Current Therapeutic Research 95 (2021) 100655

Contents lists available at ScienceDirect

Current Therapeutic Research

journal homepage: www.elsevier.com/locate/curtheres

The Role of Nitric Oxide in the Efficacy of Adenosine, Lidocaine, and Magnesium Treatment for Experimental Hemorrhagic Shock in Rats

Hayley L. Letson, PhD*, Geoffrey P. Dobson, PhD, FAHA

Heart, Trauma, and Sepsis Research Laboratory, College of Medicine & Dentistry, James Cook University, Townsville, Queensland, Australia

ARTICLE INFO

Article history:
Received 12 October 2021
Accepted 12 November 2021

Key words:
endothelium
hemorrhagic shock
nitric oxide
nitric oxide synthase
resuscitation

ABSTRACT

Background: Nitric oxide (NO) plays multiple roles regulating the central nervous, cardiovascular, and immune systems.
Objective: Our aim was to investigate the role of NO in the efficacy of hypertonic saline (7.5% sodium chloride [NaCl]) adenosine, lidocaine, and magnesium (ALM) to improve mean arterial pressure (MAP) and heart rate following hemorrhagic shock.
Methods: One hundred one male Sprague-Dawley rats (mean [SD] weight = 425 [6] g) were randomly assigned to 20 groups (groups of 4–8 rats each). Hemorrhagic shock (MAP < 40 mm Hg) was induced by 20-minute pressure-controlled bleeding (~40% blood volume), and the animal was left in shock (MAP = 35-40 mm Hg) for 60 minutes. The NO synthase (NOS) inhibitor L-NAME was administered with a 0.3-mL bolus of different combinations of 7.5% NaCl ALM active ingredients and hemodynamic parameters were monitored for 60 minutes. A number of specific NOS and NO inhibitors were tested.
Results: We found that 7.5% NaCl ALM corrected MAP after hemorrhagic shock. In contrast, the addition of L-NAME to 7.5% NaCl ALM led to a rapid fall in MAP, sustained ventricular arrhythmias, and 100% mortality. Saline controls receiving 7.5% NaCl with N^G-nitro-L-arginine methyl ester (L-NAME) showed improved MAP with no deaths. None of the specific NOS and NO inhibitors mimicked L-NAME's effect on ALM. The addition of inducible NOS inhibitor 1400W to 7.5% NaCl ALM failed to resuscitate, whereas the NO scavenger PTIO and the PI3K inhibitor wortmannin reduced MAP recovery during 60-minute resuscitation.
Conclusions: The ability of 7.5% NaCl ALM to resuscitate appears to be linked to 1 or more NO-producing pathways. Nonspecific NOS inhibition with L-NAME blocked ALM resuscitation and led to cardiovascular collapse. More studies are required to examine NO site-specific contributions to ALM resuscitation. (*Curr Ther Res Clin Exp*. 2022; 82:XXX–XXX)

Introduction

Hemorrhage is responsible for 30% to 40% of early trauma mortalities in the civilian population and up to 50% of deaths on the battlefield.[1] Over the past 3 decades, resuscitating bleeding patients with large fluid volumes of crystalloids/colloids has led to poor outcomes.[2] The current resuscitation guidelines for hemorrhage without suspected brain injury suggest the use of smaller fluid volumes and a target mean arterial blood pressure (MAP) of 60 to 65 mm Hg (permissive hypotension) to prevent rebleeding and secondary complications.[1] We have been developing a small-volume fluid therapy for prehospital use comprising hypertonic saline with adenosine, lidocaine, and magnesium (ALM).[3,4] Hypertonic saline assists ALM to increase blood pressure into the permissive hypotensive range, and the combination has been shown to increase survival after hemorrhagic shock by improving cardiac function, reducing inflammation, correcting coagulopathy, and preventing ischemia-reperfusion injury.[1,3,5–11] We have also shown that the ALM combination is key to permissive hypotensive resuscitation and protection; not the individual active ingredients adenosine, lidocaine, and magnesium or other combinations.[1,3,12]

Nitric oxide (NO) is a ubiquitous signalling messenger molecule involved in diverse pathophysiologic processes such as neurotransmission, inflammatory and immune responses, and regulation of cardiovascular function.[13] The regulation of myocardial function by

* Address correspondence to: Hayley Letson, PhD, Heart, Trauma, and Sepsis Research Center, College of Medicine and Dentistry, James Cook University, 1 James Cook Dr, Townsville, QLD 4811 Australia.
E-mail address: hayley.letson@jcu.edu.au (H.L. Letson).

https://doi.org/10.1016/j.curtheres.2021.100655

NO is important for the maintenance of myocardial calcium ion homeostasis, relaxation and distensibility, and protection from arrhythmias and the sympathetic-induced stress response.[13–15] Thus, the fine NO balance between production and synthesis maintains cardiac function, vasculature patency, arterial blood pressure, endothelial function, and tissue perfusion.[16] Because NO is pivotal in regulating the cardiovascular, central nervous and immune systems,[14,15,17] and hypertonic saline at 7.5% sodium chloride (NaCl) ALM has been shown to modulate and protect these systems, our aim was to investigate the role of NO in the efficacy of 7.5% NaCl ALM resuscitation following severe hemorrhagic shock in a rat model. Our hypothesis is that addition of the nonspecific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), to ALM therapy will influence its resuscitation potential. L-NAME has been shown to resuscitate and increase MAP in multiple preclinical animal models of hemorrhage via its arteriolar vasoconstrictive effects.[15,18,19] We will examine the role of specific NO inhibitors to further understand the underlying mechanism of ALM resuscitation.

Methods

Animals and ethics

Male Sprague Dawley rats (weight = 425 [6] g) were obtained from our institutional breeding colony, and housed in a 14/10 hour light/dark cycle with free access to food and water ad libitum. Animals were heparinized with 2,500 IU Heparin Sodium (Hospira, Lake Forest, Illinois) and anesthetized intraperitoneally with 100 mg/kg sodium thiopentone (Thiobarb, Rutherford, Australia). Anesthetic was administered as required throughout the protocol. The study was approved by our institutional Animal Ethics Committee (No. A1148) and conforms to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition; 2013) and the Guide for Care and Use of Laboratory Animals (8th edition; 2011). As per the Australian code, the 3Rs (ie, replacement, reduction, and refinement) have been applied in this study. Hemorrhage results in the activation of multiple body systems that cannot be accurately mimicked using an alternative in vitro method (ie, replacement). Power analysis was conducted to determine the smallest number of animals necessary to achieve the study's aims (see Statistical Analysis) (ie, reduction), and depth of anesthesia was continuously monitored to ensure animal well-being (ie, refinement).

Surgical protocol

The surgical procedure has been described previously.[1,12] Following anesthesia, a tracheotomy was performed, and animals were ventilated on humidified room air at 90 strokes/minute with positive end expiratory pressure of 1 cm, and tidal volume of 5 mL/kg (Harvard Small Animal Ventilator, Holliston, Massachusetts, USA). Temperature was monitored throughout with a rectal probe. Temperature was left to drift, with no thermal support provided during surgery, bleeding, or resuscitation. The left femoral vein and artery were cannulated using PE-50 tubing for drug infusions and hemodynamic monitoring (Powerlab; ADInstruments, Bella Vista, New South Wales, Australia), and the right femoral artery was cannulated for blood-letting. All cannulae contained heparinized saline (1000 U/mL saline). Lead II electrocardiogram was attached for BioAmp recording (ADInstruments). Following surgical instrumentation, anesthetized animals had a 10-minute baseline equilibration period (Figure 1). Animals were excluded from study if they were difficult to anesthetize; experienced complex arrhythmias during preparation, stabilization, or within the shock period; or were hemodynamically unstable before phlebotomy.

Experimental design

Rats (n = 101) were randomly assigned to 1 of 20 groups (see the Table 1). 7.5% NaCl was used as the vehicle for all groups. Study groups 1 through 12 examined the effect of the nonspecific NO synthase (NOS) inhibitor L-NAME on 7.5% NaCl ± ALM resuscitation. Doses were 30 mg/kg (40 mg/mL) L-NAME, 1 mM (0.26 mg/mL) adenosine, 3 mM (0.8 mg/mL) lidocaine, and 2.5 mM (0.3 mg/mL) magnesium sulfate, as per previous studies.[1,9,18,20] Study groups 13 through 20 examined the effect of specific NOS inhibitors and modulators, including wortmannin (1 mg/kg),[21] oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) (2 mg/kg),[22,23] aminoguanidine hydrochloride (AMG) (20 mg/kg),[24] 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (10 mg/kg),[25] N-[3-aminomethyl]benzyl] acetamidine (1400W) (10 mg/kg),[26] *S*-methyl-L-thiocitrulline (SMTC) (1 mg/kg),[27] ARL17477 dihydrochloride hydrate (ARL17477) (1 mg/kg),[28] and 1-(2-trifluoromethylphenyl) imidazole (TRIM) (1 mg/kg)[29] (see the Table 1 for group sizes and specifics).

Shock protocol

Hemorrhagic shock was induced by withdrawing arterial blood to MAP of 35 to 40 mm Hg. Blood-letting started at ~1 mL/min before decreasing to ~0.4 mL/min. Phlebotomy was continued for 20 minutes and then rats were left in shock for 60 minutes with blood withdrawal or infusion to ensure MAP remained between 35 to 40 mm Hg (Figure 1). The average shed volume was 10.6 (0.2 mL) and represented an average blood loss of 40.3% (0.6%) (calculated from $[(0.06 \times \text{body weight [g]}) + 0.77]$[30]), with no difference between groups. At the end of shock, rats were injected with 0.3 mL treatment bolus (~3%–4% of shed volume) into the femoral vein over a 10-second period, and were monitored for a further 60 minutes. Hemodynamic parameters were monitored throughout the study, including heart rate (HR), systolic pressure, diastolic pressure, and MAP. Death time was defined as the point of last detectable electrical activity on lead II electrocardiogram. Ventricular arrhythmias, including premature ventricular contractions, and episodes of bigeminy, salvos, and ventricular tachycardia, were identified by an investigator blinded to treatment groups using the Lambeth convention as previously outlined in Canyon and Dobson.[31]

Statistical Analysis

SPSS Statistical Package 24 was used for all statistical analysis (IBM; St Leonards, New South Wales, Australia). All values are expressed as mean (SEM). Data normality was assessed numerically with Shapiro-Wilk test. ANOVA was used to evaluate parametric data with Tukey honestly significant difference or Dunnett post-hoc test dependent on Levene's homogeneity of variance. Survival was assessed using the Kaplan-Meier method with a log-rank test for comparison between treatment groups. Statistical significance was defined as $P < 0.05$. A priori power analysis was conducted using G*power[3] program (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) to determine sample size to minimize Type 1 errors (MAP = 15 minutes resuscitation; n = 4; Cohen's $d = 3$; Critical $t = 2.45$; α error probability = 0.05; Power (1-β err prob) = 0.94).

Results

Survival

The addition of L-NAME to 7.5% NaCl ALM led to 100% mortality (mean [SD] survival time = 19.4 [4.5] minutes; $P < 0.001$)

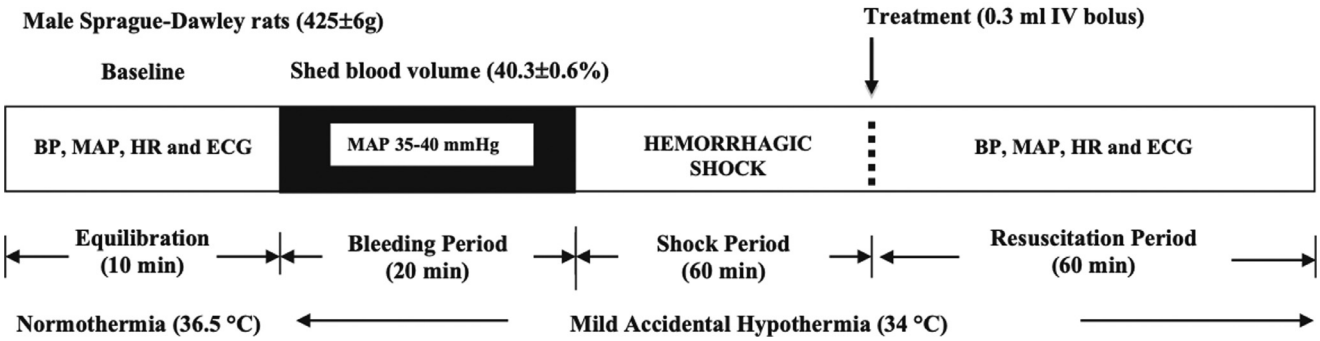

Figure 1. A schematic of the pressure controlled in vivo rat protocol of hemorrhagic shock. Shed blood volume was taken over a 20-minute period to maintain mean arterial blood pressure of 35 to 40 mm Hg (40.3% [0.6%] blood loss), and the rat remained in shock for a period of 60 minutes before resuscitation (0.3 mL intravenous fluid bolus). See Methods for treatment group details.

(Figure 2). All other groups survived the 60-minute resuscitation period following 20-minute blood loss and 60-minute shock (Figures 2**A** and 2**B**). The early cardiovascular collapse in 7.5% NaCl ALM + L-NAME animals was associated with extensive ventricular arrhythmias (65.5 [1.5] arrhythmic episodes) compared with no ventricular arrhythmias detected in 7.5% NaCl ALM alone-treated animals.

Effect of L-NAME on MAP and HR in ALM and controls

Small-volume 7.5% NaCl ALM improved MAP to the permissive hypotensive range following hemorrhagic shock from 38 to 64 mm Hg at 60 minutes (67% baseline) (Figure 3**A**), whereas HR remained at a constant 240 to 260 bpm (73%–79% of baseline) (Figure 3**B**). In contrast, in the presence of L-NAME, MAP decreased toward baseline at 5 minutes (10 mm Hg) and HR decreased to 112 bpm. MAP and HR approached 0 at 30 minutes (Figures 3**A** and 3**B**). The effect of L-NAME to cause cardiovascular collapse did not occur if it was added after 60 minutes of 7.5% NaCl ALM resuscitation. There was no change in MAP (66 mm Hg 15 minutes after 30 mg/kg L-NAME IV administration), no arrhythmias, and no mortality (n = 3, data not shown), indicating lethality only occurs when ALM and L-NAME are administered together after shock.

In contrast to 7.5% NaCl ALM, the saline control (7.5% NaCl alone) failed to resuscitate, with MAP only increasing to 53 mm Hg at 10 minutes before falling below 40 mm Hg at 60 minutes (Figure 3**C**). When L-NAME was added to 7.5% NaCl alone, MAP increased from shock values by up to 1.7-fold over the 60 minutes, and HR decreased by ~17% (Figures 3**C** and 3**D**).

Effect of L-NAME on 7.5% NaCl with different combinations of ALM active ingredients

Control animals (7.5% NaCl) receiving adenosine, lidocaine, or magnesium alone with L-NAME increased MAP during resuscitation (Figure 4**A**). When controls received the combinations of AM, AL, or LM with L-NAME, MAP was corrected early at 5 minutes before slowly declining at different rates over 45 minutes, with LM dropping MAP to 15 mm Hg at 60 minutes (Figure 4**A**). In contrast, 7.5% NaCl AL, 7.5% NaCl magnesium, or 7.5% NaCl alone (no L-NAME) did not resuscitate. HR was defended across all groups relative to their shock values with the exception of 7.5% NaCl ALM + L-NAME (after 5 minutes) and 7.5% NaCl LM + L-NAME (after 45 minutes) (Figure 4**B**).

Effect of specific NOS inhibitors on MAP resuscitation with 7.5% NaCl ALM

In contrast to L-NAME, which led to cardiovascular collapse, the addition of other NOS or NO inhibitors to 7.5% NaCl ALM did not have this effect (Figure 5). At 5 minutes of resuscitation, the presence of NOS inhibitors SMTC, ARL17477, and AMG, and NO scavenger PTIO increased MAP by 1.4- to 1.8-fold compared with 7.5% NaCl ALM alone (Figure 5**A**). In contrast, ODQ, TRIM, and wortmannin had little effect on MAP recovery with 7.5% NaCl ALM, whereas the addition of the inducible NOS inhibitor 1400W failed to resuscitate from shock (Figure 5**A**). After the initial MAP increase, the AMG and PTIO groups fell toward or below shock values (Figure 5**A**). After 5 minutes, 7.5% NaCl ALM with wortmannin failed to improve MAP.

None of the specific NO inhibitors reduced HR similar to 7.5% NaCl ALM + L-NAME, which produced significantly lower heart rates from 15- to 60-minute resuscitation compared with all other groups (Figure 5**B**). All groups maintained a relatively constant HR across 60-minute resuscitation, except for the NO scavenger PTIO, which fell by 17% from 252 bpm at 45 minutes to 209 bpm at 60 minutes. The neuronal NOS inhibitors TRIM and ARL17477 produced the highest HRs (15%–23% and 8%–12% higher than 7.5% NaCl ALM alone, respectively) (Figure 5**B**). Similar to its failure to improve MAP, the addition of 1400W to 7.5% NaCl ALM reduced HR by ~15% over the course of resuscitation, compared with 7.5% NaCl ALM alone.

Discussion

We report that the efficacy of 7.5% NaCl ALM to resuscitate into the protective permissive hypotensive range and prevent ventricular arrhythmias after severe hemorrhagic shock is completely abrogated in the presence of the nonselective inhibitor of NOS L-NAME. The addition of L-NAME to 7.5% NaCl ALM led to 100% mortality, whereas controls receiving 7.5% NaCl (no ALM) with L-NAME corrected MAP with no deaths. It appears that the resuscitation efficacy of 7.5% NaCl ALM is linked to a mechanism involving 1 or more NO-producing pathways. Using a variety of specific NOS and NO inhibitors, we report that no inhibitor mimicked 7.5% NaCl ALM L-NAME's effect to elicit cardiovascular collapse.

7.5% NaCl ALM resuscitation appears to be NO-dependent

Similar to previous studies, and in contrast to 7.5% NaCl alone or in combination with individual adenosine, lidocaine, and magnesium actives, 7.5% NaCl ALM was the only treatment to resuscitate and maintain MAP in the permissive hypotensive range over

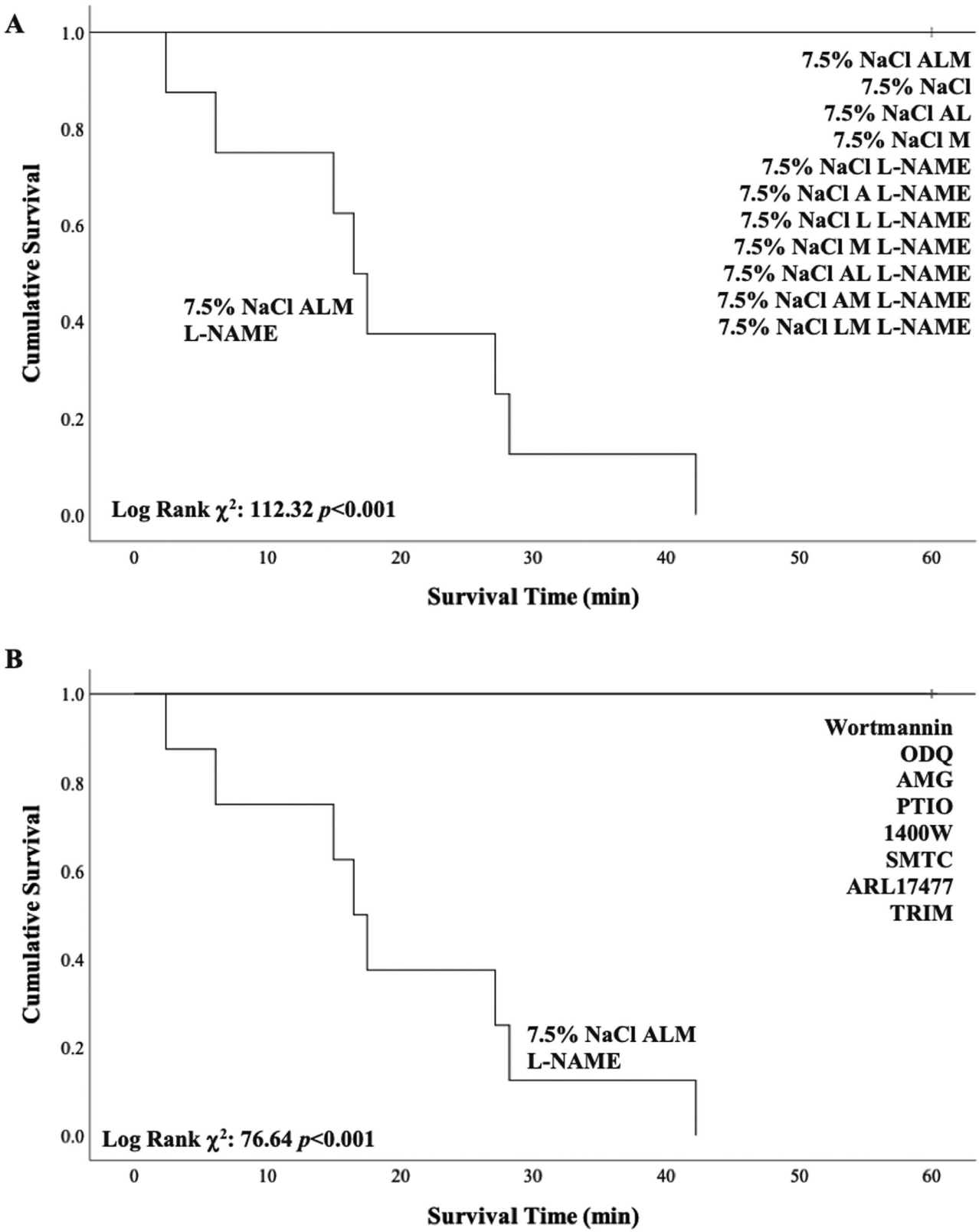

Figure 2. Kaplan-Meier survival curves for (A) N^G-nitro-L-arginine methyl ester (L-NAME) study and (B) nitric oxide/nitric oxide synthase (NO/NOS) inhibitor study. One hundred percent of 7.5% sodium chloride (NaCl) adenosine, lidocaine, and magnesium (ALM) + L-NAME-treated animals died during the 60-minute resuscitation period (mean survival time = 19.4 [4.5] minutes; $P < 0.001$). A = adenosine; AL = adenosine and lidocaine; AM = adenosine and magnesium; AMG = aminoguanidine; L = lidocaine; LM = lidocaine and magnesium; M = magnesium; ODQ = 1*H*-[1,2,4] Oxadiazolo[4,3-*a*] quinoxalin-1-one; PTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 1400W = N-[3-aminomethyl]benzyl] acetamidine; SMTC = *S*-methyl-L-thiocitrulline; ARL17477 = ARL17477 Dihydrochloride hydrate; TRIM = 1-(2-trifluoromethylphenyl) imidazole.

Table 1
Experimental groups and doses.

Group	Name	Abbreviation	Size	Dose (mg/mL)	Description
1	Saline control	7.5% NaCl	n = 7	0.075	Vehicle for all groups
2	Adenosine + lidocaine + magnesium	ALM	n = 8	A: 0.26; L: 0.8; M: 0.3	Novel resuscitation fluid therapy
3	Adenosine + lidocaine	AL	n = 8	A: 0.26; L: 0.8	ALM resuscitation therapy individual actives
4	Magnesium	M	n = 8	0.3	
5	N^G-nitro-L-arginine methyl ester	L-NAME	n = 4	40	Non-specific NO synthase inhibitor[45]
6	Adenosine + lidocaine + magnesium + N^G-nitro-L-arginine methyl ester	ALM + L-NAME	n = 8	A: 0.26; L: 0.8; M: 0.3; L-NAME: 40	Effect of nonspecific NO synthase inhibitor on ALM resuscitation
7	Adenosine + N^G-nitro-L-arginine methyl ester	A + L-NAME	n = 4	A: 0.26; L-NAME: 40	Effect of nonspecific NO synthase inhibition on individual actives and combinations of ALM fluid therapy
8	Lidocaine + N^G-nitro-L-arginine methyl ester	L + L-NAME	n = 4	L: 0.8; L-NAME: 40	
9	Magnesium + N^G-nitro-L-arginine methyl ester	M + L-NAME	n = 4	M: 0.3; L-NAME: 40	
10	Adenosine + lidocaine + N^G-nitro-L-arginine methyl ester	A + L + L-NAME	n = 4	A: 0.26; L: 0.8; L-NAME: 40	
11	Adenosine + magnesium + N^G-nitro-L-arginine methyl ester	A + M + L-NAME	n = 4	A: 0.26; M: 0.3; L-NAME: 40	
12	Lidocaine + magnesium + N^G-nitro-L-arginine methyl ester	L + M + L-NAME	n = 4	L: 0.8; M: 0.3; L-NAME: 40	
13	Adenosine + lidocaine + magnesium + wortmannin	ALM + Wortmannin	n = 4	A: 0.26; L: 0.8; M: 0.3; wortmannin: 1.33	Effect of phosphatidylinositol-3-kinase inhibitor on ALM resuscitation. PI-3-kinase activation and protein kinase B/Akt signaling increases eNOS activity[21]
14	Adenosine + lidocaine + magnesium + 1*H*-[1,2,4] oxadiazolo[4,3-*a*] quinoxalin-1-one	ALM + ODQ	n = 4	A: 0.26; L: 0.8; M: 0.3; ODQ: 2.67	Effect of selective inhibition of NO-sensitive guanylyl cyclase which mediates cardiovascular and platelet actions of NO[22,23] on ALM resuscitation
15	Adenosine + lidocaine + magnesium + aminoguanidine hydrochloride	ALM + AMG	n = 4	A: 0.26; L: 0.8; M: 0.3; AMG: 26.7	Effect of iNOS selective inhibitor[24] on ALM resuscitation
16	Adenosine + lidocaine + magnesium + 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide	ALM + PTIO	n = 4	A: 0.26; L: 0.8; M: 0.3; PTIO: 13.3	Effect of NO scavenger[25] on ALM resuscitation
17	Adenosine + lidocaine + magnesium + N-[3-aminomethyl]benzyl] acetamidine	ALM + 1400W	n = 4	A: 0.26; L: 0.8; M: 0.3; 1400W: 13.3	Effect of iNOS selective inhibitor[26] on ALM resuscitation
18	Adenosine + lidocaine + magnesium + *S*-methyl-L-thiocitrulline	ALM + SMTC	n = 4	A: 0.26; L: 0.8; M: 0.3; SMTC: 1.33	Effect of nNOS selective inhibitor[27] on ALM resuscitation
19	Adenosine + lidocaine + magnesium + ARL17477 Dihydrochloride hydrate	ALM + ARL17477	n = 4	A: 0.26; L: 0.8; M: 0.3; ARL17477: 1.33	Effect of nNOS selective inhibitor[28] on ALM resuscitation
20	Adenosine + lidocaine + magnesium + 1-(2-trifluoromethylphenyl) imidazole	ALM + TRIM	n = 4	A: 0.26; L: 0.8; M: 0.3; TRIM: 1.33	Effect of nNOS and iNOS selective inhibitor[29] on ALM resuscitation

NO = nitric oxide; eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase.

60-minute monitoring (Figures 3 and 4).[1] A key finding of the present study was that NO inhibition with L-NAME in the presence of 7.5% NaCl ALM led to a rapid and lethal hypotensive state following hemorrhagic shock (Figures 2, 3, and 5). This was surprising because 7.5% NaCl L-NAME (no ALM) significantly increased MAP (Figure 3**C**), and L-NAME has been shown to be a powerful resuscitative agent in a number of preclinical models.[15,18,19] We further showed that L-NAME with other combinations (adenosine, lidocaine, magnesium, AM, and AL) resuscitated with 100% survival (Figures 2 and 4). However, in terms of our development of a new ALM fluid therapy, we have previously shown that the individual actives or AM, LM, and AL combinations are not optimal,[1,4,12,32] and despite L-NAME's well-known ability to increase MAP, it has been shown to have multiple adverse effects in animals and human beings on liver and kidney function.[18,33–35]

The underlying mechanisms of why inhibiting NOS with L-NAME abolishes ALM's resuscitative effect in our model is not currently known. When we examined single and dual combinations of ALM active ingredients, we found that after 15 minutes, LM with L-NAME combination contributed to 7.5% NaCl ALM + L-NAME's effect, but it did not explain the precipitous fall in MAP at 5 minutes (Figures 4 and 5). It was only when the 3 components—adenosine, lidocaine, and magnesium—were present that MAP plummets to zero in the presence of L-NAME, which suggests a role for NO for 7.5% NaCl ALM resuscitative actions. The possible NO mechanisms for cardiovascular collapse include loss of vascular tone, loss of cardiac function, and/or a major defect in the central nervous system (eg, nucleus tractus solitaries [NTS]) controlling cardiovascular function.

We believe a direct effect of NO on vascular tone and cardiac function are unlikely candidates for the cardiovascular collapse because removing the effect of NO by blocking its synthesis with L-NAME would result in constriction (higher MAP) and have a positive inotropic effect,[18,36] not the opposite as we found (Figure 3**A**). Similarly, specifically blocking NO actions through cyclic guanosine monophosphate (cGMP) with ODQ would increase MAP because elevation of cGMP in cardiomyocytes or intact heart is associated with a negative inotropic effect.[37,38] Blocking cGMP-dependent NO functions using ODQ added to 7.5% NaCl ALM improved MAP; however, substituting ODQ with L-NAME dropped MAP implying cardiovascular collapse mechanisms are not directly leveled at vascular tone or cardiac function. The finding that HR remained steady or dropped slightly despite a decrease in blood pressure during the bleed period is typical of rats, and has been reported by us,

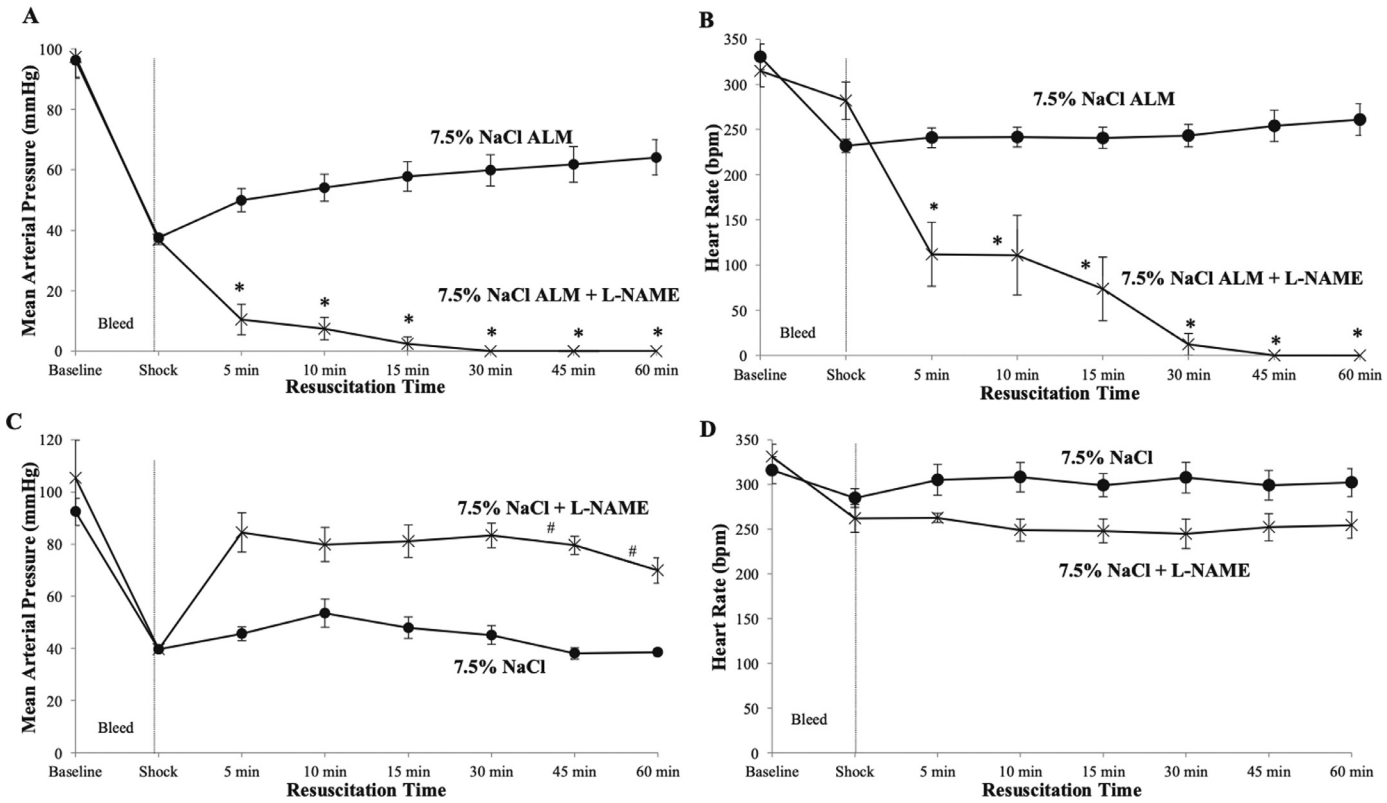

Figure 3. Mean arterial pressure (MAP) and heart rate (HR) at baseline, after 60-minutes of shock, and during 60-minute resuscitation period for 7.5% sodium chloride (NaCl) adenosine, lidocaine, and magnesium (ALM) and 7.5% NaCl ALM + N^G-nitro-L-arginine methyl ester (L-NAME) groups (A and B) and 7.5% NaCl and 7.5% NaCl + L-NAME groups (C and D). Values are presented as mean (SEM). $^*P < 0.05$ compared with 7.5% NaCl ALM; $^\#P < 0.05$ compared with 7.5% NaCl.

and others, in hemorrhagic shock models.[1,9,11] The maintenance of lower HRs in some groups during 60-minute resuscitation compared with baseline (Figure 4**B**), may be due to the differential bradycardic effects of adenosine, lidocaine, and magnesium[1,39]; however, the rapid and profound bradycardic effect observed with 7.5% NaCl ALM L-NAME was likely due to a central nervous system effect leveled at the sinatrial node as part of irreversible shock (MAP < 20 mm Hg). This question requires further study.

A likely candidate for the cardiovascular collapse from 7.5% NaCl ALM with L-NAME is an effect in the NTS located in the medulla. L-NAME is known to cross the blood-brain barrier to the NTS, where both endothelial and neuronal forms of NOS are expressed.[40,41] The medullary NTS integrates convergent information from the body's organs and regulates sympathetic and parasympathetic outflows, and itself is the site of substantial modulation by NO,[14,42,43] adenosine,[44–47] and sodium ion fast-channel modulating drugs, such as lidocaine.[48] For example, activation of adenosine receptors and NO pathways in the NTS has been shown to differentially inhibit or reset the baroreflex control of MAP, HR, and renal sympathetic nerve activity.[44,47,49] Bilateral microinjections of lidocaine (and γ-Aminobutyric acid type A (GABA-A) receptor agonists) into the NTS have also been shown to increase MAP in alpha-chloralose-anesthetized control rats.[50] Similarly, Wang et al[48] have also shown that the tonic blockade of cardiac sympathetic afferent reflex by epicardial lidocaine in chronic heart failure experiments can reduce the activity of the NTS chemoreceptive neurons, and alter sympathetic outflows to the heart, and possibly other organs. We conclude that the cardiovascular collapse after administration of 7.5% NaCl ALM + L-NAME appears to be linked to a complex interaction between NO and ALM in the NTS, a proposal that requires further investigation.

Contribution of different NOS isoforms to the observed protective effect of ALM

Our study also found that MAP increased at 5 minutes of resuscitation when specific neuronal NOS inhibitors SMTC or ARL17477 were added to 7.5% NaCl ALM, which was opposite to 7.5% NaCl ALM + L-NAME (Figure 5**A**). These data support Copp et al's[51] earlier studies showing that SMTC increases peripheral vasoconstriction in animal models. Similarly, as previously mentioned, MAP increased in the presence of the selective guanylyl cyclase inhibitor ODQ, which is consistent with the study of Olson et al[29] who showed that it reversed NO-induced vasorelaxation.[29] MAP was also corrected to different degrees with neuronal and inducible NOS inhibitor TRIM,[52] and inducible NOS inhibitor AMG (Figure 5**A**). Following an initial increase, MAP fell 48% with NO scavenger PTIO, and 25% when endothelial NOS activity was blocked through phosphatidylinositol 3-kinase inhibition with wortmannin, suggesting continued NO availability is required for ALM resuscitation. This is consistent with previous studies demonstrating a beneficial effect of NO synthesis after hemorrhagic shock.[34] MAP did not increase from shock values when 7.5% NaCl ALM was combined with inducible NOS inhibitor, 1400W, which was different from the large increases in a rat model reported in the study of Kan et al[18] (MAP = 104 mm Hg), and with L-NAME alone (108 mm Hg).

We therefore conclude that the cardiovascular collapse after administration of the nonselective NOS inhibitor L-NAME with 7.5% NaCl ALM does not appear to involve NO produced by neuronal NOS pathways but may have involved a partial contribution from inducible NOS (Figure 5). However, no NO or NOS inhibitor mimicked the rapid fall in MAP or mortality found with 7.5% NaCl ALM with L-NAME. On the basis of our data it is possible that the car-

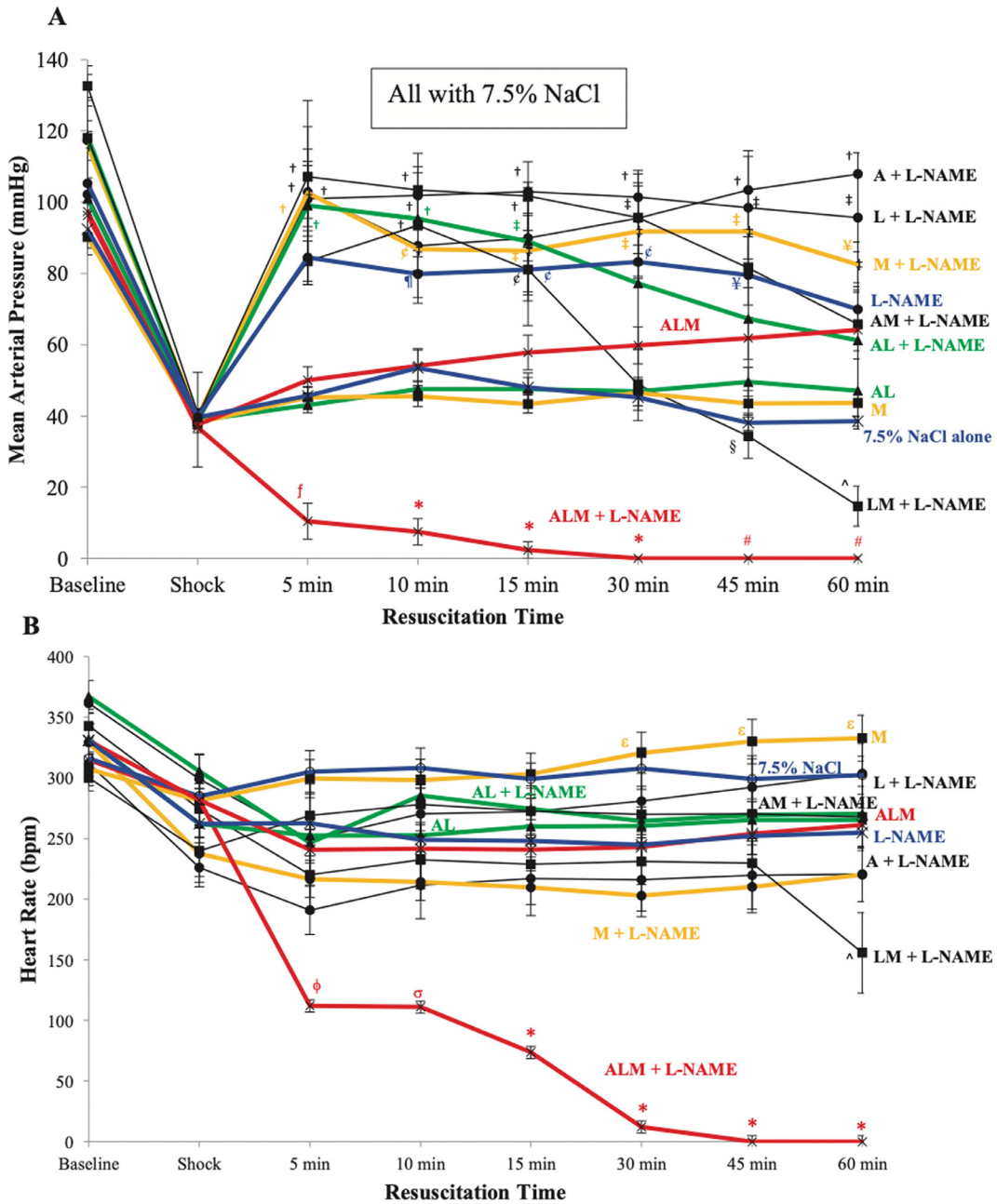

Figure 4. (A) Mean arterial pressure (MAP) and (B) heart rate (HR) at baseline, after 60-minutes of shock, and during 60-minute resuscitation period for 7.5% sodium chloride (NaCl) alone; 7.5% NaCl with adenosine, lidocaine, and magnesium (ALM), adenosine and lidocaine (AL), and magnesium (M); 7.5% NaCl + N^G-nitro-L-arginine methyl ester (L-NAME); and 7.5% NaCl + L-NAME with adenosine (A), lidocaine (L), M, AL, adenosine and magnesium (AM), lidocaine and magnesium (LM), and ALM. Values are presented as mean (SEM). $^{f}P < 0.05$ compared with all groups except 7.5% NaCl alone and 7.5% NaCl AL; $^{*}P < 0.05$ compared with all groups; $^{\#}P < 0.05$ compared with all groups except 7.5% NaCl LM + L-NAME; $^{\dagger}P < 0.05$ compared with 7.5% NaCl, 7.5% NaCl AL, 7.5% NaCl M, and 7.5% NaCl ALM groups; $^{\S}P < 0.05$ compared with 7.5% NaCl M; $^{¥}P < 0.05$ compared with 7.5% NaCl; $^{\epsilon}P < 0.05$ with 7.5% NaCl M and 7.5% NaCl AL; $^{\ddagger}P < 0.05$ compared with 7.5% NaCl, 7.5% NaCl AL, and 7.5% NaCl M; $^{\S}P < 0.05$ compared with 7.5% NaCl + L-NAME, 7.5% NaCl A + L-NAME, 7.5% NaCl L + L-NAME, and 7.5% NaCl M + L-NAME; $^{\wedge}P < 0.05$ compared with 7.5% NaCl + L-NAME, 7.5% NaCl A + L-NAME, 7.5% NaCl L + L-NAME, 7.5% NaCl M + L-NAME, and 7.5% NaCl ALM; $^{\phi}P < 0.05$ compared with all groups except 7.5% NaCl A + L-NAME and 7.5% NaCl M + L-NAME; $^{\sigma}P < 0.05$ compared with 7.5% NaCl L-NAME, 7.5% NaCl L + L-NAME, 7.5% NaCl AL + L-NAME, 7.5% NaCl AM + L-NAME, and 7.5% NaCl LM + L-NAME; $^{\epsilon}P < 0.05$ compared with 7.5% NaCl A + L-NAME, 7.5% NaCl M + L-NAME, and 7.5% NaCl LM + L-NAME.

diovascular collapse involved endothelial NOS inhibition because it has been reported that L-NAME has 2000 times more selectivity for endothelial NOS than inducible NOS.[53] Unfortunately, there are no specific endothelial NOS inhibitors at present that can be solubilized for safe animal administration to test this hypothesis. The use of another nonspecific NOS inhibitor, N5-(1-Iminoethyl)-*L*-ornithine (L-NIO) dihydrochloride, was considered to support the L-NAME findings; however, the dose for effective inhibition in a rat (20 mg/kg) would require 26.67 mg/mL, which exceeds the solubility limit of 24.61 mg/mL. Another potential limitation of the present study was the variation in selectivity of the NOS inhibitors tested, especially in the in vivo environment where anesthesia, artificial ventilation, and heparinization may affect drug pharmacokinetics. Further experiments are required to tease apart the underlying NO-specific mechanisms to understand the nature of the L-NAME effect in the presence of hypertonic ALM resuscitation fluid, and loss of whole-body protection. This includes measurement of circulating NO and levels in heart muscle and vascular tissue (eg, NOS protein expression) before, during, and following shock.

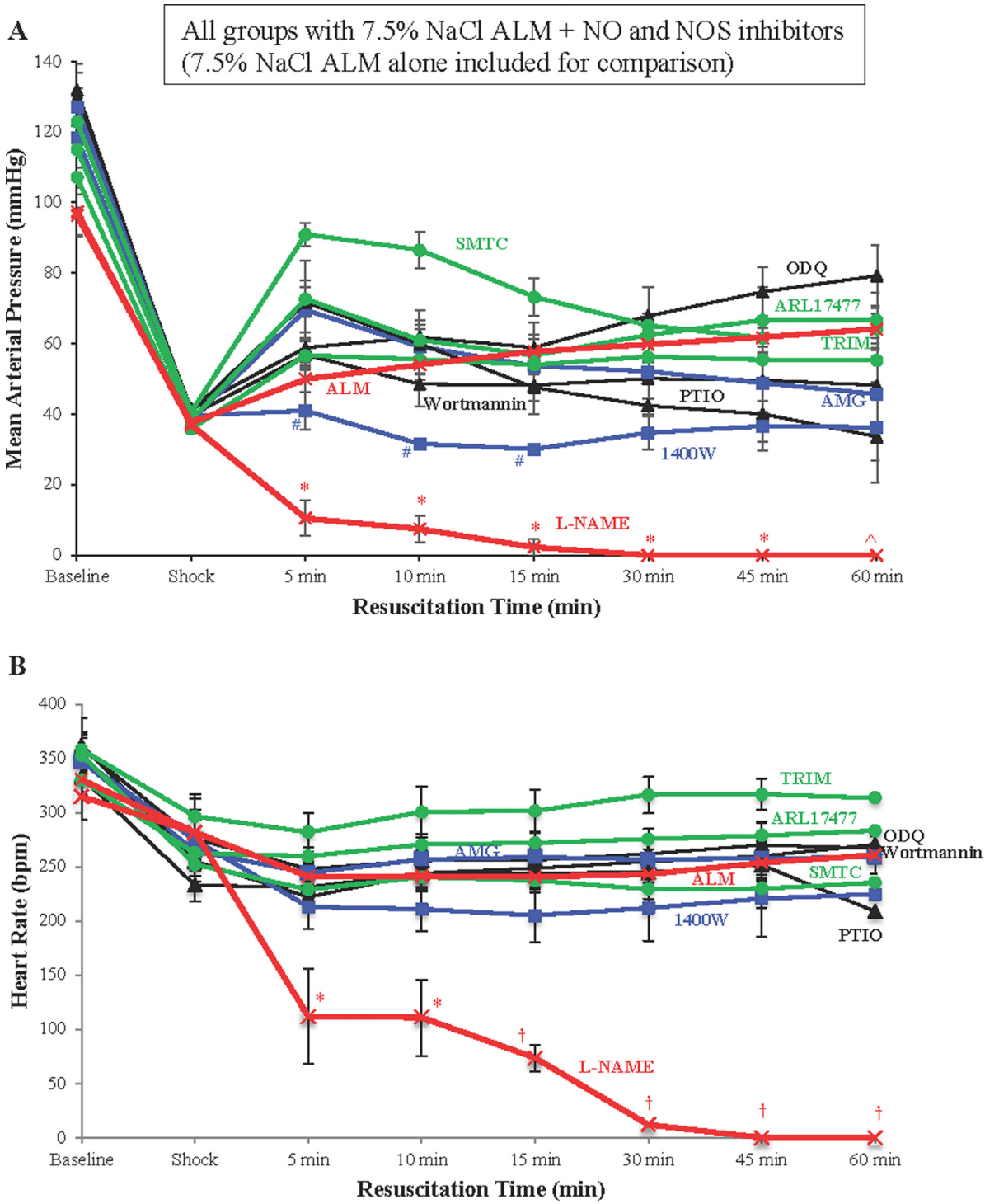

Figure 5. (A) Mean arterial pressure (MAP) and (B) heart rate (HR) at baseline, after 60-minutes of shock, and during 60-minute resuscitation period for 7.5% sodium chloride (NaCl) adenosine, lidocaine, and magnesium (ALM) alone, and 7.5% NaCl ALM with N^G-nitro-L-arginine methyl ester (L-NAME), wortmannin, 1*H*-[1,2,4] Oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ), aminoguanidine (AMG), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), N-[3-aminomethyl]benzyl] acetamidine (1400W), *S*-methyl-L-thiocitrulline (SMTC), ARL17477 dihydrochloride hydrate (ARL17477), and 1-(2-trifluoromethylphenyl) imidazole (TRIM). Values are presented as mean (SEM). Inducible nitric oxide synthase (iNOS) selective inhibitors (1400W and AMG) are highlighted in blue, whereas neuronal nitric oxide synthase (nNOS) selective inhibitors (SMTC, ARL17477, and TRIM) are highlighted in green. $^{*}P < 0.05$ compared with all groups except 1400W; $^{\wedge}P < 0.05$ compared with all groups except 1400W and PTIO; $^{\#}P < 0.05$ compared with SMTC; $^{\dagger}P < 0.05$ compared with all groups.

Conclusions

The resuscitation efficacy of 7.5% NaCl ALM appears to be linked to 1 or more NO-producing pathways. Using a variety of NOS and NO inhibitors, we report that nonspecific NOS inhibition with L-NAME blocked ALM resuscitation and led to cardiovascular collapse. Future work will examine NO site-specific contributions and actions to support cardiovascular function, and possible involvement of the central nervous system.

Conflicts of Interest

G. Dobson is the inventor of the ALM concept for cardiac surgery, trauma, and sepsis. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

CRediT authorship contribution statement

Hayley L. Letson: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Geoffrey P. Dobson:** Conceptualization, Supervision, Methodology, Resources, Visualization, Writing – original draft.

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