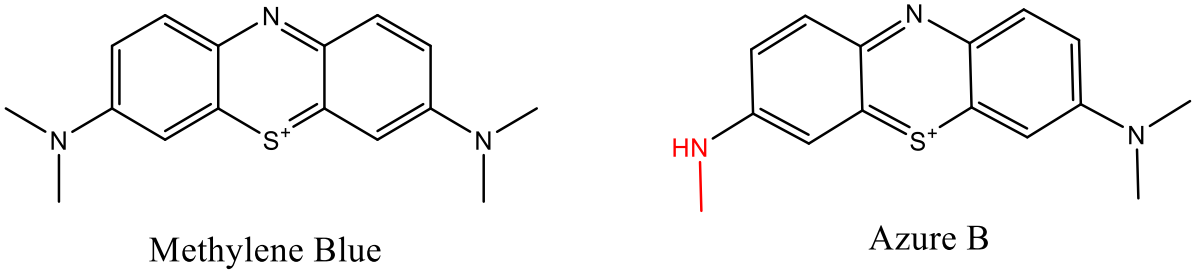

Figure 1. Methylene blue and azure B as its de-methylated metabolite. Azure B seems to be responsible for some pharmacological effects of MB.

in the liver mitochondria of cholestatic rats (Figure 3). The effects of MB on mitochondrial function was not dose-dependent in most parameters evaluated in the current study (Figure 3).

4. Discussion

Cholestasis is a clinical complication which could be induced by various etiologies (1, 2). Severe and untreated cholestasis could lead to cirrhosis and liver failure. Oxidative stress and its

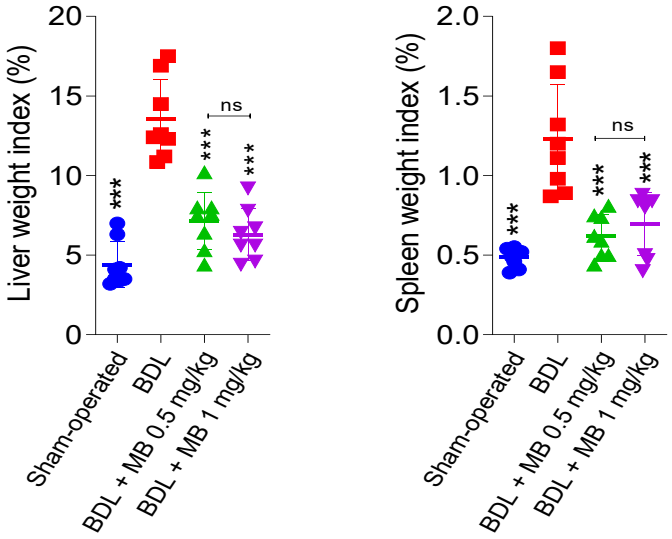

Figure 2. Organ weight indices in cholestatic rats. MB: Methylene blue; DL: Bile duct ligated. Data are given as mean±SD (n=8).
***Indicate significantly different as compared with the BDL group (P<0.001).
ns: not statistically significant.

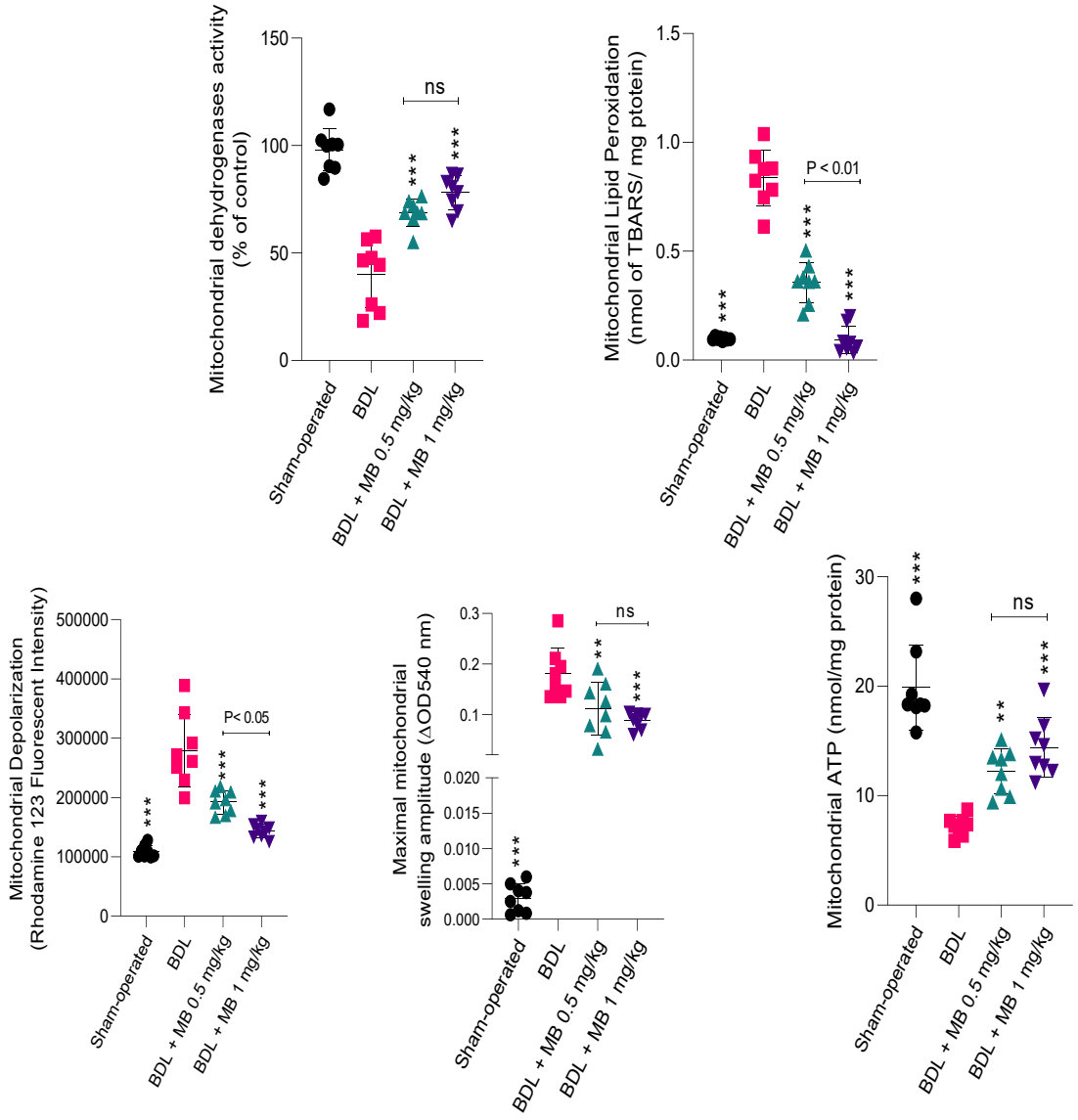

Figure 3. Evidence of improvement in liver mitochondrial indices in bile duct ligated (BDL) rats treated with methylene blue (MB). ATP: Adenosine triphosphate; TBARS: Thiobarbituric acid reactive substances. Data are given as mean±SD (n=8).
Asterisks indicate significantly different as compared with the BDL group ($^{**}P<0.01$, $^{***}P<0.001$).

associated events could play an essential role in cholestasis with different etiologies (5, 9, 11, 12). Although there are no specific sources for ROS and oxidative stress in cholestasis, cellular mitochondria seem to play a role in this complication (3, 6, 7, 9, 16, 17, 81, 82). Hence, agents that affect mitochondria-born ROS might influence liver injury during cholestasis. MB is a mitochondrial active agent that, at low concentrations, could regulate mitochondria-facilitated ROS formation.

Although no adverse effects of MB have been reported has been reported in humans (≈300 mg/day) (33, 83). The beneficial effects of MB are provided at low doses of this compound (Figure 4) (49, 84). High doses of MB not only are not helpful but also might deteriorate cell function by inhibiting vital enzymes as well as severely impairing mitochondrial function (84). The effects of MB on liver function and histopathological alteration also has been previously reported by Aksu *et al.* (85). The authors revealed that MB treatment decreased ROS formation and liver histopatho-

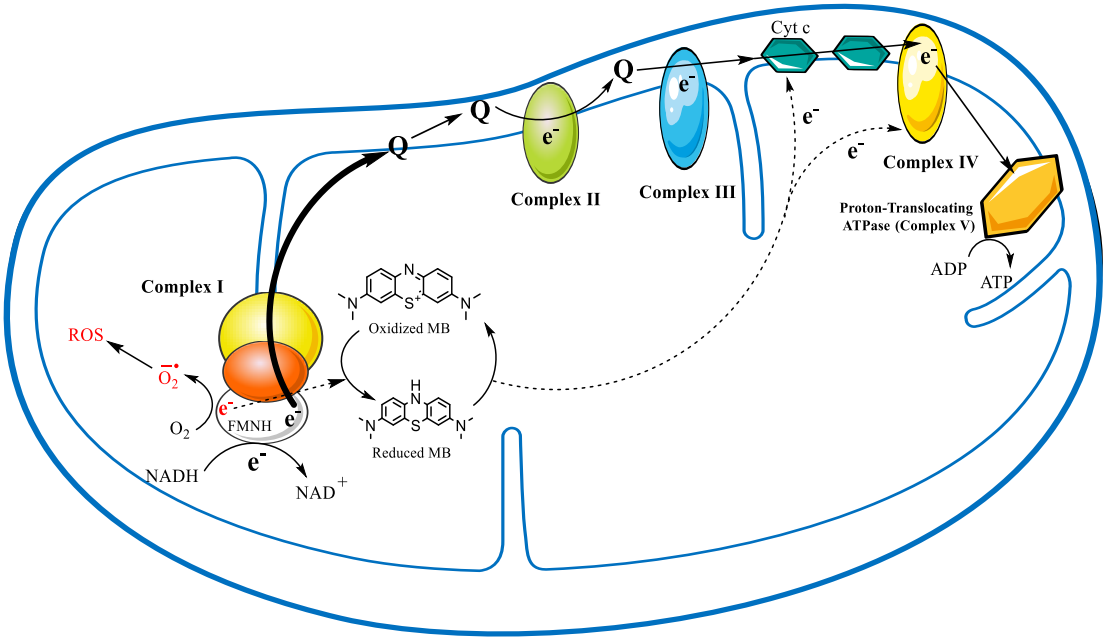

Figure 4. Schematic representation of the possible effects of methylene blue for the enhancement of mitochondrial function. Low concentrations of methylene blue (MB; 0.5, 1, and 5 µM) could directly transfer electron (e^-) from complex I of electron transport chain (ETC) to cytochrome c and complex IV. Therefore, this mechanism bypasses ETC-facilitated reactive oxygen species (ROS) production by ETC (e.g., by complex II). It has been found that higher MB concentrations (e.g., >5 µM) deteriorate mitochondrial function by enhancing mitochondrial permeabilization and a significant decrease in ATP production. Q: Co-enzyme Q; FMNH: Flavin mononucleotide. This schematic representation was inspired by, and draw based on the mechanism of MB action in cellular mitochondria represented in the manuscript: Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. DOI: 10.1096/fj.07-9610com

logical changes and fibrosis (85). However, there is no molecular mechanism for the source of ROS or the role of mitochondria in their investigation. In the current study, we evaluated the effect of MB on hepatocyte mitochondria as its primary site of action.

The antioxidant activity of MB seems to be mediated through interaction with the mitochondrial electron transport chain (Figure 4) (49). The cycling between the oxidized and reduced forms of MB plays a vital role in its mitochondria regulatory properties (Figure 4). It has been found that a Flavin-dependent enzyme in complex I of the mitochondrial electron transport chain (ETC) use NADH to reduce oxidized MB to reduced MB (leucomethylene blue; MBH2) (Figure 4) (49). On the other hand, cytochrome c and complex IV of ETC deoxidizes MBH2 to MB (Figure 4) (49). Therefore, electron transport through ETC is bypassed. ETC is a significant source of ROS. Hence, this could be one of the primary mechanisms for the positive effects of low concentrations of MB in mitochondria. On the other hand, some investigations mentioned that MB could play an essential role in mitochondrial biogenesis (86). This process improves mitochondrial energy metabolism and will decrease ROS produced by damaged and old mitochondria (86).

Interestingly, it has also been found that MB could enhance cellular antioxidant defense mechanisms through the activation of the nuclear factor erythroid-2-related factor 2 ((Nrf2) signaling pathway (86). Nrf2 is responsible for the expression of different cellular antioxidant defense enzymes. Hence, MB boosts cellular antioxidant defense mechanisms.

As previously mentioned, the effects of MB on mitochondrial function are restrictedly dose-dependent (87). Visarius *et al*. reported that increasing concentrations of MB provoked mitochondrial impairment and severe mitochondrial permeabilization (87). On the other hand, low concentrations of this chemical (e.g., 0.5, 1, and 5 µM) stimulated mitochondrial respiration and enhanced

energy production (Figure 4) (87, 88).

Several studies also mentioned that MB administration could slow the development of neurodegenerative diseases (e.g., Alzheimer's disease) (33, 34, 89, 90). Interestingly, it has been reported that MB could mitigate the aggregation of tau proteins and amyloid β in Alzheimer's disease (33, 91). A formulation of MB even went under clinical trial (Rember™) for Alzheimer's disease (33, 92). Interestingly, it has been found that MB could facilitate fatty acids oxidation in the hepatocytes mitochondria (87). Hence, this compound might found new applications in liver diseases. Fatty liver is a prevalent hepatic disorder which could lead to other complications such as liver fibrosis (93). Some studies mentioned that the therapeutic effects of MB might be associated with its demethylated metabolite, azure B (Figure 1). However, many other investigations mentioned that the effects of MB, especially its effects on cellular mitochondria, are associated with MB (Figure 1). All these data mention the potential clinical use of MB in various diseases.

Conclusively, based on the data obtained from the current study, the antioxidative effects of MB reported in previous models of liver injury might be associated with the effects of this compound on mitochondrial function, enhancement of mitochondrial energy production, decreasing mitochondria-facilitated ROS formation, and inhibition of cell death mediators from hepatocytes mitochondria might play a role in its hepatoprotective and antifibrotic effects during cholestasis.

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Conflict of Interest

None declared.

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