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TECHNICAL REPORT

Diagnostic and Treatment Innovations for Mass Casualties

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14. ABSTRACT
It was proposed to define the very early molecular response to soft tissue and vascular injury. That information was applied to develop innovative diagnostic technologies and targeted pharmacologic and genetic therapies. The research strategy consisted of two main goals: 1) quantify the very early inflammatory response to cardiovascular/soft tissue trauma to define an injury severity predictive panel of biomarkers to be incorporated into a sensor and 2) design and test novel targeted therapeutic approaches using pharmacologic and gene delivery systems to accelerate and/or stabilize reparative processes leading to greater injury survivability. Experiments were designed to understand of the role of ubiquitin in trauma by testing ubiquitin as a novel protein therapeutic for the treatment of severe injuries and to further develop an automatic hemostatic vector for treatment of hemorrhage in combat injuries.

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inflammation; trauma; animal models; pharmacologic delivery system; gene delivery system

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UNIT CONVERSION TABLE

U.S. customary units to and from international units of measurement*

U.S. Customary Units	Multiply by → / ← Divide by†		International Units
Length/Area/Volume			
inch (in)	2.54	$\times 10^{-2}$	meter (m)
foot (ft)	3.048	$\times 10^{-1}$	meter (m)
yard (yd)	9.144	$\times 10^{-1}$	meter (m)
mile (mi, international)	1.609 344	$\times 10^{3}$	meter (m)
mile (nmi, nautical, U.S.)	1.852	$\times 10^{3}$	meter (m)
barn (b)	1	$\times 10^{-28}$	square meter (m^2)
gallon (gal, U.S. liquid)	3.785 412	$\times 10^{-3}$	cubic meter (m^3)
cubic foot (ft^3)	2.831 685	$\times 10^{-2}$	cubic meter (m^3)
Mass/Density			
pound (lb)	4.535 924	$\times 10^{-1}$	kilogram (kg)
unified atomic mass unit (amu)	1.660 539	$\times 10^{-27}$	kilogram (kg)
pound-mass per cubic foot (lb ft^{-3})	1.601 846	$\times 10^{1}$	kilogram per cubic meter (kg m^{-3})
pound-force (lbf avoirdupois)	4.448 222		newton (N)
Energy/Work/Power			
electron volt (eV)	1.602 177	$\times 10^{-19}$	joule (J)
erg	1	$\times 10^{-7}$	joule (J)
kiloton (kt) (TNT equivalent)	4.184	$\times 10^{12}$	joule (J)
British thermal unit (Btu) (thermochemical)	1.054 350	$\times 10^{3}$	joule (J)
foot-pound-force (ft lbf)	1.355 818		joule (J)
calorie (cal) (thermochemical)	4.184		joule (J)
Pressure			
atmosphere (atm)	1.013 250	$\times 10^{5}$	pascal (Pa)
pound force per square inch (psi)	6.984 757	$\times 10^{3}$	pascal (Pa)
Temperature			
degree Fahrenheit (^oF)	$[T(^oF) - 32]/1.8$		degree Celsius (^oC)
degree Fahrenheit (^oF)	$[T(^oF) + 459.67]/1.8$		kelvin (K)
Radiation			
curie (Ci) [activity of radionuclides]	3.7	$\times 10^{10}$	per second (s^{-1}) [becquerel (Bq)]
roentgen (R) [air exposure]	2.579 760	$\times 10^{-4}$	coulomb per kilogram (C kg^{-1})
rad [absorbed dose]	1	$\times 10^{-2}$	joule per kilogram (J kg^{-1}) [gray (Gy)]
rem [equivalent and effective dose]	1	$\times 10^{-2}$	joule per kilogram (J kg^{-1}) [sievert (Sv)]

*Specific details regarding the implementation of SI units may be viewed at http://www.bipm.org/en/si/.

†Multiply the U.S. customary unit by the factor to get the international unit. Divide the international unit by the factor to get the U.S. customary unit.

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Diagnostic and Treatment Innovations for Mass Casualties

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Introduction:

Improvised and conventional weapons cause modern battlefield and mass civilian trauma. Both types of weapons cause blast and thermal injuries, but improvised weapons are designed to spew large amounts of shrapnel that result in peripheral blunt and multiple puncture wound injuries to the cardiovascular system and peripheral soft tissues. These injuries induce both local and systemic inflammatory responses leading to organ dysfunction remote from the sites of injury that can ultimately affect prognosis. However, the initial severity of peripheral blunt and multiple puncture wound injuries is often difficult to ascertain and thus often underestimated. The primary objective of this project was to develop a device consisting of a surface acoustic wave (SAW) sensor mated to a biofluid collection system to provide on-site injury severity assessment and triage. In order to achieve this objective, a robust analysis of the very early molecular response to these peripheral blunt and multiple puncture wound injuries was required to define a panel of biomarkers that correlate and ultimately predict injury severity. This panel of biomarkers would then be incorporated into the sensor. An additional objective was the design of a system to initiate wound stabilization and treatment in the field. The early humoral response to injury was measured and a potential set of biomarkers released into the plasma in murine models simulating blunt and multiple puncture wound injuries, confirmed in porcine models, and ultimately clinical studies, of trauma of varying complexity and severity was indentified. A prototype SAW sensor and biofluid collection systems was developed and validation studies begun. Pharmacologic and gene delivery development programs were initiated. The work funded by DTRA continued and extended the biomarker identification and validation studies and the pharmacologic and gene delivery therapeutic discovery programs.

This project was an extension of work conducted from 2006-2011, for triage of peripheral blunt and multiple puncture wound injuries from battlefield trauma. The main hypothesis to be tested was that a systematic assessment of candidate biomarkers would be able to define a molecular pattern to discriminate various degrees of trauma severity and outcome during the pre-hospital phase. The overall objectives were to: identify a panel of biomarkers that can be incorporated into a point-of-care device and identify novel therapeutic strategies and delivery systems that can improve battlefield and mass casualty trauma care. Rapid detection of the severity of cardiovascular/soft tissue trauma will lead to more effective triage/treatment and ultimately better survivability amongst those who sustain these types of injuries. This was accomplished by the following goals of this multi-year project: 1) Determine, quantify, and model the release kinetics of the very early immunologic and inflammatory responses to cardiovascular/soft tissue trauma employing murine trauma studies in order to define an injury severity predictive panel on biomarkers that can be incorporated into a sensor Biofluid collection system currently under development. 2) Design and test novel therapeutic approaches, including pharmacologic and gene delivery systems to accelerate stabilization/reparative processes.

Task 1: Quantify and model the release kinetics of the very early immunologic and inflammatory response to cardiovascular/soft tissue trauma employing murine trauma studies in order to obtain novel insights into early genetic, humoral, and cellular regulation of acute responses to injury in order to define an injury severity predictive panel of biomarkers that can be incorporated into a sensor.

Task 2: Design and test novel therapeutic approaches using targeted pharmacologic and gene delivery systems in rodents and swine to accelerate and/or stabilize reparative processes leading to greater injury survivability.

Background and Significance:

The accurate initial assessment of the severity of injury is a pivotal prerequisite to optimize available resources and provide adequate care to the trauma victim. Thus, a diagnostic tool that improves the initial triage at the scene of injury will ultimately improve survival and reduce trauma related disabilities resulting from both battlefield-related injuries and civilian sector mass trauma injuries.

On the battlefield, blunt trauma is often accompanied by multiple penetrating injuries, compounded by cavitation, thermal, acceleration, and barotrauma (pressure) effects. Battlefield trauma occurs under extreme conditions, such as hostile fire and lack of medical equipment and supplies. Finally, soldiers with minimal medical training typically provide initial triage and emergency aid. Civilian trauma victims share many of the features of battlefield injuries, especially those related to mass trauma due to high-energy explosives. The initial triage is difficult because even life threatening injuries are not obviously apparent and their diagnosis requires a detailed emergency workup. Thus, development of a device that will facilitate triage on the battlefield will also have civilian applications.

The body's response to injury is relatively independent of the type of injury, but demonstrates variation among individuals. This response involves a complex interaction of genomic, proteomic, cellular, organ, and ultimately systemic multiple organ responses [1-3]. This interaction can induce the systemic inflammatory response syndrome (SIRS) that in some individuals can progress to the multiple organ dysfunction syndrome (MODS) involving organs often remote from the site of trauma [4]. The genomic and proteomic cascades leading to MODS and why it occurs in some individuals despite equivalence in severity of injury is a subject of a number of large multi-center studies. In particular, the National Institutes of Health funded Glue Grant entitled: Inflammation and the Host Response to Injury (www.gluegrant.org), (we are a consortium member) is studying the genomics and proteomics of the reparative processes after trauma, burns and sepsis, but is addressing a different time frame. They are collecting blood from human trauma victims beginning 12 hours after injury. Thus, our study is effectively the "front end" of the Glue Grant.

Identification of biomarkers that are released immediately after injury during the pre-hospital phase (0-60 min) could lead to a biologic assessment of the trauma severity. If used as a diagnostic tool in the pre-hospital setting, it could greatly improve survival probability and reduce morbidity, since severely injured trauma victims could rapidly be identified and provided with optimal care, thus reducing irreversible secondary organ damage.

During the past 15 years, multiple studies reported on trauma biomarkers (cytokines, complement factors, coagulation factors) determined at hospital admission [1-7], but only a few studies are available that investigated possible trauma biomarkers during the pre-hospital phase [8-10]. Several pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1), IL-6, IL-8, IL-12 or macrophage inflammatory protein (MIP), are either not detectable, not elevated above the normal range, or peak several hours after trauma, and therefore appear not to be suitable for this purpose. In contrast, for several anti-inflammatory cytokines or soluble cytokine receptors, (e.g. sTNF-R p55, IL-10, transforming growth factor ß or IL-1 RA), peak levels have been detected at hospital admission. Although their release kinetics during the pre-hospital phase is largely unknown, these molecules are potential candidates. Another possible candidate molecule is complement factor C3a. It was reported that C3a plasma levels are significantly higher in non-survivors than in survivors after severe blunt trauma, if determined on-scene, within a few minutes after injury [8]. Furthermore, elevated concentrations of molecules such as heat shock proteins, ubiquitin and proteasomes have recently been identified in plasma after trauma and critical illness [11-13], but their early release kinetics and predictive values are unknown.

It has become clear that a systematic assessment of possible candidate biomarkers is necessary in order to identify and define a molecular pattern that discriminates various degrees of trauma severity and outcome and that has the potential to be used as a diagnostic tool during the pre-hospital phase for civilian or battlefield trauma. In addition, the predictive panel of biomarkers will likely include not only those that rise rapidly, but also those whose levels are undetectable early after trauma. Because circulating cytokines and other candidate biomarkers can increase from causes other than trauma (e.g. exercise, infection), the predictive values of a candidate panel will have to be tested under conditions other than trauma. Although identification of a single "ideal" biomarker is unlikely, it is expected that a biomarker panel composed of at least 3-5 biomarkers that

fulfill most of the requirements for optimal trauma biomarkers, and from which a combined score can be derived and used to improve the initial trauma triage will be defined.

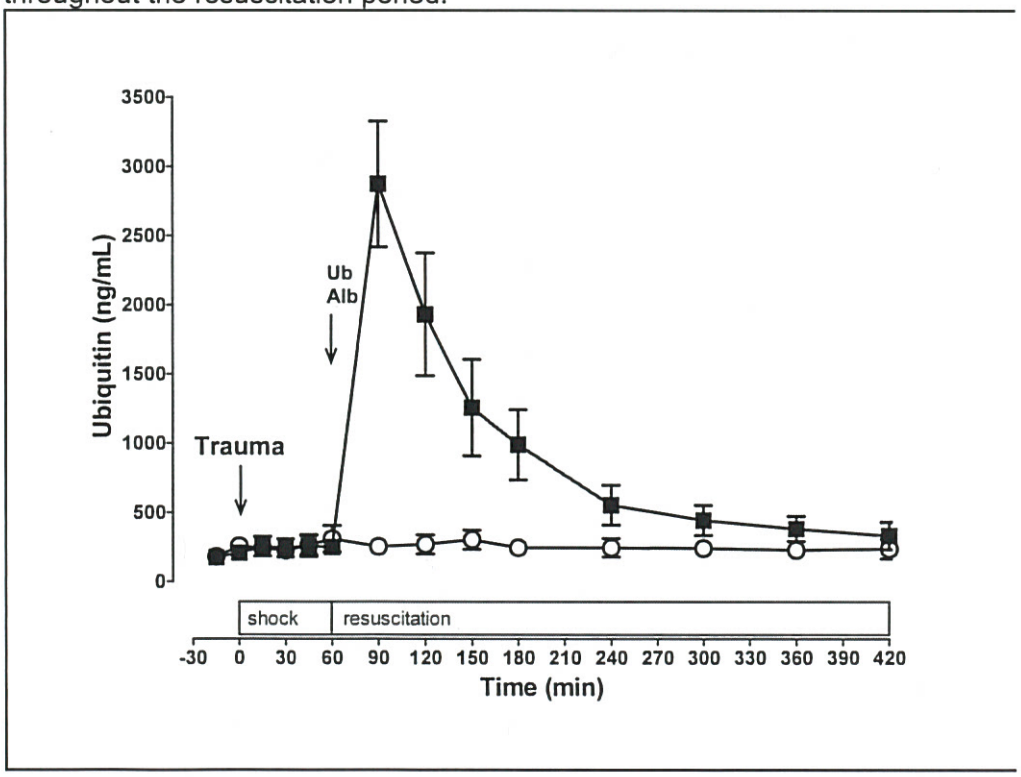
Task 1: Quantify and model the release kinetics of the very early immunologic and inflammatory response to cardiovascular/soft tissue trauma employing murine trauma studies in order to obtain novel insights into early genetic, humoral, and cellular regulation of acute responses to injury in order to define an injury severity predictive panel of biomarkers that can be incorporated into a sensor.

A. Identification of regulatory response genes and markers of inflammatory responses to cardiovascular and soft tissue injury (University of South Florida, Tampa, FL):
The mouse models of graded blunt force and multiple puncture wound injury to the hindlimbs were developed and validated.

Task 2: Design and test novel therapeutic approaches using targeted pharmacologic and gene delivery systems in rodents and swine to accelerate and/or stabilize reparative processes leading to greater injury survivability.

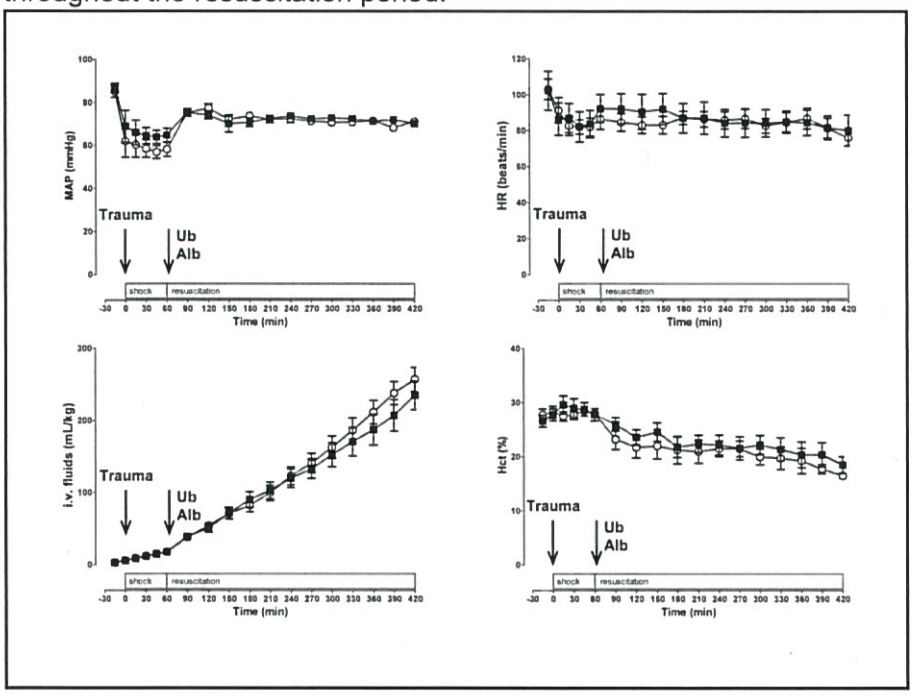
A. Test the therapeutic efficacy of intravenous (IV) ubiquitin in porcine trauma models (M Majetschak, J Romero, Loyola University Chicago, Maywood, IL):
The effects of exogenous ubiquitin were tested in a novel and clinically relevant pig polytrauma model. This model consisted of bilateral femur fractures plus blunt chest trauma. Unlike most other models, this model does not depend on an additional hemorrhage component to produce the typical physiological response to trauma. After a traumatic shock period of 1 hour (t=0-60 min), animals were treated according to human standard of care (Advanced Trauma Life Support, ATLS, guidelines). Immediately before initiation of resuscitation according to ATLS guidelines (t=60 min), animals received either 1.5 mg/kg ubiquitin (n=8) or the same dose of albumin (n=9; control protein) as an intravenous (i.v.) bolus injection (total volume: 150 ml) within 5-10 min. The overall observation and treatment period was 7 hours post trauma. One animal in the control group died at t=400 min. This animal demonstrated cardio-circulatory failure (progressive decrease in blood pressure despite fluid resuscitation). All animals in the ubiquitin group survived the observation period. The ubiquitin plasma concentrations of the animals are shown in Figure 1. Consistent with research findings in previous studies, ubiquitin plasma concentrations reached 2873 ± 454 ng/ml at t=90 min and decreased with a half-life of ~60 min.

Figure 1: Ubiquitin plasma concentrations. Arrows indicate time points of trauma (femur fractures plus blunt chest trauma) and drug administration (closed squares: Ubiquitin; open circles: Albumin). Data are mean ± SEM. Ubiquitin was measured by ELISA. Shock period: no intervention except ventilation with FiO_2 of 0.21 and positive end-expiratory pressure (PEEP) 0 cm H_2O. Resuscitation: Fluid resuscitation according to ATLS guidelines. From t=60-120 min and t=120-420 min, FiO_2 was 1 and 0.4, respectively. PEEP was 5 cm H_2O throughout the resuscitation period.



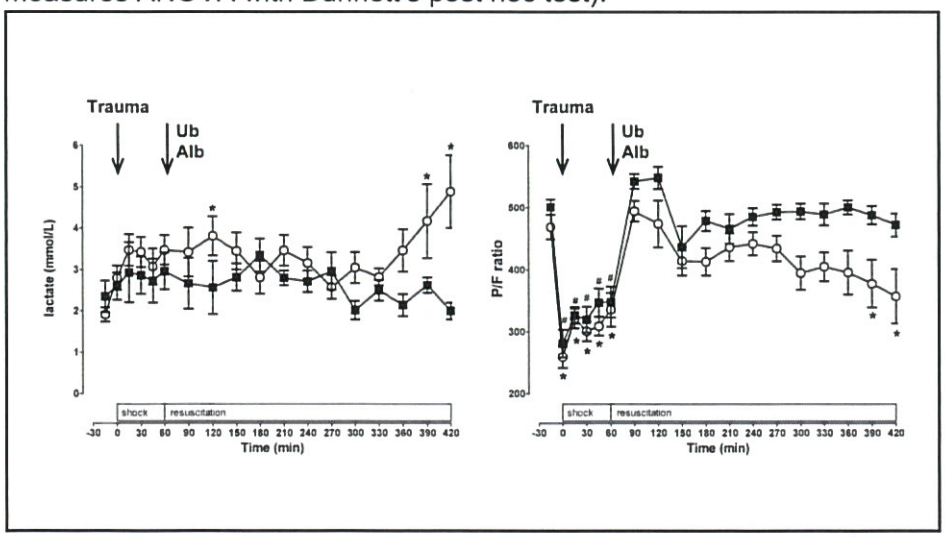
All animals showed a decrease in mean arterial blood pressure during the shock phase (Figure 2). This was accompanied by a decrease in heart rate, which was interpreted to correspond to cardiac contusion after blunt chest trauma. However, none of the animals developed cardiac arrhythmia during the experimental procedure. With fluid resuscitation, mean arterial blood pressure could be maintained at the resuscitation target value of 70 mmHg. Cumulative i.v. fluid requirements and hematocrit values were comparable in both groups.

Figure 2: Mean arterial blood pressure (MAP), heart rate (HR), i.v. fluid requirements and hematocrit (Hct). Arrows indicate time points of trauma (femur fractures plus blunt chest trauma) and drug administration (closed squares: Ubiquitin; open circles: Albumin). Data are mean ± SEM. Shock period: no intervention except ventilation with FiO_2 of 0.21 and Peep 0 cm H_2O. Resuscitation: Fluid resuscitation according to ATLS guidelines. From t=60-120 min and t=120-420 min, FiO_2 was 1 and 0.4, respectively. PEEP was 5 cm H_2O throughout the resuscitation period.



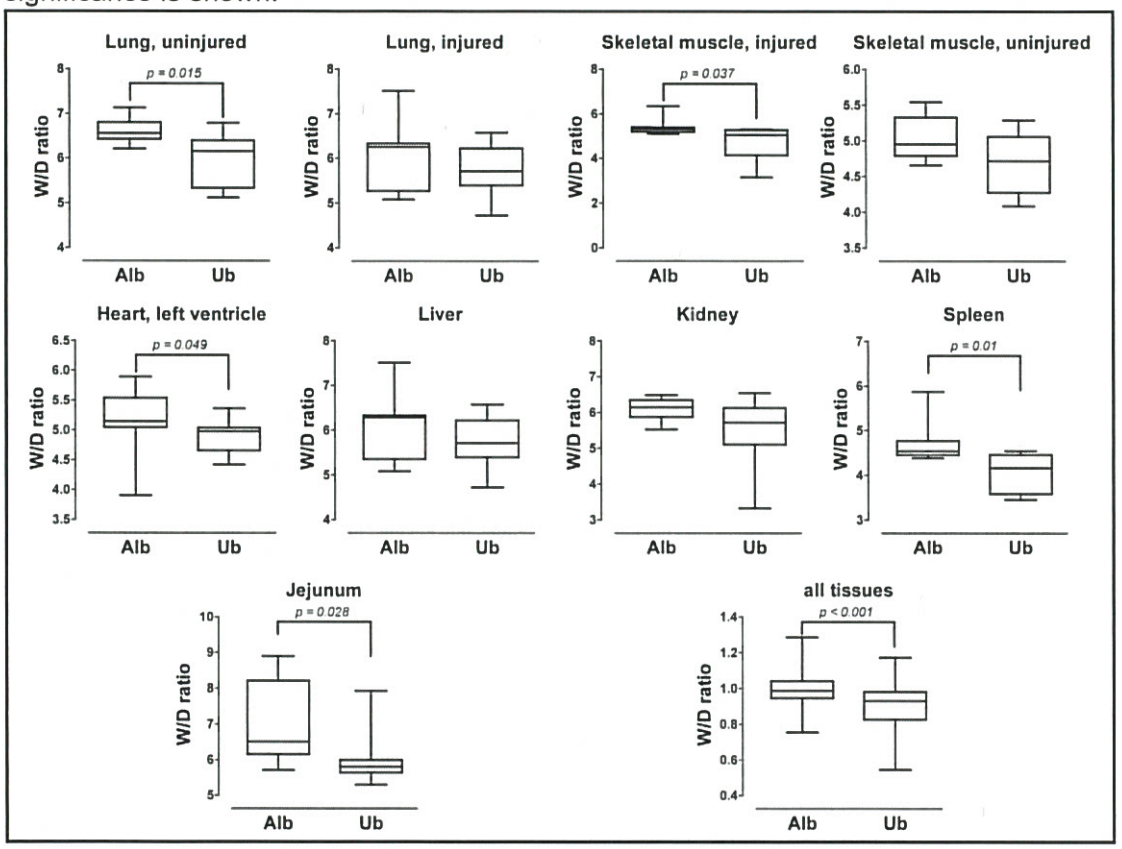
With ubiquitin treatment, lactate levels normalized during resuscitation, whereas lactate concentrations increased significantly in the control group at the end of the observation period (Figure 3, left). In addition, animals in the control group showed a progressive decrease in P/F ratios during resuscitation, which was prevented with ubiquitin (Figure 3, right panel).

Figure 3: Lactate levels and P/F ratios. Arrows indicate time points of trauma (femur fractures plus blunt chest trauma) and drug administration (closed squares: Ubiquitin; open circles: Albumin). Data are mean ± SEM. Shock period: no intervention except ventilation with FiO_2 of 0.21 and PEEP 0 cm H_2O. Resuscitation: Fluid resuscitation according to ATLS guidelines. From t=60-120 min and t=120-420 min, FiO_2 was 1 and 0.4, respectively. PEEP was 5 cm H_2O throughout the resuscitation period. *: Albumin group; $p<0.05$ vs. baseline (repeated measures ANOVA with Dunnett's post hoc test). #: Ubiquitin group, $p<0.05$ vs. baseline (repeated measures ANOVA with Dunnett's post hoc test).



At the end of the experiment, wet-weight dry-weight (W/D) ratios were lower with ubiquitin in all tissues that were analyzed: the uninjured left lung and injured right lung, uninjured and injured skeletal muscle, heart, liver, kidney, spleen, jejunum (Figure 4). These differences reached statistical significance for uninjured lung, injured skeletal muscle, heart, spleen and jejunum, and for the overall effect of ubiquitin in all tissues (Figure 4, bottom row, right panel; W/D ratios normalized to 1 for each individual tissue).

Figure 4: Wet-weight dry-weight (W/D) ratios in various tissues. W/D ratios were determined at t=420 min. Alb: Albumin group. Ub: Ubiquitin group. To assess the global effect of ubiquitin on W/D ratios in all tissues, the W/D ratios in individual organs from animals of the control group were normalized to 1 and the relative change with ubiquitin treatment calculated (bottom row, right panel). Boxes extend from the 25th to 75th percentile. The horizontal line shows the median. Error bars show the minimum/maximum. The level of statistical significance is shown.



Taken together, these data showed that ubiquitin preserved lung function, reduced organ edema formation and reduced metabolic consequences after severe blunt trauma in a clinically relevant large animal model of polytrauma.

The investigators already provided proof of ubiquitin's therapeutic efficacy after two important types of injury (extremity and brain trauma, with/without hemorrhage). In addition, the current study demonstrates, for the first time, clinically relevant treatment effects of ubiquitin after polytrauma with lung contusion. Therefore, these data indicate that ubiquitin could be administered after severe trauma in general and provide a strong rationale to develop the use of ubiquitin-supplemented resuscitation fluids as a new standard of care on the battlefield and in the civilian arena.

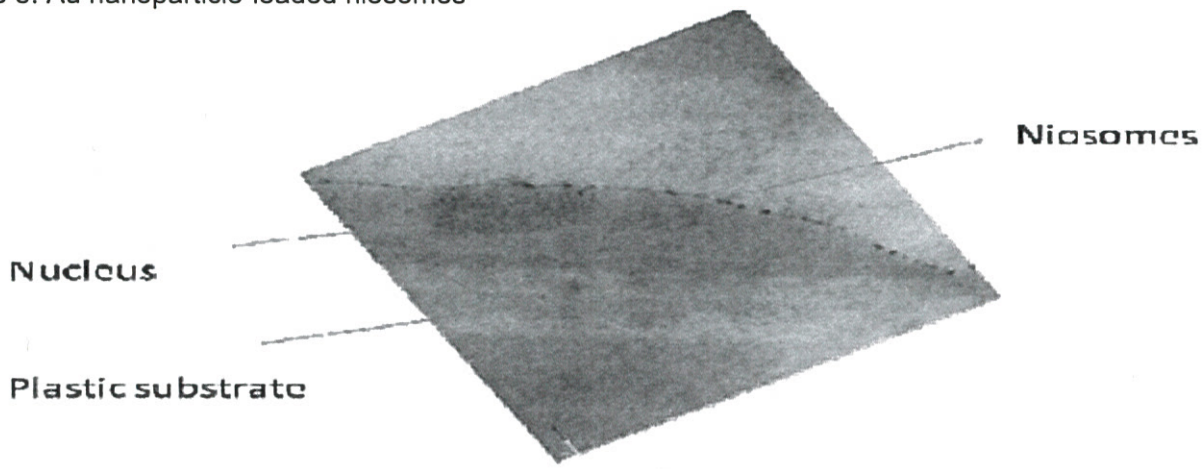
The tissues from the described study were analyzed for additional markers of organ injury and inflammation, stimulating leukocytes to determine ubiquitin's effects on immune functions and also analyzing differential blood counts for possible effects of ubiquitin. The manuscript is in preparation.

Task 2: Design and test novel therapeutic approaches using targeted pharmacologic and gene delivery systems in rodents and swine to accelerate and/or stabilize reparative processes leading to greater injury survivability.

B. Immuno-niosomes targeted to inflammation (JA Elliott, BR Flam, University of South Florida, Tampa, FL):

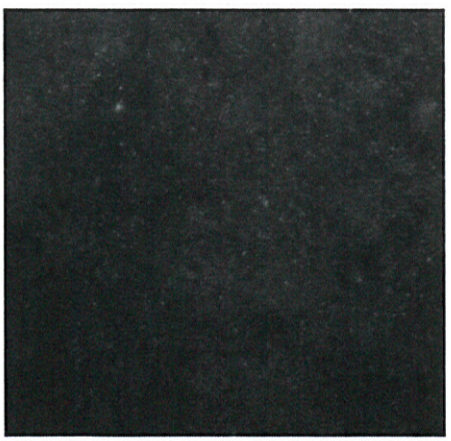
Progress was made in the development of the immuno-niosomes of nanometer dimension and validation of their capacity to attach to inflammatory unregulated receptors, specifically CD44, on the cell surface. Static binding to inflammatory receptors on fixed bovine aortic endothelial cells (BAECs) was demonstrated using ~200 nm gold (Au) nanoparticle-loaded CD44 conjugated immuno-niosomes. The BAECs were treated with 10 ng/ml TNF-α, a cytokine, to cause expression of the CD44 inflammatory receptor on the cell surface. Transmission electron microscopy (TEM) was used to image the Au nanoparticle-loaded CD44 conjugated immuno-niosomes (INs) attached to BAEC grown on plastic cover slips (Figure 5).

Figure 5: Au nanoparticle-loaded niosomes



From the TEM results it was noted that the cell surface was nearly saturated with Au nanoparticle-loaded CD44 conjugated INs. Verification of the binding density of INs to BAEC under static conditions was performed using large 450 nm diameter, 5 mM carboxyfluorescein fluorescent dye-loaded CD44 INs. Time-dependent and concentration-dependent experiments verified attachment efficiency was well above previous determinations. The time-dependent experiments using 30, 60 and 120 minute attachment times showed saturation at the shortest time point (30 minutes) using a IN concentration of 5 mM total lipid. The large 450 nm niosome provided a more intense fluorescent signal compared to 200 nm niosomes, based on the increase in the volume of fluorescent dye. The BAEC nuclei were stained with the nuclear stain, 4',6-diamidino-2-phenylindole (DAPI) shown in blue and bound groups of the carboxyfluorescein-loaded CD44 INs were visualized as green (Figure 6).

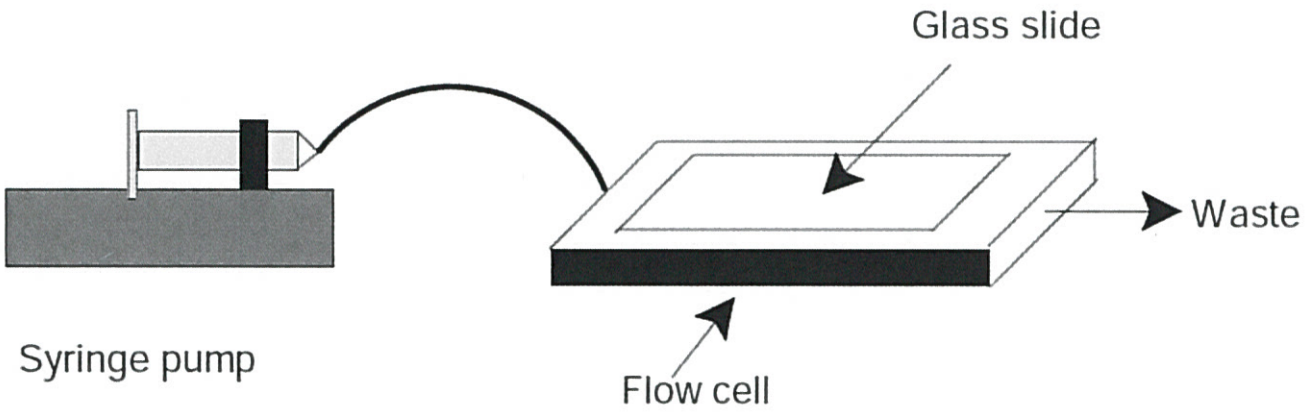
Figure 6: Carboxyfluorescein-loaded CD44 INs bound to BAECs under static conditions (30 minutes)



Binding in flow with fixed cells

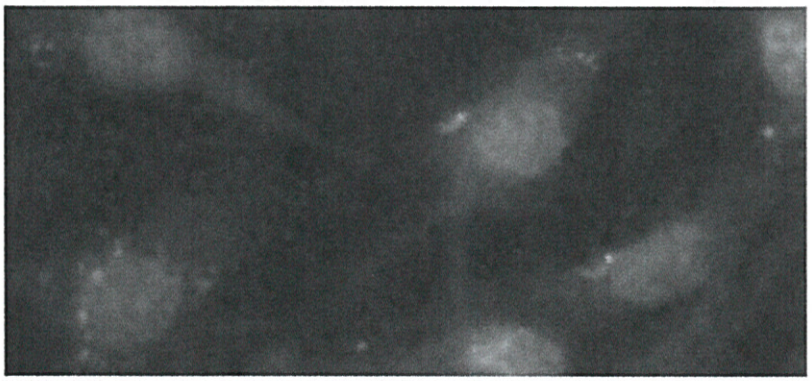
BAEC, grown on glass slides, were treated with 10 ng/ml of TNF-α for 24 hours to cause inflammatory CD44 cell surface expression. BAEC were chemically fixed with glutaraldehyde. The glass slide was placed on top of the flow chamber cell side down and connected to a syringe pump with a 60 cc syringe filled with 0.01M phosphate buffered saline solution (PBS) (Figure 7).

Figure 7: Experimental layout



The carboxyfluorescein-loaded CD44 INs were injected in a 3 ml bolus injection after 10 ml of PBS had flowed through the system allowing the remaining 50 ml of PBS to elute behind the IN sample washing any unbound niosomes into waste. The image of the INs binding to BAEC is shown in Figure 8.

Figure 8: Carboxyfluorescein-loaded CD44 INs (yellow dots) bound to BAECs under flow conditions



Niosome surface enhancement

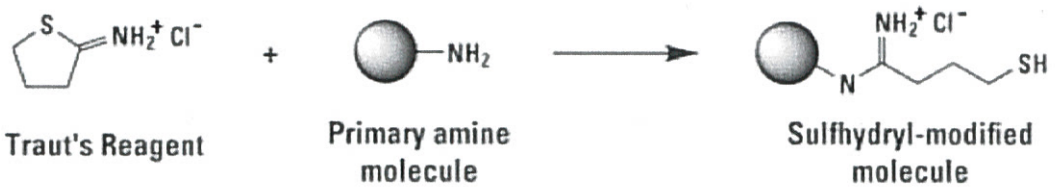
Polyethylene glycol (PEG) is a family of polymers made from repeating chains of ethylene oxide with a molecular weight below 20,000 grams per mole. Including reactive groups at one or both of the terminal ends of PEG can functionalize the non-toxic, hydrophilic polymer. It was first covalently attached to proteins by Abuchowski et al. in 1977 [14] and was shown to have an effect on immunogenicity and circulation life. Modification of the liposomal lipid membrane with PEG was initiated by the work of Blume and Cevc [15], Klibanov et al. [16], Allen and colleagues [17, 18], Lasic and Martin [19] and Torchilin et al. [20]. The PEGylated liposome design using cyanuric chloride (CC) as a linker molecule for antibody attachment improved in vitro binding to 1.8 times greater than vesicles without the PEG-linker motif [21].

Our previous niosome design used Tween surfactant with the linker molecule CC attached to the head group to provide an attachment site for antibodies [22]. The PEG chain provided improved binding performance in flow and added steric stability to the vesicle as previously shown for liposomes [18-21].

Antibody thiolation

Traut's Reagent (2-iminothiolane) was used to increase the number of available sulfhydryl groups for antibody-niosome conjugation. Traut's reagent reacted with primary amines and introduced sulfhydryl groups (thiolation) to those amines and maintained the antibody charge properties. Maintenance of the antibody charge was necessary for activity of the thiolated antibody-niosome. When Traut's reagent reacted with the primary amine(s) on the antibody, a spacer arm with free sulfhydryl group was introduced (Figure 9).

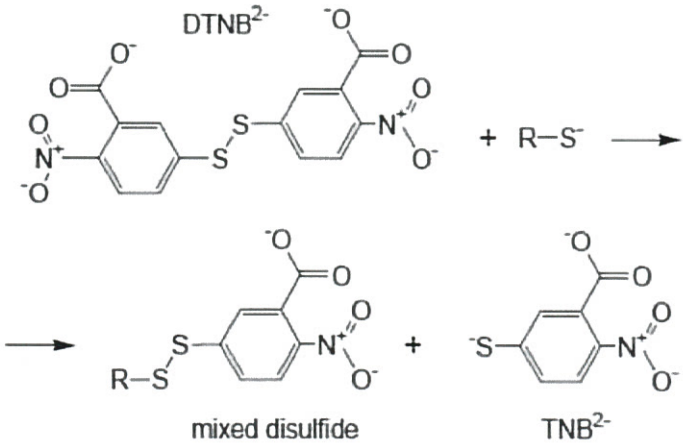
Figure 9: Reaction scheme of Traut's Reagent antibody (primary amine-containing molecule); (Thermo Fisher Scientific Inc.; http://www.piercenet.com/browse.cfm?fldID=02040121)



Quantitation of antibody thiolation

Ellman's reagent (5,5'-dithiobis(2-nitrobenzoate); DTNB) was used to quantitate thiolation of the antibody. Ellman's reagent reacts with free sulfhydryl groups in aqueous solution at neutral pH to produce a yellow product measurable at 412 nm (Figure 10). Cysteine hydrochloride, a sulfhydryl containing amino acid, was used to create a standard curve. Antibody containing samples and cysteine hydrochloride standards were mixed with Ellman's reagent in 0.1 M sodium phosphate, 1 mM EDTA, pH 8 (reaction buffer), incubated at room temperature for 15 min and absorbance was measured at 412 nm according to the manufacturer's instructions (Thermo Fisher Scientific manual 0311.2).

Figure 10: Reduction of Ellman's reagent; (Thermo Fisher Scientific Inc.; http://www.piercenet.com/browse.cfm?fldID=02040121)



Task 2: Design and test novel therapeutic approaches using targeted pharmacologic and gene delivery systems in rodents and swine to accelerate and/or stabilize reparative processes leading to greater injury survivability.

C. Vigilant vector hemostat (Keck Graduate Institute, Claremont, CA):
Goal: The goal was to make a gene delivery system that could be administered before combat duty to give soldiers 7 days protection from bleeding to death from hemorrhage in small blood vessels injury, internally or externally. The challenge was to develop a system that would start to produce Factor VII for localized coagulation within the golden hour. A system switched on by hypoxia was established to generate Factor VII in blood vessels at the site of bleeding where the oxygen levels rapidly fall. The system is named the "Vigilant Vector Hemostat" because: 1) it is not activated unless there is bleeding plus hypoxia; and 2) it causes coagulation locally with small amounts of endogenously produced Factor VII. One of the main goals was to increase the amplitude of Factor VII expression and to speed up the onset of action. Previously, it was established that there were significant increases in Factor VII gene expression within two hours after hypoxia induction (1% oxygen). The overall goal was to reduce the Factor VII gene expression time to minutes rather than hours.

A technical problem of accurately measuring the dissolved oxygen levels in the cell culture medium was encountered. The technical problem was to define exactly when the 1% oxygen threshold was reached. Hypoxia conditions were achieved using a modular incubator chamber (Billups-Rothenberg, Inc.) and by gassing repeatedly with 1% O_2 /5% CO_2 /94%N_2 for 5 minutes, tightly sealing the chamber and then incubating at 37°C for 24 hours. The time after reduction of oxygen was the start point, but data showed that it actually took 2-2.5 hours to achieve this equilibrium of a stable level of hypoxia in the medium. This meant that the time to activation of gene expression was already within the desired time range of 0-60 minutes. Therefore, another method was used to determine oxygen levels in situ. A color-coded strip was placed in each sealed modular incubator chamber. The anaerobic indicator strip was blue in normoxic conditions and turned colorless under hypoxic conditions (Figure 11). A GasPak EZ Anaerobe Pouch System (BD Diagnostic Systems) was also tested to determine if the equilbrium time of hypoxia in the experimental systems was better than the Modular Incubator Chamber.

Figure 11: A. Anaerobic indicator strip (blue) in Modular Incubator Chamber; B. GasPak EZ Anaerobe Pouch System (BD Diagnostics, www.bd.com/ds).
A.

B.

Further development of the vigilant vector hemostat:
The hemostat switch was originally constructed using the GAL4-ODD-p65 gene construct (MI Phillips and YL Tang, 2015, US patent number 9,040,676) that produced a fusion protein, which increased in concentration as oxygen levels were reduced. The fusion protein bound to an upstream binding sequence in front of the Factor VII gene. The more binding by the fusion protein, the more expression of Factor VII was achieved.

Conclusion from cell-based studies
A feasible system to reduce death from combat injuries due to hemorrhage from multiple wounds was developed. Although the vector cannot protect against massive, rapid bleeding from torn arteries, it should be able to protect smaller vessels and prevent slow bleeding to death. The concept is that the Vigilant Vector Hemostat should be particularly helpful to combatants who are wounded in a situation that requires 1 or more hours to get them treatment. One would also expect that the vector would improve recovery because the bleeding would stop sooner. In the course of this study a new gene switch for hypoxia was invented, an invention for demonstrated gene modification for Factor VII expression and a Vigilant Vector Hemostat.

In vivo testing of Vigiliant Vector Hemostat: (University of South Florida, Tampa, FL)
Using the model of blunt force and multiple puncture wound injuries to the mouse hindlimb(s) developed at the University of South Florida, testing was performed to determine if the vector reduced bleeding caused by blunt high impact injury and multiple needle injury. The high impact injury models the effects of IEDs. Multiple needle tip injury induces slow bleeding.

Working hypothesis: A drop in oxygen concentration caused by bleeding will activate the Vigilant Vector (VV) plasmid and Factor VII (FVII) will be produced directly at the site of injury.

Future plans using in vivo models
The optimal final plasmid DNA concentration for in vivo transfection should be determined to ensure that all the plasmid DNA is available to enter the cells. Plasmid DNA should be injected multiple times over the course of several days prior to injury to ensure high enough concentrations. Plasmid DNA should be injected via tail vein to increase the amount available to transfect the cells.

Task 2: Design and test novel therapeutic approaches using targeted pharmacologic and gene delivery systems in rodents and swine to accelerate and/or stabilize reparative processes leading to greater injury survivability.

D. Gene delivery for trauma-related limb ischemia (University of South Florida, Tampa, FL):

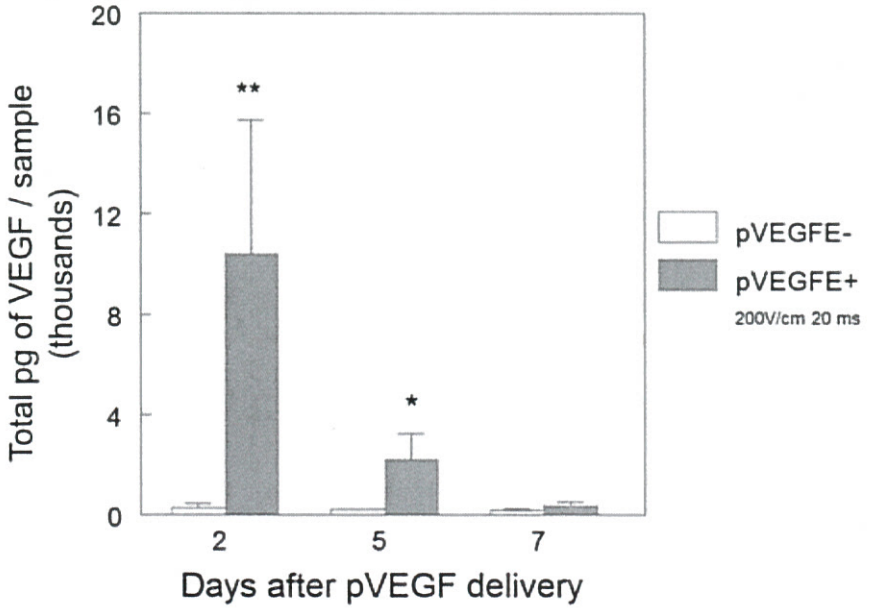
Development of the hindlimb ischemia model

Animal models of hindlimb ischemia are often used to evaluate potential therapeutic approaches for restoring limb blood flow. There are several surgical approaches in the literature for creating rodent models of hindlimb ischemia. Several of these approaches were evaluated in the rat, and the approach that achieved the largest decrease in limb perfusion, determined by Laser Doppler Imaging, was used for this study. To create this model of hindlimb ischemia, the saphenous artery was ligated distally of the bifurcation of the femoral artery into the saphenous and popliteal arteries. Next, the femoral artery was ligated adjacent to the inguinal ligament and proximally and distally to the bifurcation of the superficial epigastric artery and vein. The superficial epigastric artery and vein were also ligated at two sites and cut between these sites. The femoral artery was then cut between the ligations adjacent to the inguinal ligament and proximal to the superficial epigastric artery and vein. Finally, the femoral artery was dissected free from the point of the distal ligation of the saphenous artery to the ligation distal to the bifurcation of the superficial artery and vein. This model of hindlimb ischemia successfully decreased postoperative perfusion to 40% of the preoperative, or baseline, perfusion.

Delivery of plasmid vascular endothelial growth factor (VEGF)

The original proposal was to evaluate if delivery of a plasmid encoding VEGF (pVEGF) to the skin of the ischemic hindlimb by in vivo electroporation (EP) using the conformable electrode applicator would increase limb perfusion. VEGF expression, mRNA stability and translation are increased under hypoxic conditions and decreased under normoxic conditions. Despite the substantial decrease in perfusion achieved in the hindlimb ischemia model, indicating the muscles of the limb were effectively rendered ischemic, oxygen levels in the skin of the ischemic limb did not sufficiently decrease to allow for efficient VEGF expression. Other studies have reported the oxygen level in ischemic skin is equivalent to the oxygen level in non-ischemic muscle. To confirm that the low level of VEGF expression observed in the skin after delivery of pVEGF was not due to the specific plasmid utilized in this study, pVEGF was delivered to the gastrocnemius muscle by injection alone (pVEGFE-) and injection with EP (pVEGFE+) (200 V/cm, 20 ms, 8 pulses) using a previously characterized four needle electrode. There was a significant increase in VEGF expression with pVEGFE+ compared to pVEGFE- in the gastrocnemius muscle for five days after delivery (Figure 12). These results confirmed the hypothesis that the low levels of VEGF expression observed when delivering pVEGF to the skin was due to decreased VEGF stability resulting from normal oxygen levels in the skin.

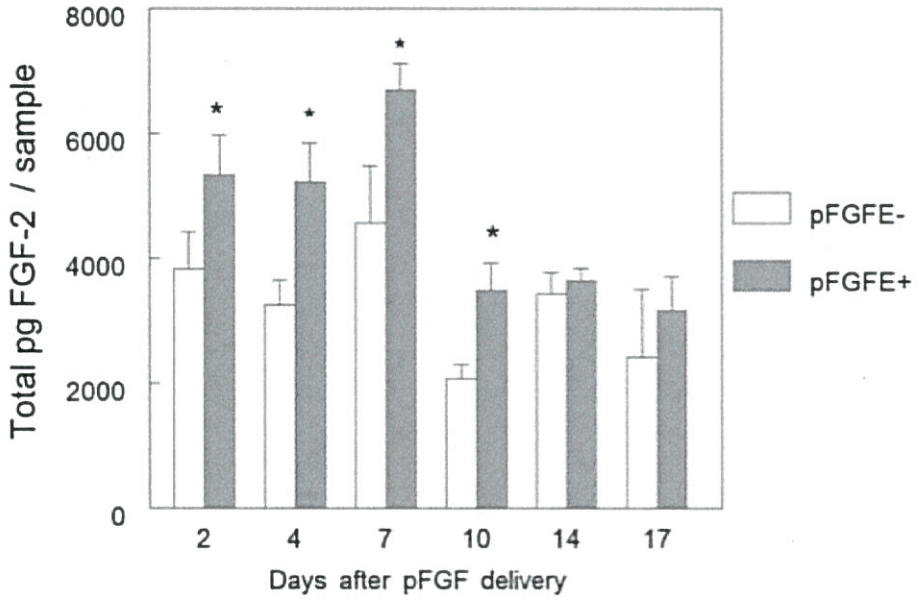
Figure 12: pVEGFE+ significantly increased VEGF expression in the gastrocnemius muscle for 5 days after delivery compared to pVEGFE- (** $p<0.01$, * $p<0.05$)



Delivery of plasmid fibroblast growth factor-2 (FGF-2)

FGF-2 increases neovascularization by regulating angiogenic pathways similar to those regulated by VEGF, but oxygen levels do not regulate the expression and stability of FGF-2. Delivery of FGF-2 to ischemic limbs by other gene therapy approaches has successfully increased limb perfusion in both animal models and clinical trials. For these reasons experiments were designed to evaluate if electrically-mediated intradermal delivery of plasmid FGF-2 (pFGF) with the conformable electrode (pFGFE+) could significantly increase FGF-2 expression compared to injection of pFGF alone (pFGFE-) and if the resulting increase in FGF-2 would increase perfusion in the rat model of hindlimb ischemia. FGF-2 expression kinetics resulting from pFGFE+ and pFGFE- were first determined in the skin of the rat flank. pFGFE+, using the delivery conditions of 300 V/cm and 150 ms pulses, significantly increased FGF expression for ten days after delivery (Figure 13). The skin in the rat hindlimb is much thinner than the skin of the rat flank and pFGF was delivered to the hindlimb ischemia model at 250 V/cm, 150 ms pulses. These conditions resulted in equivalent levels of expression and the expression kinetics as observed when delivering pFGF to the skin of the flank.

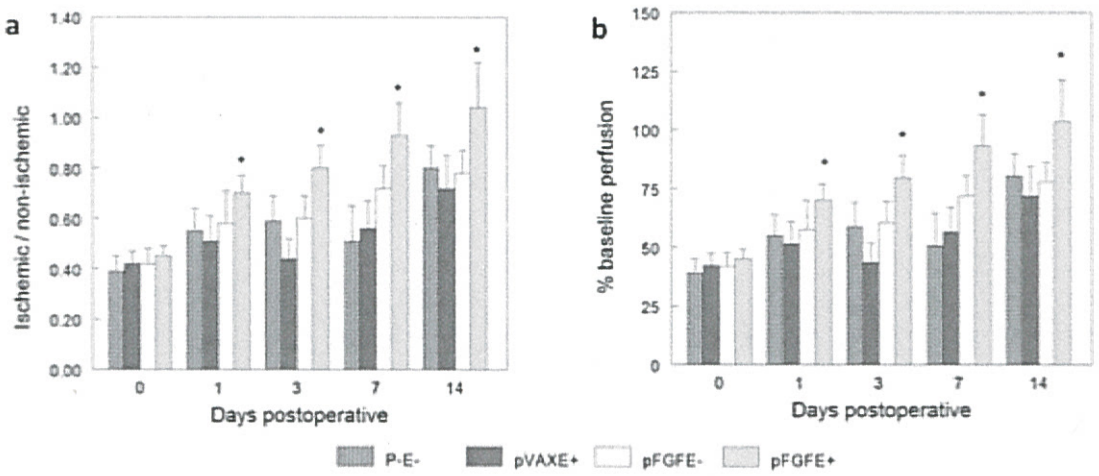
Figure 13: pFGFE+ significantly increased FGF-2 expression compared to pFGFE- for 10 days after delivery (* $p<0.05$)



Delivery of plasmid FGF-2 to the ischemic hindlimb increased perfusion

Four treatment groups were established to assess if pFGFE+ could increase perfusion in the hindlimb ischemia model. In addition to pFGFE+, perfusion was also assessed for pFGFE-, delivery of the vector backbone lacking the FGF-2 cDNA with EP (pVAXE+), and no treatment (P-E-). Treatment was administered at two sites on the day of surgery to induce hindlimb ischemia. Laser Doppler Imaging was used to assess perfusion at baseline, immediately postoperatively but before treatment and at days 1, 3, 7 and 14 postoperatively. Perfusion was expressed as a ratio of perfusion in the ischemic limb to the non-ischemic limb and the percent recovery of baseline perfusion was determined. pFGFE+ increased perfusion beginning at day 1 postoperatively compared to all control groups. Perfusion in the pFGFE+ group remained higher than all controls throughout the study (Figure 14). These results indicated that pFGFE+ was a potential non-viral and non-invasive approach to restore perfusion to ischemic limbs. This study was continued to determine if the observed increase in perfusion with pFGFE+ correlated with increased neovascularization in the ischemic limb.

Figure 14: pFGFE increases perfusion. pFGFE+ increased perfusion beginning at day 1 postoperatively. (a) ischemic/non-ischemic ratio (b) recovery of baseline perfusion (* $p<0.05$ compared to all controls).



Appendix A: Meeting abstract/poster presentation

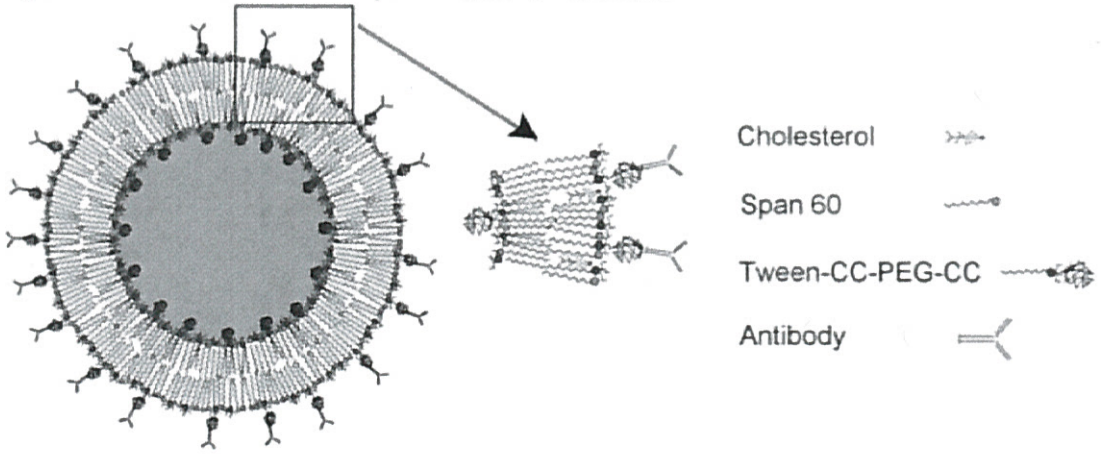
"In Vivo Imaging of Vesicles Loaded with Near Infrared Dye Targeted to CD44 on Inflamed Endothelial Cells in a Mouse Model of Atherosclerosis"
Authors: Elliott JA*, Muffly K, Strom JA, Flam B
Poster presentation by JA Elliott at the Scientific Sessions at the American Heart Association Meeting, November 13, 2010, McCormick Place, Chicago, IL.
Published in Circulation, 23 November 2010; 122: A20505

Introduction:
Atherosclerosis
Atherosclerosis, is a chronic inflammatory and degenerative disease that develops silently over several decades until symptoms are detected from restricted blood flow caused by the irreversible remodeling of the arterial walls. Inflammation is upregulated in stages 2-6 and can provide a binding target for imaging agents used for diagnosis and provide a site-specific target for therapeutic targeted vesicles.

Adhesion Molecules
Vascular inflammation-mediated processes associated with atherosclerosis induce endothelial cells (ECs) to overexpress cell adhesion molecules (CAMs) on their surface. CAMs, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), are transmembrane glycoproteins involved in leukocyte recruitment, adhesion and transmigration, and thus have been widely studied as potential targets for drug delivery vesicles. CD44 is another adhesion receptor protein involved in inflammation-mediated signal transduction that holds promise as a target for drug delivery since it is upregulated on inflammatory, endothelial, and smooth muscle cells involved in the initiation and progression of atherosclerosis.

Targeted Vesicles
A niosome is a synthetic self-assembling transport vesicle made from polyethylene glycol (PEG)-modified nonionic surfactants creating a bi-layer membrane surrounding an aqueous core. Site specific targeting is achieved by the conjugation of antibodies to the functionalized surfactant creating the PEGylated immuno-niosome (IN) shown in Figure 1.

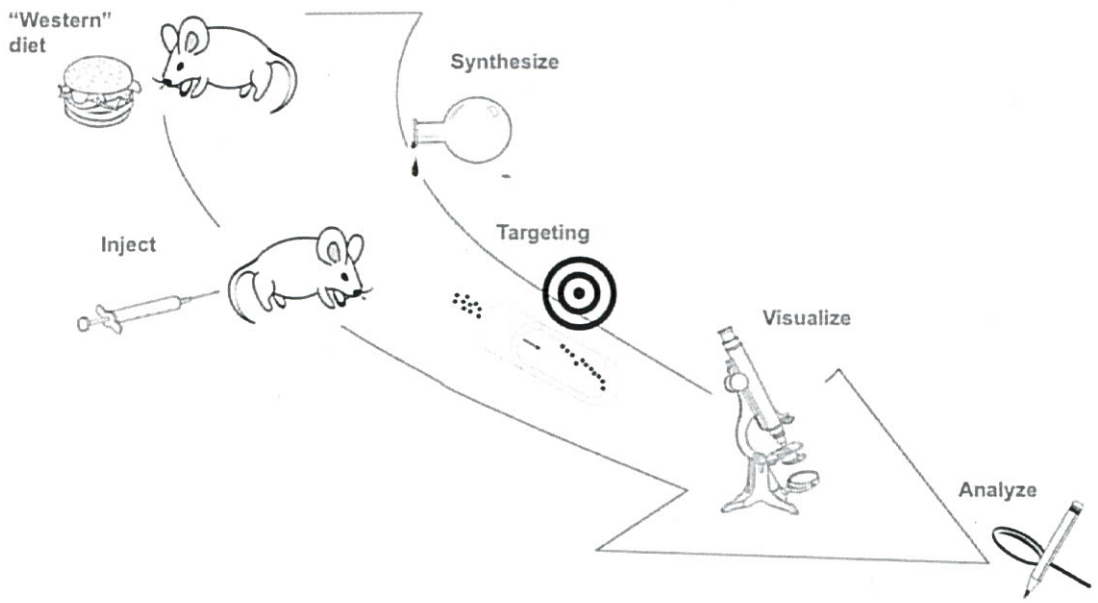
Figure 1: Schematic of a PEGylated immuno-niosome



Study objective and hypothesis:
CD44, an adhesion molecule overexpressed in atherosclerosis, provides a site for targeted drug delivery. The hypothesis was that a novel surfactant vesicle loaded with near infrared dye (IRDye-800CW) and conjugated with anti-CD44 antibodies could target dysfunctional endothelium in the apoE -/- mouse and could increase vesicle circulation time.

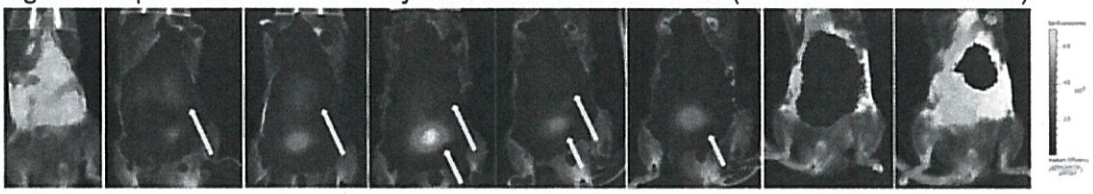
Methods and Results:

Figure 2: Schematic of experimental design for in vivo experiments



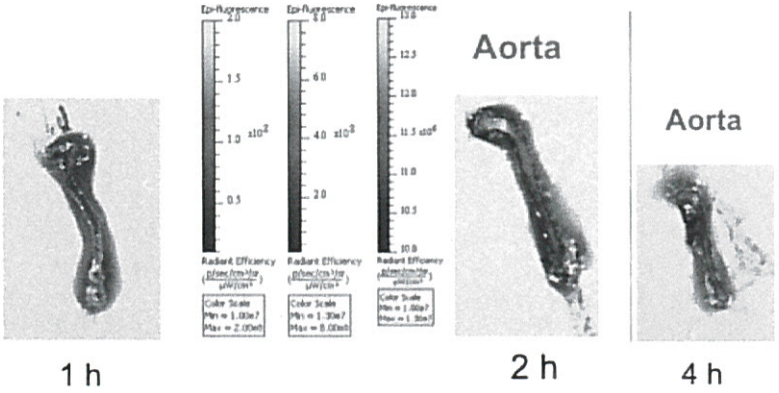
On day 31, whole body epi-fluorescent imaging was performed using an IVIS 200 Spectrum small animal imager (Caliper Life Sciences) at 0, 0.5, 1, 2, 4, 8, 26 and 52 h post-injection using 745/800 nm (excitation/emission) filters (Figure 3). Extended vesicle circulation time (arrows) was observed in the organs at 1 h and persisted until 12 h, with the peak signal intensity between 1-4 h.

Figure 3: apoE -/-: 0.5 nmol IRDye-800CW-PEG-CD44-IN (31 d on "Western" diet)



The mice were fed a lipid-rich or standard rodent diet for an additional 7 days. On day 38, whole body imaging was performed at 0, 1, 2 and 4 h post-injection (Figure 4). The organs were imaged ex vivo at the 1, 2 and 4 h time points (n=2/time point), (Figure 4; aorta shown).

Figure 4: apoE -/-; 38 d "Western" diet; 0.5 nmol IRDye-800CW-PEG-CD44-IN (1, 2, 4 h post-injection)



Summary:

These preliminary results showed our ability to target inflamed aortic endothelial cells in an atherosclerotic mouse using a vesicle with extended circulation time. The CD44 vesicle design represented a valuable tool for: 1) imaging atherosclerotic plaques and 2) targeting encapsulated therapeutic agents to areas of inflammation.

Appendix B: List of personnel receiving pay from the research effort

University of South Florida, Tampa, FL
Goldman, Allan L. (costshare): Principal Investigator for University of South Florida
Strom, Joel A.: previous Principal Investigator for University of South Florida
Anderson, Sandra L.: Senior Faculty Research Administrator
Bhansali, Shekhar: Professor
Bhethanabotla, Venkat R.: Professor
Carney, Michael J.: Technical & Professional Staff
Elliott, John A.: Predoctoral Fellow (2008-2010), Postdoctoral Fellow (2010-2011)
Ferraro, Bernadette: Predoctoral Fellow
Flam, Brenda R.: Postdoctoral Fellow (2008-2010), Research Assistant Professor (2010-2011)
Hua, Jianhong: Technical & Professional Staff
Jaroszeski, Mark J.: Associate Professor
Osborn, Barbara A.: Technical & Professional Staff
VanAuker, Michael D.: Assistant Professor

Keck Graduate Institute, Claremont, CA
Phillips, M. Ian: Principal Investigator for Keck Graduate Institute
Qian, Keping: Senior Scientist
Shen, Leping: Researcher

Loyola University Chicago, Maywood, IL
Majetschak, Matthias: Principal Investigator for Loyola University Chicago
Romero, Jacqueline: Research Specialist I

Appendix C: Publications/Meeting abstracts/Presentations

1. Elliott JA, Flam B, Muffly K, Strom JA, Hood E, VanAuker MD. Targeted drug delivery with PEGylated immuno-niosomes. In 25th Southern Biomedical Engineering Conference 2009, 15 – 17 May 2009, Miami, Florida, USA, 24: 363-366, Springer.
2. Elliott JA, Flam BR, Muffly KE, Strom JA, Hood ED, VanAuker MD. PEGylation of CD44-conjugated niosomes enhances their delivery to and uptake by inflamed vascular endothelium. In revision.
3. Ferraro B, Cruz YL, Coppola D, Heller R. 2009. Intradermal delivery of plasmid VEGF(165) by electroporation promotes wound healing. Mol Ther 17(4): 651-657.
4. Elliott JA, Muffly K, Strom JA, Flam BR. 2010. In vivo imaging of vesicles loaded with near infrared dye targeted to CD44 on inflamed endothelial cells in a mouse model of atherosclerosis. (Poster presentation at Scientific Sessions 2010, November 15, 2010, Chicago, IL) Circulation 122: A20505.
5. Ferraro B, Cruz YL, Baldwin M, Coppola D, Heller R. 2010. Increased perfusion and angiogenesis in a hindlimb ischemia model with plasmid FGF-2 delivered by noninvasive electroporation. Gene Therapy 17: 763-769, doi:10.1038/gt.2010.43.
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7. Baker TA, Bach HH, Romero J, Strom JA, Gamelli RL, Majetschak M. 2011. Ubiquitin improves lung function and reduces edema formation in a clinically relevant polytrauma model. Shock 35(1): 37-37.
8. Baker TA, Romero J, Bach HH, Strom JA, Gamelli RL, Majetschak M. 2012 Systemic release of cytokines and heat shock proteins in porcine models of polytrauma and hemorrhage. Crit Care Med 40(2): 876-885.

Appendix D: Interactions/Transitions, Discoveries, Honors/Awards

Meetings:
Three meeting presentations of work directly related to the grant have occurred.

DTRA site visits to University of South Florida:
March 19, 2010
March 18, 2011

DTRA site visit to Loyola University, Chicago:
March 21, 2011

Consultations:
No consultations have occurred.

Transitions:

a. The insights gained from the humoral response to injury can help understand the transition from the systemic inflammatory response syndrome to the development of multiple organ failure, a dreaded complication of battlefield trauma. These insights can be applied to other conditions where trauma-induced inflammation plays an important role, e.g. traumatic brain injury.
b. The research from this project has identified ubiquitin as a potential anti-inflammatory agent.
c. This project has helped develop three forms of targeted therapies:
    i. Immunoniosomes targeted to inflammation. This work can be expanded to include atherosclerosis and other inflammation –mediated diseases.
    ii. The vigilant hemostatic vector has the potential to automatically treat the diffuse types of hemorrhage that occur with multiple puncture wound injury.
    iii. The angiogenic gene delivery system has potential applications to all forms of vascular injury.
d) New discoveries:
    i. A Vigilant Vector Hemostat with gene modification for Factor VII expression to stop bleeding from injured blood vessels.
    ii. Novel modifications to PEGylated niosomes to improve stability and targetability

Honors/Awards:

a. Bernadette Ferraro
"Intradermal Delivery of Plasmids Encoding Angiogenic Growth Factors by Electroporation Promotes Wound Healing and Neovascularization"
Dissertation defense: March 20, 2009
PhD in Medical Sciences awarded May 2009
Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL
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b. Research Assistant Professor faculty appointment in Internal Medicine at USF effective for Brenda Flam, PhD, February 19, 2010.

c. John A. Elliott
"PEGylation of Niosomes"
Dissertation defense: November 16, 2009
PhD in Chemical Engineering awarded May 2010
Department of Chemical and Biomedical Engineering, College of Engineering, University of South Florida, Tampa, FL
UMI 3424467; Copyright 2010 by ProQuest LLC

Courses taught:
none

Appendix E: Intellectual property submissions

DD-882 forms for University of South Florida, Keck Graduate Institute and Loyola University Chicago are attached.

Prime account: University of South Florida, HDTRA1-08-1-0080, #6123-1070-00 (Period of performance: April 1, 2008 – March 31, 2011)

2. Subcontractor: Keck Graduate Institute, # 6123-1070-00-A (Period of performance: April 1, 2008 – December 31, 2009)

3. Subcontractor: Loyola University Chicago, # 6123-1070-00-B (Period of performance: April 4, 2008 – March 31, 2011)

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