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ORIGINAL ARTICLE

Effect of Methylene Blue on a Porcine Model of Amlodipine Toxicity

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Abstract

Introduction Calcium channel blocker (CCB) overdoses cause significant morbidity and mortality. Dihydropyridine CCBs cause peripheral vascular dilation and at high doses cardiac dysfunction. Amlodipine, a dihydropyridine, causes peripheral vasodilation from release of nitric oxide (NO) in addition to calcium channel blockade; NO scavenging is a potential treatment. Methylene blue (MB) inhibits NO directly and inhibits NO production. We compared the effects of MB versus norepinephrine (NE), with time to death as the primary outcome, in a porcine amlodipine toxicity model.

Methods Animals were anesthetized and instrumented, and an amlodipine infusion was administered to mimic oral overdose. After 70 minutes, each group was resuscitated with normal saline. Animals in each group were then randomized to receive either MB or NE. Hemodynamic parameters, including mean arterial pressure and cardiac output, were recorded every 10 minutes. The primary outcome was survival time (Kaplan-Meier analysis and log-rank test).

Results Interim analysis after 15 animals (7 MB, 8 NE) revealed that MB was clearly not superior to NE. Overall, 1 of 7 animals in the MB group survived to 300 minutes compared with 2 of 8 animals in the NE group. The median survival time was 100 minutes for the MB group and 177 minutes for the NE group. Survival time did not differ by group (log-rank test $p = 0.29$).

Conclusion In this porcine model of amlodipine toxicity, methylene blue did not improve survival time compared with norepinephrine. Whether methylene blue is beneficial in combatting distributive shock in amlodipine toxicity remains unclear and requires further study.

Keywords Amlodipine · Overdose · Methylene blue · Cardiovascular toxin · Pig

Introduction

Cardiovascular medication overdose is associated with significant morbidity and mortality. The American Association of Poison Control Centers' National Poison Data System has reported that serious outcomes related to cardiovascular drug exposures are rapidly increasing. Calcium channel blockers (CCBs) were associated with 5.2% of death cases in the 2017 report [1]. CCBs affect the heart and peripheral vasculature in varying amounts depending on type. Drugs in the dihydropyridine (DHP) class of CCBs impart their therapeutic effect via peripheral vasodilation. In overdose, their effect remains primarily via peripheral vasodilation causing distributive shock, but there is loss of class specificity in severe cases; they can also cause cardiac toxicity in this context [2]. Despite maximal pharmacologic therapy for DHP overdoses, some cases still result in refractory shock and subsequent death. Studies have established the utility of calcium, vasopressors, high-dose insulin (HDI), lipid emulsion therapy, and extracorporeal life support, but there is not one particular standard of care for a DHP-poisoned patient [3–7]. There is a need to investigate these therapies further as well as other treatment options.

HDI is often used in both beta-blocker and CCB overdoses as it has been shown to improve cardiac output, perfusion, and mortality in animal models [3]. While maximizing cardiac output in these overdoses is helpful, there is often a need for improvement in systemic vascular resistance (SVR) to combat the distributive component of the shock. Adding a vasopressor, such as norepinephrine, after already establishing HDI therapy can increase SVR and has been shown in an animal model of beta-blocker toxicity to improve cerebral

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oxygenation [8]. Vasopressors, such as norepinephrine and epinephrine, have also been suggested as therapy exclusive of HDI in the context of CCB overdose, with some controversy [9, 10].

Amlodipine, a CCB in the DHP class, exerts the expected effect of vasodilatory shock in overdose. However, this is likely mediated by release of nitric oxide (NO) in addition to L-type calcium channel blockade [11]. Methylene blue has been suggested as an alternative treatment for DHP overdose as it may limit nitric oxide–mediated vasodilation [12–14]. In this study, we utilized a newly developed porcine model of amlodipine toxicity [15] to compare time to death between animals treated with methylene blue or traditional vasopressor therapy (norepinephrine).

Methods

Animal Preparation

Our institution approved all protocols. We performed the experimental protocol in a secured animal care facility that is US Department of Agriculture licensed and accredited with the American Association for Accreditation of Laboratory Animal Care.

We based the animal anesthetizing and instrumentation for monitoring on previous animal experiments performed by this investigative group [8, 16–18]. We sedated and anesthetized fifteen healthy Yorkshire Duroc cross swine (median weight of 40.9 kg) with a combination of ketamine and xylazine. The animals remained anesthetized throughout the protocol using a combination of nitrous oxide and isoflurane. The animals' response to a brief toe pinch during induction of anesthesia was assessed to ensure adequate sedation. Once adequately sedated, over-sedation was avoided to minimize cardiovascular-depressant effects.

We then performed a tracheostomy and the animals were placed on a ventilator. Mechanical ventilation was maintained throughout the study at Vt = 10 mL/kg, f = 15, PEEP = 5, and FiO_2 = 50%. FiO_2 was adjusted to maintain oxygen saturation > 90%. Hypoxia was defined as oxygen saturation < 90% and if an animal became hypoxic, the FiO_2 was increased accordingly. Continuous electrocardiogram monitoring occurred for the duration of the protocol. Furthermore, we maintained baseline temperature externally. We placed a Swan-Ganz catheter via the right external jugular vein to monitor the cardiac output as determined by a thermodilution technique. This was followed by placement of a femoral arterial line for arterial blood gas and pH determinations, which occurred every 30 minutes, as well as continuous blood pressure monitoring. We gained femoral venous access to facilitate venous blood sampling in addition to fluid and medication administration. Finally, we placed a suprapubic urinary catheter in each animal.

Once anesthetized and instrumented, we then paused for a stabilization period of 30 minutes prior to making baseline measurements and initiating the amlodipine toxicity. We continually monitored animal hemodynamics (CO, CVP, calculated SVR, calculated pulmonary vascular resistance, MAP, BP, and HR). Point-of-care testing occurred every 30 minutes (iSTAT CG8+, Abbott Laboratories, Chicago, IL) to quantify arterial sodium, potassium, chloride, ionized calcium, hematocrit, pH, pCO_2, pO_2, and HCO_3. This laboratory monitoring was done for in-study treatment, if needed, and was therefore not included in the analysis.

Induction of Toxicity

We started the amlodipine infusion at 0.25 mg/kg/hour and increased by 0.25 mg/kg/hour every 30 minutes. This increasing dose protocol was selected to simulate ongoing absorption of drug as would occur in an oral overdose (recognizing the difficulty of adequately modeling an oral overdose with an intravenous drug), but more importantly to simulate an exceptionally toxic model that would require the use of alternative antidotes beyond accepted standard of care. Furthermore, our research group has had success with previous models utilizing propranolol in a stepwise increasing dose fashion. Based on a pilot study, it was determined that animals began to display signs of toxicity at 0.75 mg/kg/hour, which occurs at approximately 60 minutes after the start of infusion of amlodipine [15]. The initial goal point of toxicity was defined in the pilot study as a 25% decrease in MAP multiplied by CO. The animals began showing signs of amlodipine toxicity prior to reaching the goal point of toxicity (including tachycardia and hypotension) and quickly became very sick, sometimes beyond the point of resuscitation. Given the lethal model, we chose to begin the antidote administration 70 minutes after the start of the amlodipine infusion, as this was when animals began showing signs of toxicity (tachycardia) but would still allow time for antidotal therapy prior to becoming hypotensive and ultimately expiring. At that time, all animals were rapidly given a 20 mL/kg bolus of NS. After fluid resuscitation, we administered the rescue drug (MB or NE), which was assigned randomly prior to the start of each experiment. The investigator preparing and initiating the drug was blinded to the hemodynamics of the animal, whereas the investigator recording hemodynamics was blinded to which rescue drug was used in each experiment. The rescue drug bag as well as tubing was covered in foil to maintain blinding since MB is easily identified based on color.

We administered a 2 mg/kg bolus followed by an infusion of 0.1 mg/kg/hour to the animals randomized to treatment with MB. Animals randomized to NE were started on an infusion of 0.1 mcg/kg/minute which was rapidly titrated until a

MAP of greater than 55 mmHg was sustained or a maximum rate of 0.5 mcg/kg/minute was achieved. Once maximized, the animal received no further interventions over the course of the protocol. The investigator administering the treatment drugs titrated a normal saline infusion to keep a constant volume infusion rate in each subject of 125 mL/hour for both of the groups. The protocol concluded at time of death or 5 hours from the start of the amlodipine infusion, whichever came first. If the animals survived to the end of the protocol, we euthanized them.

Statistical Analysis

The primary outcome was time to death. Survival times were compared using a Kaplan-Meier analysis, and the two groups were compared with the log-rank test. The study was powered at 80% to detect a hazard ratio of 0.2 (MB vs. NE), assuming a two-sided log-rank test with alpha = 0.05. We used data from our previous experience to guide the probable mortality rate in the NE arm (roughly 35% mortality per hour). There is very little published data on the effects of methylene blue to be used in a predictive fashion. One study [14] examined methylene blue versus placebo as treatments for amlodipine poisoning in rats. In that study, rats with methylene blue lived longer (an average of 109 minutes versus 42) and more of them survived the study (33% versus 7%). Our own estimate of the hazard ratio is roughly based on these data. Nine animals per group were required for adequate power. Descriptive statistics were used to compare the baseline characteristics of each group.

Results

Baseline characteristics for weight and secondary hemodynamic variables at the time of toxicity (cardiac output, mean arterial pressure, and heart rate) are summarized in Table 1.

Although not a part of the initial design, an interim analysis was encouraged by our Animal Care and Use Committee given some unexpected funding concerns and a sense among the research group that survival was limited across all the animals (and therefore unlikely to be different between the groups in such a small study). A statistician conducted the analysis after 15 of the initially planned 18 animals were completed (7 MB and 8 NE). This revealed that, for the primary outcome, MB was clearly not superior to NE. Furthermore, it would have been impossible to achieve statistical significance for the MB hazard ratio with the addition of 3 pigs, regardless of the outcome of those subjects. Therefore, in accordance with appropriate animal protection and guidance from the review board, the study was ended prematurely. Overall, 1 of 7 (14%) animals in the MB group survived to 300 minutes compared with 2 of 8 (25%) animals in the NE group. The median survival time was 100 minutes for the MB group and 177 minutes for the NE group. Survival time did not differ by group (log-rank test $p = 0.29$); survival was longer in the NE group, but this was not statistically significant. Survival curves are presented in Fig. 1.

Individual hemodynamic data is presented in Figs. 2, 3, 4, and 5; Fig. 2 depicts heart rate as a function of time, Fig. 3 depicts MAP as a function of time, Fig. 4 depicts cardiac output as a function of time, and Fig. 5 depicts SVR as a function of time.

Table 1 Baseline hemodynamic and weight characteristics at time of toxicity.

Variable	NE		MB		
	Mean	SEM	Mean	SEM	*p* value
Weight (kg)	43.2	1.8	41.6	1.2	0.48
Cardiac output (L/minute)	3.8	0.3	4.6	0.7	0.34
Heart rate (bpm)	81.3	3.0	84.7	7.3	0.67
Mean arterial pressure (mmHg)	58.3	3.9	63.3	3.8	0.38

SEM standard error of the mean

Discussion

Our animal study suggests methylene blue performs no better than norepinephrine for treatment of amlodipine toxicity. The optimal treatment of DHP CCB overdoses remains unclear. Although high-dose insulin therapy is quite effective and has been shown to be non-inferior to vasopressors in DHP overdose [16], adjunctive therapies are frequently required to augment the profound shock seen in severe cases. For amlodipine specifically, choosing treatment options is even more difficult because of the additional mechanism of NO-mediated vasodilation. Methylene blue, a nitric acid scavenger, has emerged as a promising therapy to address this mechanism, although evidence is limited.

CCBs slow influx of calcium through L-type calcium channels. At therapeutic doses, DHPs are selective for peripheral vasculature and have much less effect on the myocardium. Phenylalkylamines (verapamil) are selective for the myocardium, and therefore will depress both the sinoatrial node and atrioventricular node, while also decreasing contractility. They have very little effect on peripheral vasculature. Benzothiazepines (diltiazem) behave similar to verapamil, but have less ability to depress myocardial contractility [6]. Though these 3 subsets of CCBs are distinctly different from each other at therapeutic doses, they lose specificity in overdose, which can result in both distributive and cardiogenic shock across the entire CCB drug class, leading to death [2].

In studies of canine arteries exposed to CCBs, amlodipine (not nifedipine or diltiazem) increased vasodilatory effects by stimulating the release of nitric oxide (NO) in addition to the L-type calcium channel blockade [11]. The pathway is unclear,

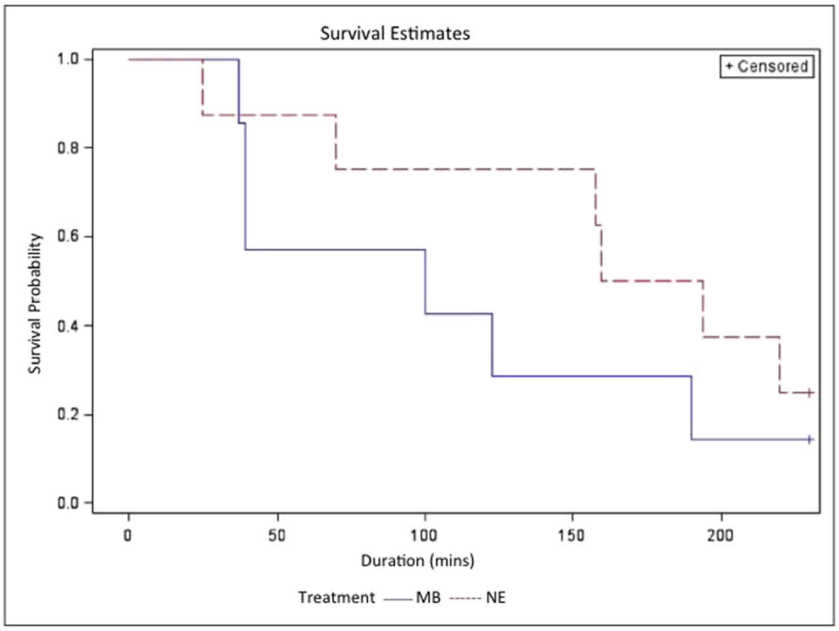

Fig. 1 Survival curve. No significant difference by log-rank test, $p = 0.29$.

but it is thought to be mediated by the inhibition of kinin breakdown. These two mechanisms directly affect vasodilation of peripheral vasculature, making patients vulnerable to refractory hypotension despite maximal supportive care and treatment with calcium, fluids, high-dose insulin, and vasopressors.

Methylene blue (MB) has been proposed as a possible treatment for inappropriate peripheral vasodilation, such as in anaphylaxis and sepsis. These conditions are at least in part potentiated by release of NO in peripheral vasculature [19, 20]. MB has been shown to inhibit the final pathway of NO production by inhibiting guanylyl cyclase and also directly inhibiting NO and endothelial NO synthase [12]. In theory, this inhibition by MB may limit further NO-mediated vasodilation, thereby improving SVR. Despite the convincing mechanism, in this porcine model of amlodipine toxicity, we were unable to show a survival benefit using methylene blue.

Our study showed that in this very toxic model—with an overall survival to the end of the protocol of only 20%—methylene blue performed no better than norepinephrine with regard to overall survival. Furthermore, the total time of survival of the animals treated with methylene blue was nearly half of those treated with norepinephrine, although given the small group size, this difference did not meet statistical significance.

This negative result is instructive in that it highlights the importance of a multi-faceted approach to the treatment of severe CCB overdoses. One of the initial studies on high-dose insulin showed that high-dose insulin therapy is superior to other therapies (including vasopressors and glucagon) in verapamil, a more cardiac-specific calcium channel blocker [21]. A review of the literature on this topic indicates that high-dose insulin is the superior pharmacologic therapy [3], and a more recent review made the same conclusion [4]. Even in the initial case report that described the successful use of methylene blue for a refractory case of amlodipine toxicity, providers had previously administered high-dose insulin therapy (albeit at a relatively low dose), norepinephrine, calcium

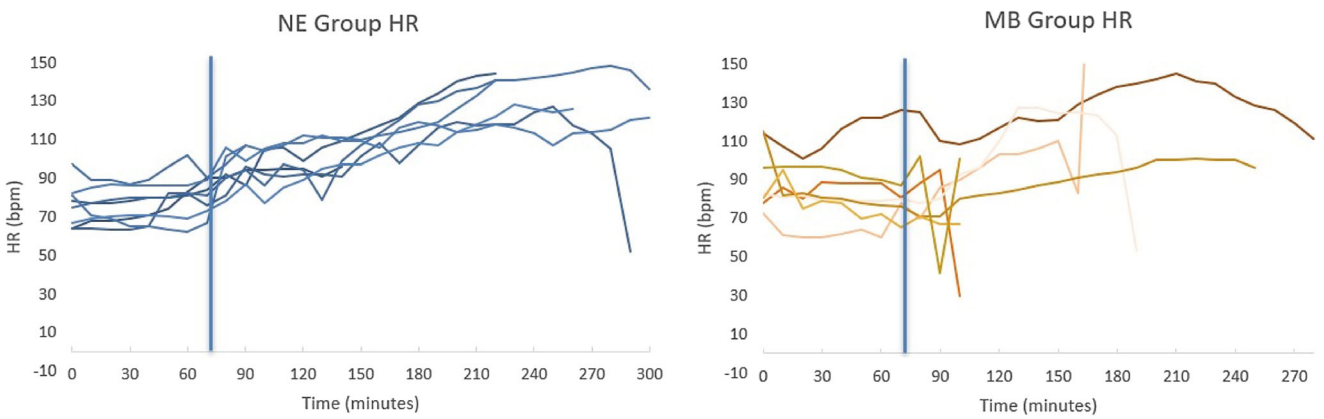

Fig. 2 Heart rate time graphs for norepinephrine-treated swine (left) and methylene blue–treated swine (right). The vertical line at 70 minutes denotes initiation of the antidote.

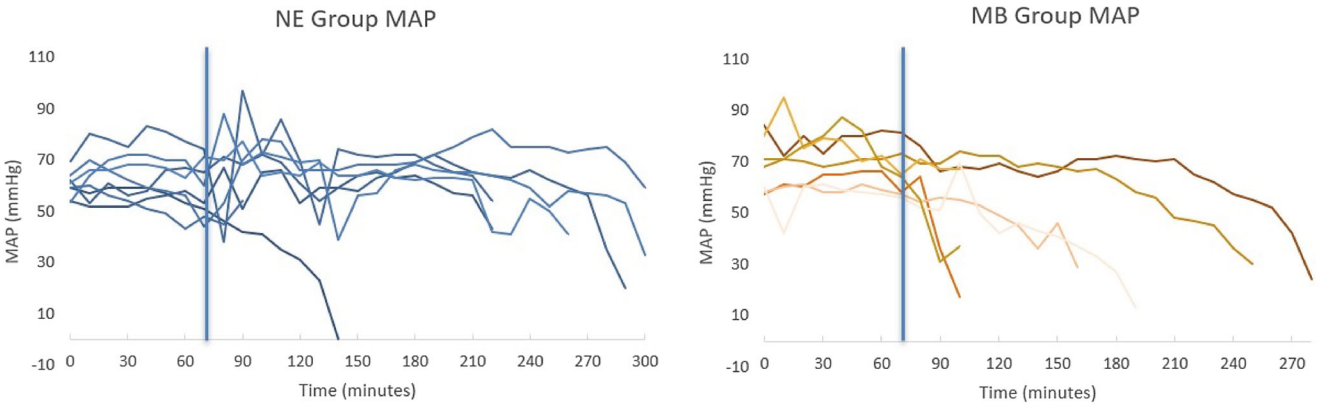

Fig. 3 Mean arterial pressure versus time graphs for norepinephrine-treated swine (left) and methylene blue–treated swine (right). The vertical line at 70 minutes denotes initiation of the antidote.

boluses, and dopamine [13]. The methylene blue was given 16 hours after ingestion with improvement seen within an hour. The temporal association with improvement is intriguing but by no means clear evidence of causation. Clearly, more study is required to definitively answer the question as to whether methylene blue is effective in toxin-induced shock.

There was no statistically significant difference in survival between MB and NE. As shown in Figs. 2, 3, 4, and 5, there was also no significant difference in the secondary hemodynamic parameters. As NE is a current standard therapy and there was no placebo comparison in this study, more study is needed. This suggests that methylene blue should at least be considered as an adjunctive therapy until further research can offer more conclusive recommendations.

Limitations

The interpretation of this study's results is limited by several important factors. Most importantly, this study is a porcine model and as such the generalizability to human physiology is limited. However, swine are recognized as excellent large-animal models for the human cardiovascular system, as they share similar anatomic and physiologic characteristics to humans. Furthermore, multiple porcine studies on toxin-induced shock have resulted in generalizable results to humans and have successfully changed the treatment standard of care [16, 17].

The optimal dosing of intravenous amlodipine to cause toxicity in swine is unknown. In the pilot portion of the study, we extrapolated the LD_{50} of amlodipine from a rat study [14] to determine a presumed toxic dose in swine [15]. We ultimately created a very toxic model that may not be characteristic of the level of toxicity seen in humans. Furthermore, simulating ongoing gastric absorption by increasing the dose as we did is an approach that has not been extensively studied. Likewise, the dose of methylene blue for refractory shock is not well established. Many dosing protocols have been suggested, but there is no consensus on which is optimal [22, 23].

The use of intravenous amlodipine presents several other limitations that became apparent during the study. The decision to use intravenous amlodipine was made because of the unpredictable and slow nature of gastric absorption. We also wanted to control the exact amount of medication administered without needing to measure serum drug levels. Unfortunately, intravenous amlodipine is not commercially available, as it is administered clinically as an oral formulation. Therefore, we had to dissolve the amlodipine in a

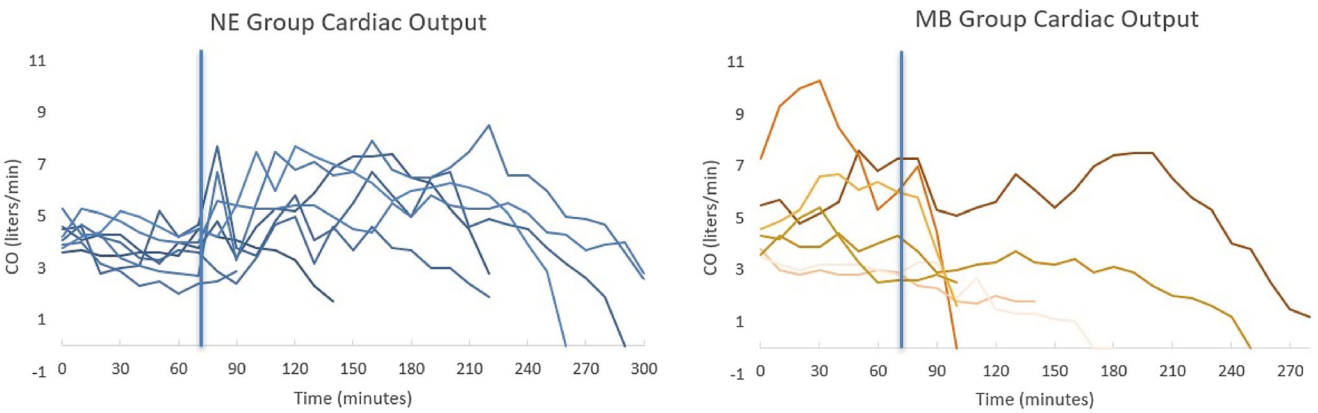

Fig. 4 Cardiac output versus time graphs for norepinephrine-treated swine (left) and methylene blue–treated swine (right). The vertical line at 70 minutes denotes initiation of the antidote.

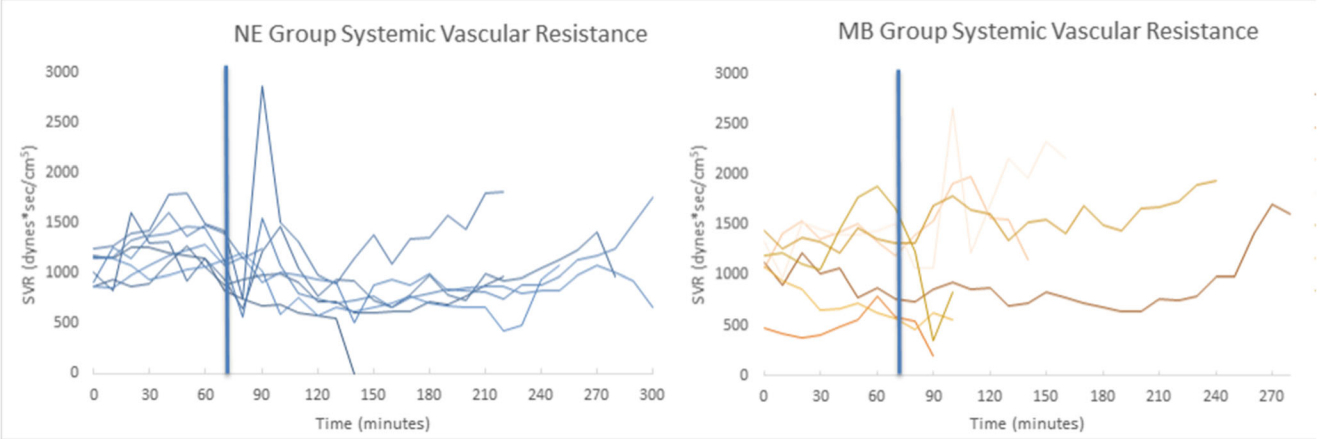

Fig. 5 Systemic vascular resistance versus time graphs for norepinephrine-treated swine (left) and methylene blue–treated swine (right). The vertical line at 70 minutes denotes initiation of the antidote.

solution that could safely be administered intravenously. This presents some difficulty as amlodipine is difficult to dissolve. It has minimal solubility in water, ethanol, and saline [24]. Dimethyl sulfoxide (DMSO) has been suggested as an effective solvent of amlodipine by several manufacturers and was used by Jang et al. in a rat study [14]. DMSO is known to cause hypotension in large doses and as such presents another important limitation to this study. However, swine and canine studies as well as the Jang rat study have shown that very large doses of DMSO are required to affect mean arterial pressure [14, 25–27]. It is unlikely that the doses of DMSO we used affected mean arterial pressure in our subjects significantly. That being said, a control animal using a DMSO placebo infusion would have been beneficial. Ultimately, our limited budget precluded this approach.

Future Directions

Given the aforementioned limitations, as well as the ongoing question as to the role of MB in the treatment of severe amlodipine toxicity, further study is required. A study utilizing MB as an adjunct after maximizing NE therapy (compared to NE alone) would be a natural successor to this study. Additionally, following other measures of tissue perfusion (especially brain tissue perfusion) is crucial in elucidating the best management of these patients. This is because when vasopressors are used in severe shock, providers typically titrate to a specific blood pressure goal, which can lead to decreased end-organ perfusion and affect survival at high doses. Our group has studied this previously in a propranolol model and found that brain tissue oxygenation is improved when vasopressors are used in combination with high-dose insulin [8]. Additionally, and as mentioned in the "Limitations" section, it would also be prudent to repeat the study with a DMSO-only arm to determine the effect that the solvent may have had, if any, on the hemodynamic parameters.

Conclusion

In this porcine model of amlodipine toxicity, methylene blue did not improve survival time compared with norepinephrine. Hemodynamic parameters were also not appreciably different between the arms. While we recognize that methylene blue will likely never be used as monotherapy for amlodipine poisoning, use of methylene blue as an adjunct is intriguing and deserves further study.

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Compliance with Ethical Standards

Our institution approved all protocols. We performed the experimental protocol in a secured animal care facility that is US Department of Agriculture licensed and accredited with the American Association for Accreditation of Laboratory Animal Care.

Conflicts of Interest None.

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