Cardioprotection by *methylene* Blue Against Epinephrine-Induced Cardiac Arrhythmias and Myocardial Injury

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Abstract: - Methylene blue is used in the treatment of vasoplegic syndrome after cardiac surgery, anaphylaxis, and septic shock refractory to epinephrine and fluid resuscitation. In this study, we investigated the potential protective effect of methylene blue on the development of cardiac arrhythmias after injection of epinephrine in rats. Methylene blue was given intraperitoneally at doses of 50 or 100 mg/kg. Cardiac arrhythmia was then induced with 10 μg/kg of epinephrine intravenously. In untreated, control rats, epinephrine caused bradycardia (96.48 ± 1.06 *vs.* 365.03 ± 0.68 beats/min), increased PR interval (0.54 ± 0.04 *vs.* 0.039 ± 0.004), RR interval (0.64 ± 0.003 *vs.* 0.16 ± 0.004 sec), shortened QTc interval (0.067 ± 0.05 *vs.* 0.1 ± 0.004 sec), increased QRS duration (0.048 ± 0.005 *vs.* 0.028 ± 0.002 sec), decreased R wave amplitude (0.3 ± 0.03 *vs.* 0.49 ± 0.04 mv), decreased the height of the ST segment (-0.0696 ± 0.004 *vs.* -0.0054 ± 0.003 mv), and caused ventricular extrasystoles (7.92 ± 0.56 *vs.* 0.5 ± 0.5). Methylene blue given at 50 or 100 mg/kg increased the heart rate, decreased RR interval, QRS duration and the drop in the ST height, increased duration of QTc interval and R wave amplitude and decreased the number of extrasystoles. The histological study showed that methylene blue protected against myocardial structural disorganization, cellular damage, necrosis, and haemorrhage between muscle fibres induced by epinephrine injection. We conclude that methylene blue dose-dependently prevented epinephrine-induced arrhythmias and cardiac muscle injury.

Key-Words: - methylene blue; epinephrine; cardiac arrhythmia; cardioprotection

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1 Introduction

Methylene blue is an autoxidizable synthetic phenothiazine dye with a wide range of clinical applications. The dye has been used in treatment of methemoglobinaemia by virtue of its redox-cycling between its blue oxidized and colorless reduced (leuco-methylene blue) states [1]. It is also administered to treat malaria [2] and for the prevention and treatment of encephalopathy associated with the alkylating drug ifosfamide in patients with cancer [3]. One of the most important

clinical uses of methylene blue, however, is its intravenous infusion in patients with vasoplegic shock occurring during cardiopulmonary bypass surgery. The vasoplegic syndrome is characterized by decreased systemic vascular resistance and severe hypotension which is refractory to therapy with vasopressors eg., catecholamines and vasopressin and intravenous administration of fluids [4,5]. This state of vasodilatory shock is caused by the development of a systemic inflammatory response and the over production of nitric oxide [6,7]. Methylene blue is an inhibitor of nitric oxide synthases and guanylyl cyclase enzymes thereby block the production of nitric oxide and antagonize its vasorelaxant effects [8,9]. This property of methylene blue is largely thought to mediate its beneficial effects in increasing the systemic vascular resistance and mean arterial blood pressure when administered in cardiosugery, obviating the need for high doses of catecholamine drugs and vasopressin [10].

The intraoperative use of epinephrine to treat hypotension associated with vasoplegic shock or other clinical states carries the risk of inducing ventricular arrhythmia and damage to the myocardium [11,12]. Epinephrine mediates its cardiovascular effects by stimulating cardiac β1- and β2- adrenergic receptors causing an increase in heart rate, myocardial contractility and increased excitability of pacemaker tissue that may result in ventricular arrhythmias. It also acts on α1-adrenergic in cardiac and vascular smooth muscle in high doses causing cardiac stimulation and increased peripheral vascular resistance [13,14].

2 Materials and Methods

2.1 Animals

Male Sprague-Dawley rats weighing 170-180 g were used in the study. Rats were obtained from the Animal House Colony of the National Research Centre. Animals were kept under temperature- and light-controlled conditions (20–22 °C and 12 h/12 h light/dark cycle) and given free access to tap water and standard laboratory rodent chow. Animal procedures followed the guidelines of the Institute ethics committee for the use of animals in experimental studies and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

2.2 Drugs and Chemicals

Methylene blue (Sigma Chemical Co., St. Louis, MO, U.S.A) and epinephrine (Nile Co., Egypt) were used in the study and freshly dissolved in saline before the experiments to obtain the necessary doses.

2.3 Experimental Groups

Rats were randomly divided into four equal groups (n=8/group) and treated as follows:
Group 1: received intraperitoneal (i.p.) saline and served as negative control.
Group 2: were given i.p. saline prior to induction of cardiac arrhythmia by intravenous injection of 10 μg/kg of epinephrine (positive control).
Group 3: were treated with i.p. methylene blue at 50 mg/kg, 30 min before the induction of arrhythmia.
Group 4: received i.p. methylene blue at 100 mg/kg, 30 min before the induction of arrhythmia.

2.4 Electrocardiography

After 30 min of drug or saline administration, rats were anesthetized with i.p injection of 45 mg/kg thiopental. ECG was recorded using the ECG Powerlab module, which consists of Powerlab/8sp and Animal Bio-Amplifier (Australia), in addition to Lab Chart 7 software with ECG analyzer.
After a steady state was established, arrhythmia was induced by intravenous injection of epinephrine at 10 μg/kg and ECG recording continued until the termination of the arrhythmia [15]. The heart rate, RR interval, PR interval, QRS interval, QT Interval, QTc, R wave amplitude, ST height, number of extrasystoles, and duration of heart block after epinephrine injection were determined.

2.5 Cardiac Histopathology

Cardiac specimens were immediately fixed in 10% formalin at room temperature, treated with a conventional grade of alcohol and xylol, embedded in paraffin and sectioned at 5 μm thicknesses. The sections were stained with haematoxylin and eosin (H&E) in order to study the histopathological changes using a light microscope (Olympus Cx 41 with DP12 Olympous digital camera).

2.6 Statistical Analysis

Data are presented as mean ± SE for measurement variables. Comparison between groups was performed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. GraphPad Prism 6 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA) was used and differences were considered

statistically significant when probability values were less than 0.05.

3 Results

3.1 Electrocardiographic Results

Following epinephrine injection, ECG recordings showed variable degrees of bradycardia and ventricular extrasystoles. Representative ECG tracings in saline control and after epinephrine injection are shown in figures 1 & 2. The ECG changes induced by epinephrine were ameliorated by prior treatment with methylene blue in a dose-dependent manner (Figure 3 & Figure 4).

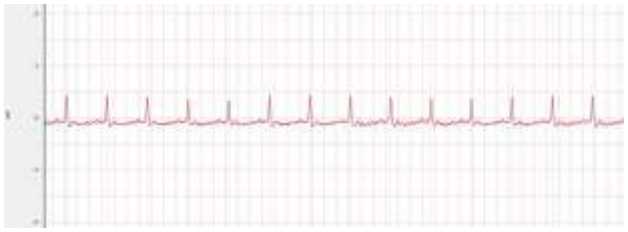

Fig. 1: Representative ECG tracing in saline control.

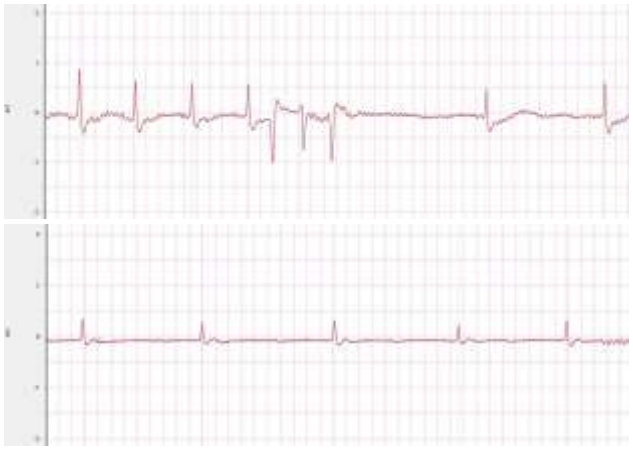

Fig. 2: Two representative ECG tracings of the changes induced by epinephrine injection. Ventricular premature beats and bradycardia.

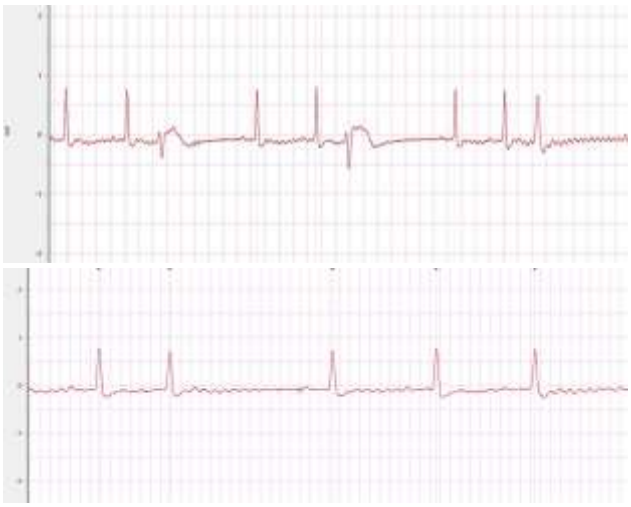

Fig. 3: Two representative ECG tracings of the effect of methylene blue at 50 mg/kg on the changes induced by epinephrine injection.

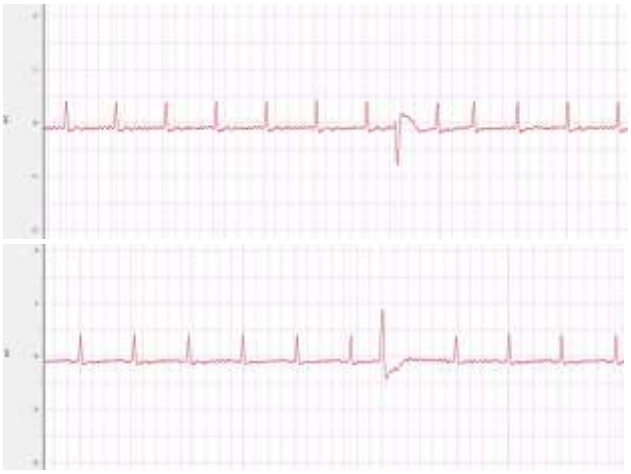

Fig. 4: Two representative ECG tracings of the effect of methylene blue at 100 mg/kg in epinephrine-treated rats.

The heart rate of saline control rats was 365.0 ± 0.68 beats/min. Epinephrine control had significantly lower heart rate by 73.6% (96.5 ± 1.06 beats/min). Methylene blue given at 50 or 100 mg/kg reversed the epinephrine-induced bradyarrhythmia and increased the heart rate by 124.5% and 130.5%, respectively, as compared to the epinephrine control group. Epinephrine injection significantly increased RR interval by 300% from 0.16 ± 0.004 sec in the saline control group to 0.64 ± 0.003 sec. Treatment with methylene blue 50 or 100 mg/kg reduced the RR interval after epinephrine-induced arrhythmia by 57.8% and 60.9%, respectively, compared to the epinephrine control group.

Rats treated with epinephrine exhibited significantly longer PR interval by 38.5% as compared to the

saline group. Methylene blue had no significant effect on PR interval in the epinephrine-treated rats. Epinephrine significantly shortened QTc interval by 35.6% as compared to the saline control. Methylene blue given at 50 or 100 mg/kg prevented the epinephrine-induced shortening of QTc interval. Epinephrine control rats exhibited longer QRS duration compared with the saline control by 71.4%. The higher dose of methylene blue brought QRS duration almost to the normal saline group value.

R wave amplitude of the epinephrine group was significantly decreased by 38.8% compared with the saline control value. However, methylene blue given at 50 or 100 mg/kg, increased R wave amplitude by 153.3% and 76.7%, respectively, compared to the epinephrine control value. It was also noted that R wave amplitude in rats treated with methylene blue at 50 mg/kg was significantly higher than in the 100 mg/kg group.

The ST height is different in rodents than humans as it is not iso-electric and appears as a shoulder emerging from QRS complex. In the epinephrine group, ST height decreased to -0.0696 ± 0.004 mv from normal saline control value of -0.0054 ± 0.003 mv. Methylene blue administered at 50 mg/kg, significantly increased the drop in ST height. In contrast, treatment with 100 mg/kg methylene blue returned the ST height to comparable value to that of the saline group value.

Methylene blue (50 or 100 mg/kg) reduced the duration of epinephrine-induced bradycardia by 60.3% and 92.0%, respectively. It also significantly reduced the number of epinephrine-induced extrasystoles by 71.6% and 89.4%. The effect of methylene blue on ECG tracings and ECG parameters is shown in Table 1, Figure 5 & Figure 6.

Table 1. Effect of methylene blue on epinephrine-induced electrocardiogram parameters and arrhythmia.

Parameter/ Group	**Normal control**	Epinephrine	Epinephrine + MB 50 mg/kg	Epinephrine + MB 100 mg/kg
Heart rate (bpm)	365.0 ± 0.68	96.5 ± 1.06*	216.6 ± 0.85^{*+}	222.4 ± 1.64$^{*+\#}$
RR interval (s)	0.16 ± 0.004	0.64 ± 0.003*	0.27 ± 0.004^{*+}	0.25 ± 0.002$^{*+\#}$
PR interval (s)	0.039 ± 0.004	0.054 ± 0.004*	0.055 ± 0.003*	0.049 ± 0.003
QT interval (s)	0.042 ± 0.003	0.053 ± 0.005	0.056 ± 0.003	0.049 ± 0.006
QTc interval (s)	0.104 ± 0.004	0.067 ± 0.006*	0.13 ± 0.004^{*+}	0.09 ± 0.005$^{*+\#}$
QRS duration (s)	0.028 ± 0.002	0.048 ± 0.005*	0.042 ± 0.003*	0.024 ± 0.003$^{+\#}$
R wave amplitude (mv)	0.49 ± 0.004	0.30 ± 0.003*	0.76 ± 0.004^{*+}	0.53 ± 0.005$^{*+\#}$
ST segment height (mv)	-0.005± 0.003	-0.07 ± 0.005*	-0.12 ± 0.005*	-0.02 ± 0.004$^{+\#}$
Duration of bradycardia (s)	0.0 ± 0.0	831.7 ± 16.9*	330.6 ± 6.3*	66.25 ± 15.0^{+}
Number of extrasystoles	0.0 ± 0.0	7.92 ± 0.56*	2.25 ± 0.25^{*+}	1.5 ± 0.19^{+}

MB: methylene blue. Data were expressed as mean ± SE (n = 12). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. *p<0.05: significantly different from normal control group. +p<0.05: significantly different from epinephrine control group. #p<0.05: significantly different from the MB 50 mg/kg group.

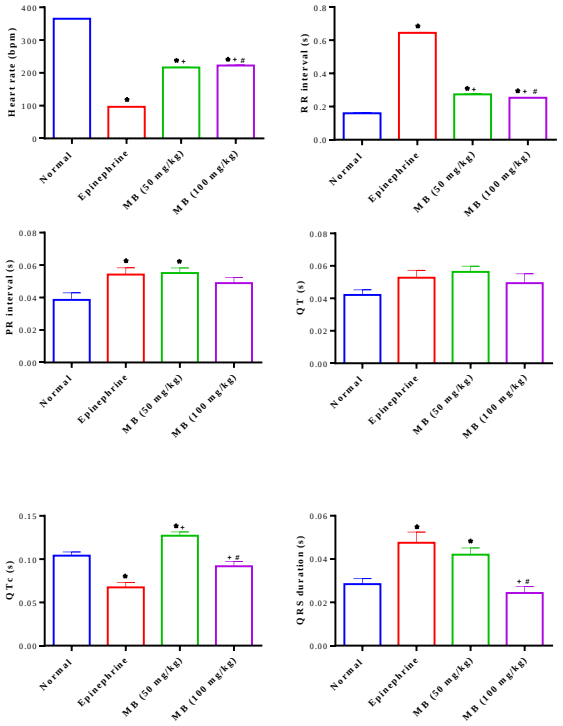

Figure 5. Effects of treatment with methylene blue (MB) on the epinephrine-induced changes in heart rate, RR interval, PR interval, QT, QTc, and QRS duration. *p<0.05: significantly different from normal control group. +p<0.05: significantly different from epinephrine control group. #p<0.05: significantly different from MB 50 mg/kg group.

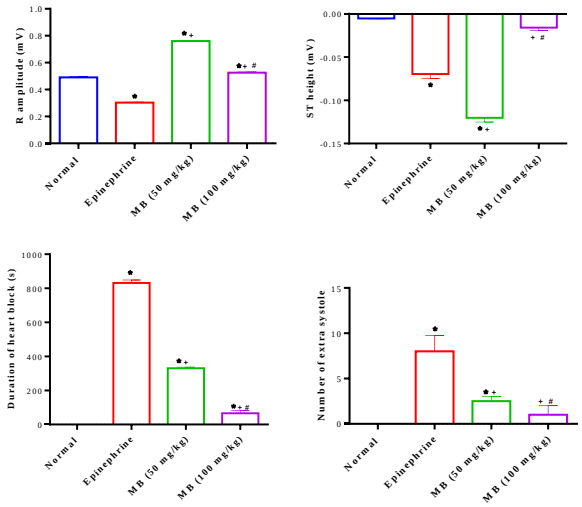

Fig. 6: Effects of treatment with methylene blue (MB) on the epinephrine-induced changes in R wave amplitude, ST wave height, duration of bradycardia and number of ventricular extrasystoles. *p<0.05: significantly different from normal control group. +p<0.05: significantly different from epinephrine control group. #p<0.05: significantly different from MB 50 mg/kg treatment group.

3.2 Histopathological Results

In the saline control group, the myocardium was formed of longitudinally striated cardiac myocytes with central oval pale nuclei. They were joined together by intercalated discs and appeared branching and anastomosing forming muscle sheets. In-between the cardiac myocytes, there was a delicate layer of connective tissue with well-demonstrated blood vessels (Fig.7A). In the adrenaline only group, the myocardium demonstrated structural disorganization with features of inflammation and cellular damage, necrosis, widening of the intercellular spaces with active fibroblasts and thickened delicate connective tissue. There were many dilated, congested blood vessels, haemorrhage between muscle fibres, and pyknotic nuclei (Fig. 7B).

Sections of the adrenaline and methylene blue at 50 mg/kg group presented mild to moderate features of myocardial lesion with dilated congested blood vessels, inflammatory cells infiltrations, mild deeply stained (pyknotic) nuclei and mild thickened delicate connective tissue (Fig. 7C). Whereas, sections of the adrenaline and methylene blue at 100 mg/kg group showed nearly normal histological architecture, except for the presence of dilated congested blood vessels, mild thickened delicate

connective tissue, and very few shrunken darkly stained nuclei (Fig. 7D).

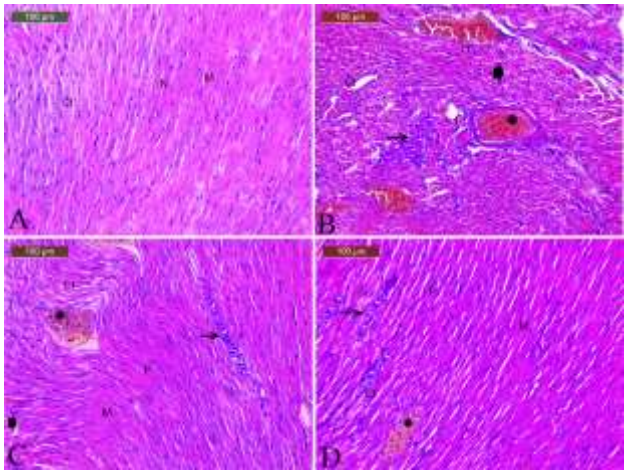

Fig. 7: Representative photomicrographs of (A): Saline control showing normal histological architecture of cardiac myocytes (M), most appear longitudinally with rounded vesicular centrally located nuclei (N), in-between the cardiac myocytes, there was a delicate layer of connective tissue. (B) Adrenaline control showing disorganization cardiac structure, with features of inflammation (arrow), necrosis (arrowhead) with widening of the intercellular spaces with active fibroblasts (F), thickened delicate connective tissue (Ct), dilated, congested blood vessels (Star), haemorrhage (H) between muscle fibres, and pyknotic nuclei (P). (C) Adrenaline and methylene blue 50 mg/kg showing moderate features of myocardial lesion with mild cellular inflammation (arrow), mild necrosis (arrowhead), pyknotic nuclei (P), slight congested blood vessels (star). (D) Adrenaline and methylene blue 100 mg/kg showing most of cardiac muscle fibres appearing more or less normal. Few areas still show cellular inflammation (arrow), mild necrosis (arrowhead), pyknotic nuclei (P), and slight congested blood vessels (star).

4 Discussion

The results of the present study showed that epinephrine-induced arrhythmia and ECG changes were reduced by prior with methylene blue. The i.v. administration of 10 µg/kg epinephrine caused severe bradycardia and first degree heart block as well as polymorphic ventricular premature beats. The ECG showed increased RR, PR and QTc intervals, QRS widening, a decrease in R wave height and ST segment. Methylene blue prevented these epinephrine-induced changes accompanied by marked decrease in the duration of heat block and number of ventricular extrasystoles. The study supports and extends previous work in which methylene blue protected against arrhythmias and restored contractility in mouse cardiac myocytes after intoxication with hydrogen sulfide [16].

The mechanism of epinephrine-induced arrhythmia and cardiac muscle injury is thought to involve direct stimulation of β-adrenoceptors in cardiomyocytes resulting in increased cyclic AMP and intracellular Ca^{2+}, while stimulation of α-adrenergic receptors in coronary arteries induces coronary spasm and myocardial ischemia. Added to this are the effects evoked by the oxidation products adrenochromes and oxyradicals [17]. In the perfused rat heart preparation, the leakage of lactate dehydrogenase induced by epinephrine is prevented by the β1-adrenoceptor antagonist atenolol [18]. It has also been suggested that epinephrine induces cardiac arrhythmia partly by the local release of acetylcholine i.e. a cholinergic mechanism is involved [19]. Moreover, intracerebroventricular (i.c.v.) injection of dynorphin caused an increase in the threshold for epinephrine-induced ventricular arrhythmias which is opposed by i.c.v. or i.v. atropine sulfate, suggesting mediation by central cholinergic mechanisms [20]. Other studies showed that the antiarrhythmic action of bradykinin [15] or ATP-sensitive potassium channel opener nicorandil [21] against epinephrine arrhythmia is mediated by nitric oxide.

Methylene blue has been used in treatment of hypotension and reduced systemic vascular resistance i.e., vasoplegic shock, that occurs during cardiopulmonary bypass despite intravenous norepinephrine and fluids [10]. This condition is thought to be caused in part by initiation of a systemic inflammatory response resulting in increased production of reactive oxygen metabolites, cytokines, and increased production of nitric oxide by the enzyme nitric oxide syntheses (NOS) [6,7]. The administration of ethylene blue in the event of cardiopulmonary bypass results in improved hemodynamic, reduced the requirement of vasopressin agents and decreased serum lactate levels [22]. Methylene blue was also reported to increase mean arterial pressure and improve cardiac function in septic shock [23]. The beneficial effect of methylene blue is largely attributed to counteracting the vasorelaxant action of the excessively released nitric oxide [10, 24]. Methylene blue inhibits nitric oxide production through inhibition of nitric oxide synthases [9]. Moreover, methylene blue binds to the heme moiety of the enzyme guanylate cyclase. In this way, the

dye prevents the activation of guanylyl cyclase, the increase in cyclic guanosine 3'5' -monophosphate and the subsequent vascular relaxation [9, 24]. It is not clear, however, that this action of methylene blue in inhibiting nitric oxide production have accounted for its antiarrhythmic effect reported herein.

Methyblue is highly lipophilic and readily crosses the blood brain barrier [25] and exert neuroprotective effects [26,27]. The dye and its metabolites are also a reversible and competitive inhibitor of both acetylcholinesterase and butyrylcholinesterase [28-30]. In view of data suggestive of a cholinergic mechanism being involved in epinephrine arrhythmia [19,20], an intriguing possibility is that the above action of methylene blue may underlie at least in a part its antiarrhythmic properties.

Oxidative stress has been involved in the genesis of cardiac arrhythmias [31,32]. In rats with epinephrine arrhythmia, the glutathione precursor N-acetyl-L-cysteine or vitamin E were reported to decrease the duration and increase the time of onset of arrhythmias, possibly by reducing the level of the oxidation products of catecholamines aminochromes [33]. These products increase intracellular Ca^{2+}, induce coronary spasm, depletion of high energy stores, subcellular alterations and cause ventricular arrhythmias and myocardial cell damage [17]. Methylene blue has antioxidants effects. It was shown to inhibit the enzyme xanthine oxidase and the subsequent generation of superoxide radicals [34]. The dye is a redox-cycling agent, a blue cation which is reduced by nicotinamide adenine dinucleotide phosphate (NADPH) or thioredoxin to give the uncharged and colorless leucoMB to be re-oxidized by O_2 [24] Methylene blue reduces the formation of brain mitochondrial reactive oxygen radicals and thus protects and enhances mitochondrial function and affords neuroprotection [35]. It also enhances the electron transport chain, thereby promoting oxygen consumption [36]. In cardiac mitochondria isolated from diabetic and normal rat hearts, methylene blue improved mitochondrial respiratory function [37]. The dye was also shown to restore ATP levels in cardiomyocytes exposed to toxic concentrations of hydrogen sulfide [16]. The above mentioned effects of methylene blue may explain its antiarrhythmic and cardioprotective effects reported in the present study.

5 Conclusion

We conclude that the epinephrine-induced bradyarrhythmia, ventricular premature beats and myocardial injury is prevented by prior administration of methylene blue. The mechanism is not clear but may involve antioxidant action and improved mitochondrial function. Further work is needed in order to elucidate the exact mechanism by which methylene blue exerts its antiarrhythmic and cardioprotective effects.

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Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

Omar Abdel-Salam, Marawan Abd El Baset, and Amany Sleem designed the study. Marwan Sayed conducted the experiments. Enayat Omara performed the histopathology and its interpretation. Omar Abdel-Salam prepared the manuscript. Omar Abdel-Salam, Marawan Abd El Baset, Amany Sleem and Enayat Omara approved the final version of the manuscript.

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Conflict of Interest

The authors have no conflict of interest to declare.