# EFFECTS OF SANGUINATE ON SYSTEMIC AND MICROCIRCULATORY VARIABLES IN A MODEL OF PROLONGED HEMORRHAGIC SHOCK

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ABSTRACT—**Background:** Hemorrhage and its complications are the leading cause of preventable death from trauma in young adults, especially in remote locations. To address this, deliverable, shelf-stable resuscitants that provide therapeutic benefits throughout the time course of hemorrhagic shock and the progressive ischemic injury it produces are needed. SANGUINATE[*] is a novel bovine PEGylated carboxyhemoglobin-based oxygen carrier, which has desirable oxygen-carrying and oncotic properties as well as a CO moiety to maintain microvascular perfusion. **Objectives:** To compare the crystalloid (Lactated Ringer's Solution; LRS), and the colloid (Hextend[†]) standards of care with SANGUINATE in a post "golden hour" resuscitation model. **Methods:** Rats underwent a controlled, stepwise blood withdrawal (45% by volume), were maintained in untreated hemorrhagic shock state for >60 min, resuscitated with a 20% bolus of one of the three test solutions, and observed till demise. Parameters of tissue oxygenation ($P_{ISF}O_2$), arteriolar diameters, and mean arterial pressure (MAP) were collected. **Results:** SANGUINATE-treated animals survived significantly longer than those treated with Hextend and LRS. SANGUINATE also significantly increased tissue $P_{ISF}O_2$ 2 h after resuscitation, whereas LRS and Hextend did not. SANGUINATE also produced a significantly higher MAP, which was hypotensive compared to baseline, that endured until demise. **Conclusions:** Resuscitation with SANGUINATE after prolonged hemorrhagic shock improves survival, MAP, and $P_{ISF}O_2$ compared with standard of care plasma expanders. Since the pathologies of hemorrhagic shock and the associated systemic ischemia are progressive, preclinical studies of this nature are essential to determine efficacy of new resuscitants across the range of possible times to treatment.

KEYWORDS—Hemoglobin-based oxygen carrier, hemorrhage and resuscitation, ischemia, microcirculation, phosphorescence quenching microscopy, SANGUINATE, shock

## INTRODUCTION

Despite advancements in tourniquets and wound dressings for prompt control of active bleeding, hemorrhage, and the state of shock it produces remains a leading cause of death in both civilian and military populations (1, 2). A major contributor to mortality due to hemorrhagic shock is the delay between the point of injury (3) and resuscitative care (4, 5). This critical period of shock is characterized by hypotension, reduced blood flow (ischemia), and systemic hypoxia that worsens with duration. The result is a cumulative and potentially insurmountable oxygen debt causing irreversible tissue/organ injury and death. Guidelines have indicated that transit times to resuscitative care should be no longer than 60 min (6), which is based on

the "golden hour" of resuscitation. As such, many research efforts utilize hemorrhagic shock durations shorter than 60 min leaving dearth of studies focused on the >60 min time to treatment scenario as discussed by Bjerkvig et al. (7).

The ideal resuscitation solution in the trauma setting provides volume support as well as oxygen-carrying capacity, which is currently lacking in non-blood containing solutions. Thus, it maintains circulatory function without introducing harm into an already sensitive, pathological state of shock until blood component therapy and definitive hemostasis are available. Resuscitation solutions are most needed in remote locations where blood availability is minimal. Therefore, research efforts have begun to focus on settings involving prolonged hemorrhagic shock (PHS) due to acute blood loss (6). In recent years, this need has been recognized in academic circles (7), and from funding agencies such as the Department of Defense's Combat Casualty Care Research Program's $47M support for the "Prolonged Field Care" initiative, which highlights the importance of new studies on extended evacuation time and the need "to develop new solutions to provide for prolonged Damage Control Resuscitation" (8). Earlier attempts at developing oxygen-carrying resuscitative agents failed to provide clinical improvements and may have contributed to arteriolar vasoconstriction and systemic hypertension (9).

A preclinical rodent model of PHS was developed that reflects clinical scenarios where patients suffering from hemorrhagic shock experience an extended delay in care resulting in

[*]SANGUINATE is the registered trademark of Prolong Pharmaceuticals, LLC.
[†]Hextend is the registered trademark of BioTime Inc.

cumulative ischemic damage. Three resuscitation solutions were evaluated using this PHS model: Lactated Ringer's solution (LRS), Hextend, and SANGUINATE, a bovine PEGylated car-boxyhemoglobin-based oxygen carrier. LRS is a clinically used crystalloid and Hextend is a colloid plasma volume expander, neither of which are oxygen carriers. SANGUINATE has been tested in multiple animal models of acute ischemia (10, 11). It has completed phase I clinical trials (12), and is currently in multiple phase 2 clinical studies (NCT02672540, NCT02323685, NCT02658162, NCT02600390, NCT02411708). SANGUI-NATE does not induce vasoconstriction and instead has been shown to improve cerebral collateral perfusion (11). This unique capacity to deliver oxygen while maintaining circulatory dynam-ics suggest SANGUINATE may provide a useful alternative to crystalloid or red blood cell transfusions in the setting of pre-hospital resuscitation. Additionally, SANGUINATE has been found to mitigate ischemia reperfusion injury (10), which makes it an enticing candidate for resuscitation either during or after the "golden hour."

## MATERIALS AND METHODS

### Animals

The following protocol and experimental procedures were approved by the SoBran Biosciences Inc. IACUC (Protocol # SON-001-2015) and are consistent with the National Institutes of Health guidelines for the humane treatment of laboratory animals, as well as the American Physiological Society's Guiding Principles in the Care and Use of Animals. Study subjects (Fig. 1) were male Sprague–Dawley rats (300–350 g; Harlan, Indianapolis, Ind).

### Surgical preparation

Animals were induced with 1% to 5% isoflurane in medical air for initial pre-operative preparation and cannulations. An intravascularly delivered anesthetic, alfaxalone acetate (Alfaxan; Schering-Plough Animal Health, Welwyn Garden City, UK), was continuously infused at 0.1 mg/kg/min through a femoral vein cannula for maintenance of anesthesia during the remainder of the experiment. A femoral artery cannula was connected to a pressure transducer for monitoring of systemic circulatory variables with a multichannel physiological monitoring system (BIOPAC MP-150; BIOPAC Systems, Goleta, Calif). The carotid artery and jugular vein were cannulated for blood withdrawal and resuscitant infusions, respectively. Arterial cannu-las were kept free of clots with heparinized phosphate-buffered saline (PBS; 20 IU heparin/mL). A tracheal tube was inserted to maintain airway patency; however, animals continued to inspire room air and were not artificially ventilated. After experimentation, animals were euthanized with a lethal dose of Euthasol (150 mg/kg, pentobarbital component, intravenously; Delmarva, Midlothian, Va).

### Spinotrapezius preparation

The rat spinotrapezius muscle was exteriorized as described by Gray (13) with some modification to accommodate measurements of microvascular activity and interstitial oxygen tension on a thermostable animal platform adapted for intravital and phosphorescence quenching microscopy (PQM) as described here (14, 15) and further in the supplemental methods section, http://links.lww.com/SHK/A687. Animals and exteriorized spinotrapezius muscle—isolated from atmospheric contamination by a transparent barrier film—were maintained at 37°C during experimentation, which was monitored by rectal probe (BIOPAC; Part # SS7L; BIOPAC Systems).

### Microcirculatory parameters

Observation and measurement of the exteriorized spinotrapezius preparation were carried out with an intravital microscope (Axio Imager A2m; Carl Zeiss, Göttingen, Germany) configured for trans-illumination through a 20×/0.8 objective (Plan-APOCHROMAT; Zeiss, Jena, Germany) and custom modified for PQM. Trans-illumination was used to select measurement sites, establish appropriate focal planes, and verify flow conditions. Measurements of arteriolar diameter in the spinotrapezius muscle were made using a 10×/0.25 objective
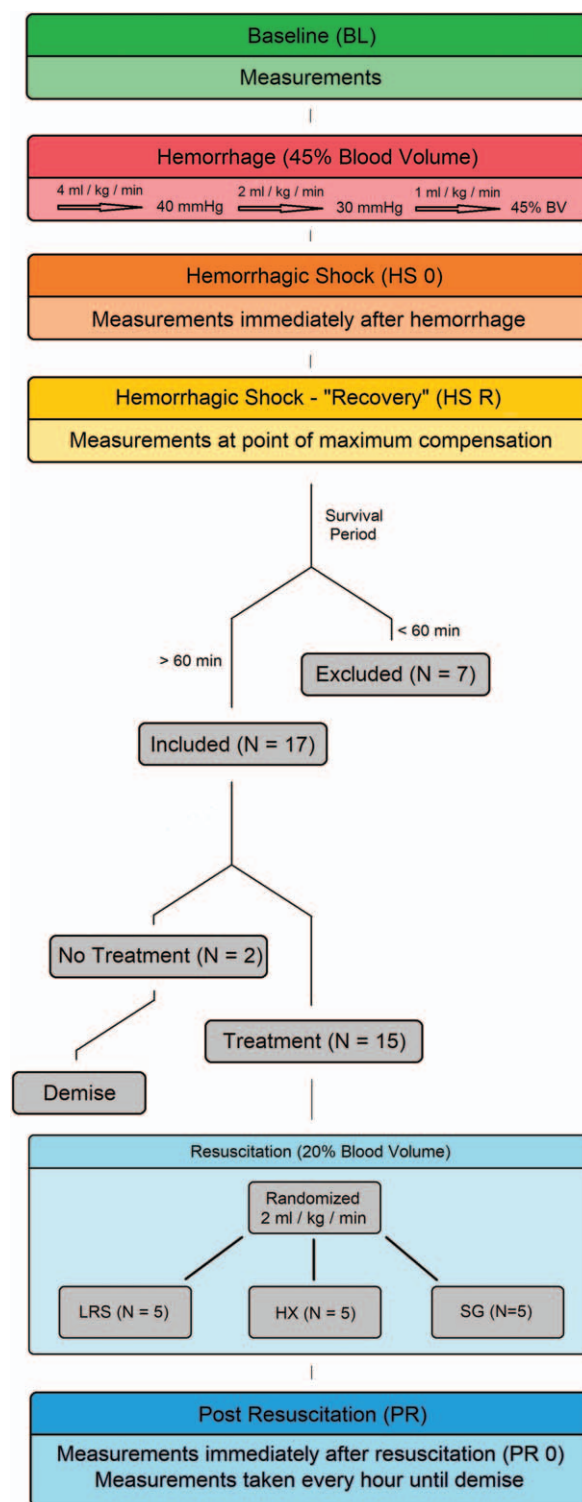


Fig. 1. **PHS Protocol Flow Chart.** Male Sprague–Dawley rats were surgically prepared before entering the protocol at "baseline." "Measure-ments" refers to systemic cardiovascular parameters collected through a femoral artery pressure transducer (BIOPAC), microvascular interstitial oxy-genation ($P_{ISF}O_2$) collected via phosphorescence quenching microscopy, and microvascular imaging using bright field microscopy and a digital camera to measure arteriolar diameters. Both continuous and discrete measurements are reported at the time points indicated in the schematic. Animals meeting the inclusion criteria of surviving a hemorrhagic shock of >60 min were placed into either treatment or no treatment (sham) groups. The first two animals were assigned to Sham, the rest were assigned to randomized resuscitation treatment: LRS (Lactated Ringer's Solution), HX (Hextend), and SG (SAN-GUINATE).

### TABLE 1. Resuscitant comparison

| Test agent | Plasma expanding | Oncotic pressure | Oxygen transfer |
|---|---|---|---|
| LRS | Yes | No | No |
| Hextend | Yes | Yes | No |
| SANGUINATE | Yes | Yes | Yes |

SANGUINATE's utility as a resuscitation solution following prolonged hemorrhagic shock was compared to two standards of care with different properties: (1) LRS, which is a crystalloid that functions as a plasma expander, and (2) Hextend, which is a colloid that functions as a plasma expander and has a sufficient oncotic pressure to retain fluid within the vasculature. SANGUINATE provides both of these functions, in addition to its oxygen transfer properties.

(N-Achroplan; Zeiss). The objective was focused in the diametral plane and the image displayed on an ultra-high definition screen (XBR43X830C; Sony, Tokyo, Japan). Images of the microvasculature were captured in real-time by a color CMOS camera (ACE ACA2040–90UC; Basler, Berlin, Germany) and stored digitally. Internal (luminal) vessel diameters for second-order arterioles were measured by converting from pixels to micrometers using a calibration based on pixelization of a standard microscope micrometer (Stage Micrometer; Zeiss). Arterioles between 40 and 80 μm were selected for assessment of vasoactivity.

### Phosphorescence quenching microscopy

Phosphorescence quenching for the detection of oxygen in biological systems was originally described by Wilson et al. (16, 17). Adaptation of this technology for microscopic measurements of the interstitial oxygen tension ($P_{ISF}O_2$) in the exteriorized spinotrapezius preparation as a determinant of "tissue oxygenation" are described elsewhere (15, 18, 19). A specific focus on our equipment and methodology is included in the supplemental methods section, http://links.lww.com/SHK/A687.

Briefly, a palladium porphyrin "oxygen probe" (Oxyphor R0; Frontier Scientific, Newark, Del) bound to bovine serum albumin was topically applied to the tissue and allowed to diffuse into the spinotrapezius muscle's interstitium. For measurements, the oxygen probe was excited by a xenon flash lamp (L11969; Hamamatsu Photonics, Hamamatsu, Japan), in an octagonal region 300 μm in diameter at a frequency of 1 Hz. The excitation light pulse was passed through a filter cube consisting of a narrow-band filter (525 CWL Narrowband; Edmund Optics, Barrington, NJ), a dichroic mirror (567 nm DMLP Longpass; Thorlabs, Newton, NJ), and a wide-band filter (Longpass Cut-on >650 nm; Thorlabs) for selective collection of phosphorescence emission. Three interstitial sites were measured per animal. The phosphorescence signal was collected by a photomultiplier tube (R9110; Hamamatsu Photonics) and routed through a custom-built signal processor, collected by a data acquisition device (NI PCIe-6361; National Instruments, Austin, Tex) and stored digitally on a computer. Each phosphorescence decay curve (which could be thought of as an exponential decay curve) was fitted to a rectangular distribution model presented in this paper (20) from which individual partial pressure of oxygen (PO₂) values for $P_{ISF}O_2$ were calculated. Calibration of our PQM equipment and oxygen probe was performed as described by Golub and Pittman (21). For additional information, please see the supplemental information, http://links.lww.com/SHK/A687, describing our PQM technique.

### Resuscitation solutions

Lactated Ringer's Solution (LRS; Hospira Inc., Lake Forest, Ill) is a crystalloid electrolyte solution that is hypo-oncotic to blood and contains no specific oxygen carrier (Table 1).

Hextend (6% hetastarch in lactated electrolyte solution; Hospira Inc.) is a colloid resuscitation solution that is iso-oncotic to blood, but contains no specific oxygen carrier.

SANGUINATE (PEGylated carboxyhemoglobin bovine; Prolong Pharmaceuticals, LLC, South Plainfield, NJ) is a multi-functional, large-volume parenteral that provides fluid and colloidal resuscitation in addition to oxygen gas transfer properties, which have been described in-depth elsewhere (10, 11).

### Experimental protocol

Animals were subjected to a controlled blood withdrawal that allowed for temporary recovery and long-lasting hemorrhagic shock, but was ultimately lethal. Inclusion into the study required animals to tolerate the blood withdrawal

and survive for >60 min without treatment or intervention. Exclusion was animal demise <60 min or evidence of adverse events associated with surgery or blood withdrawal that would have caused interruptions in the process. Seven animals were excluded from the study due to premature demise.

Measurements of mean arterial pressure (MAP), pulse pressure (PP), heart rate (HR), and temperature were recorded continuously through the BIOPAC data acquisition equipment. Imaging of arteriolar diameters and measurement of interstitial oxygen tension ($P_{ISF}O_2$) were conducted at discrete time points throughout each experiment. Continuous data were plotted to align with those discrete time points. Baseline (BL) measurements of MAP, PP, HR, arteriolar diameter, and $P_{ISF}O_2$ were recorded once the animal had stabilized from surgery (15–30 min).

Animals were hemorrhaged at stepwise rates of 4.0 mL/kg/min to an MAP of 40 mmHg, then 2.0 mL/kg/min to an MAP of 30 mmHg, and finally 1.0 mL/kg/min until 45% of the blood volume was removed (see Fig. 1). Blood volume was calculated as body weight × 0.06 + 0.77 based on measurements reported by Lee and Blaufox (22). This blood withdrawal was controlled and facilitated by a syringe pump (Genie Touch™, Kent Scientific, Torrington, Conn) to ensure rates and volumes were standardized between experiments. Measurements at the end of hemorrhage ($HS_0$), point of max recovery of MAP during hemorrhagic shock ($HS_R$), and every 60 min post-resuscitation ($PR_n$) were made using identical sites and methods as BL and all other time points. The "point of max recovery" was assessed as a peak plateau in MAP after hemorrhage. All animals demonstrated hemorrhagic compensation with a gradual rise in MAP, which eventually plateaued and then began to decline over the HS phase. $HS_R$ measurements were made at the onset of this plateau.

The animals remained in the HS phase until "near demise" that was defined as an MAP below 22 mmHg, and an HR above 300 BPM. Sham animals (untreated) then expired within a minute after "near demise," while treatment animals were resuscitated with various test solutions (Fig. 1).

A hypovolemic resuscitation (2.0 mL/kg/min infusion of 20% of the estimated blood volume) was performed using one of the three test solutions. At the end of resuscitation, hourly measurements were made with the first marked as time point post-resuscitation at time point 0 min ($PR_0$). The observation phase then lasted for 8 h or until the animal expired.

### Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Kaplan–Meyer was used for the analysis of survival with post-hoc Log-rank Mantel–Cox. One-way ANOVA (Prism 6, GraphPad Software Inc, San Diego, Calif) was used to detect changes within groups over the experimental time course. In cases where a significant difference ($P < 0.05$) was detected, an appropriate multiple comparison test (Tukey's HSD) was conducted. Comparisons between groups was conducted using a Two-way ANOVA where appropriate and further analyzed by Tukey's HSD multiple comparison test when ($P < 0.05$).

## RESULTS

### Prolonged hemorrhagic shock phase

All animals were treated identically during the PHS (pre-resuscitation) phase. All animals that met the study inclusion criteria (Fig. 1) responded and progressed similarly through hemorrhage into hemorrhagic shock. Furthermore, all "baseline" variables herein were statistically not significant among treatment groups suggesting that the randomly selected animals came from a similar population.

MAP was recorded continuously throughout each experimental time course and analyzed discretely to pair with other, discontinuous measurements. The average baseline MAPs for PHS-induction were: LRS 131 ± 5.3 mmHg, Hextend 126 ± 4.6 mmHg, and SANGUINATE 139 ± 9.2 mmHg. Hemorrhage significantly reduced MAPs to 28 ± 0.5, 26 ± 0.8, 27 ± 1.4, and 28 ± 1.2 mmHg, respectively. Following $HS_0$ MAP began to recover, reaching peak values of 59 ± 7.0, 55 ± 8.1, 57 ± 6.7, 75 ± 5.2 mmHg, respectively, which were significantly higher than $HS_0$ for all groups at $HS_R$ (Table 2).

Following hemorrhage, the physiological impact and development of hemorrhagic shock was assessed for other variables at $HS_R$ since it provided the best assessment of peripheral tissue

TABLE 2. **Systemic variables: mean arterial pressure, pulse pressure, heart rate**

| Measurement | BL | HS$_0$ | HS$_R$ | PR$_0$ | PR$_{60}$ | PR$_{120}$ | PR$_{180}$ | PR$_{240}$ |
|---|---|---|---|---|---|---|---|---|
| **Sham** | | | | | | | | |
| MAP | 122 ± 18.5 | 28 ± 0.5 | 86 ± 3.5 | | | | | |
| PP | 52 ± 2.1 | 15 ± 4.9 | 66 ± 14.5 | | | | | |
| HR | 413 ± 56.0 | 429 ± 12.0 | 462 ± 7.5 | | | | | |
| **LRS** | | | | | | | | |
| MAP | 131 ± 5.3 | 26 ± 0.8**** | 61 ± 7.8****$_{ββ}$ | 36 ± 2.4****,† | | | | |
| PP | 51 ± 4.7 | 13 ± 1.1** | 54 ± 10.4$_{ββ}$ | 28 ± 4.9† | | | | |
| HR | 433 ± 19.8 | 437 ± 16.8 | 409 ± 29.5 | 351 ± 27.7 | | | | |
| **Hextend** | | | | | | | | |
| MAP | 126 ± 4.6 | 27 ± 1.4**** | 58 ± 3.3****$_{ββββ}$ | 46 ± 3.4****$_{ββ}$ | 38 ± 4.9**** † | | | |
| PP | 46 ± 2.5 | 10 ± 1.5**** | 45 ± 7.1$_{ββββ}$ | 27 ± 2.0* † | 22 ± 4.9* † | | | |
| HR | 451 ± 14.2 | 442 ± 30.3 | 437 ± 24.5 | 407 ± 24.6 | 514 ± 43.5 | | | |
| **SANGUINATE** | | | | | | | | |
| MAP | 139 ± 9.2 | 28 ± 1.2**** | 76 ± 5.2****$_{ββ}$ | 68 ± 4.5****$_{β}$ | 96 ± 5.8**$_{ββββ}$ | 83 ± 8.3****$_{βββ}$ | 66 ± 19.6**** | 68 ± 7.0**** |
| PP | 46 ± 2.1 | 14 ± 2.1** | 64 ± 7.8$_{ββββ}$ | 61 ± 5.0$_{ββββ}$ | 66 ± 6.8$_{ββββ}$ | 62 ± 6.3$_{βββ}$ | 53 ± 14.8$_{ββ}$ | 69 ± 0.0$_{ββ}$ |
| HR | 435 ± 14.0 | 420 ± 14.8 | 415 ± 13.6 | 376 ± 15.2 | 471 ± 19.1 | 470 ± 19.5 | 425 ± 13.4 | 389 ± 13.0 |

Systemic variables for mean arterial pressure (MAP; mmHg), pulse pressure (PP; mmHg), and heart rate (HR; BPM) are presented here as mean ± SEM. One-way ANOVA was used for determinations of significance and Tukey's HSD for intragroup comparisons. Comparisons to BL, HS$_0$, and HS$_R$ are reported. Redundant comparisons such as BL-HS$_0$, and HS$_0$-BL, are not illustrated.
BL indicates baseline; HS$_0$, end of hemorrhage; HS$_R$, hemorrhagic shock; PR$_0$, post-resuscitation at time point 0 min; PR$_{60}$, 60-min post-resuscitation; PR$_{120}$, 120-min post-resuscitation; PR$_{180}$, 180-min post-resuscitation; PR$_{240}$, 240-min post-resuscitation.
*,**,***,****indicate $P < 0.05$, 0.01, 0.001, and 0.0001 compared with BL, respectively.
β,ββ,βββindicate $P < 0.05$, 0.01, 0.001, and 0.0001 compared with HS$_0$, respectively, unless previously noted.
†,††,†††,††††indicate $P < 0.05$, 0.01, 0.001, and 0.0001 compared with HS$_R$, respectively, unless previously noted.

$P_{ISF}O_2$ and recovery of microcirculatory perfusion at the point of maximal "compensatory response." Table 2 describes this increased MAP at HS$_R$, which is associated with a numeric bump in $P_{ISF}O_2$ for all groups *versus* HS$_0$ (Fig. 2).

Figure 2 shows baseline $P_{ISF}O_2$ was significantly (not illustrated) reduced by hemorrhage similarly for individual groups as follows: LRS 50 ± 4.2 to 3 ± 2.1 mmHg, Hextend 52 ± 2.6 to 5 ± 2.5 mmHg, and SANGUINATE 60 ± 4.5 to 3 ± 1.7 mmHg, which indicated the spinotrapezius muscle was in a state of severe hypoxia and ischemia. All subsequent time points were significantly lower than baseline. $P_{ISF}O_2$ recovered slightly, but not significantly, by the post-hemorrhage recovery (HS$_R$; LRS: 13 ± 4.4 mmHg, Hextend: 8 ± 3.9 mmHg, and SANGUINATE: 6 ± 2.5 mmHg). Assessment at this peak recovery of MAP and, ostensibly, microvascular perfusion, indicated that the state of severe hypoxia was persistent—all measured tissues remained well within the realm of hypoxia (<25 mmHg)—throughout the hemorrhagic shock phase.

PP declined significantly from baselines of 51 ± 4.7, 46 ± 2.5, and 46 ± 2.1 mmHg to 13 ± 1.1, 10 ± 1.4, and 14 ± 2.1 mmHg for LRS, Hextend, and SANGUINATE, respectively, following withdrawal of 45% estimated blood volume. By HS$_R$, it had recovered to 54 ± 10.4, 45 ± 7.1, and 64 ± 7.8 mmHg, respectively, none of which were significantly different from baseline, and then progressively deteriorated until "near demise."

Baseline values for HR were 433 ± 20, 451 ± 14, and 435 ± 14 BPM for LRS, Hextend, and SANGUINATE groups, respectively. Heart rates did not significantly vary between groups over the measurement time course of the PHS phase, but did show changes consistent with increases to preload (Table 2). Arterioles contracted from baselines of 58 ± 2.3,

54 ± 3.0, and 60 ± 2.7 μm to 55 ± 2.3, 44 ± 2.8, and 40 ± 3.7 μm following hemorrhage for LRS, Hextend, and SANGUINATE, respectively. The only significant change detected was for the SANGUINATE group of animals between baseline and HS$_0$. By HS$_R$, arteriolar diameters were not significantly different from baseline at 55 ± 2.1, 49 ± 2.0, and 49 ± 3.8 μm for LRS, Hextend, and SANGUINATE,
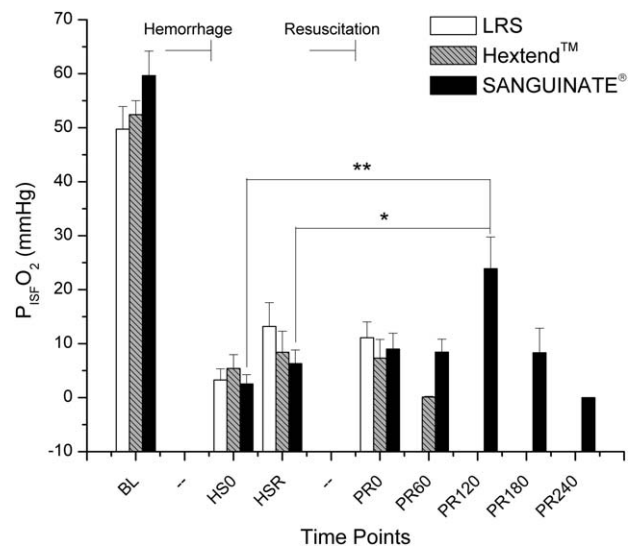


Fig. 2. **Peripheral tissue oxygenation ($P_{ISF}O_2$).** Intra-group statistical analysis was performed by one-way ANOVA with Tukey's HSD for determination between time points. Skeletal muscle oxygenation was significantly lower at all time points across all groups compared to baseline (not illustrated). After resuscitation, $P_{ISF}O_2$ was significantly higher than HS$_0$ and HS$_R$ at PR$_{120}$ for SANGUINATE treated animals. An intergroup comparison could not be made at PR$_{120}$ due to animal mortality in LRS and Hextend treated groups. *$P < 0.5$, **$P < 0.01$.
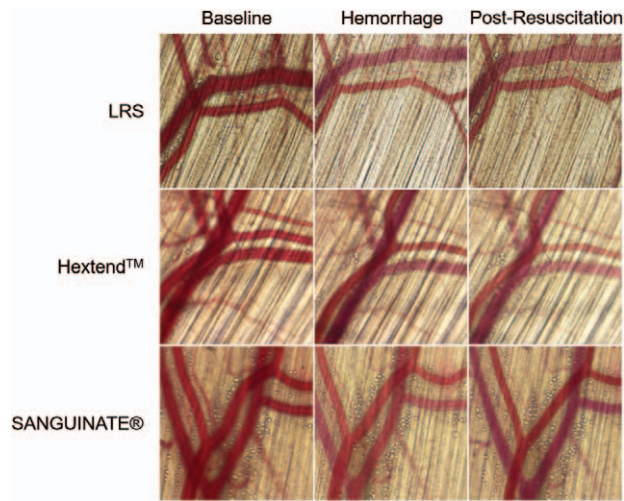
FIG. 3. **Microvasculature panel.** Representative changes to the micro-circulation are illustrated here from Baseline (BL), immediately after hemor-rhage ($HS_0$), and immediately after treatment ($PR_0$) for LRS, Hextend, and SANGUINATE treated animals. Images were recorded at $10\times$ magnification. Noteworthy features include: (1) a colorimetric blue-shift of arterial blood during hemorrhage that persists after treatment with LRS and Hextend, but appears mitigated after treatment with SANGUINATE; and (2) no visible differences in vessel diameter between groups, which were not statistically different from each other.

respectively. See Figure 3 for representative images of micro-vascular progression through hemorrhage and resuscitation.

MAP steadily declined after maximal recovery until demise in Sham (untreated; N = 2; see Table 2) animals, which occurred approximately $103 \pm 25$ min after hemorrhage. Death per this protocol was gauged to occur shortly ($\sim 1$ min) after MAP reached 22 mmHg. PP below $\sim 10$ mmHg was another indication of impending demise. Animals remained viable and responsive to resuscitation with their MAPs in the low 20 s if their HRs remained above 300 BPM. This point of "near demise" was used to determine when resuscitative fluids would be administered. This served to base resuscitation on the animal's proximity to physiological collapse rather than an arbitrary time point to minimize variation between animals.

### Post-resuscitation phase

Treatment groups were resuscitated at "near demise," as defined above, by a continuous infusion rate of 2.0 mL/kg/min with LRS (N = 5), Hextend (N = 5), or SANGUINATE (N = 5) and observed until demise. Treatments were plotted against survival time in a Kaplan–Meier plot (Fig. 4) and show a significant separation between LRS, Hextend, and SANGUINATE. LRS showed the least post-resuscitation survival benefit with a mean survival time of $16 \pm 3.4$ min. Hextend extended survival to $76 \pm 11.1$ min, which was significantly greater than LRS. Resuscitation with SANGUINATE produced the longest survival time of $193 \pm 33.4$ min, which was significantly improved over Hextend and LRS.

Figure 2 shows that resuscitation did not have an immediate effect on $P_{ISF}O_2$ for any group. Following the completion of the resuscitation phase, $PR_0$ $P_{ISF}O_2$ values for LRS, Hextend, and SANGUINATE were $11 \pm 2.9$, $7 \pm 3.5$, and $9 \pm 2.9$ mmHg, respectively. After 60 min post-resuscitation ($PR_{60}$), LRS-

treated animals had expired and Hextend treated animals showed non-detectable levels of $P_{ISF}O_2$. Animals resuscitated with SANGUINATE, however, reported a value of $8 \pm 2.4$ mmHg, and although it was not significantly different from $HS_0$ or $HS_R$, it marked the beginning of a trend towards an increase in $P_{ISF}O_2$ at the 120-min time point ($PR_{120}$). Only SANGUINATE-resuscitated groups survived to and beyond the $PR_{120}$ time point, so measurements could only be compared to earlier time points for other groups. At $PR_{120}$, SANGUI-NATE $P_{ISF}O_2$ was significantly higher ($24 \pm 5.9$ mmHg) than time points $HS_0$ ($3 \pm 1.7$ mmHg) and $HS_R$ ($6 \pm 2.5$ mmHg). $P_{ISF}O_2$ then declined steadily at later time points to the point of demise.

LRS-resuscitated animals showed no improvement in MAP following infusion ($PR_0$) compared with $HS_0$ and were signifi-cantly depressed compared to $HS_R$. MAP immediately began to decline when the infusion pump was stopped and all animals had expired prior to $PR_{60}$. Hextend produced a greater response in MAP reaching $46 \pm 3.4$ mmHg by $PR_0$, which was signifi-cantly higher than $HS_0$, and not significantly different than $HS_R$. By $PR_{60}$, MAP was statistically no higher and trending lower than $PR_0$. All Hextend treated animals expired prior to $PR_{120}$. SANGUINATE produced a MAP of $68 \pm 4.5$ mmHg by $PR_0$, which was significantly higher than $HS_0$, and not statisti-cally different from $HS_R$. MAP then continued to rise to a peak of $96 \pm 5.8$ by $PR_{60}$ and declined steadily thereafter until demise. All post-resuscitative MAPs across groups were sta-tistically lower than baseline (Table 2).

PP for LRS-treated animals was not improved by resuscita-tion ($PR_0$) compared to $HS_0$ and significantly worse than $HS_R$. Hextend produced a numerical rise from $HS_0$, but remained significantly lower than $HS_R$ by $PR_0$. Pressure then trended downwards by $PR_{60}$ and further to animal demise. Both post-resuscitative time points were significantly lower than baseline. Resuscitation with SANGUINATE produced a significantly higher PP compared with $HS_0$, and was not significantly different compared with baseline and $HS_R$. PPs remained at
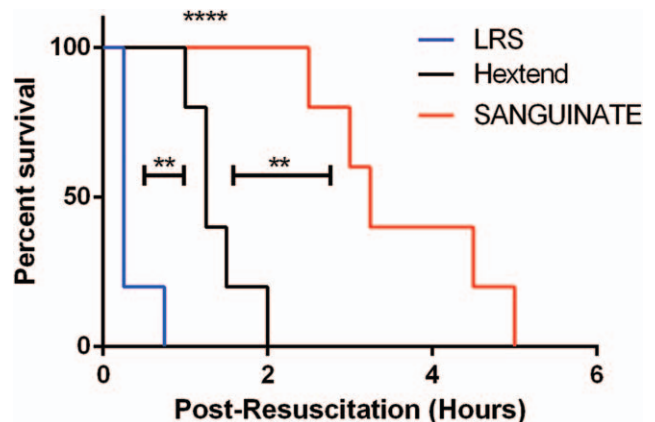


FIG. 4. **Post-resuscitation survival.** Treatment groups were assessed for duration of survival from the end of resuscitation. Both Hextend and SANGUINATE showed significant increases in survival *versus* LRS and from each other. **Between groups indicate significance level of $P < 0.01$ for those comparisons. ****Indicates $P < 0.0001$ result from overall Log-rank Mantel–Cox test.

baseline levels for the remainder of the experimental time course (Table 2).

No significant changes to HR were detected for any groups (Table 2). At time point $PR_0$, immediately following the infusion of resuscitant, HR had trended downward for all groups. By $PR_{60}$, however, HR had trended upwards for both Hextend and SANGUINATE groups (LRS animals did not survive to this time point).

Following hypovolemic resuscitation ($PR_0$) arteriolar diameters were not significantly different from baseline in all three treatment groups (LRS: $57 \pm 4.4$, Hextend $49 \pm 3.5$, SANGUINATE: $51 \pm 3.1$ $\mu$m). Additionally, there were no differences between groups at $PR_0$. Visual differences in the microcirculation at $PR_0$ are presented in Figure 3.

## DISCUSSION

A novel model of PHS was developed and employed as a first assessment of SANGUINATE as a trauma resuscitant in comparison with other standards-of-care. The setting of severe hemorrhagic shock followed by prolonged systemic hypoxia, which has persisted past the "golden hour" of resuscitation, was chosen due to a report of SANGUINATE's therapeutic benefit during ischemia/reperfusion injury (10). This protracted ischemic state was designed to be lethal, but sensitive to the different constituents of resuscitative solutions in the following robust and unique battery of systemic and microcirculatory measurements: post-resuscitative survival, tissue oxygenation ($P_{ISF}O_2$), MAP, vasoactivity, and other cardiovascular parameters. Resuscitative outcomes between a non-oxygen-carrying crystalloid (LRS), a non-oxygen-carrying colloid (Hextend), and SANGUINATE an oxygen-carrying bovine PEGylated carboxyhemoglobin were compared. Overall, SANGUINATE extended survival times *versus* Hextend, which in turn outperformed LRS. Key parameters associated with an increased survival were resuscitant impact on $P_{ISF}O_2$ and MAP without a detectable vasoconstrictive effect on the rat skeletal muscle microvasculature.

A hypovolemic resuscitation, equivalent to two 500 mL bags of Hextend or one 1 L bag of LRS in human patients, was chosen due to an increasing amount of supporting clinical data (23, 24) combined with the logistical practicality of reduced transfusion volumes in remote regions. It extended post-resuscitative survival without the confounding consequences of a volumetric overload. Differences between LRS, Hextend, and SANGUINATE were most noticeable in the early MAP response. LRS failed to have a substantial reticence within the vasculature, which was indicated by peak resuscitation MAP occurring when the infusion pump was stopped at the end of reperfusion. Map then trended downwards from that point forward. Hextend and SANGUINATE, however, were better able to maintain blood pressure following the cessation of the infusion, likely due to their oncotic properties. SANGUINATE in turn produced a longer lasting MAP and PP response than Hextend. Overall, the MAP profile was predictive of survival, with LRS animals dying shortly after the end of reperfusion and Hextend animals surviving longer, which is consistent with clinical and animal studies investigating the impact of crystalloid *versus* colloidal fluid resuscitation as reviewed by Butler et al. (25). SANGUINATE animals then endured the longest, which indicates the presence of an additional effect on top of its oncotic properties. SANGUINATE impact on PP and MAP occurred without appreciable impact on HR. That suggests a possible increase in cardiac output (but this was not directly measured). Further study of SANGUINATE impact on myocardial function is indicated.

The PHS model is a lethal protocol due to prolonged ischemia during the hemorrhagic shock phase. Post-resuscitation $P_{ISF}O_2$ was significantly elevated in SANGUINATE-treated animals at the 2-h mark, which likely contributed to increased survival times for SANGUINATE-treated animals. Previous experience with introducing SANGUINATE into a hemorrhaged and acutely ischemic animal shows a quick $P_{ISF}O_2$ response in peripheral tissues, as would be expected from an oxygen carrier that enhances oxygen delivery (data unpublished). The difference with the PHS model was the extent of the ischemic injury, which we speculate had two major effects on peripheral $P_{ISF}O_2$: (1) it created a substantial oxygen debt, which increased oxygen demand, and (2) it damaged oxidative machinery, which reduced oxygen debt repayment efficiency.

The oxygen debt is classically described as the cumulative burden of the imbalance in oxygen delivery to oxygen demand. It is a drive to replenish cellular stores of adenosine triphosphate (ATP) and phosphocreatine (for review, see (7)). Replenishing ATP after a prolonged period of hypoxia in skeletal muscle requires a functioning tricarboxylic acid cycle and mitochondrial electron transport chains as well as oxygen. Following both ischemic and reperfusion injury cellular mitochondria are damaged, thus slowing down ATP generation (26) and protracting repayment of the oxygen debt. The dysfunctional machinery still binds oxygen, but much of it is decoupled from ATP synthesis or shunted to reactive oxygen species production (27). Thus, $P_{ISF}O_2$ could remain in hypoxic ranges during resuscitation, since although oxygen is still being processed, the inefficiency keeps oxygen demand higher than delivery. We can speculate that reperfusion with Hextend may have produced a non-detectable increase in oxygen delivery to peripheral tissues by mass effect of higher MAP on microvascular perfusion, but only SANGUINATE impact on $P_{ISF}O_2$ was detectable, which is consistent with its oxygen transfer properties. Further studies including clearance of blood lactate may better determine SANGUINATE effect on systemic oxygen debt. Additionally, characterizing reperfusion injury in the context of resuscitation after PHS may help further delineate different oxygen resuscitant formulations.

Shunting of blood to critical organs provides another mechanism to mask minor increases in oxygen delivery from $P_{ISF}O_2$ measurements in peripheral tissues, making characterization of the immediate post-reperfusion period difficult. Although there was no statistically significant change in oxygen tension between time points immediately following the reperfusion phase (Fig. 2), SANGUINATE treated animals showed a steady increase in $P_{ISF}O_2$, which became significant by $PR_{120}$. Due to a lack of survival in LRS and Hextend groups,

comparisons *versus* SANGUINATE could not be made at the $PR_{120}$ time point. This difference in oxygen delivery to tissues is a likely candidate for the enhanced survival times seen with SANGUINATE.

### Limitations

This report describes a preclinical study of PHS, which was performed in rats to approximate, but not actuate human conditions of hemorrhagic shock resulting from trauma with protracted times to treatment. Although the study does directly examine tissue oxygenation, supporting variables such as: lactate, $ScvO_2$, total hemoglobin, methemoglobin, arterial and venous oxygen contents, and blood flow were not assessed. Additionally, this model made use of a progressive, controlled bleed *versus* an uncontrolled bleed that would be seen clinically (see Fig. 1).

## SUMMARY

Hemorrhage and its complications are the leading cause of preventable death from trauma in young adults, and survival is dependent on prompt hemorrhage control and restoration of tissue oxygenation (28–31). Ideal resuscitation scenarios occur within minutes of hemorrhage control, but extended time to definitive treatment can sometimes be unavoidable, resulting in complications. As great as the need for new resuscitants, so is the need for their evaluation in different resuscitative scenarios such as: acute and protracted hemorrhagic shock. Here we present a novel model of PHS that investigates an extreme scenario of lethal, protracted hemorrhagic shock to provide a sensitive platform for resuscitant performance in key cardiovascular and microvascular areas. The addition of $P_{ISF}O_2$ data provides a key parameter by which the extent of injury and potential for outcomes can be assessed. Tissue oxygenation is the gold standard for assessing the overall combined functioning of the cardiopulmonary system. Unfortunately, direct measurements of peripheral tissue $P_{ISF}O_2$ are currently limited to animal models due to technological constraints. The PHS rat model provides a perspective on how severe hypoxia is generated in terms of volume and rate of blood lost and how it might compare to cardiovascular variables assessed on the field and monitored in the clinic. This study is also the first assessment of SANGUINATE as a trauma resuscitant and demonstrates that it improves vital indicators of outcomes in cases of protracted hemorrhagic shock compared to standard of care. The encouraging data from this preclinical study suggest that SANGUINATE be evaluated against standard of care in larger animal models and perhaps humans for resuscitative efficacy.

## REFERENCES

1. Jurkovich GJ, Greiser WB, Luterman A, Curreri PW: Hypothermia in trauma victims: an ominous predictor of survival. *J Trauma* 27(9):1019–1024, 1987.
2. Kauvar DS, Lefering R, Wade CE: Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. *J Trauma* 60(6 suppl):S3–S11, 2006.
3. Tieu BH, Holcomb JB, Schreiber MA: Coagulopathy: its pathophysiology and treatment in the injured patient. *World J Surg* 31(5):1055–1064, 2007.
4. Crandall M, Sharp D, Unger E, Straus D, Brasel K, Hsia R, Esposito T: Trauma deserts: distance from a trauma center, transport times, and mortality from gunshot wounds in Chicago. *Am J Public Health* 103(6):1103–1109, 2013.
5. Esposito TJ, Maier RV, Rivara FP, Pilcher S, Griffith J, Lazear S, Hogan S: The impact of variation in trauma care times: urban versus rural. *Prehosp Disaster Med* 10(3):161–166, 1995. discussion 166-7.
6. Kotwal RS, Howard JT, Orman JA, Tarpey BW, Bailey JA, Champion HR, Mabry RL, Holcomb JB, Gross KR: The effect of a golden hour policy on the morbidity and mortality of combat casualties. *JAMA Surg* 151(1):15–24, 2016.
7. Bjerkvig CK, Strandenes G, Eliassen HS, Spinella PC, Fosse TK, Cap AP, Ward KR: ''Blood failure'' time to view blood as an organ: how oxygen debt contributes to blood failure and its implications for remote damage control resuscitation. *Transfusion* 56(suppl 2):S182–S189, 2016.
8. Joint Program Committee 6. Combat Casualty Care Research Program: $47 Million For Prolonged Field Care. ProlongedFieldCare.org, 2017. Available at: http://prolongedfieldcare.org/2016/04/08/47-million-for-prolonged-field-care/.
9. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM: Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA* 299(19):2304–2312, 2008.
10. Ananthakrishnan R, Li Q, O'Shea KM, Quadri N, Wang L, Abuchowski A, Schmidt AM, Ramasamy R: Carbon monoxide form of PEGylated hemoglobin protects myocardium against ischemia/reperfusion injury in diabetic and normal mice. *Artif Cells Nanomed Biotechnol* 41(6):428–436, 2013.
11. Klaus JA, Kibler KK, Abuchowski A, Koehler RC: Early treatment of transient focal cerebral ischemia with bovine PEGylated carboxy hemoglobin transfusion. *Artif Cells Blood Substit Immobil Biotechnol* 38(5):223–229, 2010.
12. Abuchowski A: PEGylated bovine carboxyhemoglobin (SANGUINATE): results of clinical safety testing and use in patients. *Adv Exp Med Biol* 876:461–467, 2016.
13. Gray SD: Rat spinotrapezius muscle preparation for microscopic observation of the terminal vascular bed. *Microvasc Res* 5(3):395–400, 1973.
14. Golub AS, Pittman RN: Thermostatic animal platform for intravital microscopy of thin tissues. *Microvasc Res* 66(3):213–217, 2003.
15. Nugent WH, Song BK, Pittman RN, Golub AS: Simultaneous sampling of tissue oxygenation and oxygen consumption in skeletal muscle. *Microvasc Res* 105:15–22, 2016.
16. Rumsey WL, Vanderkooi JM, Wilson DF: Imaging of phosphorescence: a novel method for measuring oxygen distribution in perfused tissue. *Science* 241(4873):1649–1651, 1988.
17. Vanderkooi JM, Maniara G, Green TJ, Wilson DF: An optical method for measurement of dioxygen concentration based upon quenching of phosphorescence. *J Biol Chem* 262(12):5476–5482, 1987.
18. Song BK, Nugent WH, Moon-Massat PF, Auker CR, McCarron R, Pittman RN: Effects of top-loading a zero-link bovine hemoglobin, OxyVita, on systemic and microcirculatory variables. *Mil Med* 178(5):570–577, 2013.
19. Song BK, Nugent WH, Moon-Massat PF, Pittman RN: Effects of a hemoglobin-based oxygen carrier (HBOC-201) and derivatives with altered oxygen affinity and viscosity on systemic and microcirculatory variables in a top-load rat model. *Microvasc Res* 95:124–130, 2014.
20. Golub AS, Popel AS, Zheng L, Pittman RN: Analysis of phosphorescence in heterogeneous systems using distributions of quencher concentration. *Biophys J* 73(1):452–465, 1997.
21. Golub AS, Pittman RN: Barometric calibration of a luminescent oxygen probe. *J Appl Physiol (1985)* 120(7):809–816, 2016.
22. Lee HB, Blaufox MD: Blood volume in the rat. *J Nucl Med* 26(1):72–76, 1985.
23. Dutton RP, Mackenzie CF, Scalea TM: Hypotensive resuscitation during active hemorrhage: impact on in-hospital mortality. *J Trauma* 52(6):1141–1146, 2002.
24. Morrison CA, Carrick MM, Norman MA, Scott BG, Welsh FJ, Tsai P, Liscum KR, Wall MJ Jr, Mattox KL: Hypotensive resuscitation strategy reduces transfusion requirements and severe postoperative coagulopathy in trauma patients with hemorrhagic shock: preliminary results of a randomized controlled trial. *J Trauma* 70(3):652–663, 2011.
25. Butler FK, Holcomb JB, Schreiber MA, Kotwal RS, Jenkins DA, Champion HR, Bowling F, Cap AP, Dubose JJ, Dorlac WC, et al.: Fluid resuscitation for hemorrhagic shock in tactical combat casualty care: TCCC guidelines change 14-01—2 June 2014. *J Spec Oper Med* 14(3):13–38, 2014.
26. Sato A, Kataoka M, Kuwabara Y, Kimura M, Seo Y, Masaoka A: Ischemic injury of the small intestine studied by 31P-MRS. *J Surg Res* 61(2):373–378, 1996.
27. Baudry N, Laemmel E, Vicaut E: In vivo reactive oxygen species production induced by ischemia in muscle arterioles of mice: involvement of xanthine

oxidase and mitochondria. *Am J Physiol Heart Circ Physiol* 294(2):H821–H828, 2008.

28. Carson JL, Noveck H, Berlin JA, Gould SA: Mortality and morbidity in patients with very low postoperative Hb levels who decline blood transfusion. *Transfusion* 42(7):812–818, 2002.

29. Eastridge BJ, Mabry RL, Seguin P, Cantrell J, Tops T, Uribe P, Mallett O, Zubko T, Oetjen-Gerdes L, Rasmussen TE, et al.: Death on the battlefield (2001–2011): implications for the future of combat casualty care. *J Trauma Acute Care Surg* 73(6 suppl 5):S431–S437, 2012.

30. Holcomb J, Caruso J, McMullin N, Wade CE, Pearse L, Oetjen-Gerdes L, Champion HR, Lawnick M, Farr W, Rodriguez S, et al.: Causes of death in US Special Operations Forces in the global war on terrorism: 2001–2004. *US Army Med Dep J*; 2007;24–37, 2007.

31. Kelly JF, Ritenour AE, McLaughlin DF, Bagg KA, Apodaca AN, Mallak CT, Pearse L, Lawnick MM, Champion HR, Wade CE, et al.: Injury severity and causes of death from Operation Iraqi Freedom and Operation Enduring Freedom: 2003–2004 versus 2006. *J Trauma* 64(2 suppl):S21–S26, 2008. discussion S26-7.