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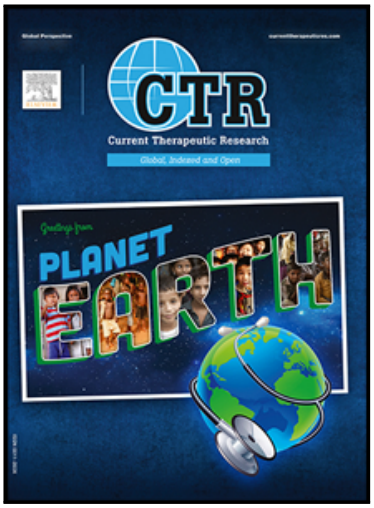

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PII: S0011-393X(21)00033-3
DOI: https://doi.org/10.1016/j.curtheres.2021.100655
Reference: CUTHRE 100655

To appear in: *Current Therapeutic Research*

Received date: 12 October 2021
Accepted date: 12 November 2021

Please cite this article as: Hayley L. Letson PhD , Geoffrey P. Dobson PhD FAHA , The role of nitric oxide in the efficacy of adenosine, lidocaine and magnesium (ALM) treatment of experimental hemorrhagic shock in rats, *Current Therapeutic Research* (2021), doi: https://doi.org/10.1016/j.curtheres.2021.100655

ORIGINAL RESEARCH ARTICLE

The role of nitric oxide in the efficacy of adenosine, lidocaine and magnesium (ALM) treatment of experimental hemorrhagic shock in rats

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Short Title: ALM protection involves nitric oxide synthesis

Conflict of Interest: GD is the inventor of the ALM concept for cardiac surgery, trauma and sepsis. HL has no conflicts to declare.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions: HL: Conceptualization, Methodology, Formal Analysis, Investigation, Visualization, Writing – Review & Editing. **GD**: Conceptualization, Supervision, Methodology, Resources. Visualization, Writing – Original Draft.

Abstract

Background: Since nitric oxide (NO) plays multiple roles regulating the central nervous, cardiovascular and immune systems, our aim was to investigate the role of NO in the efficacy of 7.5% NaCl adenosine, lidocaine and magnesium (ALM) to improve mean arterial pressure (MAP) and heart rate (HR) following hemorrhagic shock (HS).

Methods: Male Sprague-Dawley rats (101; 425±6g) were randomly assigned to 20 groups (n=4-n=8). HS (MAP<40mmHg) was induced by 20min pressure-controlled bleeding (~40% blood volume), and the animal was left in shock (MAP 35-40mmHg) for 60min. NO synthase (NOS) inhibitor L-NAME was administered with a 0.3ml bolus of different combinations of 7.5% NaCl ALM active ingredients and hemodynamics were monitored for 60min. A number of specific NOS and NO inhibitors were tested.

Results: 7.5% NaCl ALM corrected MAP after HS. 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 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 (iNOS) inhibitor 1400W to 7.5% NaCl ALM failed to resuscitate, while

NO scavenger PTIO and PI3K inhibitor Wortmannin reduced MAP recovery during 60min resuscitation.

Conclusions: The ability of 7.5% NaCl ALM to resuscitate appears to be linked to one or more NO-producing pathways. Non-specific 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.

Keywords: hemorrhagic shock; nitric oxide; resuscitation; nitric oxide synthase; endothelium.

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 three 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 60-65 mmHg (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 A, L or M, 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 NO is important for the maintenance of myocardial Ca^{2+} 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]. Since NO is pivotal in regulating the cardiovascular, central nervous and immune systems [14, 15, 17], and 7.5% 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 adenosine, lidocaine and magnesium (ALM) resuscitation following severe hemorrhagic shock (HS) in the rat model. Our hypothesis is that addition of the non-specific NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), to ALM therapy will impact 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 (425±6g) were obtained from the institutional breeding colony, and housed in a 14-10 hr light-dark cycle with free access to food and water *ad libitum*. Animals were heparinized (Hospira, 2,500IU) and anesthetized intraperitoneally with 100mg/kg sodium thiopentone (Thiobarb). Anesthetic was administered as required throughout the protocol. The study was approved by the institutional Animal Ethics Committee (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* (NIH, 8th Edition, 2011). As per the Australian code, the 3Rs ('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 ('replacement'). Power analysis was conducted to determine the smallest number of animals necessary to achieve the study's aims (see *Statistical Analysis*) ('reduction'), and depth of anesthesia was continuously monitored to ensure animal wellbeing ('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/min with positive end expiratory pressure (PEEP) of 1cm, and tidal volume of 5ml/kg (Harvard Small Animal Ventilator, Holliston, 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, Australia), and the right femoral artery was cannulated for blood-letting. All cannulae contained

heparinized saline (1,000U/mL saline). Lead II electrocardiogram (ECG) was attached for BioAmp recording (ADInstruments). Following surgical instrumentation, anesthetized animals had a 10min baseline equilibration period (Fig 1). Animals were excluded from study if they (i) were difficult to anesthetize, (ii) experienced complex arrhythmias during preparation, stabilization or within the shock period, or (iii) were hemodynamically unstable prior to phlebotomy.

Experimental Design: Rats (n=101) were randomly assigned to one of 20 groups (Table 1). 7.5% NaCl was used as the vehicle for all groups. Study groups 1-12 examined the effect of the non-specific NOS inhibitor L-NAME on 7.5% NaCl + ALM resuscitation. Doses were 30mg/kg (40mg/ml) L-NAME (N^G-nitro-L-arginine methyl ester), 1mM (0.26mg/ml) adenosine, 3mM (0.8mg/ml) lidocaine and 2.5mM (0.3mg/ml) $MgSO_4$, as per previous studies [1, 9 18, 20]. Study groups 13-20 examined the effect of specific NOS inhibitors and modulators, including Wortmannin (1mg/kg) [21], Oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ; 2mg/kg) [22, 23], Aminoguanidine hydrochloride (AMG; 20mg/kg) [24], 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; 10mg/kg) [25], N-[3-aminomethyl]benzyl] acetamidine (1400W; 10mg/kg) [26], *S*-methyl-L-thiocitrulline (SMTC; 1mg/kg) [27], ARL17477 Dihydrochloride hydrate (ARL17477; 1mg/kg) [28], and 1-(2-trifluoromethylphenyl) imidazole (TRIM; 1mg/kg) [29] (refer Table 1 for group sizes and specificities).

Shock protocol: Hemorrhagic shock was induced by withdrawing arterial blood to a mean arterial pressure (MAP) of 35-40mmHg. Blood-letting started at ~1ml/min before decreasing to ~0.4ml/min. Phlebotomy was continued for 20min and then rats were left in shock for 60min

with blood withdrawal or infusion to ensure MAP remained between 35 to 40mmHg (Fig 1). The average shed volume was 10.6±0.2ml and represented an average blood loss of 40.3±0.6% (calculated from [(0.06 x body weight (g)) + 0.77] [30]), with no difference between groups. At the end of shock, rats were injected with 0.3ml treatment bolus (~3-4% of shed volume) into the femoral vein over a 10sec period, and were monitored for a further 60min. 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 ECG. Ventricular arrhythmias including premature ventricular contractions (PVCs), 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). All values are expressed as mean±SEM. Data normality was assessed numerically with Shapiro-Wilks's test. Analysis of variance (ANOVA) was used to evaluate parametric data with Tukey's HSD or Dunnett's 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 to determine sample size to minimize Type 1 errors (MAP 15min resuscitation; n=4; Cohen's d=3; Critical t=2.45; α err prob=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 survival time 19.4±4.5min; $p<0.001$) (Fig 2). All other groups survived the 60min resuscitation period following 20min blood loss and 60min shock (Figs 2A and 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 to 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 64mmHg at 60min (67% baseline) (Fig 3A), while HR remained at a constant 240-260bpm (73-79% of baseline) (Fig 3B). In contrast, in the presence of L-NAME, MAP decreased towards baseline at 5min (10mmHg) and HR decreased to 112bpm. MAP and HR approached zero at 30min (Figs 3A and B). The effect of L-NAME to cause cardiovascular collapse did not occur if it was added *after* 60min 7.5% NaCl ALM resuscitation. There was no change in MAP (66mmHg 15min after 30mg/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 53mmHg at 10min before falling below 40mmHg at 60min (Fig 3C). When L-NAME was added to 7.5% NaCl alone, MAP increased from shock values by up to 1.7-fold over the 60min, and HR decreased by ~17% (Figs 3C and D).

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

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

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

In contrast to L-NAME that led to cardiovascular collapse, the addition of other NOS or NO inhibitors to 7.5% NaCl ALM did not have this effect (Fig 5). At 5min resuscitation, the presence of NOS inhibitors SMTC, ARL17477, and AMG, and NO scavenger PTIO increased MAP by 1.4 to 1.8-fold compared to 7.5% NaCl ALM alone (Fig 5A). In contrast, ODQ, TRIM and Wortmannin had little effect on MAP recovery with 7.5% NaCl ALM, while the addition of the iNOS inhibitor 1400W failed to resuscitate from shock (Fig 5A). After the initial MAP increase, the AMG and PTIO groups fell towards or below shock values (Fig 5A). After 5min, 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 15-60min resuscitation compared to all other groups (Fig 5B). All groups maintained a relatively constant HR across 60min resuscitation, except for the NO scavenger PTIO which fell by 17% from 252bpm at 45min to 209bpm at 60min. The nNOS inhibitors TRIM and ARL17477 produced the highest heart rates (15-23% and 8-12% higher than 7.5% NaCl ALM alone, respectively) (Fig 5B). 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 to 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 non-selective inhibitor of nitric oxide synthase, 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 one 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 A, L and M actives, 7.5% NaCl ALM was the only treatment to resuscitate and maintain MAP in the permissive hypotensive range over 60min monitoring (Figs 3 & 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 (Figs 2, 3 & 5). This was surprising since 7.5% NaCl L-NAME (no ALM) significantly increased MAP (Fig 3C), 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 (A, L, M, AM, and AL) resuscitated with 100% survival (Figs 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 humans on liver and kidney function [18, 33-35].

The underlying mechanisms of why inhibiting nitric oxide synthesis 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 15min 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 5min (Figs 4 and 5). It was only when the three components, ALM, 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: 1) loss of vascular tone, 2) loss of cardiac function, and/or 3) a major defect in the central nervous system (e.g., nucleus tractus solitarius, 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 (Fig 3A). Similarly, specifically blocking NO actions through 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 levelled at vascular tone or cardiac function. The finding that heart rate 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, and others, in hemorrhagic shock models [1, 9, 11]. The maintenance of lower heart rates in some groups during 60min resuscitation compared to baseline (Fig 4B), 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 CNS effect leveled at the SA node as part of irreversible shock (MAP<20mmHg). 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 Na^{2+} 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 GABA-A receptor agonists) into the NTS have also been shown to increase MAP in alpha-chloralose-anesthetized control rats [50]. Similarly, Wang and colleagues 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 [48]. 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 NO synthase isoforms to the observed protective effect of ALM

Our study also found that MAP increased at 5min resuscitation when specific nNOS inhibitors SMTC or ARL17477 were added to 7.5% NaCl ALM, which was opposite to 7.5% NaCl ALM + L-NAME (Fig 5A). These data support Copp and colleagues' earlier studies showing that SMTC increases peripheral vasoconstriction in animal models [51]. Similarly, as previously mentioned, MAP increased in the presence of selective guanylyl cyclase inhibitor, ODQ, which is consistent with the study of Olson and colleagues 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 iNOS inhibitor AMG (Fig 5A). Following an initial increase, MAP fell 48% with NO scavenger PTIO, and 25% when eNOS 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 iNOS inhibitor, 1400W, which was different from the large increases in a rat model reported in the study of Kan and colleagues (MAP 104mmHg), and with L-NAME alone (108mmHg) [18].

We therefore conclude that the cardiovascular collapse after administration of the non-selective NOS inhibitor L-NAME with 7.5% NaCl ALM does not appear to involve NO produced by nNOS pathways but may have involved a partial contribution from iNOS (Fig 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 cardiovascular collapse involved eNOS inhibition because it has been reported that L-NAME has 2000 times more selectivity for eNOS than iNOS [53]. Unfortunately, there are no specific eNOS inhibitors at present that can be solubilized for safe animal administration to test this hypothesis. The use of another non-specific NOS inhibitor, L-NIO dihydrochloride, was considered to support the L-NAME findings, however, the dose for effective inhibition in the rat (20mg/kg) would require 26.67mg/ml, which exceeds the solubility limit of 24.61mg/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 (e.g., NOS protein expression) prior to, during, and following shock.

Conclusion

The resuscitation efficacy of 7.5% NaCl ALM appears to be linked to one or more NO-producing pathways. Using a variety of NOS and NO inhibitors, we report that non-specific 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.

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Figure Legends

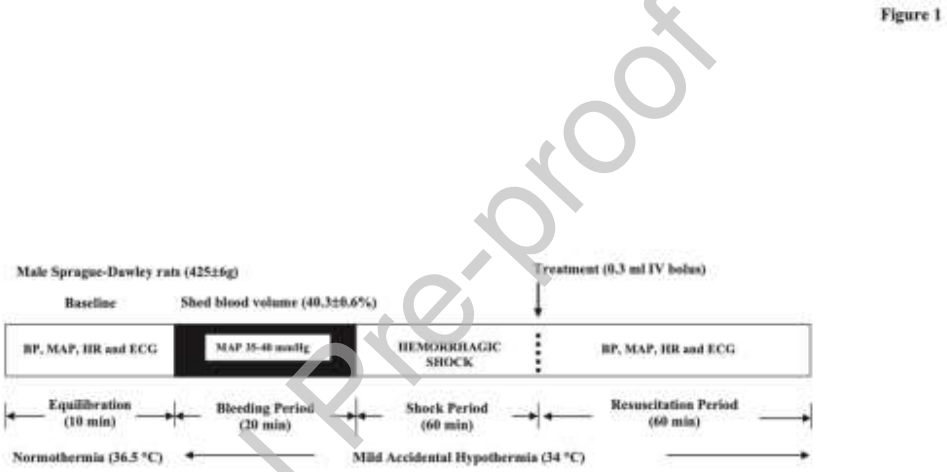

Figure 1: A schematic of the pressure controlled *in vivo* rat protocol of hemorrhagic shock. Shed blood volume was taken over a 20min period to maintain mean arterial blood pressure of 35 to 40mmHg (40.3±0.6% blood loss), and the rat remained in shock for a period of 60min prior to resuscitation (0.3ml intravenous fluid bolus). See Methods for treatment group details.

Figure 2

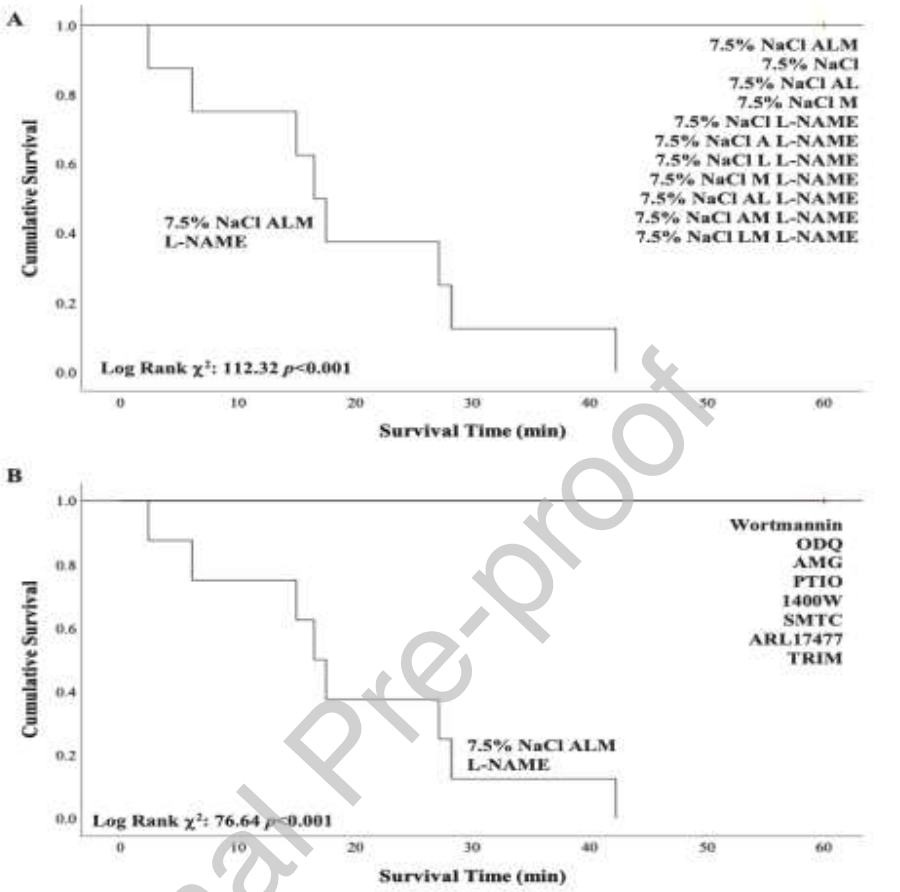

Figure 2: Kaplan-Meier survival curves for (A) L-NAME study and (B) NO/NOS inhibitor study. 100% of 7.5% NaCl ALM + L-NAME-treated animals died during the 60min resuscitation period (mean survival time 19.4±4.5min; $p<0.001$). L-NAME = N^G-nitro-L-arginine

methyl ester; NO = nitric oxide; NOS = nitric oxide synthase; ALM = adenosine, lidocaine and magnesium; A = adenosine; L = lidocaine; M = magnesium; AL = adenosine and lidocaine; AM = adenosine and magnesium; LM = lidocaine and magnesium; ODQ = 1*H*-[1,2,4] Oxadiazolo[4,3-*a*] quinoxalin-1-one; AMG = aminoguanidine; 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.

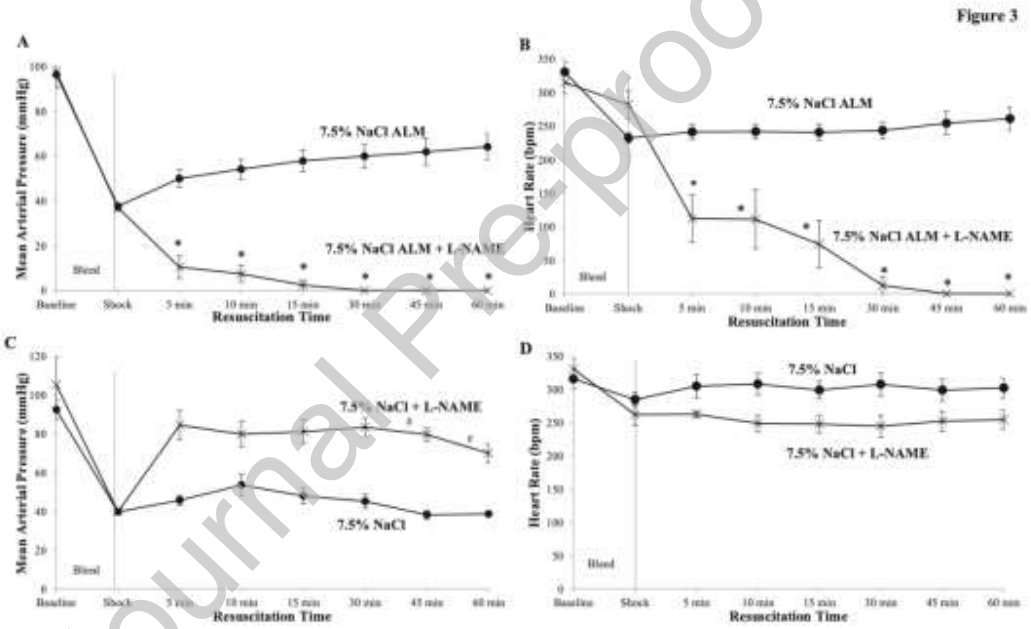

Figure 3: Mean arterial pressure (MAP, mmHg) and heart rate (HR, bpm) at baseline, after 60min shock, and during 60min resuscitation period for 7.5% NaCl ALM and 7.5% NaCl ALM + L-NAME groups (A, B) and 7.5% NaCl and 7.5% NaCl + L-NAME groups (C, D). Values represent mean±SEM. ALM = adenosine, lidocaine and magnesium; L-NAME =

N^G-nitro-L-arginine methyl ester. * p<0.05 compared to 7.5% NaCl ALM; # p<0.05 compared to 7.5% NaCl.

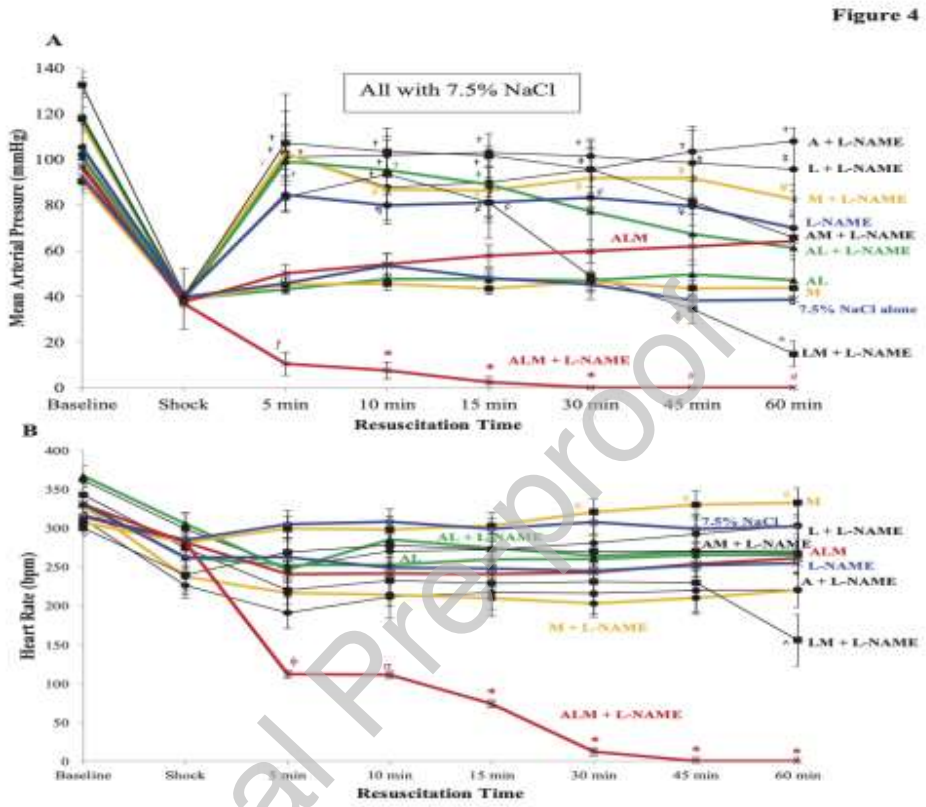

Figure 4: (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) at baseline, after 60min shock, and during 60min resuscitation period for 7.5% NaCl alone; 7.5% NaCl with ALM, AL, and M; 7.5% NaCl + L-NAME; and 7.5% NaCl + L-NAME with A, L, M, AL, AM, LM, and ALM. Values represent mean±SEM. ALM = adenosine,

lidocaine and magnesium; A = adenosine; L = lidocaine; M = magnesium; AL = adenosine and lidocaine; AM = adenosine and magnesium; LM = lidocaine and magnesium; L-NAME = N^G-nitro-L-arginine methyl ester. [f] $p<0.05$ compared to all groups except 7.5% NaCl alone and 7.5% NaCl AL; * $p<0.05$ compared to all groups; [#] $p<0.05$ compared to all groups except 7.5% NaCl LM + L-NAME; [†] $p<0.05$ compared to 7.5% NaCl, 7.5% NaCl AL, 7.5% NaCl M, and 7.5% NaCl ALM groups; [¶] $p<0.05$ compared to 7.5% NaCl M; [¥] $p<0.05$ compared to 7.5% NaCl; [¢] $p<0.05$ compared to 7.5% NaCl M and 7.5% NaCl AL; [‡] $p<0.05$ compared to 7.5% NaCl, 7.5% NaCl AL, and 7.5% NaCl M; [§] $p<0.05$ compared to 7.5% NaCl + L-NAME, 7.5% NaCl A + L-NAME, 7.5% NaCl L + L-NAME, and 7.5% NaCl M + L-NAME; [^] $p<0.05$ compared to 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; [ϕ] $p<0.05$ compared to all groups except 7.5% NaCl A + L-NAME and 7.5% NaCl M + L-NAME; [σ] $p<0.05$ compared to 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; [ε] $p<0.05$ compared to 7.5% NaCl A + L-NAME, 7.5% NaCl M + L-NAME, and 7.5% NaCl LM + L-NAME.

Figure 5

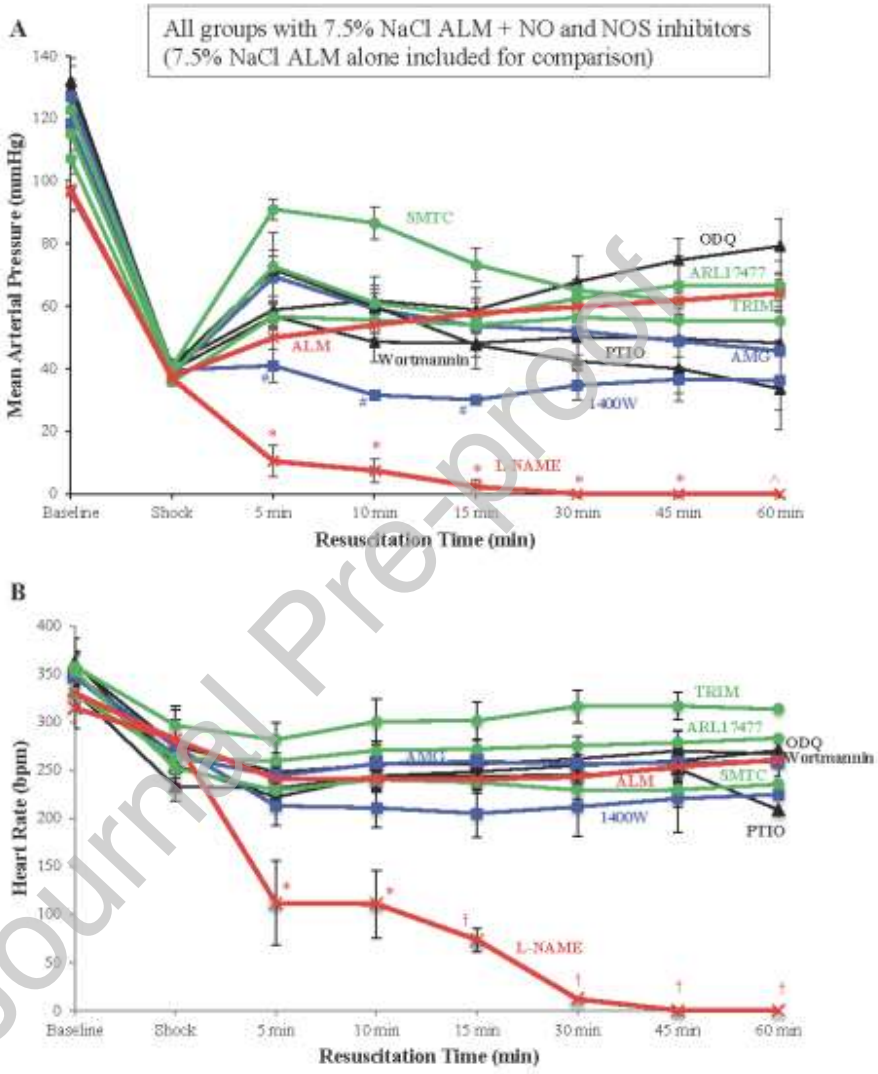

Figure 5: (A) Mean arterial pressure (MAP, mmHg) and (B) heart rate (HR, bpm) at baseline, after 60min shock, and during 60min resuscitation period for 7.5% NaCl ALM alone, and 7.5% NaCl ALM with L-NAME, Wortmannin, ODQ, AMG, PTIO, 1400W, SMTC, ARL17477, and TRIM. Values represent mean ± SEM. iNOS selective inhibitors (1400W and AMG) are highlighted in blue, while nNOS selective inhibitors (SMTC, ARL17477, and TRIM) are highlighted in green. * $p<0.05$ compared to all groups except 1400W; $^{\wedge}$ $p<0.05$ compared to all groups except 1400W and PTIO; $^{\#}$ $p<0.05$ compared to SMTC; † $p<0.05$ compared to all groups. ALM = adenosine, lidocaine and magnesium; L-NAME = N^G-nitro-L-arginine methyl ester; ODQ = 1*H*-[1,2,4] Oxadiazolo[4,3-*a*] quinoxalin-1-one; AMG = aminoguanidine; 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; NOS = nitric oxide synthase; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase.

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 non-specific 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 non-specific 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.