

Effect of valproic acid upon skeletal muscle subjected to prolonged tourniquet application

Robert Moritz ¹, Lee Mangum,¹ Chet Voelker,¹ Gerardo Garcia,¹ Joseph Wenke²

► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/tsaco-2022-001074>).

¹Combat Wound Care Group (CRT4), US Army Institute of Surgical Research, Fort Sam Houston, Texas, USA

²Orthopaedic Surgery and Rehabilitation, The University of Texas Medical Branch at Galveston, Galveston, Texas, USA

Correspondence to

Dr Robert Moritz; robert.j.moritz.ctr@health.mil

Received 12 December 2022

Accepted 16 June 2023

ABSTRACT

Background Valproic acid (VPA), a histone deacetylase inhibitor, has shown improved outcomes when used as a pharmaceutical intervention in animal studies of hemorrhage, septic shock, and combined injuries. This study was designed to investigate the ability of VPA to mitigate ischemia–reperfusion injury produced by prolonged tourniquet application to an extremity.

Methods The ischemia–reperfusion model in anesthetized rats was established using hemorrhage and a 3-hour tourniquet application. VPA was administered intravenously prior to tourniquet wear and removal. Ischemia–reperfusion injury was evaluated by investigating pathway signaling, immune modulation of cytokine release, remote organ injury, and skeletal muscle function during convalescence.

Results We found that VPA sustained Protein kinase B (Akt) phosphorylation and Insulin-like growth factor signaling and modulated the systemic release of interleukin (IL)-1 β , tumor necrosis factor α , and IL-6 after 2 hours of limb reperfusion. Additionally, VPA attenuated a loss in glomerular filtration rate at 3 days after injury. Histological and functional evaluation of extremity skeletal muscle at 3, 7, and 21 days after injury, however, demonstrated no significant differences in myocytic degeneration, necrotic formation, and maximal isometric tetanic torque.

Conclusions Our results demonstrate that VPA sustains early pro-survival cell signaling, reduces the inflammatory response, and improves renal function in a hemorrhage with prolonged ischemia and reperfusion model. However, these do not translate into meaningful preservation in limb function when applied as a pharmaceutical augmentation to tourniquet wear.

Level of evidence IV.

INTRODUCTION

Casualty demographics compiled under the Joint Theater Trauma System describe a fivefold increase in vascular injury during the Iraq and Afghanistan wars when compared with previous conflicts. Extremity wounds compose 54% of all recorded combat wounds directly resulting from hostile enemy action.^{1,2} Casualties with compressible limb injury rapidly exsanguinate, prompting the fielding of battlefield tourniquets with guidelines to use them for hemorrhage control. Since implementation, comparative studies of tourniquet recipients versus those who did not receive a tourniquet show significantly improved survival outcomes for the recipients.³ The extent of tissue injury and dysfunction distal to tourniquet application is directly related to the magnitude and duration of

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Studies investigating valproic acid (VPA) as a therapeutic in models of hemorrhagic shock have shown improved serine/threonine protein kinase (Akt) phosphorylation and survival outcomes.

WHAT THIS STUDY ADDS

⇒ VPA as a therapeutic for focal ischemia associated with prolonged tourniquet application was investigated in this study. Our findings demonstrated sustained Akt phosphorylation in skeletal muscle distal to tourniquet application, but this did not translate into improved functional outcomes.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Pharmaceutical adjuncts to prolonged tourniquet wear is a field of ongoing research. Our study informs the efficacy of histone deacetylase inhibitors for this purpose and demonstrates that Akt signaling may not have utility toward determining functional outcomes.

an ischemic episode. Tourniquet-induced skeletal muscle ischemia for periods beyond 3 hours shows not only the onset of ischemia-based injury but also exacerbated reperfusion injury and potential limb dysfunction.^{4,5} Current evacuation strategy typically enables expeditious transition between echelons of care circumventing the majority of tourniquet-based ischemic injury, though an evolving operational environment may produce scenarios which exceed the ischemic threshold of permanent injury. Perpetuating tourniquet use as a field strategy when evacuation to definitive care is delayed benefits from investigation of supplemental pharmaceutical strategies to mitigate ischemia–reperfusion injury. Early hemorrhage control sustains patient survival in the prehospital setting, and this intervention may be pharmaceutically augmented in the future to create a pro-survival phenotype to mitigate long-term tissue damage.

Valproic acid (VPA), the most widely prescribed antiepileptic drug worldwide, was first synthesized in 1882 as an analog of valeric acid and was approved for administration in 1967.^{6,7} Recently, VPA has gained renewed attention as a histone deacetylase inhibitor capable of reversing widespread deacetylation changes when supplied at dosages (150 mg/kg to 400 mg/kg) well above those required for its intended use as an antiepileptic drug.⁸ Epigenetic modulation of gene expression is

© Author(s) (or their employer(s)) 2023. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

To cite: Moritz R, Mangum L, Voelker C, et al. *Trauma Surg Acute Care Open* 2023;**8**:e001074.

an essential mechanism to cell survival and tissue homeostasis. Modification of DNA by methylation and associated histones by acetylation, methylation, ubiquitination, and sumoylation allows a cell to dynamically alter gene expression in response to a host of stimuli.^{9,10} Maintenance of histone acetylation is accomplished by histone acetyltransferases and histone deacetylases, which catalyze the addition and removal of acetyl groups. Perturbation of their activity manifests during ischemia and hemorrhagic shock. Cardiac and cerebral ischemia have been shown to produce a ~40% decrease in total histone acetylation.^{11,12} Animal studies investigating hemorrhagic shock combined with traumatic brain injury^{13–15} or sepsis^{16–18} have shown improved survival with VPA treatment in an otherwise fatal model of polytrauma. Specifically, VPA has been shown to upregulate signaling pathways that suppress the induction of apoptosis including serine/threonine protein kinase (Akt), glycogen synthase kinase-3 β , antiapoptotic members of the BCL2 family,^{19,20} mitogen-activated protein kinases (ERK/JNK), and nuclear factor κ B (NF- κ B).^{20,21} Increased activation of the Akt pathway as a proportion of phosphorylated to total Akt has been shown to correspond to improved survival outcomes in a rodent model of hemorrhagic shock.²² A systemic inflammatory response acts concurrently with injury to local tissues to exacerbate ischemia–reperfusion injury. VPA treatment has been shown to reduce levels of interleukin (IL)-1 β , IL-6, and tumor necrosis factor alpha (TNF- α) in animal models of hemorrhagic shock and septic shock.^{23,24} Furthermore, suppressing NF- κ B mediated release of IL-1 β , IL-6, and TNF- α with VPA protected mice from renal injury in a sepsis model.²⁵ Although the efficacy of VPA treatment has been demonstrated during hemorrhagic shock and polytrauma, a gap in knowledge exists as to whether VPA treatment may play a role in ameliorating tourniquet-based ischemia–reperfusion injury of the extremity during hemorrhage and resuscitation.

Our group employs a model of hemorrhage, tourniquet application, fluid resuscitation, and reperfusion that effectively recapitulates battlefield injury within an animal rodent model.^{26,27} We hypothesized that intravenous injection of VPA as a treatment within this injury model would produce increases in prosurvival Akt signaling in skeletal muscle, reductions in systemic inflammation and kidney injury, and improvements in limb function after tourniquet injury.

MATERIALS AND METHODS

Precannulated male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were shipped at ~10 weeks to 12 weeks of age (350 g to 400 g); each rat had an exteriorized catheter in the carotid artery for removal of blood. Rats acclimated for a minimum of 3 days after transport and were maintained in a 19°C to 23°C room with a 12 hours on light cycle. Rats were singly housed in shoebox cages with ad libitum access to rat chow and water prior to surgical procedures. Experimental groups were selected randomly at the date of operation. Surgical procedures and postsurgical housing were simultaneous and colocalized between groups to minimize confounding variables. Operations within this study were not amenable to blinding procedures.

Experimental protocol

Rats were subcutaneously injected with buprenorphine (1.2 mg/kg) then anesthetized with 1% to 3% isoflurane and maintained under anesthesia until fluid resuscitation was complete or 2 hour post resuscitation, at which time they were allowed to regain consciousness. Microdialysis probes were implanted bilaterally

within the tibialis anterior muscles of both hind limbs, and tail vein catheters were placed for VPA dosing or fluid resuscitation with 6% hetastarch in lactated electrolyte solution (HexTend; Pfizer, NY). Non-invasive measurement of glomerular filtration rate (GFR) was accomplished using Fluorescein isothiocyanate (FITC)–sinistrin clearance before and 72 hours after hemorrhage and injury. Hemorrhage was accomplished at a fixed volume as described in Klemcke, 2008.²⁸ Total blood volumes were calculated at 5.83 mL/100g body weight with 42.5% of calculated volumes withdrawn to reproduce severe vascular injury necessitating tourniquet application. Blood was withdrawn via the carotid artery catheter with 25% removed at a constant rate for 4 min and the remaining 75% removed at a constant rate during the subsequent 22 min.

Immediately after hemorrhage, the first dose of VPA was administered via the tail vein using a standard infuse/withdraw PHD 22/2000 syringe pump (Harvard Apparatus, Holliston, MA) for a duration of 10 min. After completion of VPA administration, an additional 10 min was permitted to allow drug perfusion to local tissues. A commercially available pneumatic digit cuff (Model DC 1.6, D.E. Hokanson, Bellevue, WA) was placed on the left hindlimbs that had been elevated and suspended from the operating table. Tourniquet pressure was increased from 250 mm Hg to 300 mm Hg using a rapid cuff inflator equipped with a pressure transducer (Model E20, D.E. Hokanson, Bellevue, WA); this pressure was maintained for a period of 3 hours. Tourniquet-induced ischemia in rat skeletal muscle during this duration has been previously shown to produce an initial complete loss of function and an approximate 80% recovery in the absence of hemorrhage.²⁹

Ten minutes prior to tourniquet release, a second dose of VPA was administered. At 3 hours of total tourniquet time, the tourniquet pressure was released, and volume-based fluid resuscitation (15 mL/kg body weight, 1 mL/kg/min) with HexTend was administered via the tail vein. Rats designated for Akt pathway signaling, cytokine release, and tibialis anterior wet/dry quantitation remained anesthetized for a 2-hour observation period after reperfusion of the hindlimb.

Rats designated for GFR and muscle function quantitation after injury regained consciousness and were returned to their cages singly housed. At 3, 7, and 21 days after injury, these rats were again anesthetized. GFR quantitation occurred at 3 days after injury, and muscle function was evaluated at all three time points. In all groups, blood samples were collected from the initial hemorrhage and immediately prior to euthanasia with plasma samples stored at –80°C; immediately after euthanasia, tibialis anterior muscles of ischemic and non-ischemic limbs were harvested, weighed, and processed in accordance with sampling requirements under each methodology.

In anticipation of attrition associated with 42.5% total blood volume hemorrhage, 10 rats were allocated to each experimental group. Experiments whose endpoint was designated at 2 hours of reperfusion were performed prior to all other experimental groups and proceeded until a predetermined threshold of $n=4$ had been met. Rats within these groups used preservation of phosphorylated Akt in skeletal muscle as a key metric to substantiate VPA dosage efficacy before continuation with experimentation at later time points. Total rats used within these groups and mortality are as follows: VPA high dosage: $n=7/2$, low dosage: $n=7/1$, and saline control: $n=7/2$. One rat was excluded from the VPA high-dosage group due to observed respiratory distress at the time of operation in accordance with a priori exclusion criteria. Experimental timelines for each methodology and their respective endpoints are shown in figure 1.

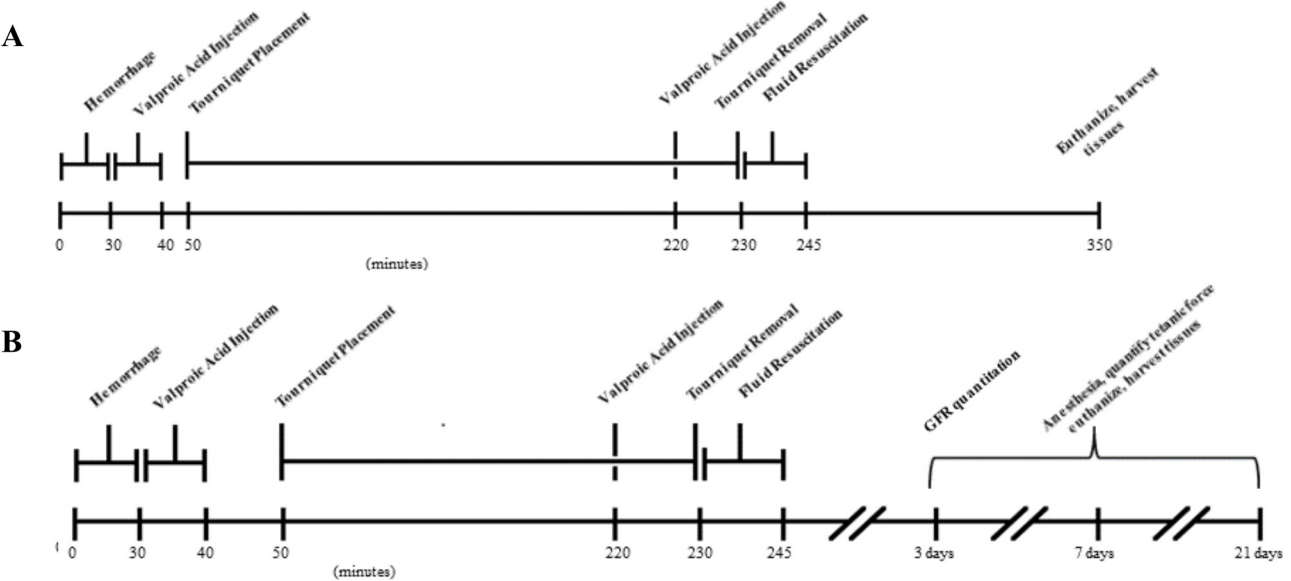


Figure 1 Experimental timelines and methodologies. Animals underwent hemorrhage of 42.5% total blood volumes with a 3-hour tourniquet application and subsequent fluid resuscitation. Tissues were harvested for systemic IL-1 β , TNF- α , IL-6, and local serine/threonine protein kinase pathway activity at 2 hours post reperfusion (A) or proceeded to days 3, 7, or 21 for glomerular filtration rate quantitation and peak isometric force production of the tibialis anterior muscles (B). IL, interleukin; TNF- α , tumor necrosis factor alpha.

Rats in experimental groups with endpoints of 3, 7, and 21 days proceeded until a minimum predetermined threshold of $n=6$, as determined by muscle function as a primary outcome, had been met. Total rats used within these groups and mortality are as follows: 3 days (VPA $n=9/2$, saline $n=8/1$), 7 days (VPA $n=8/2$, saline $n=8/1$), and 21 days (VPA $n=10/3$, saline $n=7/1$). No rats were excluded from the experimental groups at endpoints of 3, 7, and 21 days.

VPA dosing and quantitation

VPA sodium salt prepared in 0.5 mL of 0.9% saline (400 mg/kg or 300 mg/kg) was administered by standard infuse/withdraw PHD 22/2000 syringe pumps (Harvard Apparatus) during a 10 min interval at concentrations shown to inhibit histone deacetylation in rats in a dose-optimization study.³⁰ Delivery of VPA to skeletal muscle distal to tourniquet application preischemia and post reperfusion injury employed a split-dosing strategy with a 2:3 proportion (267 or 200 mg/kg) of the total dose administered 10 min prior to tourniquet inflation and 1:3 proportion (133 or 100 mg/kg) of the total dose administered 10 min prior to tourniquet release based on previous studies employing a similar dosing strategy.³⁰

CMA 20 high Molecular Weight (MW) cut-off/10 mm membrane length/100 kD cut-off microdialysis probes (CMA microdialysis, Kista, Sweden) were implanted bilaterally within the tibialis anterior muscles, and a CMA 402 dual syringe microdialysis pump perfused the probe lumens with isotonic T1 perfusion fluid containing 25 μ g/mL VPA-d6 as a retrocalibrant at a flow rate of 1.5 μ L/min. Free VPA in the interstitial space of ischemic/non-ischemic tibialis anterior muscles was sampled semi-continuously throughout the 3-hour tourniquet application and 2-hour reperfusion period to verify drug delivery to local tissues and examine dosing deficiencies post reperfusion.

Circulating free (unbound) VPA was recovered by ultrafiltration of plasma samples using Centrifree 30 kD MW cut-off UF filters (Merck Millipore, Cork, Ireland) centrifuged at 1500 Relative Centrifugal Force (RCF) for 30 min. Microdialysate and plasma ultrafiltrate samples were analyzed by Ultra-high Performance

Liquid Chromatography Mass Spectrometry (UPLC-MS/MS) using a Waters Acquity H-class ultra-performance liquid chromatography system (Waters, Milford, MA) coupled to a Xevo G2-XS quadrupole time of flight mass spectrometer (Waters, USA) equipped with an electrospray ionization source. VPA and VPA-d6 were resolved on a Phenomenex Kinetex C18 column (1.7 μ m, 2.1 \times 50.0 mm) using water-formic acid (99.9:0.1 v/v) as mobile phase A and methanol-formic acid (99.9:0.1 v/v) as mobile phase B. Isocratic elution was performed at 20:80 A:B for 5 min at a flow rate of 0.3 mL/min. The column temperature was set to 40°C. Three microliters of sample was injected on column with the autosampler held at 8°C. VPA and VPA-d6 were monitored in MS positive ion mode at m/z 143.106 and m/z 149.142, respectively. Optimized values for collision energy, cone voltage, capillary voltage, source temperature and desolvation gas flow rate were 0 (nominal), 0 (nominal), 3 kV, 80°C, and 600 L/hr, respectively. VPA concentration in samples was interpolated from the standard curve using least-squares linear regression curve fitting with 1/ \times 2 weighting. All reported microdialysate VPA concentrations were normalized to the measured recovery efficiency of each microdialysis probe. Recovery efficiency was established using the retrodialysis calibrator technique³¹ with VPA-d6 as the selected calibrator agent.

Muscle function

Tibialis anterior muscle function was quantified in vivo using procedures previously shown.³² Rats were placed in a supine position with hind limbs stabilized in a custom jig and paw secured to a footplate. The extensor digitorum longus muscle was severed to delineate force output from the tibialis anterior muscle only during measurement. The footplate was connected to a servomotor-controlled force-displacement transducer (Model 300 series, Aurora Scientific, Aurora, ON, Canada), and muscles were activated via their motor nerve using a subcutaneous electrode. Opposite ends of the electrode wires were connected to a physiological stimulator, and the crural muscles were activated with a stimulus voltage adjusted to elicit peak torque. The maximum output force of the target muscle, peak

isometric tetanic force, will be quantified and represents a relevant outcome for clinical translation of experimental therapies.³³ The servo motor input and force and displacement transducer outputs were controlled and acquired, respectively, using a PC equipped with a data acquisition board (National Instruments, Austin, TX) and custom designed LabVIEW (National Instruments, Austin, TX)-based software program.

Kidney function

Transdermal monitoring of GFR using the NIC-Kidney Device and FITC–sinistrin (MediBeacon, St. Louis, MO) quantified disparities in acute kidney injury in animals receiving 400 mg/kg VPA and saline vehicle control. FITC–sinistrin dissolved in sterile isotonic saline was administered via carotid artery catheter at 7 mg/100 g of body weight with injections of ≤ 0.5 mL. Monitoring of transdermal FITC–sinistrin fluorescence continued for a duration of 2 hours post injection.

Data downloaded from the NIC-Kidney Device was processed by MediBeacon Studio software, and the elimination kinetics of FITC–sinistrin was used to calculate GFR. Baseline GFR quantitation was performed prior to injury and after injury quantitation occurred at 72 hours post ischemia–reperfusion injury. Decrement in GFR between baseline and values after injury were evaluated for significance between VPA-treated and vehicle control animals.

Akt pathway activity and cytokine release

Tissue lysate prepared from snap-frozen tibialis anterior muscles collected after 3 hours of pneumatic tourniquet application and 2 hours of reperfusion or uninjured contralateral limbs were prepared in accordance with manufacturer recommendations for the pAkt and Akt Pathway Total 7-Plex kit (Invitrogen, Carlsbad, CA). Total and phosphorylated Akt pathway proteins were quantified concurrently on a Bio-Plex 200 System (Bio-Rad, Hercules, CA). Circulating cytokines in plasma samples were assayed by RayBiotech (Atlanta, GA) using a Quantibody Rat Cytokine Array 67 kit.

Histology

Tibialis anterior muscles fixed in 10% buffered formalin were processed for light microscopy using standard procedures for H&E staining. Samples were blinded to treatment group and procedural limb then evaluated by a histopathologist using a predetermined histological grading scale. Features evaluated by histological grading included skeletal muscle and predominant muscle changes, intermyocyte hemorrhage and edema, fibroplasia, foreign body reaction, and inflammation as indicated by the presence of neutrophils, histiocytes, and lymphocytes.

Tibialis anterior sections designated for wet:dry ratio quantitation were immediately weighed on dissection and snap-frozen in liquid nitrogen until a subsequent 48-hour lyophilization where dry weight was achieved. Levels of skeletal muscle edema over uninjured tissue are expressed as a comparative ratio of wet versus dry tissue weights.

Statistical analysis

Contralateral non-ischemic limbs served as controls for experiments quantifying muscle function and local signaling changes, whereas rats receiving saline alone served as controls for experiments quantifying systemic inflammation levels and GFR. Comparison of tetanic force output between the experimental and non-ischemic contralateral limb were evaluated for significance by one-way analysis of variance test with post hoc multiple

comparisons and Bonferroni correction at $\alpha=0.05$. Akt pathway analyte, cytokines, GFR, and aggregate injury scores were evaluated for significance using Student's t-test using GraphPad Prism V.7.03 (GraphPad Software, San Diego, CA). For these comparisons, non-parametric statistical tests were used due to the relatively small sample sizes of each group ($n=4-7$ animals per tourniquet application). Data are presented as mean with SE of the mean or scatter plot unless otherwise noted.

RESULTS

To verify VPA penetrance into soft tissues distal to tourniquet application prior to cessation of blood flow and during the reperfusion period, free VPA was recovered from the interstitial space of both tibialis anterior muscles throughout the 3-hour ischemic period and for 2 hours after restoration of blood flow. Interstitial pharmacokinetics of VPA showing equivalent peak concentrations within experimental and contralateral limbs prior to tourniquet application are shown in [figure 2](#).

Akt pathway signaling

After 2 hours of reperfusion, total Akt from skeletal muscle distal to tourniquet placement trended toward a decline when compared with contralateral limbs. This loss approached attenuation by high-dose VPA but did not surpass a threshold of significance. Ischemia–reperfusion injury reduced phosphorylated Akt in skeletal muscle of tourniquet-injured limbs to 27.88% when compared with contralateral limbs in animals receiving saline control. In contrast, tourniquet-injured limbs in animals treated with 400 mg/kg VPA showed 101.66% of the quantified phosphorylated Akt within contralateral limbs and, surprisingly, animals receiving 300 mg/kg VPA showed a 113.99% proportion of phosphorylated Akt compared with uninjured contralateral limbs ([figure 3](#)). Whereas concentrations of phosphorylated insulin-like growth factor receptor remained relatively equivalent in VPA and saline control animals, phosphorylated insulin receptor substrate-1 (IRS-1) of tourniquet-injured limbs as a proportion of contralateral limbs showed an 89.28% increase in VPA treated animals versus those receiving saline vehicle control. Downstream of Akt signaling activation, phosphorylated p70s6K showed a 35.31% increase in VPA-treated animals over saline alone in tourniquet-injured skeletal muscle ([figure 4](#)).

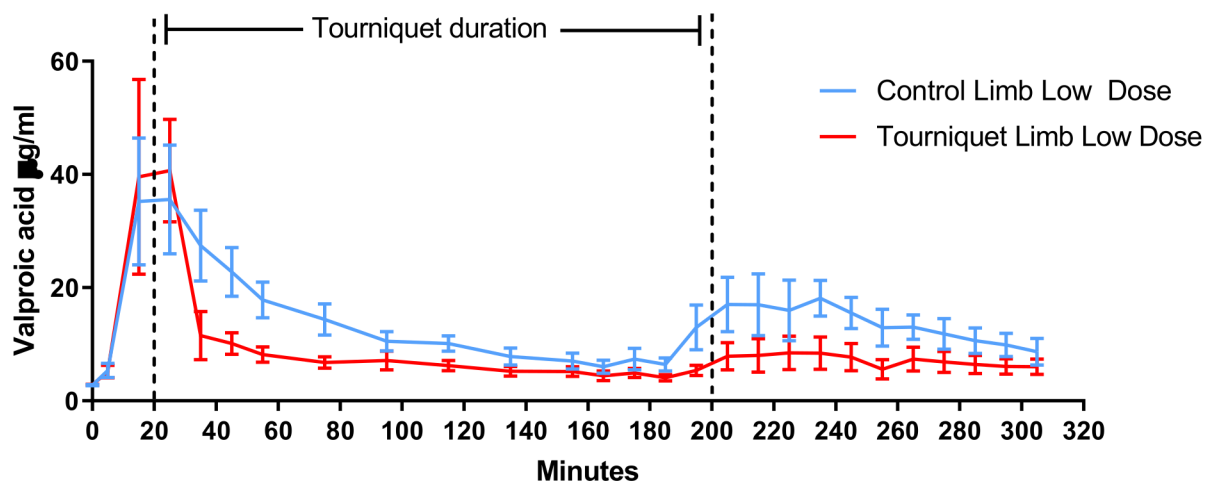
Cytokine release and kidney function

Animals receiving 400 mg/kg VPA compared with those receiving saline vehicle control showed a 91.47% reduction in plasma levels of IL-1 β , a 73.76% reduction in TNF- α , and a 45.41% reduction in IL-6 ([figure 5](#)). GFR decrement was calculated as a decline from values quantitated prior to hemorrhage and tourniquet application to 72 hours after injury. Animals receiving 400 mg/kg VPA showed a 19.21% improvement in GFR reduction when compared with animals receiving saline vehicle control ($p=0.0424$; VPA = $-7.0\% \pm 6.5$, saline = $-26.2\% \pm 7.3$).

Muscle function and histological analysis

Peak tetanic force quantitation of in vivo tibialis anterior muscles showed nearly an absence of response to stimuli at 3 days after injury and no significant difference in animals receiving VPA and saline vehicle control. At 7 days after injury, a detectable force output was established with no differences in treatment groups. A mean decrement in tetanic force output in tourniquet-injured limb versus contralateral limb of 35.05% (VPA) and 57.03% (saline vehicle control) was shown 21 days after injury. Despite recorded differences in mean values in functional limb recovery

A



B

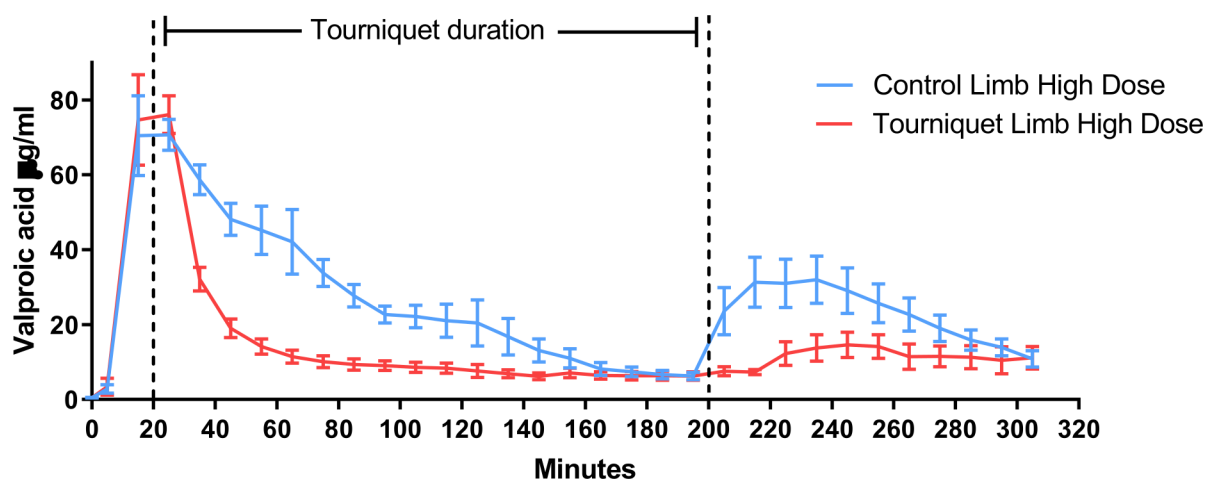


Figure 2 Free VPA in the interstitial space of rat tibialis anterior muscles. Two hundred and 100 mg/kg (n=6) (A) or 267 and 133 mg/kg (n=5) (B) of VPA were administered intravenously at 10 min prior to pneumatic tourniquet inflation and release. On tourniquet application, drug concentrations in experimental and contralateral limbs achieved comparable values. Dotted lines indicate tourniquet application and subsequent release. VPA, valproic acid.

between treatment groups at this time point, variance of quantified tetanic force output values within each group prevented interpreting these values as surpassing a threshold of significance (figure 6).

There was no significant difference with VPA treatment in elevated tibialis anterior weight to dry weight ratios ($p=0.8634$; VPA=10.8%±0.96, saline=10.5%±1.49) after 2 hours of reperfusion. Representative images from uninjured skeletal muscle and tourniquet-injured limbs throughout convalescence are shown in figure 7. Aggregate injury scores in both VPA-treated and saline-treated animals showed no difference between groups ($p=0.3734$; tourniquet: VPA=0.2±0.20, saline=0.4±0.24; uninjured: VPA=2.8±0.37, saline=3.2±0.20).

DISCUSSION

There are two main findings of this study. First, VPA administration at concentrations relevant to previous rat studies significantly attenuated reductions in Akt pathway signaling and

suppressed an acute inflammatory response in our injury model. Second, these beneficial effects did not translate into improvements in physiological function of the skeletal muscle of the affected limb.

Akt pathway activity was examined based on previous observations that VPA treatment at these concentrations successfully modulated protein expression and signaling, increased Akt phosphorylation, and improved survival outcomes in rat models of hemorrhagic shock.^{16 30 34} Given the mechanistic overlap in hypovolemic shock manifesting as a global ischemic event and the localized ischemia distal to tourniquet application occurring typically concurrent to significant blood loss, VPA was selected as a candidate for this study with Akt phosphorylation as an indicator of a prosurvival phenotype that allows for comparison with previously published findings.

There was a trend toward loss of total Akt after ischemia-reperfusion injury but high variability between experimental animals prevented meeting a threshold of statistical significance

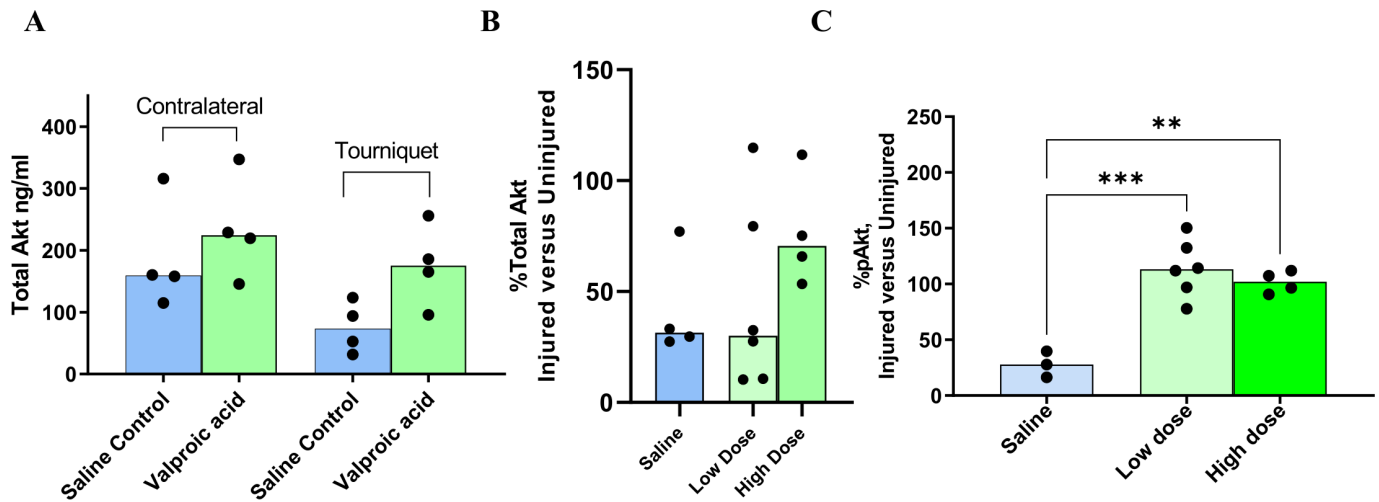


Figure 3 Total and phosphorylated Akt protein in rat tibialis anterior muscles. (A) Total Akt measured in tourniquet-injured limbs receiving saline vehicle control compared with contralateral limbs in animals receiving VPA at 400 mg/kg. (B) Total Akt protein in tourniquet-injured limbs as a proportion of contralateral limbs in animals receiving VPA at low (300 mg/kg), high (400 mg/kg), or saline vehicle control. (C) Phosphorylated Akt protein in tourniquet-injured limbs quantified as a percentage of contralateral limbs was significantly less in animals receiving saline vehicle control when compared with animals receiving low and high dosages of VPA. ** $P < 0.01$, *** $P < 0.001$. Akt, serine/threonine protein kinase; VPA, valproic acid.

when administering high dosages of VPA versus saline vehicle control ($p = 0.0905$). Despite a possibly depleted pool of Akt protein available for signal transduction, VPA treatment at both low and high dosage concentrations ameliorated the reduction of quantified phosphorylated Akt in tourniquet-injured limbs when compared with contralateral limbs. Akt phosphorylation is therefore reduced by ischemia–reperfusion injury, but VPA acts to restore this signaling pathway.

Akt activation integrates a host of transmembrane receptor–ligand-mediated stimuli whose influential pathways include insulin and insulin-like growth factor. Delineating a singular mechanism of Akt activation was not within the scope of this study; however, a preserved activation of IRS-1, whose upstream regulation of Akt is well characterized,³⁵ suggests that this may be a means of maintaining Akt signaling in the context of ischemia–reperfusion injury. Likewise, phosphorylated p70s6K protein acts as an indicator of mammalian target of rapamycin (mTOR) pathway activity, whose activation downstream of Akt promotes cell growth, proliferation, and inhibits autophagy.³⁶ Given reports of autophagy inhibition with VPA treatment after

injury³⁷ and the preservation of p70s6K signaling within this study, an autophagy-mediated mechanism producing a decrement in total Akt is suggested.

Acute ischemia and subsequent reperfusion injury modulating release of inflammatory mediators is well characterized.^{38,39} VPA suppression of inflammatory mediator release has been shown in animal models of hemorrhagic shock and septic shock,^{17,22} and our results likewise demonstrate an amelioration of systemic cytokine levels by VPA in the combined injury model studied herein.

Furthermore, kidney function as assessed by GFR measurement at 72 hours after injury was decreased in saline-treated rats but was restored by VPA administration. This finding is similar to previous demonstrations of sustained survival of renal cells and preservation of renal function by VPA in animal models of hemorrhagic or septic shock.²⁴ To our knowledge, this is the first study that demonstrates a beneficial effect of VPA on renal function in a hemorrhage model that includes clinically relevant hemorrhage control measures (eg, prolonged tourniquet application) that produce significant tissue damage.

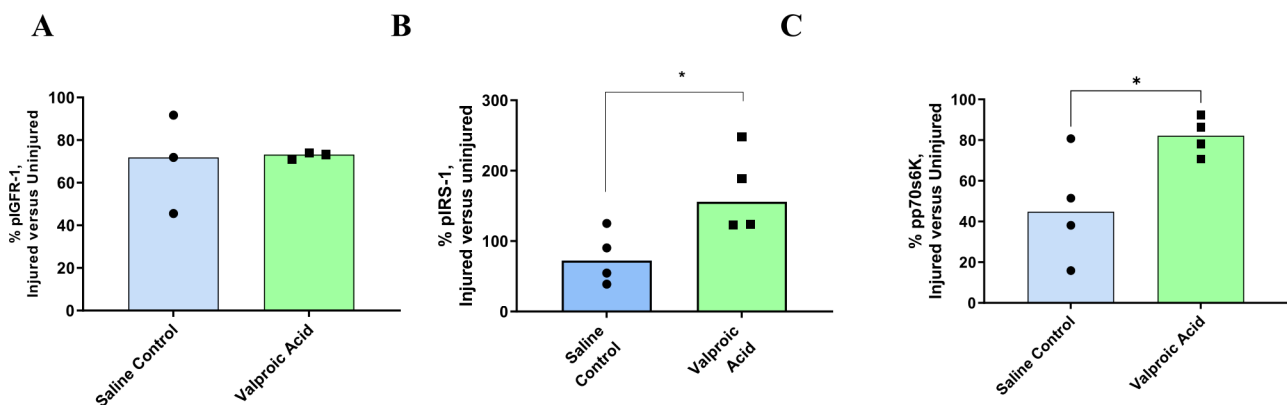


Figure 4 Akt pathway activity. Phosphorylated Akt pathway proteins in tibialis anterior muscles subject to 3 hours of ischemia and 2 hours of reperfusion quantified as a percentage of contralateral limbs. (A) Animals treated with 400 mg/kg valproic acid showed no significant difference in phosphorylated insulin-like growth factor receptor-1, (B) whereas phosphorylated insulin receptor substrate-1 (C) and phosphorylated p70s6K were significantly increased. * $P < 0.05$. Akt, serine/threonine protein kinase.

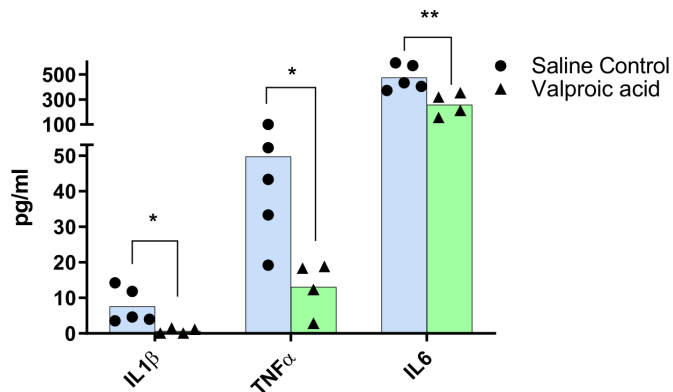


Figure 5 Immunomodulating effect of VPA. Circulating plasma samples collected after 3 hours of hind limb ischemia and 2 hours of reperfusion in animals receiving either saline vehicle control or 400 mg/kg VPA were evaluated for acute cytokine release. Significant reductions in IL-1 β , TNF- α , and IL-6 were observed with VPA administration. * $P < 0.05$, ** $P < 0.01$. IL, interleukin; TNF- α , tumor necrosis factor alpha; VPA, valproic acid.

Extending our study throughout the duration of convalescence, however, did not demonstrate that an early preservation of Akt signaling nor immune modulation translated into meaningful functional improvements of the limb. Simultaneous with evaluation of cell signaling, edema within tibialis anterior muscles after reperfusion of both treatment and control groups was elevated to comparable levels. Physiological events appreciable by light microscopy, including myocyte degeneration and loss, the formation of necrosis, and eventual myocytic repopulation, proceeded on course regardless of treatment. Substantiating these observations were an absence of significant skeletal muscle functional recovery over non-treated limbs when quantitating force output under *in vivo* stimulated tetanic contraction. These latter findings greatly limit the utility of VPA as a pharmaceutical augmentation to prolonged tourniquet application.

This study is not without limitations. Tourniquet application inherently creates conditions where intravenously dosed drugs become sequestered from the circulating blood. Differences in pharmacokinetic profiles between limbs are evident in figure 1. Decreasing quantities of VPA within the interstitial fluids of muscles distal to tourniquet application, however, suggest a mechanism of cellular sequestration and procession to the drug's molecular target. Additionally, our administering VPA via a more characterized intravenous route and using Hextend as fluid

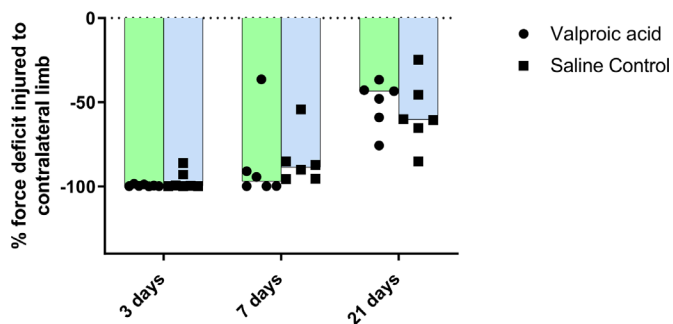


Figure 6 Tetanic force deficit of tourniquet injured and matched contralateral tibialis anterior muscles with VPA treatment. Force output with *in vivo* stimulated tetanic contracture after 3, 7, and 21 days post hemorrhage, 3-hour tourniquet wear, and 2 hours of reperfusion with 400 mg/kg VPA treatment or saline vehicle control. VPA, valproic acid.

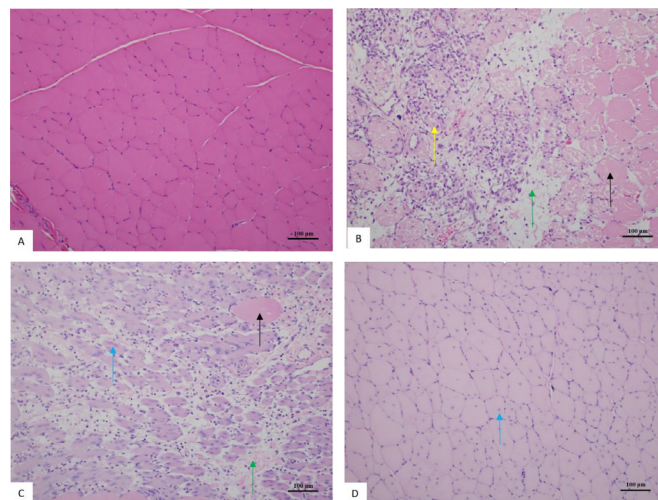


Figure 7 Histological changes in tibialis anterior muscles after tourniquet injury. (A) Normal skeletal muscle. (B) Three days after injury showing myocyte degeneration (black arrow), necrosis (yellow arrow), and loss (green arrow) (VPA). (C) Seven days after injury showing myocyte regeneration exhibited by internalized nuclei (blue arrow), loss (green arrow), and few degenerative myocytes (black arrow) (saline vehicle control). (D) Twenty-one days after injury showing myocyte regeneration (blue arrow) with no degeneration, necrosis, or loss (VPA). All images are $\times 100$. VPA, valproic acid.

resuscitation are less translatable to field care scenarios. Hextend was recently removed from military clinical practice guidelines, and our future studies will reflect more current resuscitative practices. Inclusion of intravenous administration was selected to facilitate detection of improved outcomes which, once verified, would prompt refinement of delivery methods. Furthermore, this study sought to test conditions where a 3-hour tourniquet application would be required for hemorrhage control, which produced a persistent functional deficit that did not attenuate with VPA administration. These data do not preclude a therapeutic benefit of VPA administration with a less profound ischemic injury when a tourniquet is applied for a shorter duration.

CONCLUSIONS

Tourniquet application for extended durations and associated reperfusion injuries cause a substantial loss in prosurvival cellular signaling manifesting in tissue loss. These findings build on previous research regarding hemorrhagic shock to determine if signaling and functional preservation are possible in the extreme conditions where tourniquet application produces complete cessation of blood flow for an extended duration. Despite compelling evidence that VPA treatment sustains Akt signaling, suppresses an acute inflammatory response, and attenuates loss in kidney function after prolonged tourniquet application with hemorrhage and subsequent resuscitation, these findings do not translate into a preservation in skeletal muscle function after injury. VPA therefore may not be appropriate as a pharmacological adjunct to prolonged tourniquet application.

Contributors RM: guarantor, conceptualization, methodology, and writing; LM: methodology; CV: project administration; GG: validation and data curation; JW: supervision and funding acquisition.

Funding This study was funded by the Combat Casualty Care Research Program (CO220017).

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. All procedures were performed in a laboratory accredited by the Association and Accreditation of Laboratory Animal Care following a protocol approved by the Institutional Animal Care and Use Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

ORCID iD

Robert Moritz <http://orcid.org/0000-0001-5995-1829>

REFERENCES

- Owens BD, Kragh JF, Macaitis J, Svoboda SJ, Wenke JC. Characterization of extremity wounds in operation Iraqi freedom and operation enduring freedom. *J Orthop Trauma* 2007;21:254–7.
- White JM, Stannard A, Burkhardt GE, Eastridge BJ, Blackburn LH, Rasmussen TE. The epidemiology of vascular injury in the wars in Iraq and Afghanistan. *Ann Surg* 2011;253:1184–9.
- Kragh JF, Walters TJ, Baer DG, Fox CJ, Wade CE, Salinas J, Holcomb JB. Survival with emergency tourniquet use to stop bleeding in major limb trauma. *Ann Surg* 2009;249:1–7.
- Gillani S, Cao J, Suzuki T, Hak DJ. The effect of ischemia reperfusion injury on skeletal muscle. *Injury* 2012;43:670–5.
- Korth U, Merkel G, Fernandez FF, Jandewerth O, Dogan G, Koch T, van Ackern K, Weichel O, Klein J. Tourniquet-induced changes of energy metabolism in human skeletal muscle monitored by Microdialysis. *Anesthesiology* 2000;93:1407–12.
- Burton BS. On the propyl derivatives and decomposition products of Ethylacetoacetate. *J Am Chem* 1882;3:385–95.
- Perucca E. Pharmacological and therapeutic properties of valproate. *CNS Drugs* 2002;16:695–714.
- Lin T, Alam HB, Chen H, Britten-Webb J, Rhee P, Kirkpatrick J, Koustova E. Cardiac Histones are substrates of Histone Deacetylase activity in hemorrhagic shock and resuscitation. *Surgery* 2006;139:365–76.
- Choudhuri S, Cui Y, Klaassen CD. Molecular targets of epigenetic regulation and effectors of environmental influences. *Toxicol Appl Pharmacol* 2010;245:378–93.
- Zhu Q, Wani AA. Histone modifications: crucial elements for damage response and Chromatin restoration. *J Cell Physiol* 2010;223:283–8.
- Taylor PB, Young BG. Effect of myocardial ischemia on uridine incorporation and Histone Acetylation. *Can J Physiol Pharmacol* 1982;60:313–8.
- Ren M, Leng Y, Jeong M, Leeds PR, Chuang DM. Valproic acid reduces brain damage induced by transient focal cerebral ischemia in rats: potential roles of Histone Deacetylase inhibition and heat shock protein induction. *J Neurochem* 2004;89:1358–67.
- Halaweish I, Bambakidis T, Chang Z, Wei H, Liu B, Li Y, Bonthron T, Srinivasan A, Bonham T, Chtraklin K, et al. Addition of low-dose valproic acid to saline resuscitation provides Neuroprotection and improves long-term outcomes in a large animal model of combined traumatic brain injury and hemorrhagic shock. *J Trauma Acute Care Surg* 2015;79:911–9.
- Jepsen CH, deMoya MA, Perner A, Sillesen M, Ostrowski SR, Alam HB, Johansson PI. Effect of valproic acid and injury on lesion size and endothelial Glycocalyx shedding in a rodent model of isolated traumatic brain injury. *J Trauma Acute Care Surg* 2014;77:292–7.
- Jin G, Duggan M, Imam A, Demoya MA, Sillesen M, Hwabejire J, Jepsen CH, Liu B, Mejjaddam AY, Lu J, et al. Pharmacologic resuscitation for hemorrhagic shock combined with traumatic brain injury. *J Trauma Acute Care Surg* 2012;73:1461–70.
- Li Y, Liu B, Fukudome EY, Kochanek AR, Finkelstein RA, Chong W, Jin G, Lu J, deMoya MA, Velmahos GC, et al. Surviving lethal septic shock without fluid resuscitation in a rodent model. *Surgery* 2010;148:246–54.
- Liu Z, Li Y, Chong W, Deperalta DK, Duan X, Liu B, Halaweish I, Zhou P, Alam HB. Creating a Prosurvival phenotype through a Histone Deacetylase inhibitor in a lethal two-hit model. *Shock* 2014;41:104–8.
- Zhao T, Li Y, Liu B, Liu Z, Chong W, Duan X, Deperalta DK, Velmahos GC, Alam HB. Novel pharmacologic treatment attenuates septic shock and improves long-term survival. *Surgery* 2013;154:206–13.
- Alam HB, Shuja F, Butt MU, Duggan M, Li Y, Zacharias N, Fukudome EY, Liu B, Demoya M, Velmahos GC. Surviving blood loss without blood transfusion in a swine poly-trauma model. *Surgery* 2009;146:325–33.
- Zhang C, Zhu J, Zhang J, Li H, Zhao Z, Liao Y, Wang X, Su J, Sang S, Yuan X, et al. Neuroprotective and anti-apoptotic effects of valproic acid on adult rat cerebral cortex through ERK and AKT signaling pathway at acute phase of traumatic brain injury. *Brain Res* 2014;1555:1–9.
- Li Y, Yuan Z, Liu B, Sailhamer EA, Shults C, Velmahos GC, deMoya M, Alam HB. Prevention of hypoxia-induced neuronal apoptosis through Histone Deacetylase inhibition. *J Trauma Acute Care Surg* 2008;64:863–71.
- Hwabejire JO, Lu J, Liu B, Li Y, Halaweish I, Alam HB. Valproic acid for the treatment of hemorrhagic shock: a dose-optimization study. *J Surg Res* 2014;186:363–70.
- Bambakidis T, Dekker SE, Sillesen M, Liu B, Johnson CN, Jin G, de Vries HE, Li Y, Alam HB. Resuscitation with valproic acid alters inflammatory genes in a porcine model of combined traumatic brain injury and hemorrhagic shock. *J Neurotrauma* 2016;33:1514–21.
- Kasotakis G, Galvan M, King E, Sarkar B, Stucchi A, Mizgerd JP, Burke PA, Remick D. Valproic acid mitigates the inflammatory response and prevents acute respiratory distress syndrome in a murine model of Escherichia coli pneumonia at the expense of bacterial clearance. *The J Trauma Acute Care Surg* 2017;82:758–65.
- Zheng Q, Liu W, Liu Z, Zhao H, Han X, Zhao M. Valproic acid protects septic mice from renal injury by reducing the inflammatory response. *J Surg Res* 2014;192:S0022-4804(14)00473-9:163–9..
- Chen XK, Rathbone CR, Walters TJ. Treatment of tourniquet-induced ischemia reperfusion injury with muscle progenitor cells. *J Surg Res* 2011;170:e65–73.
- Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. *J Trauma Acute Care Surg* 2006;60:53–11.
- Klemcke HG, Baer DG, Pankratz VS, Cox A, Cortez DS, Garrett MR, Joe B, Ryan KL. Is survival time after hemorrhage a Heritable, quantitative trait?: an initial assessment. *Shock* 2008;29:748–53.
- Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. *Cardiovascular Surgery* 2002;10:620–30.
- Bouw MR, Hammalund-Udenaes M. Methodological aspects of the use of a Calibrator in in vivo Microdialysis—further development of the Retrodialysis method. *Pharm Res* 1998;15:1673–9.
- Corona BT, Wenke JC, Walters TJ, Rathbone CR. Intramuscular transplantation and survival of freshly isolated bone marrow cells following skeletal muscle ischemia-reperfusion injury. *J Trauma Acute Care Surg* 2013;75:S142–9.
- Bedar M, Saffari TM, Friedrich PF, Giusti G, Bishop AT, Shin AY. Maximum Isometric tetanic force measurement of the tibialis anterior muscle in the rat. *J Vis Exp* 2021.
- Georgoff PE, Halaweish I, Nikolian VC, Higgins GA, Bonham T, Tafatia C, Remmer H, Menon R, Liu B, Li Y, et al. Alterations in the human Proteome following administration of valproic acid. *J Trauma Acute Care Surg* 2016;81:1020–7.
- Siddle K. Signalling by insulin and IGF receptors: supporting acts and new players. *J Mol Endocrinol* 2011;47:R1–10.
- Zarogoulidis P, Lampaki S, Turner JF, Huang H, Kakolyris S, Syrigos K, Zarogoulidis K. mTOR pathway: A current, up-to-date mini-review. *Oncol Lett* 2014;8:2367–70.
- Hao H-H, Wang L, Guo Z-J, Bai L, Zhang R-P, Shuang W-B, Jia Y-J, Wang J, Li X-Y, Liu Q. Valproic acid reduces Autophagy and promotes functional recovery after spinal cord injury in rats. *Neurosci Bull* 2013;29:484–92.
- Abu-Amara M, Yang SY, Tapuria N, Fuller B, Davidson B, Seifalian A. Liver ischemia/reperfusion injury: processes in inflammatory networks—a review. *Liver Transpl* 2010;16:1016–32.
- Frangogiannis NG, Youker KA, Rossen RD, Gwechenberger M, Lindsey MH, Mendoza LH, Michael LH, Ballantyne CM, Smith CW, Entman ML. Cytokines and the Microcirculation in ischemia and reperfusion. *J Mol Cell Cardiol* 1998;30:2567–76.
- Zacharias N, Sailhamer EA, Li Y, Liu B, Butt MU, Shuja F, Velmahos GC, de Moya M, Alam HB. Histone Deacetylase inhibitors prevent apoptosis following lethal hemorrhagic shock in rodent kidney cells. *Resuscitation* 2011;82:105–9.