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ORIGINAL INVESTIGATION

Methylene blue as an adjuvant during cardiopulmonary resuscitation: an experimental study in rats

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Abstract

Introduction: Methylene Blue (MB) has been shown to attenuate oxidative, inflammatory, myocardial, and neurological lesions during ischemia-reperfusion and has great potential during cardiac arrest. This study aimed to determine the effects of MB combined with epinephrine during cardiac arrest on myocardial and cerebral lesions.

Method: Thirty-eight male Wistar rats were randomly assigned to four groups: the sham group (SH, n = 5), and three groups subjected to cardiac arrest (n = 11/group) and treated with EPI 20 $\mu g.kg^{-1}$ (EPI), EPI 20 $\mu g.kg^{-1}$ + MB 2 $mg.kg^{-1}$ (EPI + MB), or saline 0.9% 0.2 ml (CTL). Ventricular fibrillation was induced by direct electrical stimulation in the right ventricle for 3 minutes, and anoxia was maintained for 5 minutes. Cardiopulmonary Resuscitation (CPR) consisted of medications, ventilation, chest compressions, and defibrillation. After returning to spontaneous circulation, animals were observed for four hours. Blood gas, troponin, oxidative stress, histology, and TUNEL staining measurements were analyzed. Groups were compared using generalized estimating equations.

Results: No differences in the Returning of Spontaneous Circulation (ROSC) rate were observed among the groups (EPI: 63%, EPI + MB: 45%, CTL: 40%, $p = 0.672$). The mean arterial pressure immediately after ROSC was higher in the EPI+MB group than in the CTRL group (CTL: 30.5 [5.8], EPI: 63 [25.5], EPI+MB: 123 [31] mmHg, $p = 0.007$). Serum troponin levels were high in the CTL group (CTL: 130.1 [333.8], EPI: 3.70 [36.0], EPI + MB: 43.7 [116.31] ng/mL, $p < 0.05$).

Conclusion: The coadministration of MB and epinephrine failed to yield enhancements in cardiac or brain lesions in a rodent model of cardiac arrest.

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Introduction

Current guidelines for treating cardiac arrest still recommend using epinephrine (EPI)[1] because of its association with a higher rate of ROSC. However, there is a rising controversy regarding its safety. EPI use has been associated with low cardiac output and reduced coronary perfusion after returning to spontaneous circulation[2] and myocardial lesions,[3] and its use does not affect neurological function.[4] Thus, strategies to improve resuscitation outcomes have been widely suggested; such strategies include devices that enhance compression-decompression quality to therapies that minimize injury followed by reperfusion.[5]

Methylene Blue (MB) is a heterocyclic aromatic substance with diverse clinical applications and is considered a low-evidence therapeutic option for refractory distributive shock in which Nitric Oxide (NO) upregulation prevails. MB restores vascular tone by inhibiting induced NO synthase and enzyme-soluble guanylate cyclase and reducing the vascular response to cyclic Guanosine Monophosphate (cGMP), which is involved in vasodilation mechanisms.[6] In addition, MB mimics xanthine oxidase action by avoiding the conversion of oxygen into superoxide, lowering oxidative stress due to its actions as a free-radical scavenger.[7,8]

MB applied in experimental models of cardiac arrest showed promising results. In a porcine model of cardiac arrest, MB improved overall survival and reduced oxidative stress and neuronal injury when associated with hypertonic saline.[9] Another porcine study evaluated the use of MB associated with vasopressin, and neurological lesions after cardiac arrest were decreased.[10] However, these studies were performed under vasopressin use, and the effects of the association of MB with EPI on organ protection and ROSC rates are unknown.

Since EPI is the first-line treatment for cardiac arrest,[1] this study aimed to determine the effects of the association between MB and EPI on cardiac and brain lesions of rats that returned to spontaneous circulation after induced cardiac arrest.

Methods

The University of São Paulo Medical School Ethics Committee for Research Project Analysis approved the experimental protocol under the number 021/16. Animals were treated humanely in accordance with the ethical principles advocated by The Brazilian College of Animal Experimentation.

Groups and treatments

Male Wistar rats (12–20 weeks of age, 386 grams $\pm$ 60 grams) were maintained under standard conditions, and food and water were provided *ad libitum*. Rats (n = 38) were assigned to four groups by simple randomization. The first group was the Sham (SH) group. The other three groups were subjected to cardiac arrest and treated as follows: epinephrine (EPI, n = 11), treated with epinephrine 20 mcg.kg^{-1}, diluted in saline to a total volume of 0.2 ml; epinephrine and methylene blue (EPI + MB, n = 11), animals received epinephrine 20 mcg.kg^{-1} and MB 2 mg.kg^{-1}, diluted in saline to a total volume of 0.2 mL; and control (CTL, n = 11), treated with saline 0.2 ml. As CPR started, the specific treatment was administered and repeated every 3 minutes, as advocated by the American Heart Association (AHA) protocol.[1] The MB dose was limited to 3 doses due to its potential toxicity. The SH group (n = 5) was anesthetized for four hours, and all surgical procedures were performed except for Ventricular Fibrillation (VF) and cardiopulmonary resuscitation.

Cardiac arrest model

General anesthesia was induced with 5% isoflurane followed by intubation with a 14 Fr catheter and mechanical ventilation (8 ml/kg tidal volume, 60 cycles/min respiratory rate, and oxygen fraction 100%, Harvard Apparatus, Inc., Holliston, MA, USA). Venous access was obtained by catheterizing the right internal jugular vein. A catheter was applied in the right ventricle to induce ventricular fibrillation, and the left femoral artery was cannulated with a PE10 catheter for invasive pressure monitoring and blood sampling. Heart rate and blood pressure were recorded using the Biopac MP-120 system (Biopac Systems Inc, EUA).

The cardiac arrest protocol was previously described by Lamoureux L et al, 2015.[11] Briefly, ventricular fibrillation was induced by 1 mA and 60 Hz and maintained for 3 minutes. When the absence of pulsatile arterial pressure was detected, mechanical ventilation was interrupted, and the anoxia persisted for 8 minutes before starting CPR (3 minutes of electrical stimulation and 5 minutes of untreated ventricular fibrillation).

CPR was provided through an automatic external compressor to maintain a 200/minute heart rate and a maximum compression depth of 1.2 cm. Mechanical ventilation was set to provide a 25–30 cycles/minute Respiratory Rate (RR). VF and pulseless ventricular tachycardia were treated with an electrical currency of 7J. The total CPR duration was limited to 20 minutes. The initial two cycles lasted 3 minutes each, and the following cycles lasted one minute[12] (Fig. 1).

Return of Spontaneous Circulation (ROSC) was defined as the presence of a pulsatile arterial wave, and data were analyzed when ROSC occurred with a mean Blood Pressure (BP) of 25 mmHg for 10 minutes. Mechanical ventilation was reassumed with an increased RR (60 cycles/min), and isoflurane was restarted if muscular movement occurred, or BP increased above basal levels. Blood samples were collected at baseline (initial), 10 minutes after ROSC, and after 4 hours of observation (final).

After ROSC, the animal was declared nonsurviving if BP decreased below 25 mmHg for more than 5 minutes. For the nonsurviving and euthanized rats, (anesthetics overdose) blood was collected from the abdominal aorta, and organs were harvested for *in vitro* analysis.

Serum analysis

Blood gas analysis and electrolyte (glucose, sodium, potassium, calcium, and lactate) measurements were performed after arterial cannulation, 10 minutes after ROSC, and at the end of the experiment. Troponin I levels were quantified at death or four hours after ROSC using the ADVIA Centaur TnI-Ultra® kit (Siemens Healthcare Diagnostics, Tarrytown, New York, USA) following the manufacturer's protocols.

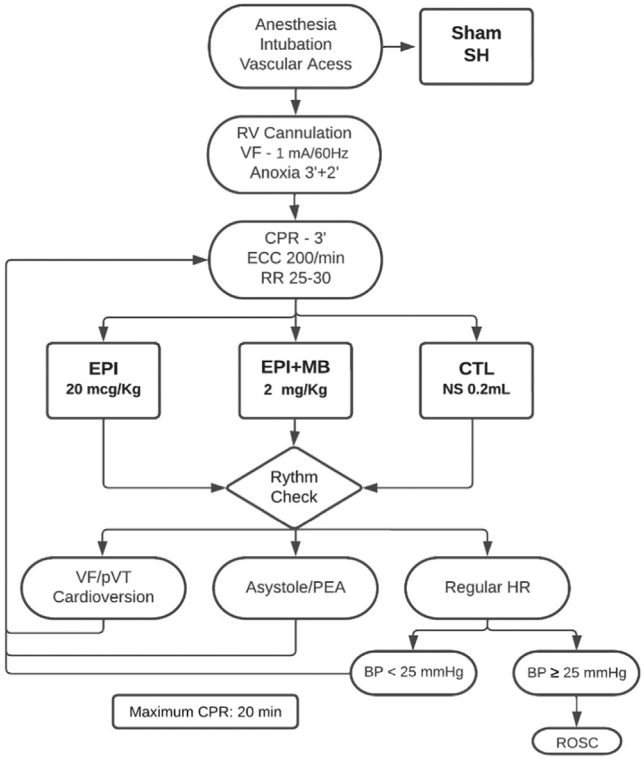

Figure 1 Cardiopulmonary resuscitation protocol. RV (Right Ventricle), VF (Ventricular Fibrillation), CPR (Cardiopulmonary Resuscitation), RR (Respiratory Rate), pVT (pulseless Ventricular Tachycardia), PEA (Pulseless Electrical Activity), ROSC (Return of Spontaneous Circulation). Rats experienced only surgical intervention (SH, n = 11), or animals were subjected to CA and treated with epinephrine (EPI, n = 11), epinephrine and MB (EPI + MB, n = 11), or saline (CTL, n = 11).

Histopathology

After aortic exsanguination, the heart and brain were harvested, infused with 4% paraformaldehyde for 24 hours, dehydrated with an alcohol gradient, and embedded in paraffin. Slices of 5 μm were stained with Harris hematoxylin and eosin and evaluated by a blinded pathologist.

Brain tissue was evaluated for gliosis and red neurons, which consist of cytoplasmic reorganization, eosinophilia, cell body retraction, and nuclear pyknosis. Coagulation necrosis was assessed in the heart tissue and was characterized by fiber eosinophilia, loss of striations, and cytoplasmic vacuolization.

Immunohistochemistry

TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) was applied to evaluate cerebral and cardiac injury. Tissue sections of 4 μm were placed on silanized slides (Sigma Chemical Co, St Louis, Missouri, USA) and subjected to deparaffinization using baths of xylene, alcohol, distilled water, and Phosphate-Buffered Saline (PBS).

The recovery of antigenic sites was obtained by proteinase K and peroxidase blockage by 0.3% H_2O_2 in methanol. The slices were washed with distilled water and PBS before incubation with 50 μL of TUNEL mixture in a humid chamber for 1 hour and a peroxidase converter for 30 minutes. The slices were washed with PBS and incubated with Diaminobenzidine (DAB) for 10 minutes at room temperature, followed by counterstaining with methyl green. A blinded pathologist evaluated 25 fields from brain tissue, left ventricle, and right ventricle using 100 $\times$ magnification.

Thiobarbituric Acid Reactive Substances (TBARS)

Oxidative stress was assessed by thiobarbituric acid reactive substance levels in the brain and heart tissue; TBARS is a lipid peroxidation marker. An aliquot of protein solution (0.2 ml) was extracted from the tissue and diluted in 0.8 ml of distilled water. Trichloroacetic acid 17.5% (ATC — 1 ml) and thiobarbituric acid (Ph 2, 1 ml) were added to the solution, incubated at -80°C for 20 minutes, and then placed on ice. Subsequently, 1 ml of 70% ATC was added, and the mixture was incubated for 20 minutes. The samples were centrifuged at 2000 rpm for 15 minutes, and the optical density of the supernatant was measured at 534 nm against a "blank" reagent on a spectrum photometer. The tissue levels of TBARS are expressed as nmoL/prot/ml.

Statistical analysis

The sample size was detected to identify a 15% difference in troponin-I value among groups, based on literature findings.[13-15] An 80% power at an α level of 0.05 for a two-tailed test indicated 9 animals per group. The sample size was determined set at 11 animals per group to account for losses.

Pooled data were analyzed in R ("R Core Team [2022]. R is a language and environment for statistical computing available from the R Foundation for Statistical Computing, Vienna, Austria") using GeePack. The data are reported as median values with range. Because the sample sizes were small, continuous variables were analyzed among time points, groups, and interactions using Generalized Estimating Equations (GEE) with Tukey's post hoc analysis for multiple comparisons. Categorical variables, such as mortality and histological data, were analyzed using the Chi-Square and Fisher's test; $p < 0.05$ was considered to indicate a statistical significance.

Results

The median resuscitation duration did not differ among groups (EPI: 3 [7.5] min; EPI + MB: 6 [3] min; CTL: 13 [4] min, p = 0.091), and the mortality rate did not vary between groups (EPI: 37% EPI + MB: 55; CTL: 60%, p = 0.647).

Histological, TUNEL, and troponin data are presented in Table 1. The troponin values were significantly higher in groups subjected to cardiac than in the SH group, and there were no significant differences among the cardiac arrest groups. The oxidative stress evaluation through TBARS levels did not indicate differences among the groups subjected to cardiac arrest (Fig. 2).

Concerning myocardium histological evaluation, the EPI +MB group displayed 40% ischemic lesions. Furthermore, the

Table 1 Values of troponin levels, TUNEL values, and anatomopathological evaluation of heart and brain tissues.

Groups	Heart			Brain	
	Troponin (ng/ml)	TUNEL	H&E [1] (%)	TUNEL	H&E [2] (%)
EPI	3.82 [a] (1.3-214.6)	15 [a] (12.9-24.7)	0	12.8 (6.6-19.2)	33
EPI+MB	43.72 [a] (5.9-200.6)	11.8 (6.9-19.6)	40	8.7 (3.9-17.2)	80
CTL	130.1 [a] (10.5-398)	19,1 [a] (12-24.6)	0	12.3 (6.6-22.2)	75
SH	0.021 (0.00-0.82)	10 (9.4-13.2)	0	5.5 (1.7-11.9)	0
p	0.013	0.071		0.308	0.349

Troponin and TUNEL values are expressed as medians (ranges). Hematoxylin and eosin staining of cardiac tissue
[1] (H&E) was used to assess the presence of vacuolization.
[2] H&E staining of brain tissue indicated the existence of red neurons, suggesting hypoxic damage. Rats experienced only surgical intervention (SH, n = 11), or animals were subjected to CA and treated with epinephrine (EPI, n = 11), epinephrine and MB (EPI + MB, n = 11), or saline (CTL, n = 11).
[a] $p < 0.05$ vs. the SH group.

apoptosis rate determined by immunohistochemical assessment was lowest in the EPI + MB group, and it was comparable to that in the SH group (Fig. 3). The cerebral histological evaluation showed red neurons in all animals subjected to cardiac arrest, and the immunohistochemical evaluation showed no differences among the groups (Table 2).

The blood pressure and heart rate measured during CPR are presented in Table 2. Despite methylene blue usage, the groups treated with EPI had significantly higher BP and coronary perfusion after ROSC. However, at the end of the experiment, no significant differences were detected among the groups.

Animals subjected to cardiac arrest exhibited lower pH (EPI: 7.0 [0.17]; EPI + MB: 7.07 [0.08]; CTL: 7.09 [0.09]; SH: 7.42 [0.01], $p < 0.001$) and higher lactate levels (EPI: 70.2 [46]; EPI + MB: 94.6 [36.9]; CTL: 77.8 [18.7]; SH: 24.3 [5.6], $p < 0.001$) than SH rats, but there were no significant differences among the cardiac arrest groups. Blood glucose levels at ROSC were higher in cardiac arrest groups than in the SH group, but there were no significant differences among the groups (EPI: 432 [83]; EPI + MB: 424 [22]; CTL: 332 [53]; SH: 188 [14], $p < 0.001$).

Discussion

The data indicate that the administration of MB in conjunction with epinephrine did not improve cerebral and myocardial tissue protection following ROSC. Furthermore, MB did not elevate the rates of ROSC or exert any influence on the duration of CPR or the hemodynamic parameters.

MB acts by inhibiting soluble nitric oxide-stimulated guanylyl cyclase, which decreases NO levels.[16] Therefore, MB has been attributed to improving the vasoactive drug response and has been used frequently in situations where systemic vascular resistance is low, such as sepsis and anaphylaxis.[17,18]

Post-cardiac arrest syndrome comprises hypoxic-ischemic brain injury, myocardial dysfunction, and systemic ischemia and reperfusion response.[19] The association among these components activates coagulation and immune pathways, leading to intravascular volume depletion, marked vasodilation, endothelial injury, and microcirculation abnormalities. Therefore, postcardiac arrest syndrome is characterized by hypotension due to myocardial dysfunction and decreased vascular resistance.[20,21]

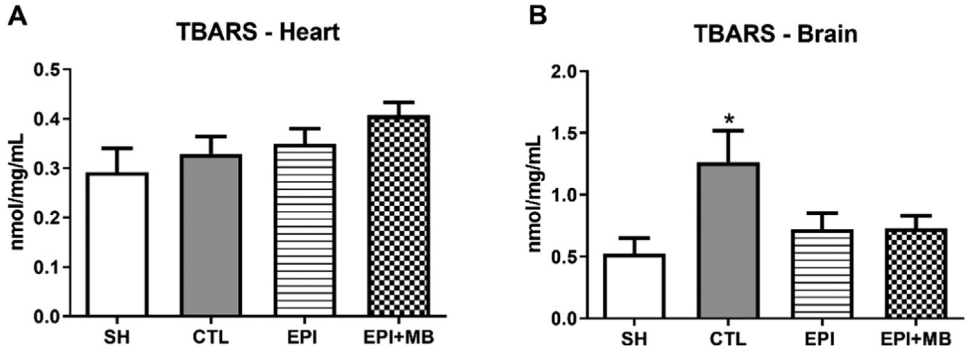

Figure 2 Levels of Thiobarbituric Acid Reactive Species (TBARS) ($nmoL.mg^{-1}.ml^{-1}$) in heart and brain tissue of animals subjected to the surgical procedure only (SH, n = 11) and rats subjected to CA and treated with Epinephrine (EPI, n = 11), epinephrine and MB (EPI + MB, n = 11), or saline (CTL, n = 11). *$p < 0.05$.

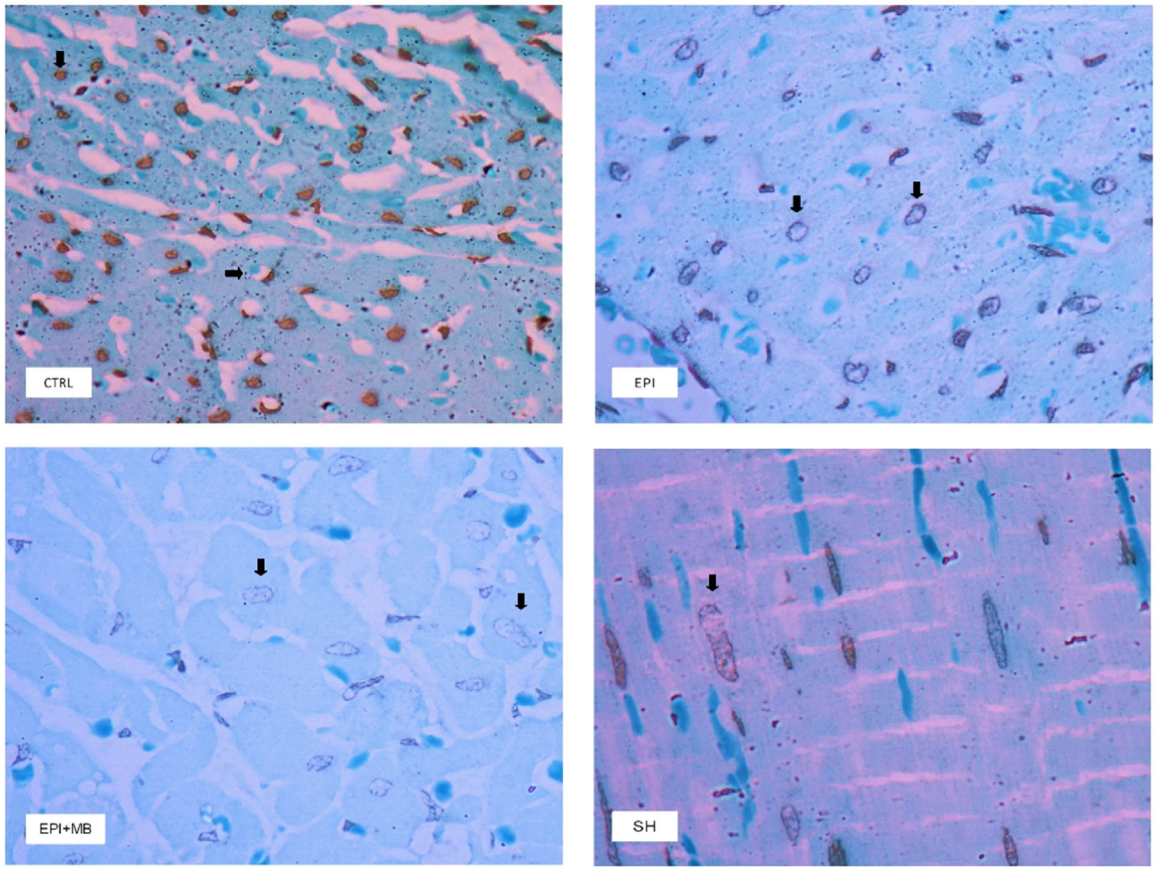

Figure 3 TUNEL staining of heart tissue (original magnification 1000 ×) from animals subjected to the surgical procedure only (SH, n = 11) and rats subjected to CA and treated with epinephrine (EPI, n = 11), epinephrine and MB (EPI + MB, n = 11), or saline (CTL, n = 11). The images depict a scarcity of striations and nuclei, along with peripheral chromatin, as demonstrated by the arrows.

Decreased vascular resistance is observed in postcardiac arrest syndrome and during cardiac arrest. EPI is advocated as the first-line treatment[1] since EPI causes vasoconstriction by stimulating α1 receptors in vascular smooth muscle. This leads to an increase in the diastolic aortic pressure, which increases coronary perfusion pressure and cerebral perfusion pressure.[22] EPI use is not exempt from complications since excessive plasma concentrations of EPI might increase myocardial oxygen demand, leading to myocardial dysfunction, troponin elevations, and a higher risk of arrhythmias such as ventricular tachycardia and ventricular fibrillation.[3] In addition, extra EPI levels might decrease cerebral microcirculation perfusion and induce circulatory mismatch in the lungs.[22,23]

Accordingly, therapeutic measurements to reduce EPI levels are appreciated. MB has a potential effect on cardiac

Table 2 Values of mean BP, diastolic BP (mmHg), and heart rate.

		CTL	EPI	EPI+MB	SH
Mean BP	Initial	83.0 [7.6]	83 [10] [a]	85 [16] [a]	70.5 [2.5]
	ROSC	30.5 [5.8]	63 [25.5] [b,d]	123 [31] [b]	—
	Final	67.5 [28.6] [c]	77.0 [11]	67.0 [12] [c]	69.0 [5.7]
Diastolic BP	CPR	17.5 [1.9]	27.0 [16.5] [b]	32.0 [14.5] [b]	—
HR	Initial	267 [35.5]	276 [53.5]	267 [109]	204 [43]
	ROSC	250 [53.6]	270 [67.5]	254 [66.2]	—
	Final	211 [56.5]	211 [56.5]	290 [60]	200 [24.4]

Values reflect the mean and its standard deviations. Rats underwent surgical manipulation only (SH, n = 11), or rats underwent CA treated with epinephrine (EPI, n = 11), epinephrine and MB (EPI + MB, n = 11), or saline (CTL, n = 11). Variables were measured at baseline (initial), 10 minutes after ROSC, and after 4 hours of observation (final).

[a] $p < 0.05$ vs. the SH Group
[b] $p < 0.05$ vs. the CTL group
[c] $p < 0.05$ vs. ROSC
[d] $p < 0.05$ vs. initial.

arrest, not only by its improvement in the vasopressor response but also by its potential neuroprotective effects.[24] Under hypoxic conditions, MB can sustain ATP production by redirecting electrons in the mitochondrial transport chain while lowering oxidative stress due to its actions as a free-radical scavenger.[7] MB also promotes cytochrome C oxidase activity and increases cerebral perfusion to mismatched areas while downregulating apoptosis.[7,25] Therefore, MB could reduce oxidative stress and the inflammatory response associated with ROSC and might increase the vasoactive drug response, making it a promising therapy during and after cardiac arrest.

Previous studies have demonstrated the neurological and myocardial protection effects of MB after ischemia-reperfusion events. In an experimental model of global cerebral ischemia, MB treatment prevented ischemic alterations and promoted ATP generation, leading to neuroprotection and functional improvement.[25] In a pig model of extended circulatory arrest, the coadministration of MB with a hyperoncotic solution decreased oxidative, myocardial, and neurologic injury.[9] Indeed, MB induced neuroprotection after cardiac arrest in pigs by hindering blood-brain barrier disruption, brain edema, myelin damage, and albumin leakage.[26] Previous studies suggest that MB can induce better hemodynamic stability and myocardial protection in ischemia and reperfusion injury situations.[10,27] An experimental study of ischemia-reperfusion injury after cardiac arrest showed that MB reduces anoxic cardiac injury and decreases troponin I levels.[28] Indeed, MB improved coronary perfusion and reduced myocardial injury in an experimental model of cardiac arrest.[9]

These studies suggest that MB is a promising therapy to reduce cerebral and cardiac ischemia after cardiac arrest. However, our results did not show an increase in diastolic coronary perfusion pressure, a surrogate for coronary perfusion pressure, or decreased cardiac arrest duration, and the histological analysis did corroborate this absence of benefit. In the studies above, vasopressin was used as the first line of treatment, and epinephrine was only given after two shocks. This differs from the procedure of this study, which followed the AHA recommendations and used epinephrine as the first-line treatment.[1] This implies that the use of epinephrine during CPR may outweigh any possible advantages of MB.

One possible explanation is that epinephrine increases NO production, which overcomes the ability of MB to suppress NO synthesis. Epinephrine can evoke the phosphorylation of eNOS. Additionally, β1-, β2-, and β3-adrenoceptors are functionally expressed in endothelial cells and are coupled to the activation of the NO/cGMP vasodilator pathway.[29] The epinephrine dose used in cardiac arrest is considered high, and experimental studies showed increased cardiac troponin levels in healthy pigs just by using a cardiac arrest epinephrine dose.[3] Thus, the amount of NO production induced by epinephrine might be over the capability of MB suppression. In fact, a retrospective study in patients with distributive shock showed that MB was less effective in cases with severe tissue hypoxia and a high anaerobic metabolism rate, a prevalent condition after cardiac arrest.[30] Thus, we believe that the MB dose might not have been high enough to reverse the systemic I/R injury observed after ROSC. However, increasing the MB dosage could cause toxicity, such as methemoglobinemia.

This study had many limitations. First, although we performed oxidative stress analysis, we did not address the efficacy of MB on NO-synthetase suppression, which could compromise the real effects of MB in this clinical setting. Second, this study had a limited sample size. Since this model had a high mortality rate (60%), which is similar to that reported in previous studies,[11] a large number of animals would be needed to address a clinical outcome (i.e., mortality), making the study unfeasible. We acknowledge that further studies are required to confirm our findings, especially those on mortality.

Conclusion

The coadministration of methylene blue and epinephrine as a combined therapeutic approach failed to yield enhancements in cardiac or brain lesions in a rodent model of cardiac arrest.

Conflicts of interest

The authors declare no conflicts of interest.

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