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14. ABSTRACT This is a novel study of high dose parenteral vitamin C (VitC) in a swine model of combined hemorrhagic shock and tissue trauma that simulates the course of a combat casualty by exhibiting the components of the lethal triad of acidosis, coagulopathy and hypothermia. The major goal of Year 2 of this project was to refine the treatment with intravenous VitC. In Phase 2, treatments were given immediately following injury (at the start of resuscitation) and at 2 hours post-resuscitation (Resuscitation time 4h). In Phase 3, treatments were given immediately following injury (at the start of resuscitation), at 2 and 4 hours post-resuscitation (Resuscitation time 6h). Histological staining (H & E) of lungs from saline treated swine showed extensive hemorrhage, septal edema, protein leak and exuberant infiltration of inflammatory cells. Significant hemorrhage and cellular damage were also evident in liver and kidney sections. Treatment with VitC (200mg/kg) was associated with a lower degree of histological tissue injury and a significantly reduced ALI score. Treatment with VitC also reduced the expression of pro-inflammatory mediators in lungs, liver and kidneys. Preliminary lipidomic analysis showed that VitC at 200mg/kg shifted the metabolic perturbation closer to the baseline indicating a net conservation of the circulating lipidome in the presence of VitC. Preliminary proteomic analysis revealed that VitC controls ADAMTS13 expression through unknown mechanisms and maintains an anti-coagulant phenotype in organ beds via the ADAMTS13-VWF axis. In sum, our data suggest that intravenous VitC at 200mg/kg appears to significantly ameliorate the inflammatory status and trauma induced coagulopathy in this model.

15. SUBJECT TERMS Intravenous vitamin C, hemorrhagic shock and tissue trauma, trauma induced coagulopathy, platelet dysfunction, histological staining, pro-inflammatory mediators, coagulation markers

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1. Introduction: This is a novel study of high dose parenteral vitamin C (VitC) in a swine model of combined hemorrhagic shock and tissue trauma that simulates the course of a combat casualty by exhibiting the components of the lethal triad of acidosis, coagulopathy and hypothermia. Casualty care for hemorrhagic shock and trauma involve varying degrees of inflammatory up-regulation and variable elements of coagulopathy associated with accumulating oxidative stress. This study investigated the therapeutic effects of parenteral vitamin C on inflammation and coagulation in a large animal model of hemorrhagic trauma/shock with a goal towards improving outcomes including mortality and multiple organ dysfunction.

2. **Keywords**: Intravenous vitamin C, hemorrhagic shock and tissue trauma, trauma induced coagulopathy, platelet dysfunction, histological staining, pro-inflammatory mediators, coagulation markers.

3. **Accomplishments**:

Major Goals of the Project:

During Year 2, the goal was to refine the effective parenteral dose of VitC and the accumulation of reproducible data regarding the effectiveness of the treatment. Treatment effectiveness of IV VitC were assessed based on analysis of:

a. Plasma coagulation biomarkers (tissue factor, von Willebrand factor, thrombomodulin, activated protein C, fibrinogen, and plasminogen activator inhibitor).
b. Changes in viscoelastic properties of blood (thromboelastography [ROTEM])
c. Platelet function (platelet shear modulus and platelet aggregometry, flow cytometry for surface glycoprotein expression for CD62P [P-selectin])
d. Expression of pro-inflammatory biomarkers (IL-6, IL-8)
e. Histological changes to lung, liver and kidney
f. Proteomic analysis of plasma for identification of novel circulating proteins, and
g. Lipidomic analysis of plasma for characterization of the lipidome in this model.

Major accomplishments under these goals:

1. Major activities:

Phase 2 studies: Following the development of the definitive trial, we moved to refine the treatment with intravenous vitamin C (Phase 2). In Phase 2, swine received 2 infusions of treatment, which was saline, vitamin C at 50mg/kg or vitamin C at 200mg/kg. The treatments were given immediately following injury (at the start of resuscitation) and at 2 hours post-resuscitation. Animals were euthanized after 4 hours of resuscitation.

Phase 3 studies: Following the development of the definitive trial, and the Phase 2 trial, we moved to further refine the treatment with intravenous vitamin C (Phase 3). In Phase 3, swine received 3 infusions of treatment, which was saline, vitamin C at 50mg/kg or vitamin C at 200mg/kg. The treatments were given immediately following injury (at the start of resuscitation), at 2 hours post-resuscitation and at 4 hours post-resuscitation. The resuscitation phase was extended from 4 hours to 6 hours.

Basic Methodology (see flow chart diagram below, Figure 1)

Instrumentation phase

- Animals acclimated 1-3 weeks prior to surgery; animals fasted 18 hours prior to surgery
- Animals were sedated with telazol IM, intubated, mechanically ventilated, and maintained at surgical anesthetic plane with isoflurane (balance medical air 21%); pre-surgical analgesia given IV
- Animals were instrumented for hemodynamic monitoring and allowed to stabilize for 30 min. 'Normal' acid-base status confirmed before proceeding. 'Baseline' acid-base and coagulation variables taken at this point.
- Vital signs (heart rate, SpO2, core temperature), anesthetic plane, and ventilator settings were monitored at least every 5 min for the duration of the experiment.
- Injury phase (2 hours) consisted of 4 parts:
  - Hypothermia induction: base pad cooled to induce core temperature of 33°C in approximately 30 to 40 min
  - Liver ischemia/reperfusion injury: Liver venous plexus exposed by laparotomy and I/R induced by application of ligature for 15 min followed by a release period of 5 min, repeated 3 times
  - Unilateral femur fracture with captive bolt gun (allowed to bleed freely)
  - Controlled arterial hemorrhage to maintain MAP 35-40 mmHg for at least 1 hour following soft tissue/extremity/ischemia injuries
- ABG, metabolites and electrolytes measured every 20 min

Resuscitation/monitoring phase

- Active rewarming to target core temperature of 36-37°C
- Abdominal closure, wound packing, other bleeding control
- Hespan bolus to 500 mL. (in definitive trial, treatment intervention i.e. saline control, or one of two doses of vitamin C will be administered at this point)
- ABG, metabolites and electrolytes measured every 30 min
- Coagulation variables taken at resuscitation time 0, 15 min, 2 hours, 4 hours, and 6 hours.

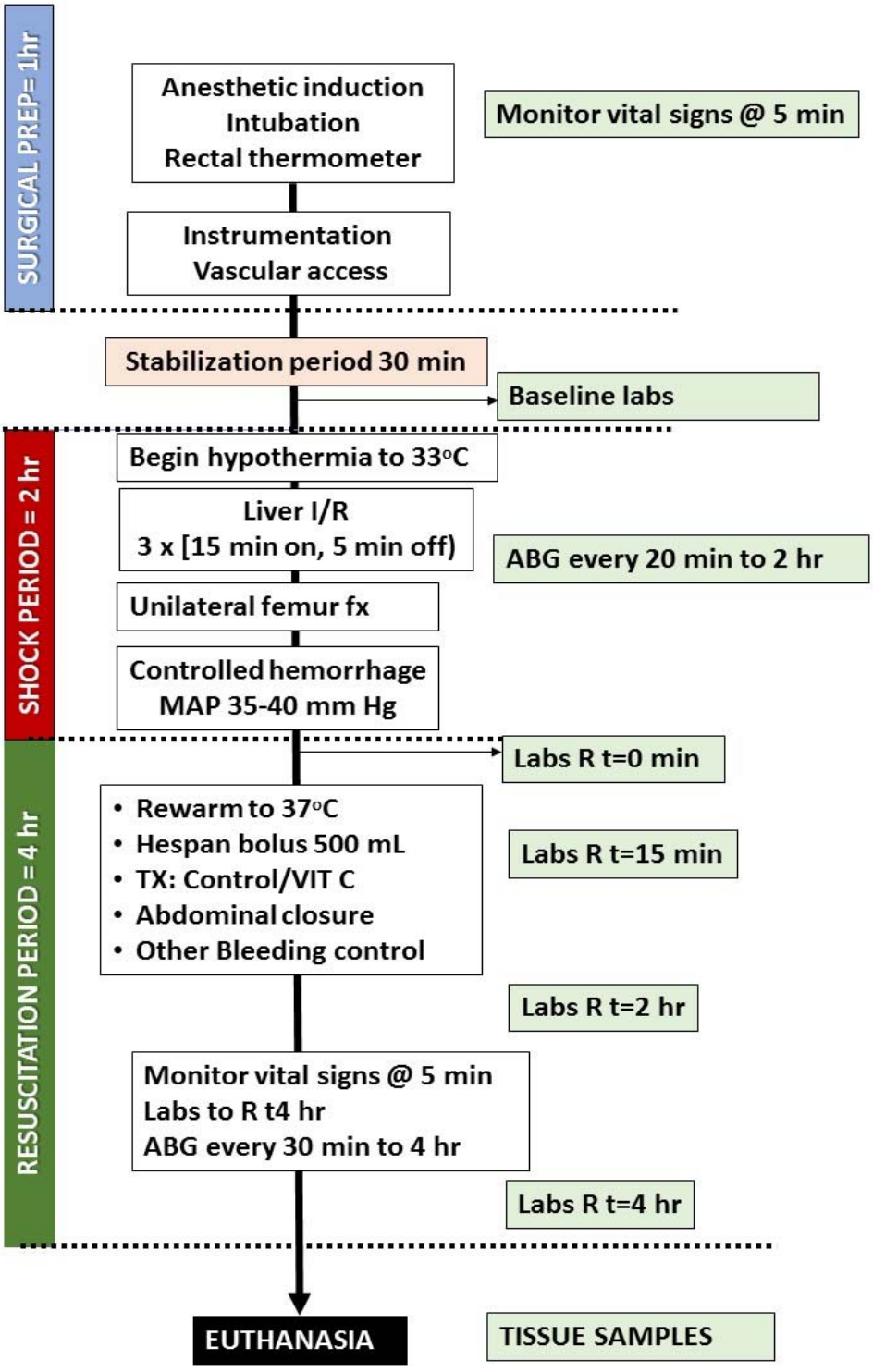


Figure 1: Flow Chart for development of reproducible traumatic injury/shock- hemorrhage swine model

Terminal phase

- Animal euthanized under deep anesthesia with Euthasol IV
- Tissue samples (lung, liver and kidney) obtained for histology and molecular characterization

Phase 2 conducted: Six (6) animals from each arm of the study were used during phase 2. Hemodynamic analysis as well as molecular and functional analysis have been completed following opening of the blind.

Phase 3 conducted: Six (6) animals from each arm of the study were used during phase 3. Hemodynamic analysis as well as molecular and functional analysis have been completed following opening of the blind.

2. Specific Objectives:

a. Assess whether resuscitation with the different doses of VitC are well tolerated by the animal following hemorrhagic trauma and shock
b. Assess the dose of VitC that maximally attenuates circulating pro-inflammatory biomarkers.
c. Assess the dose of VitC that optimally restores hemostatic integrity
d. Assess the mechanism by which VitC prevents onset of multiple organ dysfunction in the setting of hemorrhagic trauma and shock.

3. Significant results:

Phase 2 trial results: Treatment of swine with either dose of intravenous vitamin C was safe and well-tolerated with no aberrant hemodynamic or physiological changes evident following intervention with vitamin C. From a treatment standpoint, there were no major changes in hemodynamic and physiological parameters when compared to Phase 1 studies (single treatment). Molecular changes in various organs were indicative of evolution of an anti-inflammatory and anti-coagulant phenotype following administration of intravenous vitamin C.

Plasma VitC (Figure 2): At baseline, swine in all 3 groups had a plasma VitC level of 78.5µM. Hemorrhagic shock (HS) alone induced a slight increase in circulating plasma VitC to 116.2µM (data not shown). This is the expected response of the animal to injury/stress. Animals in the placebo (saline) group had plasma VitC levels decline slightly (87.3µM, non-significant) over the rest of the resuscitation period (4 hours). In the Lo group, following administration of the bolus of 50mg/kg VitC, plasma levels reached a peak of 981µM at 15 minutes post resuscitation. These levels were maintained with a second treatment at 2h, but declined over the next 2 hours to 516µM. In the Hi group, following administration of the bolus of 200mg/kg VitC, plasma levels reached a peak of 2392µM at 2:15 minutes post resuscitation. These levels declined to 1520µM. This suggests that ongoing injury/oxidative stress results in a significant consumption/destruction of plasma VitC. Alternately, VitC is being transported from the plasma to the various tissues for maintenance of organ function or being excreted via urine.

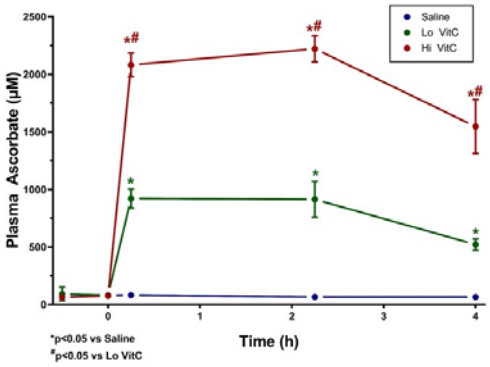


Figure 2: Plasma ascorbate levels from Phase 2 studies

Lung, liver and kidney mRNA expression of inflammatory and pro-/anti-coagulant genes: The physiological environment in which trauma induced coagulopathy (TIC) arises is a complex mixture of inflammation, coagulation, and cellular dysfunction. TIC is characterized by significant pro-inflammatory events such as nuclear factor-kappa B (NFkB) activation, cytokine expression, and neutrophil infiltration. In order to assess coagulopathy and inflammation at a molecular level, we

examined the mRNA expression of key mediators of coagulation and inflammation. As seen in Figure 3, treatment with VitC reduced the expression of the key pro-inflammatory mediators IL-1β, IL-8 and TNFα in lungs. Similar results were obtained from liver and kidneys (data not shown). This suggest that intravenous VitC attenuated the pro-inflammatory response in multiple organs during resuscitation.

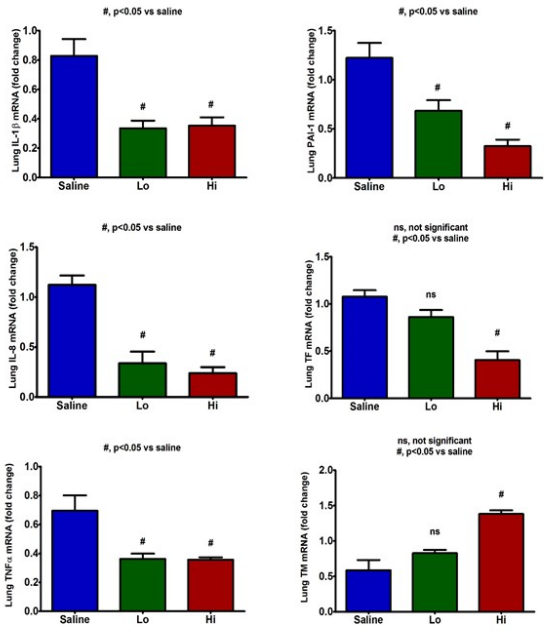


Figure 3: Inflammatory and coagulant biomarker expression from lungs at 4h post-resuscitation in Phase 2

On similar lines, treatment with VitC attenuated the lung and kidney mRNA expression of plasminogen activated inhibitor-1 (PAI-1) and tissue factor (TF) (Figure 3). PAI-1 induction in other models of HS and resuscitation was shown to be deleterious to survival. In addition to promotion of thrombosis with subsequent tissue damage, PAI-1 was shown to play a role in damaging endothelia and hepatocytes, independent of fibrin deposition. In contrast, loss of PAI-1 protected livers from injury. TF is an NFkB driven pro-inflammatory and pro-coagulant protein. As previously demonstrated by us, it is likely that VitC attenuated TF expression by repression of the transcription factor NFkB. In contrast, VitC treatment increased the mRNA expression of thrombomodulin (TM) in lung and liver (Hi dose only). TM plays a key role in the generation of endogenous activated protein C (aPC). The anticoagulant properties of aPC are derived by its degradation of activated factors V and VIII. aPC also has significant anti-inflammatory properties. Investigators have shown that blocking aPC function in a murine model of TIC led to rapid mortality with massive intravascular thrombosis. Our data suggest that by inducing TM expression, VitC may promote endogenous aPC to prevent intravascular thrombosis and perhaps improve survival. Similar results were obtained from liver and kidneys (data not shown).

Histological changes in lungs: After euthanasia, one lobe of liver and left kidney were removed and fixed in 10% formalin. One lobe of the left lung was removed and inflated with 10% formalin at a constant pressure of 20cm H_2O. After 7 days, tissue was removed from formalin and random sections were cut for processing and paraffin embedding. After embedding, 4µm sections were cut and stained using Hematoxylin and Eosin (H&E). Lung architecture was evaluated by bright-field microscopy (x10 and x40 magnification) with an Axio imager A1 microscope, Axiocam HRc camera, and AxioVision software. Ten random tissue sections from each lung were examined in each group by a blinded investigator. For each subject, a five-point scale was applied based on the recommendation of the Official American Thoracic Society Workshop Report, 0 = minimal (little) damage, 1+ = mild damage, 2+ = moderate damage, 3+ = severe damage and 4+ = maximal damage. Damage was assessed based on a) neutrophils in the alveolar or interstitial space; b) formation of hyaline membranes; c) presence of proteinaceous debris such as

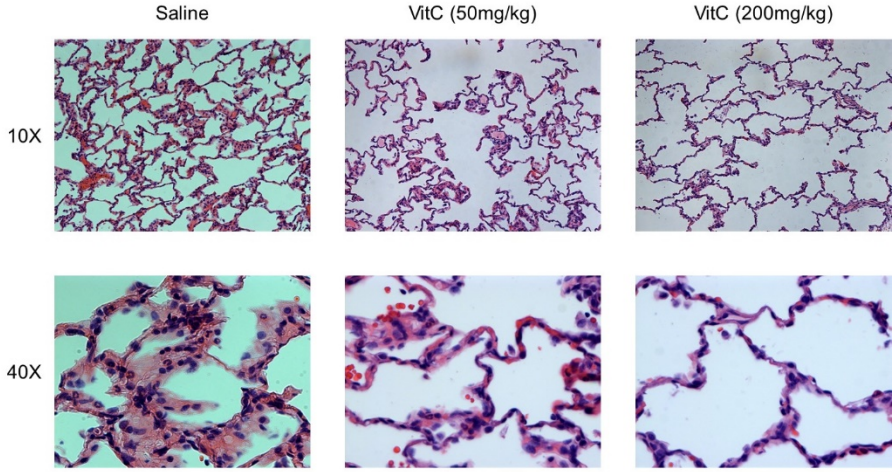


Figure 4: Representative H&E sections from lungs in Phase 2 studies

fibrin strands in the alveolar space; d) thickening of the alveolar wall and e) evidence of hemorrhage. Points were added up and expressed as median ± SE. As seen in Figures 4, treatment with VitC, particularly at 200mg/kg was associated with a lower degree of histological tissue injury.

Physiological and hemostatic changes following administration of intravenous VitC: In this model of hemorrhagic shock and trauma, hemodilution produced significant platelet dysfunction and alterations in various hemostasis parameters including a reduction in global coagulation as indicated by reductions in the ROTEM Thrombodynamic Potential Index (TPI), which is a measure of global coagulation and takes into consideration both clot onset kinetics and final clot strength. While treatment with intravenous VitC at both doses produced small trends in restoration of hemostasis parameters, none of these changes were significant at the early time point of 4 hours post resuscitation (data not shown). The full list of parameters assessed in this model can be found in the Appendix section of this report.

Phase 3 trial results: Treatment of swine with either dose of intravenous vitamin C was safe and well-tolerated with no aberrant hemodynamic or physiological changes evident following intervention with vitamin C. From a treatment standpoint, again there were no major changes in hemodynamic and physiological parameters when compared to Phase 1 studies. Molecular changes in various organs were indicative of evolution of an anti-inflammatory and anti-coagulant phenotype following administration of intravenous vitamin C.

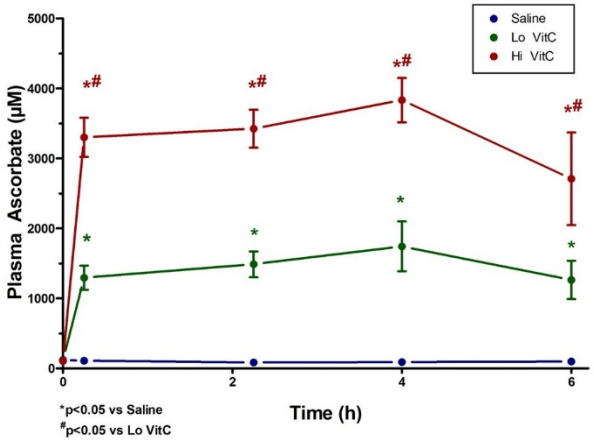


Figure 5: Plasma ascorbate levels in Phase 3 studies in swine.

Plasma VitC: Similar to Phase 2 studies, treatment with VitC resulted in significant increases in circulating VitC levels which were maintained over 4 hours. After 6 hours, the levels were >1mM in the Lo group and >2.5mM in the Hi group (Figure 5).

Lung, liver and kidney mRNA expression of inflammatory and pro-/anti-coagulant genes: As seen in Figure 6, treatment with VitC reduced the expression of the key pro-inflammatory mediators IL-1β, IL-8 and TNFα in lungs. Similar results were obtained from liver and kidneys (data not shown). This suggest that intravenous VitC attenuated the pro-inflammatory response in multiple organs during resuscitation.

On similar lines, treatment with VitC attenuated the lung mRNA expression of plasminogen activated inhibitor-1 (PAI-1) and increased TM mRNA expression (Figure 3). Our data suggest that by inducing TM expression, VitC may promote endogenous aPC to prevent intravascular thrombosis and perhaps improve survival. Similar results were obtained from liver and kidneys (data not shown).

Histological changes in lungs: As seen in Figure 7, treatment with VitC, particularly at 200mg/kg was

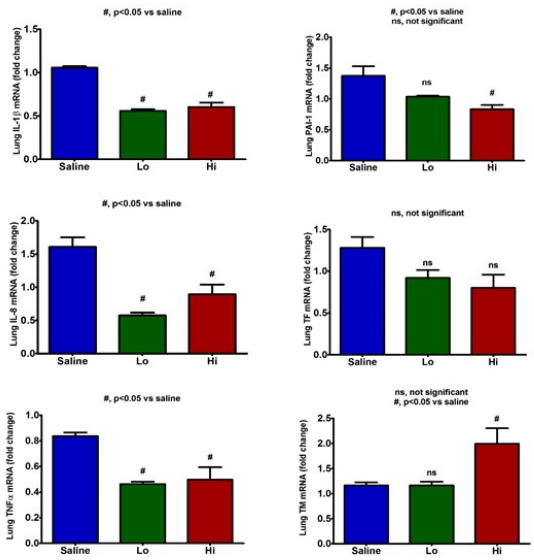


Figure 6: Inflammatory and coagulant biomarker expression from lungs at 6h post-resuscitation in Phase 3

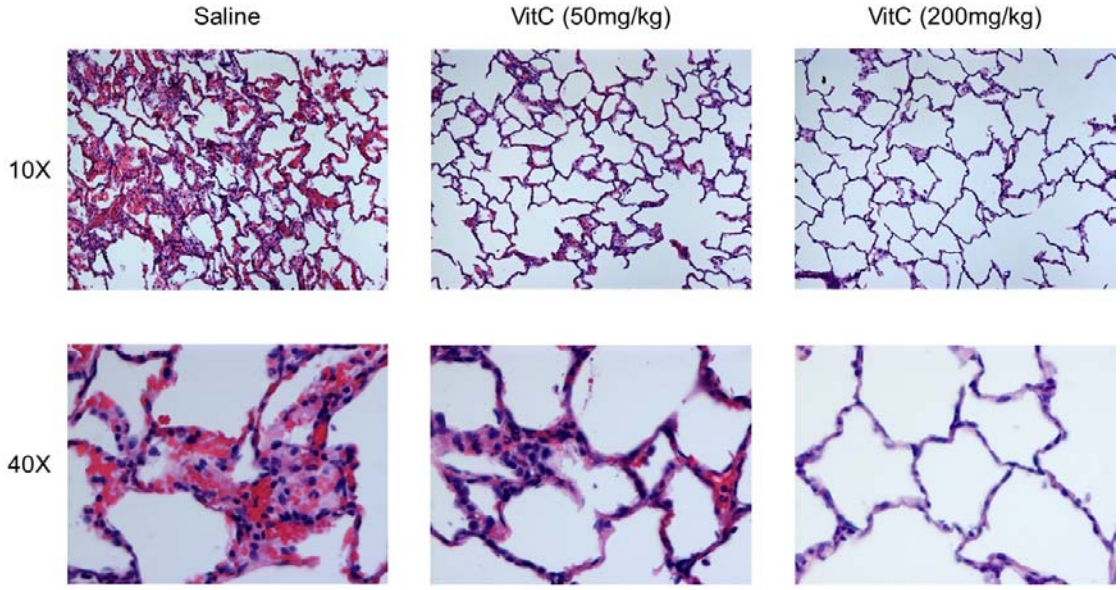


Figure 7: Representative H&E sections from lungs at 6h post-resuscitation in swine treated with intravenous VitC (50mg/kg or 200mg/kg) as compared to saline treatment at 0, 2 and 4h post-resuscitation

associated with a lower degree of histological tissue injury and a significantly reduced ALI score (Figure 8).

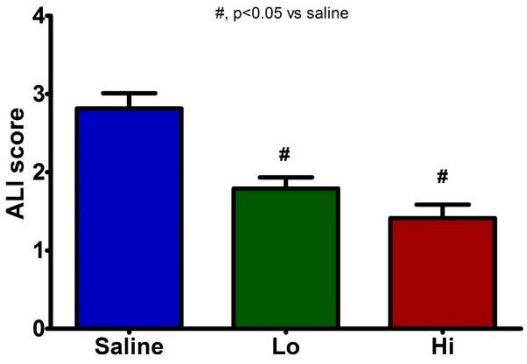


Figure 8: Acute Lung Injury (ALI) score in swine from Phase 3 studies.

Circulating cytokine levels following administration of intravenous VitC: We used ELISA to measure the circulating levels of the pro-inflammatory cytokines IL-1β and IL-8 in swine following injury and resuscitation and treatment with saline or Lo/Hi dose of intravenous VitC. While circulating levels trended lower with Hi dose VitC (200mg/kg), our results showed no significant differences in IL-1β (Figure 9) and IL-8 (data not shown) levels in swine treated with VitC. This is in contrast to the relative mRNA expression of these pro-inflammatory mediators (Figure 6). A likely explanation for this discrepancy is that the protein levels are a measure of the accumulated cytokines over the 2 hour injury period and 6 hours of resuscitation period. In contrast, the mRNA levels are a snapshot of changes occurring after 6 hours of resuscitation. These results suggest that while treatment with VitC likely altered mRNA expression levels (possibly by attenuation of the transcription factor NFκB), these changes have yet to translate to changes in the level of accumulated pro-inflammatory mediators in circulation. It is possible that a significant decline in these pro-inflammatory mediators in circulation will occur in the near future.

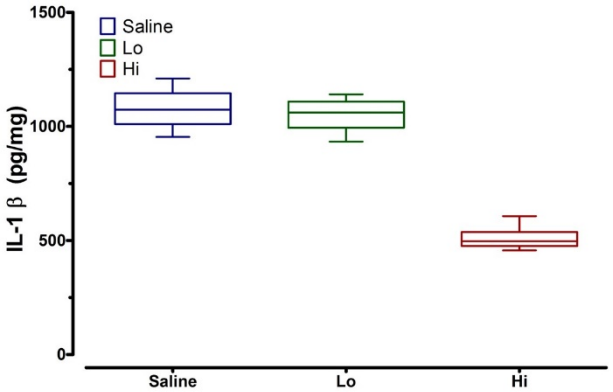


Figure 9: Plasma level of the pro-inflammatory cytokine IL-1β in Phase 3 studies

Physiological and hemostatic changes following administration of intravenous VitC: While treatment with intravenous VitC at both doses produced encouraging trends in restoration of hemostasis parameters, none of these changes were significant at the early time point of 6 hours post resuscitation

(data not shown). The full list of parameters assessed in this model can be found in the Appendix section of this report.

In conclusion, our stable repeatable model induced acute coagulopathy culminating in multiple organ injury. Treatment with VitC, in particular the Hi dose of 200mg/kg appears to significantly ameliorate the inflammatory status and TIC at a molecular level. Histologically also, treatment with 200mg/kg VitC improved lung morphology better than treatment with 50mg/kg. Finally, treatment with either dose of VitC was safe and well tolerated by swine with no deleterious changes in physiology or hemodynamics.

Other Achievements:

Alterations in Lipidome: We have completed analysis of the lipidome from Phase 1 studies. Lipids were extracted from plasma samples. Lipid extracts were analyzed via widely targeted lipidomics approach scanning for 680 different lipid species covering all of the major lipid classes. Class specific deuterated lipid standards were used in the quantitation of the lipids of interest. The resultant data were interrogated for the target lipid species and converted into concentration terms. This data were subjected to Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) to determine the separation between the baseline, and saline at 4hrs post trauma and high vitamin C at 4hrs post trauma (Figure 10). Use of Vitamin C at 200mg/kg appear to shift the metabolic perturbation closer to the baseline indicating a net conservation of the circulating lipidome in the presence of Vitamin C. Variable importance in projection was used to describe the differences in the most important lipid levels between the three time points (Figure 11). VIP scores for the top most important lipids demonstrate that in the presence of Vitamin C, the drastic decrease in the lipid species observed with saline is blunted. This is indicated by the fact that those lipids in the VIP score containing a value that is intermediate between baseline and saline at 4 hours.

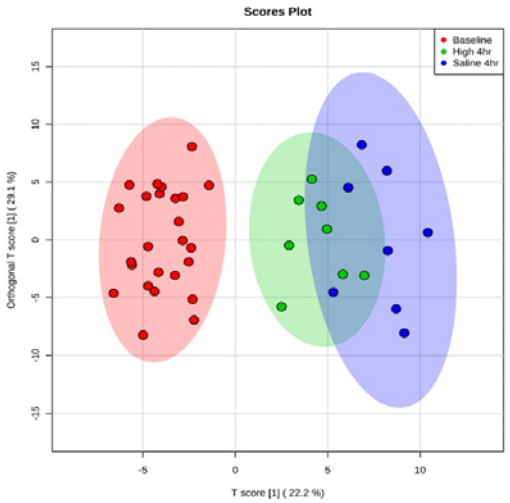


Figure 10: The OPLS-DA plot depict a significant difference between baseline and injured animals at 4 hrs. Use of Vitamin C at high dose appear to shift the metabolic perturbation closer to the baseline indicating a net conservation of the circulating lipidome in the presence of Vitamin C.

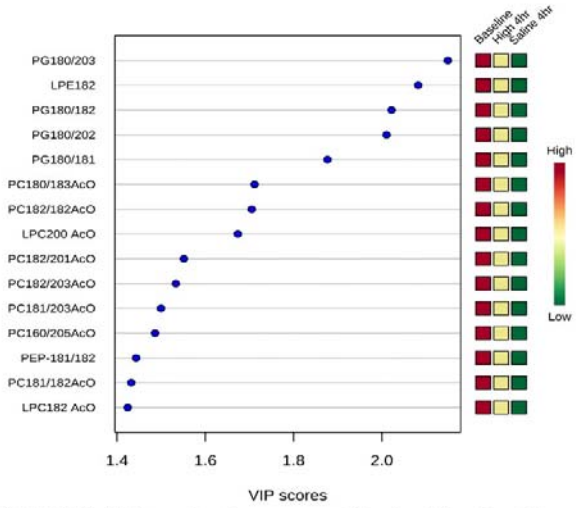


Figure 11: VIP on the Component 1 select the top 15 lipids. VIP scores for the top most important lipids demonstrate that in the presence of Vitamin C, the drastic decrease in the lipid species observed with saline is blunted. This is indicated by the fact that those lipids in the VIP score containing a value that is intermediate between baseline and saline at 4 hours.

Longitudinal plasma proteomics study of plasma from Phase 1 study:

Introduction: High-performance mass spectrometry-based proteomics (LC-MS/MS) was used to map the effect of VitC treatment as a function of time on the plasma proteome of pigs under different treatment regimens. Three treatment regimens were examined: Control (Saline), Low VitC dosage (Lo), and Hi VitC dosage (Hi). EDTA blood samples were collected from 9 pigs at Baseline, T=0min, T=15min, T=2hrs, and T=4hrs in EDTA tubes, centrifuged, and then stored at -80°C. We randomly selected longitudinal plasma samples for three pigs from each treatment

cohort to perform LC-MS/MS analysis. A total of 448 plasma proteins were identified and quantified over the 4 hour sampling interval. We are examining protein levels, extent and sites of methionine oxidation, and enriched biological function(s) including coagulation, acute phase response, and metabolism/energy production. A subset of results from this analysis is presented.

Experimental Sample Preparation. Longitudinal samples obtained from R. Natarajan Lab (N = 45 plasma samples total) were defrosted in a 37°C water bath for 5 minutes followed by albumin and IgG depletion (Millipore ProteoExtract Albumin/IgG Removal Kit; Cat 122642) using the manufacturer's recommendations. In brief, 50 µl of plasma from each animal/timepoint was diluted with 450 µl of Binding Buffer, loaded onto a pre-washed resin bed, and gravity filtered into a 1.5 ml centrifuge tube. The resin bed cartridge was washed with two 500 µl aliquots of Binding buffer to a final depleted plasma protein solution volume of 1.5 ml. Total protein for each depleted sample was determined with a BioTek Synergy H1 plate reader using the Take3 plate at 280 nm. 250 µl of depleted plasma solution from each sample was transferred to an Amicon Ultra 10 kDa molecular weight cut-off filter (Millipore; Cat UFC5010BK), centrifuged for 10 min at 15,000×g, added 400 µl Tris-HCl pH 8.1, centrifuged for 10 min at 15,000g, added 50 µl of 50 mM DTT prepared in H_2O and incubated at 56°C for 1 hr, added 100 µl 80 mM IAM in H2O and incubate in the dark for 30 minutes, centrifuged for 10 min at 15,000×g, added 350 µl of Tris-HCl pH 8.1, centrifuge for 10 min at 15,000g, add trypsin (1:50, E:S), and incubated at 37°C overnight. Samples were then eluted with 300µl of Mobile Phase A via centrifugation and stored at -80°C until removal for LC-MS/MS analysis.

Mass Spectrometer. All samples were randomized and blocked for analysis by reverse-phase high-performance tandem mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of an Eksigent Ekspert nanoLC 415 (Sciex) coupled to a Q Exactive Orbitrap (Thermo). The reverse phase trap and column consisted of a 100 µm x 5 cm trap and 100µm x 20cm Picofrit (New Objective) analytical column self-packed in house with 5 µm Magic AQ C18, 200Å stationary phase. Tryptic peptides were eluted at 350 nL/min with the following gradient: 5% B (0 – 4 min), 35% B (95 min), 75 % B (105 – 110 min), 5% B (115-120 min). Mobile phase A consisted of 98% H_2O/2% acetonitrile, 0.1% formic acid and mobile phase B consisted of 2% H_2O/98% acetonitrile, 0.1% formic acid. The electrospray emitter tip voltage was 1.5 kV in positive ion mode and the Q-Exactive inlet temperature and S-lens setting were maintained at 250°C and 50 V, respectively. Full scan (400-1600 m/z) resolution was set at 70,000 FWHM with an AGC target of 3×10^6. MS/MS was set to a resolution of 17,500 with an AGC target of 2×10^4 at 120 ms maximum inject time with a selection of the top 12 ions and a 30 second dynamic exclusion. HCD voltage was maintained at 27 NCE throughout.

Data Analysis. Proteomic datasets were processed in MaxQuant (ver. 1.5.8.3) using the Andromeda search algorithm and the Uniprot Pig Proteome FASTA database (download: 6/12/2017). Search conditions included; mass accuracy: MS = ±4.5 ppm, MS/MS = ±0.5 Da; fixed modifications: carbamidomethyl (C), variable modifications: acetyl (N-terminus) and methionine oxidation (M), and a false discovery rate (FDR) of 1%. Raw protein (i.e., tryptic peptides) intensities were $\log_2$ transformed in Perseus (ver. 1.5.8.5) and missing values imputed with normal distribution-derived values. Protein abundances are reported as either $\log_2$ transformed intensities (LFQ) or as the difference between proteins LFQ in individual samples and the LFQ_{avg} for all samples (e.g., $LFQ_{Saline\ 1,Pig\#1} - LFQ_{avg(All\ 9\ Pigs)}$).

Results: A total of 449 plasma proteins were identified and quantified in this study. A preliminary assessment of the Uniprot Pig (*Sus scrofa*) protein database shows a substantial fraction (~1/2) of

identified proteins are uncharacterized requiring us to perform BLAST analysis to identify the human homologues with the highest sequence similarity. Further, out of the ~200 proteins identified/BLAST annotated to date, we have found ~5 instances of redundant proteins in our list. Thus, we conservatively estimate the final number of unique proteins identified in this study is likely to be between 400-425 proteins. Importantly, these issues are not uncommon for unique experimental animal models and we have previous experience cleaning and aligning datasets with human genes from previous studies (e.g., LC-MS/MS of ovarian cancer in the chicken). Irrespective of the final list of proteins and their alignment with human genes, the current dataset has already yielded unique observations related to plasma protein levels as a function of VitC treatment. Here we focus on a subset of the 449 proteins involved with Coagulation but we are actively assembling other key enriched protein subsets related to Acute Phase Response, Complement System, Inflammation, Metabolism, and Proteolytic Activity.

Coagulation Proteins: A total of 20 Coagulation Factors and Associated Coagulation Proteins have been identified thus far from the 449 protein dataset (**Figure 12A**). Coagulation Factors III (F3), XII (F7), XIII (F8), and FXI (F11) were not detected whereas Fibrinogen alpha (FGA)/beta (FGB)/gamma (FGG), F2, F5, F9, F10, F12, F13A and F13B, and Factor XIV (PROC) were detected. Additional proteins associated with coagulation (via Gene Ontology) were also identified including alpha-2-macroglobulin (A2M), a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), Fibrinogen-like protein 1 (FGL1), fibronectin (FN1), prekallikrien (KLKB1), kininogen-1 (KNG1), plasminogen (PLG), antithrombin (SERPINC1), heparin Cofactor II (SERPIND1), alpha-2-antiplasmin (SERPINGF2), and von Willdebrand Factor (VWF). It is important to note that we continue to annotate the *uncharacterized* proteins in our 449 protein dataset and expect that additional Coagulation associated proteins will be identified and then added to the protein expression profile shown in **Figure 12A**. The relative protein expression heat map in **Figure 12A** displays the $\log_2$ ($LFQ_{Timepoint/Treatment}/LFQ_{avg}$) for all 20 proteins displayed over a range of -5 to 3. The majority of Coagulation Factors were unchanged although F13A did show a significant increase at 15 minutes relative to the LFQ_{avg} for all three treatments. FGL1, ADAMTS13, PLG, and VWF showed changes in relative expression over time and as a function of treatment. FGL1 levels were immediately elevated in the Saline control animals at T = 0 hrs whereas the onset of increased levels were delayed in the Lo VitC and Hi VitC treatment animals by <2 hours and <4 hours, respectively. ADAMTS13 proteolytically regulates the size and proportion of VWF microclusters in blood thereby affecting platelet adhesion and clotting. Recent studies have shown that levels of both ADAMTS13 and VWF are affected in patients with acute liver failure suggesting clinical value as a diagnostic biomarker (e.g., Hepatology, 2013, 58, 752-761). In the current study, we see a remarkable drop in ADAMTS13 levels between T = 0 and 15 minutes in the Saline treated animals relative to the VitC animals which saw stable levels for all 5 timepoints (**Figure 12B**). VWF levels progressively increased with time but

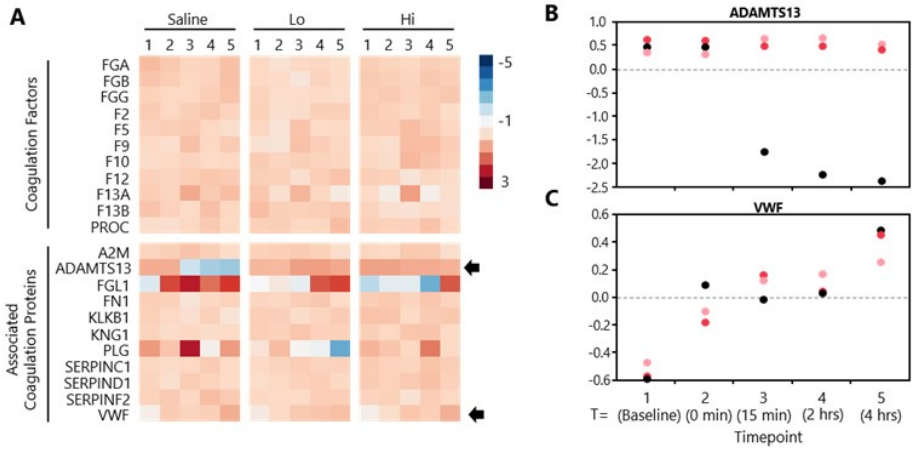


Figure 12: Summary of coagulation protein levels in swine plasma A) as a function of time and treatment condition.

no change was observed with treatment (**Figure 12C**). An emerging hypothesis is that VitC controls ADAMTS13 expression through unknown mechanisms and maintains an anti-coagulant phenotype via the ADAMTS13-VWF axis. We intend to look for additional dysregulated proteins that could support or add to the importance of this early observation.

Summary. Near term efforts are focused on completing the annotation of the full 449 protein dataset using BLAST analysis against the human database. We will then return to the quantitative data and begin comprehensively mapping protein expression levels with GO Biological Processes and Function including Acute Phase Response, Inflammation, Complement System, Metabolism (Liver and Kidney), and Proteolytic Activity. The long-term goal is to identify key enriched biological functions related to VitC dosage to establish a molecular level framework for understanding hemostasis in trauma.

Opportunities for training and professional development:

While this study was not designed to specifically provide training and professional development, it allowed the surgery technicians and the histopathologist to advance their skills. In particular, the beneficiaries of this were: Christopher Sweeney, Jacquelyn McCarter, Daniela Farkas, Evan Fowler, Paul Middleton and Matthew Ellenberg.

In addition, Jacquelyn McCarter, presented the Phase 3 aspect of the study with an abstract titled "Attenuation of Coagulopathy and Inflammation in a Swine Polytrauma model using Intravenous Vitamin C". This was accepted as an ORAL presentation at the 2017 Military Health System Research Symposium (MHSRS) in Orlando/Kissimmee, FL.

Dissemination of results:

The model development aspect of the study was presented with an abstract titled "Development Of A Novel Swine Model of Trauma-Induced Coagulopathy". This was accepted as a poster presentation at the 2016 Military Health System Research Symposium (MHSRS) in Orlando/Kissimmee, FL. It has been submitted as a manuscript. This manuscript is currently under review.

A manuscript regarding the effect of VitC from the Phase 1 studies has been submitted to Critical Care Medicine. This manuscript is currently under review.

Future Plans until the end of the No Cost Extension (NCE) Period:

- Proteomics analysis: We have completed analysis of samples from Phase 1 (see above) and results are very exciting. We anticipate that the samples will be run on the reverse-phase high-performance tandem mass spectrometer by the end of September. However, analysis of the data will take several more months. Based on our preliminary runs (Phase 1), a substantial fraction (~1/2) of identified proteins are uncharacterized requiring us to perform individual BLAST analysis to identify the human homologues with the highest sequence similarity. We anticipate unique observations related to plasma protein levels as a function of Vitamin C treatment. In addition we will actively assemble other key enriched protein subsets related to Acute Phase Response, Complement System, Inflammation, Metabolism, and Proteolytic Activity.

- Lipidomic Analysis: For lipid analysis from the Phase I studies, lipid extracts were analyzed via widely targeted lipidomics approach scanning for 680 different lipid species covering all of the major lipid classes. The resultant data were interrogated for the target lipid species and converted into concentration terms. These data were subjected to Orthogonal Partial Least Squares

Discriminant Analysis (OPLS-DA) to determine the separation between the baseline, and saline at 4 hours post trauma and high vitamin C at 4 hours post trauma. Variable importance in projection was used to describe the differences in the most important lipid levels between the three time points. Similar to the proteomics studies, we are currently extracting lipids from plasma samples from the Phase 3 studies. These samples will be run by the end of October. However the detailed analysis of the data will require a further 3 months to complete.

4. **Impact**:

Impact on the development of the principal discipline of the project:
Short-Term Impact: Immediate (2 to 5 years) applications of this project will be research-related in 4 focal areas of impact. Traditional avenues of academic sector knowledge production will include dissemination of research findings by publication in peer-reviewed journals that cover both clinical and basic research, at conferences, and in research reports. Basic data will provide comprehensive documentation of the coagulation process though the short-term shock/injury/resuscitation cycle, and will enable investigation of the potential of VitC to reduce inflammation and coagulopathy secondary to massive system trauma. The translatability potential of this research is very great. Because our team measures clinically-relevant metrics – global coagulation dysfunction, inflammation, hemodynamics – our findings will provide a rationale for proceeding to human clinical trials in the near future. Finally the methodological contribution to this area of research is novel in that it represents an extension of process quality control improvement and product optimization strategies to preclinical research to enable rapid convergence to an optimal solution. Application of these techniques, together with traditional methods of reducing bias, such as randomization and blinding, will ensure high-quality data, and be a model for other investigations of this type.

Long-Term Impact: The vision for this research is to expedite the development, clinical trial testing, and FDA certification of this potentially life-saving therapeutic, so as to enable deployment in the far-forward arena in the immediate near future. Our expectation is that intravenous preparation of VitC will prove to be a safe and effective field adjunct to current standard-of-care resuscitation products, be rapidly integrated into prehospital care protocols, and most importantly, act to minimize long-term sequelae of sepsis and MOF in surviving combat casualties.

Military Benefit: Rapid, effective resuscitation is essential for treating combat injuries with active bleeding and hypovolemia. In the far-forward arena, treatment of the wounded war-fighter is most challenging in the earliest time period following injury. Injuries are frequently multimodal (blast, penetrating, blunt), and at significant risk for infection. The amount of resuscitation fluid available in the field may be limited. Provision of large amounts of fluid is not often practical under fire or in austere environments, and optimizing allocation of scarce fluid resources according to triage criteria means that many wounded receive inadequate or no supportive fluid therapy. Logistic problems of applying stabilizing treatment in combat conditions are compounded by delayed evacuation from the hot zone. Unfortunately, this is when specific treatments need to be applied to have the greatest therapeutic benefit. Furthermore, current fluid resuscitation strategies do not reduce risk for late-term sepsis and MOF. Based on our previous calculations, low volumes of unaugmented colloids or crystalloids are unlikely to reverse or prevent severe cell and organ damage; these fluids may actually induce inflammation and coagulopathy in casualties with moderate to severe hemorrhage and polytrauma. VitC is significantly depleted in critical injury and illness; parenteral VitC administration to critically ill sepsis patients has been demonstrated to reduce the severity of end-organ damage and mortality. If the military remains committed to low-volume battlefield resuscitation, our approach – combining an approved antioxidant (VitC) with an approved colloid or crystalloid – is a realistic, cheap, and effective strategy to prevent additional mortality and morbidity, with a high probability of rapid (3-5 years) incorporation into standard of care protocols. Supplementation of resuscitation fluids already in common use would require little revision of medic skills. Instead, casualties could receive one or two IV product applications close to point of injury and/or enroute to definitive care. Product could be reconstituted with whatever approved fluid was on hand (crystalloid, colloid) and easily infused via peripheral vascular access or intra-osseous device, as established per protocol. This fluid resuscitation strategy would meet the medic's objectives of keeping the casualty alive in the short term, with the added benefit of minimizing cell damage that occurs during these early stages of traumatic injury, and which contributes to sepsis and end-organ damage in the longer term. Small volume high-dose formulations would meet logistic constraints on weight and volume.

Impact on other disciplines:

If successful, the impact of this therapeutic will be broadly applicable to a variety of critically-ill civilian surgical and medical patient populations, also at high risk for systemic inflammation and development of MOF. Current sepsis treatment involves prolonged hospital stays in the intensive care unit at very high cost (over $20 billion in 2011 alone, or over $55 million per day). Sepsis patients are hospitalized longer, more likely to be discharged to facilities other than home, and suffer high rates of readmission, resulting in additional costs of over $2 billion per year. Therefore if this product shows demonstrable reduction or prevention of runaway inflammation and TIC, early-stage intervention could be expected to greatly reduce these costs. In addition, our investigations of the cellular and molecular underpinnings of inflammatory and coagulation responses could serve as the basis for future design of "smarter", more targeted, resuscitation strategies and therapeutics. The long-term impact of successful trialing of this product will therefore be service-related because of: improvements in public health through better treatment; potentially enormous cost savings and cost containment through reductions in hospital stays and intensive care; changes in evidence-based practice (through provision of evidence affecting treatment decisions, and clinical practice), and quality of care (assuming the efficacy of this health intervention).

Currently available pharmacologic agents: Treatment for sepsis is mainly supportive, involving large amounts of fluid, antibiotics, vasopressors, corticosteroids, conservative mechanical ventilation and immunomodulatory drugs. However the major challenge of treatment is diagnosis; early recognition of sepsis is difficult, and treatment may be delayed until the condition is far advanced. In lieu of focusing on early detection, treatment with VitC close to point of injury may be a simple, cost-effective, and clinically effective alternative.

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Public Purpose: Countless medical interventions derived from military medicine have been incorporated into civilian prehospital medicine and trauma surgery. Our proposed VitC therapeutic could be readily applied to civilian patient populations characterized by compromised blood flow to organs, and thus at significant risk of developing sepsis and MOF. More than 1 million patients in the US develop sepsis each year, and over 50% die; health care costs may exceed $20B per year. Vulnerable populations include patients with hemorrhagic shock (resulting from blunt trauma, penetrating trauma, surgical mishaps, and gastrointestinal bleeds), hemodiluted surgical patients, and patients with medical emergencies, such as stroke or cardiac arrest. The prevention or reduction of sepsis and organ failure is thus of significant public health interest. Finally there is the potential for a large societal impact, if this research can prove to contribute to improvements in the care and management of the critically ill and injured by reducing morbidity and mortality, and through economic benefits resulting from direct cost savings to health care systems, and through reduction in lost productivity of a relatively young patient cohort.

5. **Changes/Problems**:

Changes in approach and reasons for change:

Our approach has remained essentially identical to that proposed in the SOW.

Actual or anticipated problems or delays and actions or plans to resolve them:

Logistic challenges: None

Technical challenges: None

Personnel challenges: None in Year 2

Changes that had a significant impact on expenditures:

The study remained financially healthy throughout Year 1 and ended up below projected budget by about $32,000. These funds will be carried over to the NCE period for completion of lipidomic and proteomic analyses and for publication charges.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

No significant changes were made in the use or care of vertebrate animals, biohazards or select agents.

6. **Products:**

Publications, conference papers, and presentations:

Christopher Sweeney, Jacquelyn McCarter, Paul Middleton, Matthew Ellenberg, Evan Fowler, Penny S. Reynolds, Erika J. Martin, Bernard J. Fisher, Donald F. Brophy, Alpha Fowler III, Bruce D. Spiess, Ramesh Natarajan. Attenuation of Coagulopathy and Inflammation in a Swine Polytrauma Model using Intravenous Vitamin C. 2017 Military Health System Research Symposium (MHSRS), Orlando/Kissimmee, FL

Website(s) or other Internet site(s):

Nothing to report

Technologies or techniques:

This study employs an operationally-standardized preclinical swine model of traumatic injury and hemorrhage developed by the PI, and extensively validated in pilot and initial definitive testing by our team. It incorporates all consensus elements of an animal coagulopathy model: significant 'multiple hit' tissue injury, a combination of controlled and uncontrolled mild to moderate hemorrhage, prolonged hypotension, hemodilution (simulating clinical resuscitation practice), hypothermia, acidosis, measurement of inflammatory markers, and assessment of anticoagulant and fibrinolytic pathways. In addition, our model applies iatrogenic injury and resuscitation procedures in the clinically relevant and appropriate chronological order (e.g. hemodilution occurs after injury). The shock/injury phase involves both hemorrhage and isolated traumatic insults, including unilateral femur fracture and solid organ injury. A novel feature of our model is the application of a standardized Pringle maneuver to impose an intermittent liver ischemia/reperfusion (I/R) injury to induce inflammation and coagulopathy. The combined trauma-hemorrhage model optimizes operational characteristics such as clinical relevance, standardization, reliability, and reproducibility.

Inventions, patent applications, and/or licenses:

Nothing to report

Other Products:

Nothing to report

7. **Participants & Other Collaborating Organizations:**

What individuals have worked on the project?

Name	Ramesh Natarajan
Project Role	Principal Investigator
Nearest person month worked	5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Development of reproducible traumatic injury/shock-hemorrhage swine model; Performed definitive Trial for intervention with intravenous vitamin C; Analyzed data; Prepared reports; Singularly responsible for conduct of project.

Name	Alpha Fowler
Project Role	Co-Investigator
Nearest person month worked	0.06
Contribution to project	Guided the evolution of the model and assisted the PI with data interpretation and analysis of treatment outcomes.

Name	Bernard J Fisher
Project Role	Co-Investigator
Nearest person month worked	1.35
Contribution to project	Measured plasma vitamin C levels and cell free DNA levels; Performed cytokine and molecular analysis for expression of genes related to inflammation and trauma induced coagulopathy; Assisted the surgical team with sample preparation and collection.

Name	Donald Brophy
Project Role	Co-Investigator
Nearest person month worked	0.12
Contribution to project	Assessed coagulation changes and platelet function in the model and following treatment.

Name	Erika Martin
Project Role	Co-Investigator
Nearest person month worked	1.2
Contribution to project	Performed assays for all coagulant-anticoagulant and fibrinolytic pathways; Assessed coagulation changes and platelet function in the model and following treatment.

Name	Jacquelyn McCarter
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	7.5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model.

Name	Christopher Sweeney
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	7.5
Contribution to project	Getting protocols approved; Training required personnel; Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model.

Name	Evan Fowler
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	6
Contribution to project	Assistance with surgeries and collection of data.

Name	Paul Middleton
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	6

Contribution to project	Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model; Responsible for intubation of swine and maintaining the ventilator

Name	Matthew Ellenberg
Project Role	Veterinary Laboratory Specialist
Nearest person month worked	1.5
Contribution to project	Development of working SOP; Performing surgeries for development of reproducible traumatic injury/shock- hemorrhage swine model; Responsible for record keeping and assisting with all aspects of surgery.

Name	Dayanjan Wijesinghe
Project Role	Assistant Professor
Nearest person month worked	0.6
Contribution to project	Performed Lipidomics analysis on plasma from swine used for injury development.

Name	Adam Hawkridge
Project Role	Assistant Professor
Nearest person month worked	0.6
Contribution to project	Performed Proteomic analysis on plasma from swine used for injury development.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. **Special Reporting Requirements**:

Collaborative Awards:

N/A

Quad Charts:

Please see after Appendix.

9. **Appendices**:

List of other hemostasis and physiological parameters investigated:

CBC Parameters:
WBC X 109/L
RBC X 1012/L
HGB g/dL
HCT %
MCV fl
MCH pg
MCHC g/dL
RDWC %
PLT x 109/L
PCT %
MPV fl
PDWC %
LY%
MON%
NE%

Coagulation Parameters:
Fib (mg/dL)
vWF:Ag (%)
aPC (%)
ATIII (%)
COLL/ADP PFA (sec)
COLL/EPI PFA (sec)
FOT (min)
PCF (kdynes)
CEM (kd/cm2)
CT NATEM (sec)
CFT NATEM (sec)
Angle NATEM (degree)
MCF NATEM (mm)
NATEM TPI
CT INTEM (sec)
CFT INTEM (sec)
Angle INTEM (degree)
MCF INTEM (mm)
INTEM TPI
CT EXTEM (sec)
CFT EXTEM (sec)
Angle EXTEM (degree)
MCF EXTEM (mm)
EXTEM TPI
Flow Cytometry for CD62p + ADP

Physiological Parameters (Bio-Pac):
MAP, PAP, CVC, CCO

Interventional Vitamin C-A Strategy for Attenuation of Coagulopathy and Inflammation in Hemorrhagic Trauma and Shock

13011003

W81XWH-15-2-0064

PI: Ramesh Natarajan **Org:** Virginia Commonwealth University **Award Amount:** $1,000,000

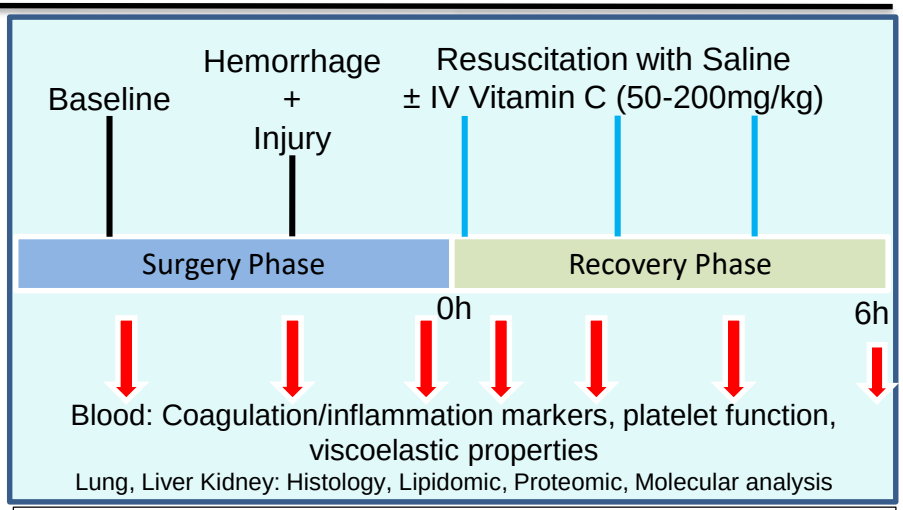
Study/Product Aim(s) – Year 2

This is a novel study of high dose parenteral vitamin C in a swine model of combined hemorrhagic shock and tissue trauma. Treatment effectiveness of IV VitC will be assessed based on analysis of:

- Plasma coagulation biomarkers
- Changes in viscoelastic properties of blood
- Platelet function
- Expression of circulating pro-inflammatory biomarkers

Approach

Our initial goal was to generate a reproducible swine model of traumatic hemorrhagic shock, tissue injury and resuscitation simulating combat casualty and examine the effects of parenteral VitC on plasma coagulation biomarkers, platelet function and expression of circulating pro-inflammatory biomarkers along with analysis of neutrophil sequestration in tissue beds as a measure of multiple organ damage (MOD). In Year 2, we will establish timing and dose of parenteral VitC administration that best reduces MOD, coagulation biomarkers and TIC.



Accomplishment: • Data analyzed from definitive trial. Preliminary manuscript on model development submitted. Started Phase 2 of definitive trial for treatment effectiveness.

Timeline and Cost

Activities CY	15	16
Generation of Trauma Model & Determination of treatment effectiveness		
Model refinement (addition of grade IV liver injury) & mechanistic studies regarding treatment effectiveness		
Estimated Budget ($K)	**$450,000**	**$550,000**

Updated: October 30, 2017

Goals/Milestones (Example)

CY17 Goal – Treatment effectiveness

- ☑ Completed 18 animals in Phase 2
- ☑ Completed 18 animals in Phase 3
- ☑ VitC attenuated onset of MODS by suppressing inflammatory markers.

Comments/Challenges/Issues/Concerns

- No new issues have arisen indicating that steps taken to date have been all encompassing to achieve successful completion of study.

Budget Expenditure to Date

Total Projected Expenditure: $1,000,000

Actual Expenditure: $968,000