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PhD THESIS

CONTRIBUTIONS TO THE ELUCIDATION OF THE ROLE OF METHYLENE BLUE IN CARDIOVASCULAR PROTECTION

ABSTRACT

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I. INTRODUCTION

Mitochondrial dysfunction and the related oxidative stress are nowadays widely accepted as the central pathomechnaisms of the vast majority of chronic disorders; thus, in the past years modulation of mitochondrial function has become an important therapeutic target. In particular, pharmacological agents able to improve mitochondrial respiratory function and/or to decrease the reactive oxygen species (ROS) generation are currently highly investigated. Among them an emerging compound is methylene blue (MB), a tricyclic phenothiazine, used for more than a century to treat a variety of disorders. Due to its redox potential, MB can modulate the mitochondrial bioenergetics and alleviatethe related dysfunction while improving the prognostic of diseases in which was administered.

KEY WORDS: methylene blue, diabetes mellitus, mitochondrial dyfunction, cellular bioenergetic profile, oxidative stress, endothelial dysfunction

AIM AND OBJECTIVES

The present PhD thesis was aimed at characterizing the effects of methylene blue in the setting of diabetes mellitus, a disease classically associated with cardiovascular oxidative stress, in which both mitochondrial and endothelial dysfunction represent important pathogenic features.

The research objectives were as follow:

1. Characterization of the effects of MB on the ***bioenergetic profile*** of a rat cardiac cell line.

2. Evaluation of the effects of MB on ***cardiac mitochondrial dysfunction*** in the setting of experimental diabetes mellitus.
3. Evaluation of the effects of MB on ***endothelial dysfunction*** in the setting of experimental diabetes mellitus.

The objectives were accomplished in **3 experimental studies:**

I. The first study evaluated the effects of MB on mitochondrial respiration and glycolysis of the rat cardiomyoblasts (H9c2) cell line.

II. The second study analyzed the effects of MB on mitochondrial respiration, reactive oxygen species (ROS) production, and calcium retention capacity, respectively, in cardiac mitochondria isolated from diabetic rats (type I of diabetes mellitus was induced by streptozotocin).

III. The third study assessed the MB effects on endothelial function and vascular oxidative stress in aortas harvested from diabetic rats.

The research methodology consisted in the *in vitro* evaluation of: i) mitochondrial function by high-resolution respirometry (Oroboros-O2K oxygraph) and extracellular flux analyzer (Seahorse XF24e) and ii) endothelial function by organ bath experiments (DMT myograph).

II. SPECIAL PART

1. ASSESSMENT OF THE EFFECTS OF METHYLEN BLUE ON CELLULAR BIOENERGETICS IN H9C2 CELLS

The present study was aimed at assessing the effects of MB in acute administration on bioenergetic and metabolic parameters with the measurement of the oxygen consumption rate (OCR) as indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR), as a measure of anaerobic glycolysis, respectively, in H9c2 rat cardiomyoblast cells by means of the extracellular flux analyzer (Seahorse XF24e).

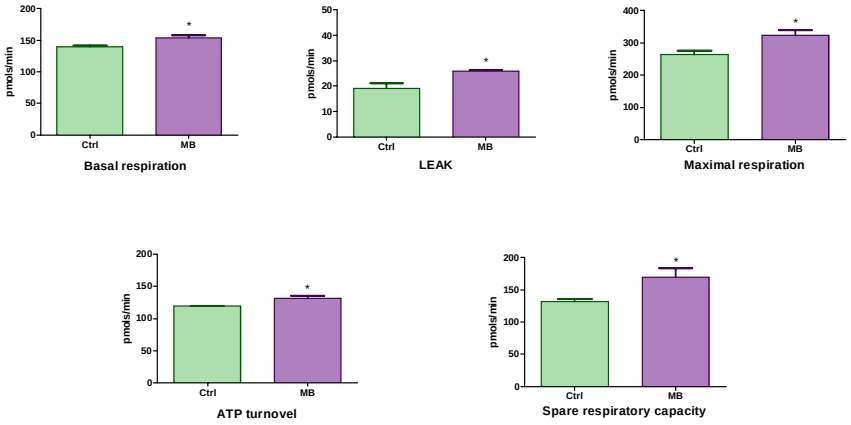

Figure 1. Acute effects of MB on OCR linked parameters. (n = 6–8/group. *p < 0.05 vs. untreated controls).

As shown in Fig. 1, submicromolar concentrations of MB (0.1 µM) exhibited a significant increase in basal respiration, proton leak, ATP turnover, maximal respiration, and spare respiratory capacity of H9c2 cardiomyoblasts as compared to the non-treated cells (Figure 1).

With respect to the compound effect on glycolysis, as for OCR, a significant increase of ECAR in MB treated vs. non-treated cells was found (Fig. 2).

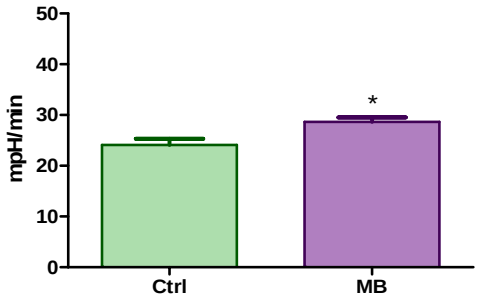

Figure 2. The acute effects of MB on ECAR. (n = 6–8/group. *p < 0.05 vs. untreated controls).

Conclusion: In H9c2 myoblasts, acute administration of 0.1 µM methylene blue elicited an increase in O2 consumption (OCR) and extracellular acidification (ECAR) rates, indicating an overall stimulatory impact on cellular bioenergetics.

2. ASSESSMENT OF MB EFFECTS ON MITOCHONDRIAL RESPIRATION AND ROS PRODUCTION IN DIABETIC RAT HEARTS

The present study was purported to characterize the effects of acute administration of methylene blue on mitochondrial respiration, H_2O_2 production, and calcium retention capacity in rat heart mitochondria isolated from healthy and streptozotocin-induced diabetic rats. Since impairment of mitochondrial respiration and increased ROS production are the major pathomechanisms underlying the progression of diabetic cardiomyopathy towards heart failure, the working hypothesis was that MB could improve mitochondrial respiration and alleviate oxidative stress in the setting of experimental diabetes.

High-resolution respirometry studies. MB elicited a substrate-independent improvement in respiratory function as demonstrated by the increase in all respiratory parameters (i.e., State 2, OXPHOS, State 4, and ETS - Electron Transport System capacity) in both non-diabetic (Figure 3 A, B) and diabetic (Figure 4 A, B) animals. Indeed, stimulation of respiration was observed for both Complex I and Complex II substrates, i.e., glutamate+malate and succinate (+ rotenone), respectively.

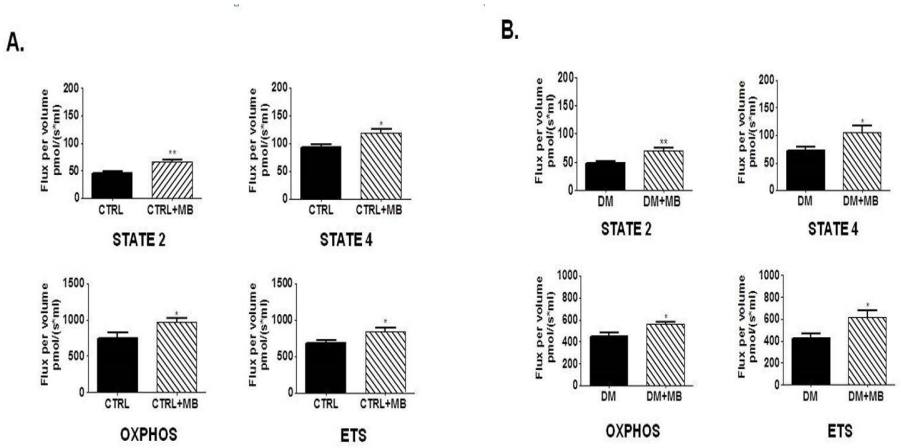

Figure 3. High-resolution respirometry data for CI-supported respiration. (A) CTRL group; (B) DM group. Values are means ± S.E.M. *p<0.05; **p<0.01.

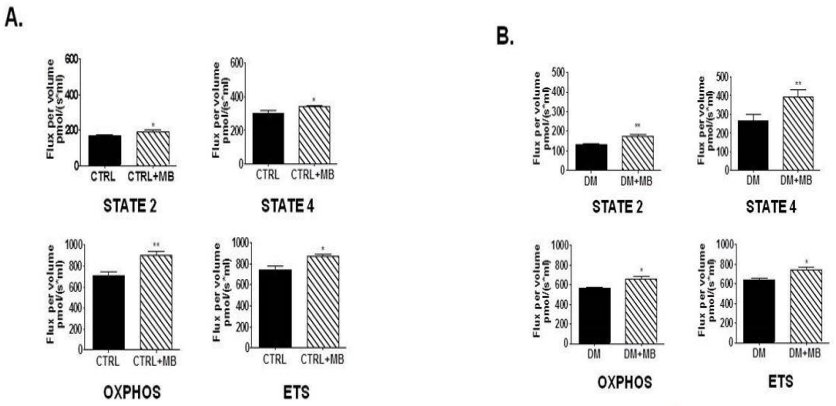

Figure 4. High-resolution respirometry data for CII-supported respiration. (A) CTRL group; (B) DM group. Values are means ± S.E.M. *p<0.05; **p<0.01.

In our hands, MB improved both basal (state 2) and ADP-stimulated respiration - as shown by an increase in OXPHOS for both substrates, most probably by serving as an additional source of electrons for the electron transport chain. In line with the hormetic behavior, it is important to emphasize that the beneficial effects of MB were observed at very low doses (i.e., 0.1 μM).

Assessment of oxidative stress. Acute administration of MB (0.1 μM) elicited an (unexpected) significant increase in H_2O_2 production in both groups (with/without diabetes) when mitochondria were energized with glutamate+malate (in diabetic group by 210%, and in non-diabetic group by 78%), whereas a significant decrease was observed when succinate (+rotenone) was used as substrate (in diabetic group by 49%, and in non-diabetic group by 53.8%) (Figure 5 A, B).

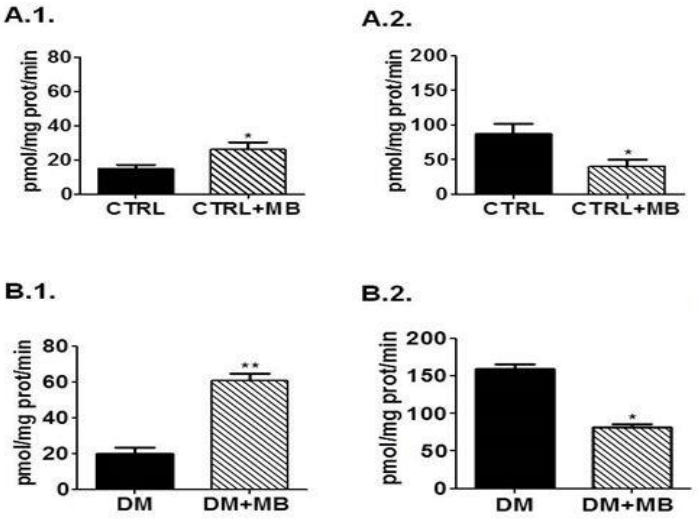

Figure 5. Assessment of mitochondrial H2O2 production in CTRL (A) and DM (B) groups. Additions are as follows: A1, B1: GM (5 mM glutamate + 5 mM malate), and A2, B2: S/Rot (5 mM succinate + 0.5 μM rotenone). Values are means ± SEM. * p<0.05, ** p<0.01.

Measurement of the Calcium Retention Capacity (CRC). A significant decrease in CRC was found, as expected, in diabetic vs. non-diabetic mitochondria regardless the absence or the presence of cyclosporine (CsA, the classic inhibitor of the mitochondrial permeability transition pore). These results are suggestive for the increased propensity of the diabetic hearts to undergo the phenomenon of permeability transition. However, the addition of MB to mitochondria isolated from either group (diabetic and non-diabetic) did not interfere with the capability of mitochondria to buffer calcium ions (Figure 6).

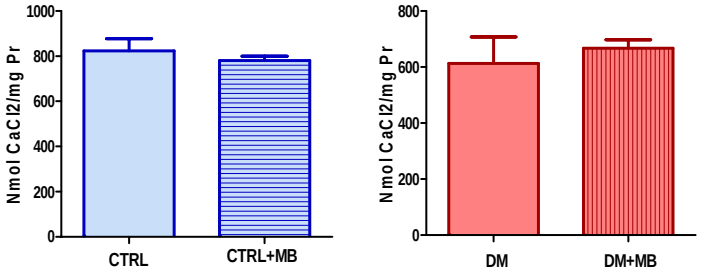

Figure 6. Assessment of calcium retention capacity data in CTRL (A) and DM (B) rats. Values are means ± SEM. *p<0.05, **p<0.01.

Conclusion: Acute *in vitro* administration of methylene blue, in submicromolar doses, improved mitochondrial respiration and elicited a dichotomic, substrate-dependent effect on ROS production in diabetes that clearly warrants further investigation in line with the highly-favored concept of ”drug repurposing”.

3. METHYLENE BLUE ALLEVIATES ENDOTELIAL DYSFUNCTION & REDUCES OXIDATIVE STRESS IN AORTAS FROM DIABETIC RATS

The present study was aimed to investigate whether MB might contribute to the improvement of vascular function and alleviation of oxidative stress in aortic rings isolated from rats with streptozotocin-induced DM. The effects of MB (0.1μM, 30min *ex vivo* incubation) on vascular reactivity in organ chamber (phenylephrine-induced contraction, acetylcholine-induced relaxation) and H_2O_2 production (assessed by FOX assay) were investigated in vascular preparations with intact endothelium and after denudation, respectively (Fig. 7).

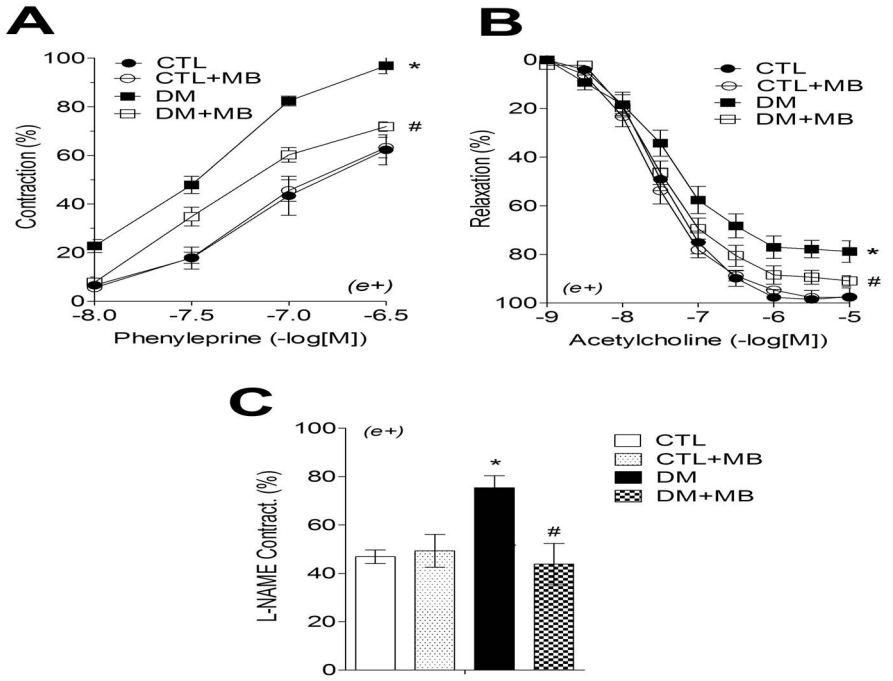

Figure 7. Assessment of the MB effects on vasomotor function in aortas with intact endothelium: (A) Phenylephrine induced contraction, (B) Acetylcholine-induced relaxation and (C) Contraction to L-NAME (10 μM) in aortic rings isolated from diabetic (DM) and control (CTL) rats in the presence vs. absence of MB (0.1 μM, 30 min incubation), n=10, *p<0.05 DM vs. CTL. #p<0.05 with and without MB.

A significant reduction in phenilephrine contraction after incubation with MB was recorded in rat aortic rings with intact endothelium harvested from diabetic vs. non-diabetc animals (Fig. 7A). We have further assessed the endothelial-dependnet relaxation at cumulative doses of acethylcholine; a significant improvement in relaxation was obtained in the presence of MB in vascular preparations from diabetic rats (Fig. 7B). Since vascular relaxation is mediated via the NO signaling pathway we further hypothesized that MB could modulate the NO bioavailability. To this aim, vascular contractility in the

presence of L-NAME (10 µM) was assessed. Since coincubation with MB significantly decreased L-NAME-contractility in aortic segments, we can speculated an increase availability of NO in the vascular walls (Fig.7C).

In line with these results, we envisaged a putative interaction between MB and the NO-dependent pathway at the endothelial layer; accordingly, we recapitulated the experiments in aortas without endothelium (denuded with CHAPS solution). As shown in Fig. 8, the presence of an intact endothelium is mandatory for the favorable effects of MB in diabetic vessels.

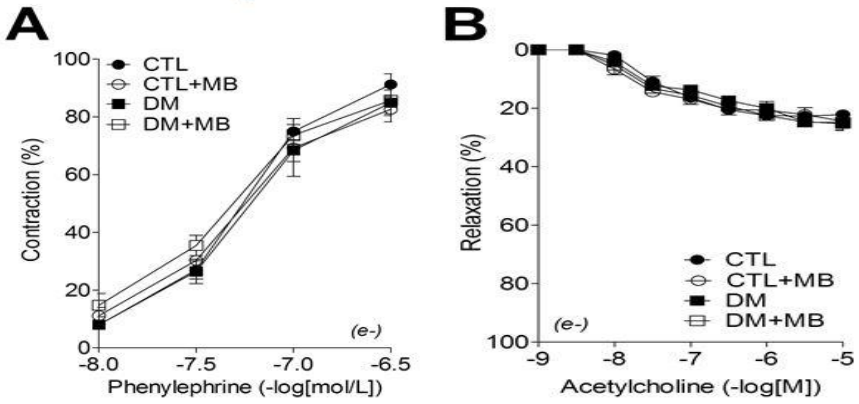

Figure 8. Assessment of the MB affects on vasomotor function in aortas with denuded endothelium: (A) Phenylephrine induced contractions, (B) Acetylcholine-induced relaxation in aortic rings isolated from diabetic (DM) and controls (CTL) rats, after endothelial denudation with CHAPS, in the presence vs. absence of MB (0.1 µM, 30 min incubation), n=10.

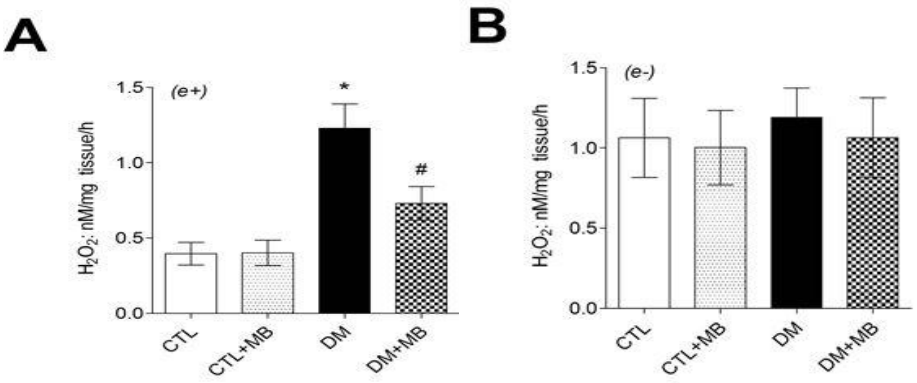

Figure 9. Assessment of the MB effects on ROS production in rat aortas. (A) FOX Assay for H2O2 measurements in rat aortic rings with endothelium and (B) denuded endothelium, isolated from diabetic (DM) and control (CTL) rats in the presence vs. absence of MB (0.1 µM, 30 min incubation), n=10, *p<0.05 DM vs. CTL. #p<0.05 with and without MB.

DM represents a pathological condition that has been systematically associated with increased ROS production. Since *ex vivo* incubation with MB was able to significantly improve the vasomotor function in studies of vascular reactivity, we further investigated whether the MB-related restoration of vascular function could be associated with the mitigation of the oxidative stress. Hydrogen peroxide (H_2O_2) was measured by means of the FOX assay. Incubation for 30 minutes with MB significantly reduced the amount of H_2O_2 in diabetic samples suggesting that, indeed, the reduction of oxidative stress contributes to the beneficial vascular effects of MB (Fig. 9A). Importantly, in aortic samples without endothelium, the beneficial effect of MB on H_2O_2 generation was abolished (Fig. 9B).

Conclusion: We have demonstrated here that experimental diabetes is associated with increased H_2O_2 production in rat aortas that elicits NO signaling impairment and subsequent endothelial dysfunction. *Ex vivo* treatment with MB was able to restore the vascular reactivity and mitigate oxidative stress in an endothelial-dependent manner. Both areas appear ripe for further study of the potential beneficial effects of MB in the clinical setting of diabetic vascular complications.

GENERAL CONCLUSIONS

In the present thesis, the contribution of methylene blue to the cellular protection at the level of cardiomyocytes and vascular endothelium was assessed; it also partially elucidated the methylene blue effects, mainly on the mitochondrial function.

Accordingly, the general conclusions of this doctoral thesis are:

1. MB exerts protective effects on cardiomyocytes by improving the cell bioenergetic profile, with the increase in both oxygen consumption and the extracellular acidification rates.
2. MB protects the cardiac mitochondrial function via the stimulation of mitochondrial respiration and modulation of H_2O_2 production, in both healthy and diabetic rats.
3. MB improves the vascular vasomotor function via an endothelium dependent mechanism.
4. MB ameliorates endothelial dysfunction and reduces the vascular oxidative stress in the setting of experimental diabetes mellitus.

ORIGINAL CONTRIBUTIONS

The original contributions of the present doctoral thesis can be resumed as follow:

1. Characterization for the first time at international level of the double modulator effect of methylene blue on cellular bioenergetics and glycolysis and the stimulation of mitochondrial respiration, in an experimental model of diabetes mellitus.
2. The first international description of the beneficial effect of methylene blue in alleviating the endothelial dysfunction and oxidative stress in an experimental model of diabetes mellitus.

LIST OF PUBLISHED PAPERS

This doctoral thesis is based on the following original articles:

1. **Privistirescu, I.A.,** Sima, A., Duicu, O.M., Timar, R., Roșca, M.G., Sturza, A., Muntean, D.M. *Methylene blue alleviates endothelial dysfunction and reduces oxidative stress in aortas from diabetic rats.* **Can J Physiol. Pharmacol** 2018; Jun 12:1-5. doi: 10.1139/cjpp-2018-0119 (ISI Journal, IF: 2.21)
2. [*]Duicu, O.M., [*]**Privistirescu, A.,** Wolf, A., Petruș, A., Dănilă, M.D., Rațiu, C.D., Muntean, D.M., Sturza, A. *Methylene blue improves mitochondrial respiration and decreases oxidative stress in a substrate-dependent manner in diabetic rat hearts.* **Can J Physiol Pharmacol** 2017; 95(11): 1376-1382. *(*Equal contribution)* (ISI Journal, IF: 2.21)
3. Duicu, O., Scurtu, I., Popescu, R., Sturza, A., Coricovac, D., Danila, M., **Privistirescu, A.,** Muntean, D. *Assessment of the effects of methylene blue on cellular bioenergetics in H9c2 cells*. **Rev Chim (Bucharest)** 2015; 66(4): 519-522. (ISI Journal, IF: 0,956)