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Evaluation of Novel Hemostatic Agents in a Swine Model of Non-Compressible Hemorrhage

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INTRODUCTION

Uncontrolled hemorrhage is the leading cause of death from wounds on the battlefield, accounting for over 50% of mortality [1]. Hemorrhage is also the second leading cause of death in civilian trauma [2]. There is a significant correlation between increased evacuation time and deaths due to combat injuries which increases proportionally with increasing evacuation time [1,3]. Of the increased deaths due to delayed evacuation, 62% are the result of hemorrhage [1]. This represents a group of casualties who bleed from wounds that are not immediately fatal. Approximately 80% of the hemorrhagic combat deaths are from wounds that are not compressible (accessible for manual pressure). Currently, there is no method available forward of the operating table that can provide hemorrhage control for non-compressible hemorrhage. In spite of the common use of pharmacologic methods for decreasing blood loss in elective surgeries and specific coagulopathies in which large blood losses are expected, the potential for use to aid hemostasis during traumatic hemorrhage has not been adequately studied. The primary innovation for the proposed study is the development of a novel approach for the treatment of non-compressible hemorrhage in battlefield casualties utilizing a mixture of highly purified blood coagulation factor(s) and phospholipid vesicles (PCPS). In the current proposal, we will ascertain the ability of factor VIIa/PCPS and factor Xa/PCPS to augment hemostasis in porcine models of uncontrolled hemorrhage. The objectives of this study are to characterize and compare the effects of combinations of selected blood coagulation proteins and phospholipids on hemorrhage, survival, coagulation function, platelet function, and fibrinolytic function after hepatic injury. In addition, we will further validate the use of our porcine model of non-compressible hemorrhage by

establishing reliable biological assays of porcine blood coagulation and the knowledge of reference standards in comparison to human values. Overall, this proposal seeks to determine the feasibility of this novel treatment with the long-term goal of ultimately developing a pharmaceutical composition that will improve hemorrhage control and reduce combat mortality.

STATEMENT OF WORK

Evaluation of Novel Hemostatic Agents in a Swine Model of Non-Compressible Hemorrhage

Work performed at Haematologic Technologies, Inc.

Task 1. Quantitate *in vitro* the level of bioequivalence of human proteins Xa and VIIa in porcine plasma.

a. Manufacture of the blood coagulation factors (human and porcine), phospholipids (PCPS), and porcine brain thromboplastin (Months 0-4).
b. Perform functional assays to determine the bioequivalence of human coagulation factors (factor Xa and factor VIIa) in a purified porcine system (Months 2-6).

Task 2. Develop a fibrinolysis assay specific to the porcine fibrinolytic system for the measurement of fibrin (ogen) degradation products (FDP's).

a. Manufacture and acquire reagents (Months 0-2).
b. Validate the assay for use with porcine plasma and establish the normal range for these variables (Months 1-6).

Work performed at US Army Institute of Surgical Research (ISR)

Task 3. Evaluate mixtures of phospholipid vesicles (PCPS) and mammalian blood coagulation factors VIIa or Xa in relative proportions and in an amount sufficient to arrest bleeding or to reduce blood loss in a swine ear bleed model (dose efficacy).

a. Utilize a swine ear bleed model developed at ISR to evaluate the potential hemostatic agents (Months 4-12).
b. Perform dose-response studies with each drug combination to determine the feasibility to generate the desired hematological alterations (Months 4-12).

Task 4. Evaluate in more detail via a dose efficacy study the formulations from specific aim 2 which displayed potential as agents in hemorrhage control.

a. Utilize the same swine ear bleed/liver injury model as in specific aim 2 to screen a range of doses for both beneficial and adverse effects (Months 12-24).
b. Approve a range of doses that include an appropriate benefit-to-risk ratio (Months 12-24).

MATERIALS AND METHODS

Work performed at Haematologic Technologies, Inc.

Purification of Blood Coagulation Factors

Several conventional procedures with modification were used for the isolation of these plasma proteins. These procedures are well defined in the literature and are routinely used at HTI to isolate proteins from human and other mammalian plasmas. Factor V was prepared by a combination of conventional and affinity techniques with minor modification from the procedures described for both human and bovine preparations [4,5]. Factor VII, factor X, and prothrombin were prepared from human or porcine plasma by barium citrate adsorption similar to the procedure described by Bajaj and coworkers for the isolation of vitamin K-dependant proteins [6,7,8]. Porcine factor Xa was prepared by activating purified factor X with the factor X activator isolated from Russell's viper venom (RVV-X). Factor Xa is purified from the activation mixture by chromatography over benzamidine-sepharose [9,10]. Fibrinogen was prepared with minor modification from the procedures described by Therorell and coworker with the addition of CL4B-sepharose chromatography [11].

Preparation of Phospholipids (PCPS)

Phosphatidylcholine (PC) and phosphatidylserine (PS) are available commercially as purified reagents. They are prepared from egg yolks and bovine brain, respectively. The PCPS lipid vesicles were prepared by a conventional and standardized protocol [12,13] that produces single compartment vesicles of uniform dimension (325-350 angstroms). The molar ratio of phosphatidylserine to phosphatidylcholine is about 1:3, based on the relative amounts of these lipids used in the preparation of the vesicles. The PCPS

vesicles can be formulated in 0.1M NaCl and 10% sucrose. The vesicles are then frozen at –70 °C and lyophilized. We are able to confirm the phospholipid concentration by phosphate assay. One phosphate head group per phospholipid molecule provides a direct measurement of phospholipid concentration.

Porcine Brain Thromboplastin

Porcine brain thromboplastin has been received as a kind gift from the laboratory of Wen Jun Martini at the ISR. This reagent will be utilized in the experimentation to determine the bioequivalence of human and porcine factor VIIa.

Functional Assays to Determine Bioequivalence

Rate of Thrombin Generation. Thrombin generation assays were used to compare the cross-species effectiveness of human factor Xa in a purified porcine system and porcine factor Xa in a purified human system. The activation of prothrombin to thrombin by prothrombinase (Factor Xa, Factor Va, PCPS, and calcium ions) was studied in the presence of dansylarginine-N- (3- ethyl-1, 5-pentanediyl) amide (DAPA). DAPA is a specific and potent inhibitor of thrombin that binds to thrombin at a stoichiometry of 1 mol of inhibitor per mol of thrombin [14]. DAPA provides a convenient continuous monitor of the progress of the prothrombinase reaction through enhanced fluorescence intensity of the thrombin DAPA complex [14-16]. Measurements of initial rates were determined with reactions containing either porcine or human components at 1.4 μM prothrombin, 3.0 μM DAPA, 20 μM PCPS, 2.5 nM Factor Va, 2.0 mM $Ca2^{+}$ in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4. The reaction was initiated by the addition of factor Xa to a final concentration of 1.0 nM. Fluorescence intensity was monitored at 565 nm with an excitation wavelength of 280 nm. Data was recorded every 0.1 seconds.

Km Determination for Porcine Prothrombin. We determined and compared the Km values for porcine prothrombin for both factor Xa species in the purified porcine prothrombinase reaction analogous to the experiments mentioned previously. Measurements of initial rates were determined with reactions containing 0.088 μM to 1.4 μM prothrombin, 3.0 μM DAPA, 20 μM PCPS, 2.5 nM Factor Va, 2.0 mM $Ca2^{+}$ in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4 was initiated by the addition of factor Xa to a final concentration of 1.0 nM. Fluoresence intensity was monitored at 565 nm with an excitation wavelength of 280 nm. Data was recorded every 0.1 seconds. Initial rates of prothrombin activation were measured by the fluorescence intensity of the DAPA-thrombin complex. Initial rates (vertical axis) are plotted against the reciprocals of the initial concentrations of prothrombin (horizontal axis). The data was fit using the Michaelis-Menten equation by least squares analysis.

Plasma-based Clotting Assays. A modified clotting assay where 100 μl of either porcine or human normal pooled plasma (NPP) is incubated at 37°C with 100 μl of 5 μM PCPS in 20 mM Tris-HCl, 150 mM NaCl, 0.01% BSA, pH 7.4. Clotting was then initiated with 200 μl of varying concentrations of either porcine or human factor Xa in 25 mM $CaCl_2$. Clot times were measured and the log of clot times are plotted against the log of the factor Xa concentration. The factor Xa concentration was varied from 0.084 nM to 86 nM. The phospholipid (PCPS) concentration was fixed at 5 μM.

Factor VIIa. Utilizing a two-stage coupled amidolytic assay we looked at the ability of human factor VII and VIIa to generate factor Xa in the presence of tissue factor. The assay is conducted where thromboplastin (source of tissue factor), human VIIa, and the chromogenic substrate Spectrozyme® fXa (American Diagnostica, Inc.) are mixed

and incubated. Factor X conversion is initiated by the addition of human or porcine factor X. Measurements of initial rates were determined with reactions containing either human factor VII or VIIa at a range of 0.23 to 30 ng/ml, 1:300 dilution of Simplastin® Excel PT reagent, 10 mM $CaCl_2$, 160 nM Factor X. Amidolytic activity was monitored at 405 nm using 200 μM Spectrozyme™ Xa substrate. For the standard curve, factor Xa generation (μg/ml) was determined by measuring initial reaction rates (mOD/min) in reactions containing either human factor Xa or porcine Xa at a range of 7.8 to 1000 ng/ml. Amidolytic activity was monitored at 405 nm with using Spectrozyme™ Xa substrate.

Antiserum Production and Purification

Production of sheep antiserum directed against porcine fibrinogen was contracted to Lampire Biological Laboratories (Pipersville, PA) by a fee for service arrangement. Standard IACUC approved immunization protocols were used. HTI provided the necessary purified antigen. Porcine fibrinogen was purified by the method described previously. Porcine Fibrinogen was >96 % clottable as measured by Clauss method using human thrombin [17].

Purification of the IgG fraction was accomplished by ammonium sulfate precipitation directly from the serum. Two salt fractionation steps (35% and 75%) are needed to obtain relatively pure IgG followed by anion-exchange chromatography. The antibody was tested functionally in both ELISA and western blot format.

FDP Assay Method 1: Reverse Passive Latex Agglutination Assay (RPLA)

Tanned red-cell hemagglutination inhibition immunoassay (TRCHII) is an established method to measure fibrin (ogen) degradation products (FDP's) as a result of

fibrinolysis [18,19]. Analysis of FDP levels provides a global assessment of increased fibrinolytic activity. This assay is based upon the agglutination of fibrinogen-coated red blood cells. These cells will normally remain suspended for a long period of time in a buffered solution, however when an appropriate amount of antibody directed toward fibrinogen is added, the cells become cross-linked and begin to agglutinate. When soluble fibrinogen or fragments of fibrinogen (such as FDP's), are added to the red cells prior to the addition of antibody, they will compete for antibody and inhibit agglutination. Thus by combining a serially diluted fibrinogen or FDP standard with a fixed concentration of red cell and antibody, a titer point can be determined which is the highest dilution of standard that gives agglutination.

Traditionally, passive agglutination tests like TRCHII have employed "tanned" red blood cells (RBC's). Presently, tests called reverse passive latex agglutination assays (RPLA) have been developed to measure a number of disease markers and chemical analytes that use microspheres (beads) to replace the RBC's [20-22]. We have adapted the protocol by using blue dyed carboxylate-modified microspheres of 0.78 microns in size (Bangs Laboratories, Inc.). The beads allow for covalent coupling of the primary amines on fibrinogen via carboxylic acid functional groups exposed at the surface polystyrene beads. As the coated beads agglutinate the clumps quickly grow in size where they scatter light much better resulting in a decrease in absorbance in the visible light spectrum. The change in scattered light vs. analyte concentration is the basis for a sensitive end-point or rate-method immunoassay [23]. The progression of agglutination is followed in the visible light range at 650 nm. This measurement can be made as an endpoint or kinetically compared to a control. As the beads agglutinate the solution

becomes more transparent to the light resulting in a decrease in absorbance. So either kinetically or endpoint one observes a decrease in OD at 650 nm over time. This type of analysis was designed to allow us to quantitate (μg/ml) a value for FDP concentration based on a FDP standard curve.

FDP Assay Method 2: Immunoassay

Many of the assays that have been developed for the measurement of FDP are based on methods that employ red cell or latex agglutination, and which utilize titer values for quantitation rather than direct reference to a standard curve. In the FDP immunoassay that we are presently developing, a standard curve is established based upon the competition between fluid-phase, purified, porcine fibrinogen-derived FDP and microtiter well-immobilized porcine fibrinogen for a fluid-phase polyclonal sheep anti-porcine Fibrinogen/FDP-peroxidase conjugate. Therefore, in a serum sample containing FDP, the color generated following the addition of chromogenic substrate (OPD) is inversely proportional to the amount of FDP in the sample.

Fibrinogen coated plates (Nunc, Maxisorp) are prepared with a 2.0 μg/ml solution of porcine fibrinogen in coating buffer (0.016M Na_2CO_3, 0.034 $NaHCO_3$, pH 9.7). Plates are blocked using 2% BSA in 0.02M Tris, 0.15M NaCl, pH 7.4. Test Serum is prepared by adding 2500 Units/ml of aprotinin followed by 100 Units/ml human thrombin to Porcine NPP. The samples are incubated overnight at room temperature. Samples are then placed at 4°C for two hours. Lastly, the samples are centrifuged and the clots are removed. The recommended dilution for the test sera is 1:10 – 1:100 to achieve a signal within the linear range of the standard curve. FDP standard is plasmin digested porcine fibrinogen. The enzymatic digest is monitored for completion by SDS-PAGE and Clauss

Fibrinogen clotting assay. The fibrinogen concentration prior to digestion is considered to be the FDP concentration. Polyclonal α-Porcine Fibrinogen/FDP-peroxidase conjugate is used as the detecting antibody at a concentration of ~70 ng/ml. Samples are incubated on the plate for 2-3 hours at room temperature (Alternatively, incubate overnight at 4°C). The plate is developed by preparing OPD (o-Phenylenediamine) substrate in substrate buffer (0.1M Citric Acid, 0.2 M Na_2HPO_4, 2 mg/ml Urea, pH 5.0). The data is analyzed by plotting absorbance at 490 nm versus FDP concentration using a 4 – parameter fit to calculate the unknown serum values.

Plasma-based Clotting Assays

Porcine and human factor Xa procoagulant activity was measured by using a modified clotting assay where 100 µl of either porcine or human normal pooled plasma (NPP) is incubated at 37°C with 100 µl of 5 µM PCPS in 20 mM Tris-HCl, 150 mM NaCl, 0.01% BSA, pH 7.4. Clotting was then initiated with 200 µl of varying concentrations of either porcine or human factor Xa in 25 mM $CaCl_2$. Clot times were measured and the results were plotted (Clot time vs. factor Xa concentration). Relative slopes were utilized in the comparison.

Drug Formulation

Purified human FVIIa and human FXa, as well as PCPS were provided as lyophilized powders. The proteins were prepared at 20 µg/ml in 20 mM HEPES, 150 mM NaCl, 1.0% PEG 8K, 0.1% HSA, pH 7.4. PCPS was prepared at 400 µM in 0.1 M NaCl and 10% sucrose. Drugs were stored at 4°C until use in experiments. The blood clotting factor/PCPS mixtures were freshly reconstituted and subsequently mixed at a ratio of

0.65 (pmole factor Xa or factor VIIa / nmole PCPS) immediately prior to animal experimentation.

Work performed at US Army Institute of Surgical Research (ISR)

Animals and Instrumentation

Crossbred commercial swine weighing 38.4 ± 0.5 kg (mean ± SEM) were used in this study. Animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. This study was approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research, Fort Sam Houston, TX. Animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1996).

Swine were fasted for 24 to 36 hours before the procedure, with water continuously available. Animals were sedated using glycopyrrolate and Telazol^{R}. Buprenorphine (0.9 mg, i.m.) was administered for analgesia. They were then and anesthetized (1-4% isoflurane in room air) and intubated using a closed circuit system with mechanical ventilation. Infusion catheters were placed occlusively in a femoral vein and a jugular vein. Maintenance fluid (lactated Ringer's; 5 ml/kg/hr) was continuously infused during the experiment. An 8.5 Fr catheter introducer was shortened to 3 cm and placed occlusively in a femoral artery for blood sampling. A Paratrend 7+ Multiparameter Sensor-catheter (Diametrics Medical Inc., Roseville, MN) was placed occlusively into a carotid artery and was attached to a Trendcare TCM 7000 blood gas monitoring system (Diametrics Medical Inc., Roseville, MN) for continuous monitoring of body temperature and blood pH. A port in the Paratrend 7+ catheter was coupled to a

continuous data collection system (MicroMed®, Louisville, KY) for monitoring blood pressure and heart rate. Laparotomy, splenectomy, and cystotomy were performed in each pig. To compensate for removal of the spleen, each animal was infused with lactated Ringer's solution at a volume equivalent to three times the spleen weight. Animals were stabilized for 20 minutes at a body temperature of 38.5 to 39.5°C, a blood pH of 7.35 to 7.45, and a mean arterial pressure (MAP) between 50 and 80 mmHg.

Mesenteric Bleeding Times

Following stabilization, a distance of 30 cm was measured from the ileo-cecal junction along the ileum, and a second mark was made 15 cm further from this point. A U-shaped plastic tube (24 cm long x 4 cm diameter) was laid under this 15 cm section of mesentery and 3 small arteries with accompanying veins were identified within this area (Figure 1). These vessels were approximately 1 mm in diameter. Each vessel was sharply transected with iris scissors and time to cessation of bleeding was measured, with 10 minutes (600 seconds) being arbitrarily chosen as the maximum BT possible. BT was then taken as an average across these triplicates, with elimination of a single value when the CV exceeded 10%. For each pig (n=18), BT was repeated along the mesentery twice more at 20 cm and 20 min intervals, as shown in Figure 2. Data from these pigs were subsequently analyzed to determine the reproducibility of this procedure in regards to time after laparotomy and location within the mesentery. To determine the ability of this BT measurement to reflect coagulation status, BT was measured in an additional 3 pigs after infusion of heparin (50, 75, and 100 IU/kg) and again after multiple doses of protamine sulfate (0.5 mg/kg) to reverse the effects of heparin.

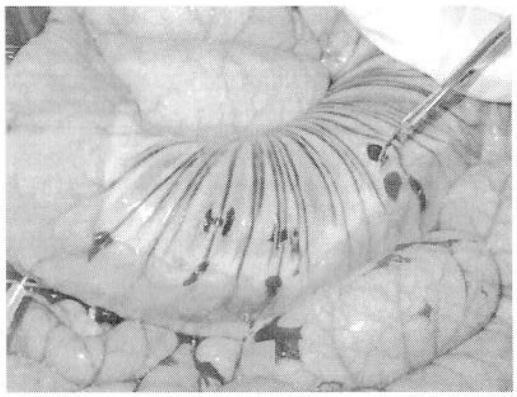
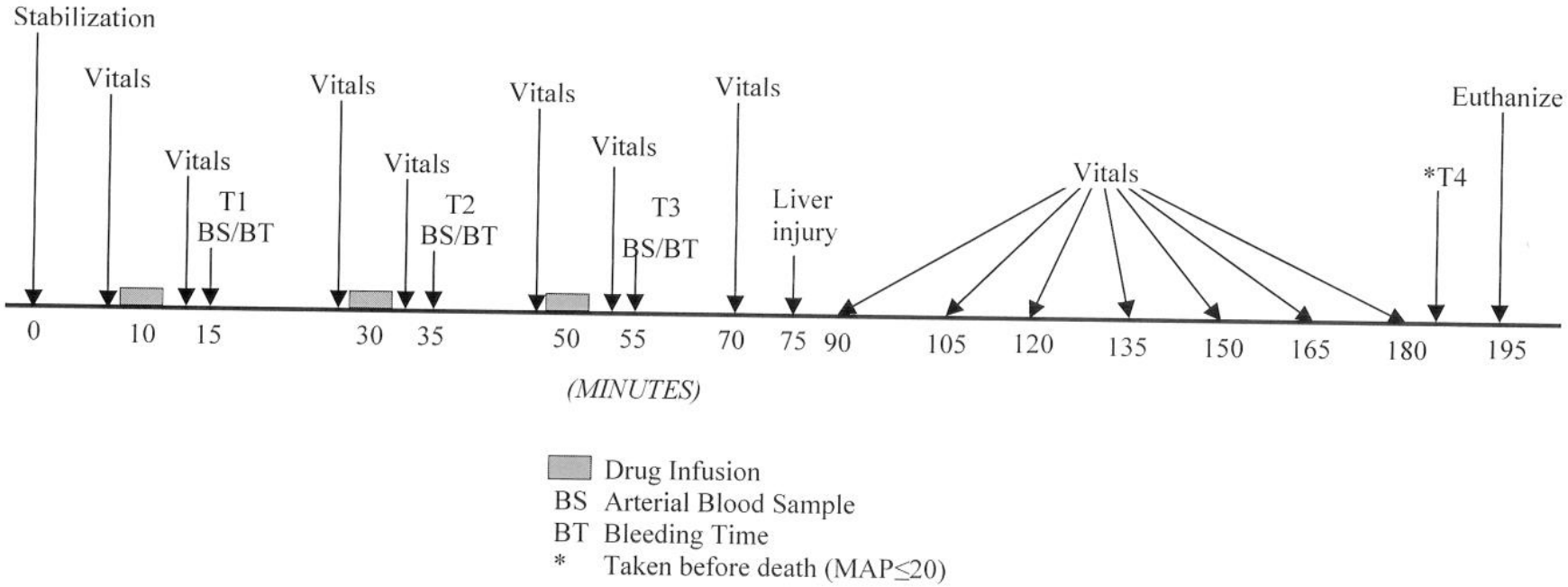


Figure 1. Performance of mesenteric BT procedure in an anesthetized pig.

Figure 2. Timeline for Drug Screening Experiment



Injury Phase

Following completion of BT determinations, a major uncontrolled hemorrhage was induced in 10 pigs. The peritoneal cavity was first suctioned and laparotomy sponges were positioned under the left medial and lateral liver lobes and within the gutters of the abdominal cavity. These sponges were clamped together for easy and immediate egress. Subsequently, the distances between the entry of the inferior vena cava into the liver and the caudal edges of both the left medial and left lateral liver lobes were measured. Each lobe was then loosely clamped approximately 45% of the distance from the caudal liver edge to the inferior vena cava. These sections were sharply cut to remove the distal aspects of each lobe. The clamps were then removed and the liver was allowed to bleed freely (Figure 3a). All sponges were swiftly removed 30 seconds after excision and the abdomen was temporarily closed. Infusion of maintenance fluid was discontinued and no resuscitation fluids were provided. Animals were continuously monitored until death or for 2 hours, at which point surviving animals were euthanized. After death, intraperitoneal blood was measured using suction into pre-weighed canisters and pre-weighed gauze sponges. Additionally, the number and size of transected vessels were measured on the excised portions of the liver (Figure 3b) and confirmed post-death on the remaining liver sections. Vessels were arbitrarily classified as being small (diameter $\leq$ 2mm), intermediate (2 mm>diameter $\leq$ 4mm) or large (diameter > 4 mm).

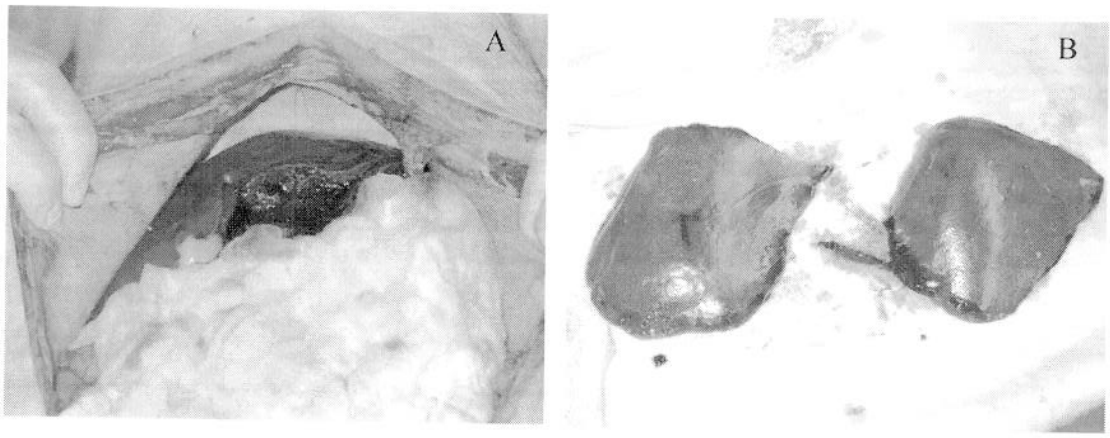


Figure 3. Panel A, freely bleeding liver following excision of approximately 45% of the left lateral and left medial lobes (Panel B).

Drug Treatments

Purified human FVIIa and human FXa, as well as PCPS were provided as lyophilized powders by investigators at Haematologic Technologies, Inc. (RHD and RJJ). Human FVIIa and Xa were obtained by fractionating plasma from normal human donors to obtain the precursor zymogen factors VII and X; these were subsequently activated to yield FVIIa and Xa. The proteins were prepared by lyophilization in 20 mM HEPES, 150 mM NaCl, 1.0% polyethylene glycol (PEG), and 0.1% HAS at a pH of 7.4. Prior to experimentation, reconstitution was accomplished by adding sterile 2.5 mM $CaCl_2$ in water. Following reconstitution, FXa or FVIIa was mixed with PCPS (reconstituted in sterile water) and diluted with a buffer solution (tris-buffered saline (TBS), 1% polyethylene glycol (PEG), pH 7.4). Our initial pig (#20) received doses of FXa-PCPS in a total volume of 8 ml; this infusion resulted in profound hypotension following the first dose and immediate death following the second. Following consultation with Dr. Michael Nesheim (who performed many of the original experiments), it was decided that the drug must be infused in larger volumes (1 ml/kg body weight); this was done in all

subsequent experiments. As these were preliminary screening experiments, a variety of doses were used (Table 1). Doses were chosen based on previous in vitro [24] and in vivo [25-27] experiments and in consultation with Dr. Nesheim. In each experiment, the initial infusion of drug consisted of an identical volume of LR (at T1) to serve as control; all T1 values reported in the tables that follow are therefore baseline levels.

Table 1. Drug treatments administered to each pig.

PIG #	Date	Treatment	Low Dose (per kg) (pmoles protein/nmoles PCPS)	High Dose (per kg) (pmoles protein/nmoles PCPS)
20*	25 Oct 04	FXa/PCPS	5.22 / 14	25.4 / 40
21	27 Oct 04	FXa/PCPS	5.22 / 14	25.4 / 40
54	15 Nov 04	FXa/PCPS	1.5 / 2.31	7.5 / 11.54
62	22 Nov 04	FXa/PCPS	2/3.86	5/7.73
102	24 Jan 05	FXa/PCPS	2.5/4.0	25.5/40
101	26 Jan 05	FXa/PCPS	2.5/4.0[b]	25.5/40[a]
103	2 Feb 05	FXa/PCPS	2.5/4.0	25.5/40
118	7 Feb 05	FXa/PCPS	2.5/4.0[b]	25.5/40[a]
121	9 Feb 05	FXa/PCPS	2.5/4.0[b]	25.5/40[a]
24	3 Nov 04	FVIIa/PCPS	9.0 / 14	25.4 / 40
55	15 Nov 04	FVIIa/PCPS	9.0 / 56	25.5 / 280
53	22 Nov 04	FVIIa/PCPS	350 / 538.5	373.8 / 569.2#
25	3 Nov 04	PCPS	14	40

*Doses given in 8 ml total volume. In all subsequent experiments, doses given in 1 mL/kg total volume.
#Values reflect an attempt to achieve a final cumulative dose of 700 pmoles VIIa using a half-life of 147 min for factor VIIa. Drug administered every 20 min.
[a,b]High dose given first followed by low dose

Blood Sampling

Blood samples were collected by inserting a 20 cm single-use catheter made from Tygon® tubing (I.D. 0.9 mm; Saint Govaine Performance, Akron OH) into the self-sealing port of the catheter introducer and gently withdrawing the blood to minimize

shear-induced platelet activation. The first 3 ml of blood was discarded. Except for thromboelastography (TEG) and activated coagulation time (ACT) samples, blood was anticoagulated with 3.2% sodium citrate at 1 part citrate solution in 9 parts blood.

Standard Laboratory Procedures

Hematocrit (Hct), hemoglobin (Hb), and platelet (PLT) counts were performed as direct measurements using the ABX Pentra 120 hematology analyzer (ABX Diagnostics, Inc., Irvine, CA). ACT was performed using the Hemochron Response (International Technidyne Corp., Edison, NJ), according to manufacturer's instructions. FVII or FX activity was measured with a one-stage clotting assay using an automated coagulation analyzer (Dade Behring BCS system, Marburg, Germany). For the assay, normal and FVII or FX deficient human plasma standards and rabbit brain thromboplastin reagent provided by the manufacturer were used. The BCS system methodology reports results in percent activity. Samples were diluted as necessary according to manufacturer specifications. Standard prothrombin time (PT; using commercial rabbit brain reagent), activated partial thromboplastin time (aPTT), and fibrinogen concentrations were determined at 37°C using an automated coagulation analyzer (Dade Behring BCS system, Marburg, Germany) according to manufacturer specifications. Thrombin-antithrombin III (TAT) concentrations were quantitated using the Enzygnost TAT micro enzyme immunoassay (Dade Behring, Marburg,Germany), which has previously been demonstrated to cross-react with porcine TAT [28].

Thromboelastography (TEG)

TEG monitors changes in the viscoelastic properties of a forming clot (see [29] for a complete description). Briefly, TEG yields various standard parameters, including

reaction time (R), coagulation time (K), α-angle, maximum amplitude (MA), and time to reach MA (MA). R reflects the period of latency from the start time to initial clot formation. K reflects the time from R until a standardized level of clot firmness is reached (amplitude = 20 mm). The α-angle is a measure of the kinetics of clot development. MA is the maximum amplitude attained and reflects the maximum firmness of the clot. Two additional TEG parameters were calculated, maximum velocity (MaxVel), and time to MaxVel (tMaxVel).

TEG was performed in the final five FXa-PCPS pigs using the model 5000 TEG (Haemoscope, Skokie, IL) as previously described [29]. Tissue factor (pig thromboplastin) was used as the agonist, with the concentration being a dilution of the original stock solution that yielded a 3 second R value time [29]. TEG was simultaneously performed using 50 µl of saline as a control to allow confirmation that the clotting observed in response to the agonist was predominantly due to agonist activity. Unaltered whole blood (300 µl) was delivered to each cup within one minute of collection to initiate clotting. TEG was performed in triplicate. The single value for each TEG parameter was derived by averaging, with elimination of a single value when the CV exceeded 10%.

Pathology

Samples from kidney, lung, mesentery, heart, and skeletal muscle were collected within 10 minutes post-mortem and fixed in formalin. All samples were embedded in paraffin, sectioned, and stained using hematoxylin and eosin, Masson's Trichrome, and phosphotungsten acid hematoxylin. Tissues were examined under light microscopy by a board-certified veterinary pathologist for evidence of disseminated intravascular coagulation (DIC) and microthrombi formation.

Statistical Analysis

Data were analyzed using the Statistical Analysis System (SAS) statistical package [30]. Survival data were analyzed using PROC FREQ and associated Fisher's Exact test. Differences among numbers of sizes of blood vessels cut were also examined using PROC FREQ with the Chi Square test. Analysis of all survival time data was conducted using the PROC LIFETEST procedure of SAS with associated Log-Rank nonparametric test. Blood loss and the percentages of lateral and medical lobes cut were analyzed using a single-way analysis of variance with pigs as a random factor (PROC GLM). All data were tested for homogeneity of variance (PROC ANOVA with associated Levene's test) and normality of distribution (PROC Univariate Normal with associated Kolmogorov-Smirnov test). Data were transformed where necessary to meet assumptions of ANOVA. All data are presented as arithmetic means ± SEM.

RESULTS

Work performed at Haematologic Technologies, Inc.

Protein Purification

We have had success in purifying to homogeneity and sufficient quantities from porcine and human plasma prothrombin, factor V, human VII, factor X, and fibrinogen (Figure 1). In addition, we were successful in generating and purifying both enzymatic forms of factor X (Xa), factor V (Va), and human factor VII (VIIa). Analysis by SDS-PAGE (Figure 4) revealed that all of the proteins obtained were essentially homogeneous (>95% pure) and migrated with an apparent molecular mass similar to their human counterparts [7,31-35].

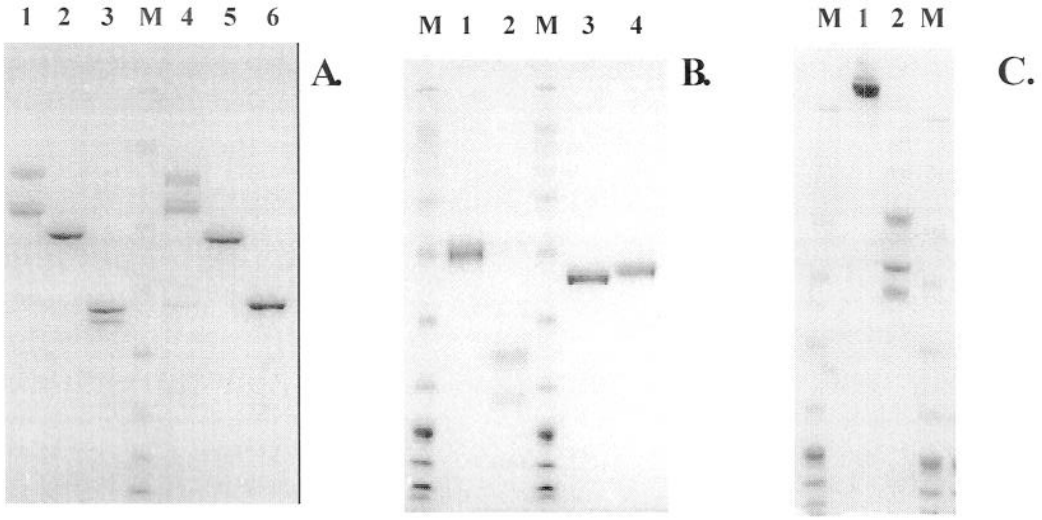


Figure 4. Purified human and porcine proteins on a coomassie blue stained 4-12% SDS-PAGE. M, Molecular weight standard, Myosin (191 kDa), Phosphorylase B (97 kDa), BSA (64 kDa), Glutamic Dehydrogenase (51 kDa), Alcohol Dehydrogenase (39 kDa), Carbonic Anhydrase (28 kDa), Myoglobin Red (19 kDa), Lysozyme (14 kDa); **(A)** Lanes 1-4 non-reduced; lane 1, human factor Va; lane 2, human prothrombin; lane 3, human Xa; lane 4, porcine Va; lane 5, porcine prothrombin; lane 6, porcine Xa. **(B)** Lane 1, human factor VII (reduced); lane 2 human factor VIIa (reduced); lane 3 human factor VII (non-reduced); lane 4 human factor VIIa (non-reduced). **(C)** Lane 1, Porcine Fibrinogen (non-reduced); lane 2 Porcine Fibrinogen (reduced).

The extinction coefficients used to determine protein concentration were inferred from the human values. In all cases we assessed the functional potential of the purified porcine proteins with respect to its human counterpart. The purified proteins were successful in rescuing human factor deficient plasma in standard clinical factor clotting assays (data not shown). Porcine Fibrinogen was >96 % clottable as measured by Clauss method using human thrombin [17].

Porcine VII purification

We have pursued a number of lines of investigation regarding porcine factor VII purification including both conventional and affinity. We have had success tracking activity during purification via human factor VII clotting activity as measured by a two-stage coupled amidolytic assay [7]. We can generate semi-purified pools of factor VII activity with minimal quantities of the protein itself.

Conventionally we made a number of attempts to purify porcine factor VII. Below is a brief summary of the techniques used to purify the protein. In general, porcine plasma was prepared by barium citrate adsorption similar to the procedures described by Bajaj and coworkers for the isolation of vitamin K-dependant proteins [6,7,8]. The DEAE-Sepharose elution profile of the barium absorbate of porcine plasma is illustrated in figure 5. The chromatogram shown represents a single attempt of which many were made using a conventional approach. Factor VII activity eluted at approximately 400 to 500 mM NaCl from fractions 130 to 160 defined by two peaks of activity. The FX-Control (RVV-X assay components without RVV-X) demonstrates that little or no factor Xa is present in the fractions containing factor VII activity. The presence of Factor Xa activity could be misleading in this two-stage coupled amidolytic assay since factor Xa is the final product generated and measured in the assay. The fractions containing factor VII activity were pooled and further fractionated using a number of chromatography techniques including anion and cation exchange, dye affinity, heparin-sepharose, and gel filtration (data not shown). At each chromatography step the activity was monitored and pooled for further purification. The end result from these attempts was a protein preparation containing a mixture of proteins (as evidenced by

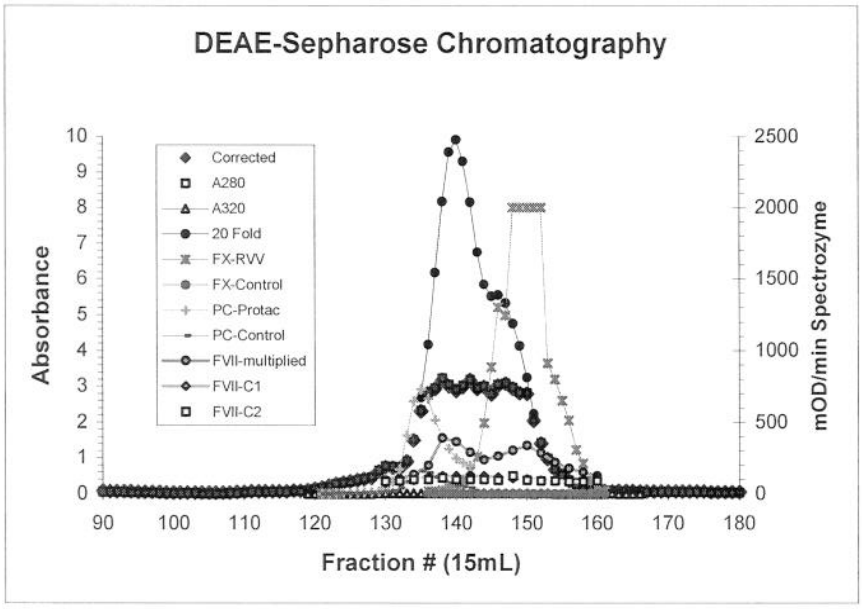


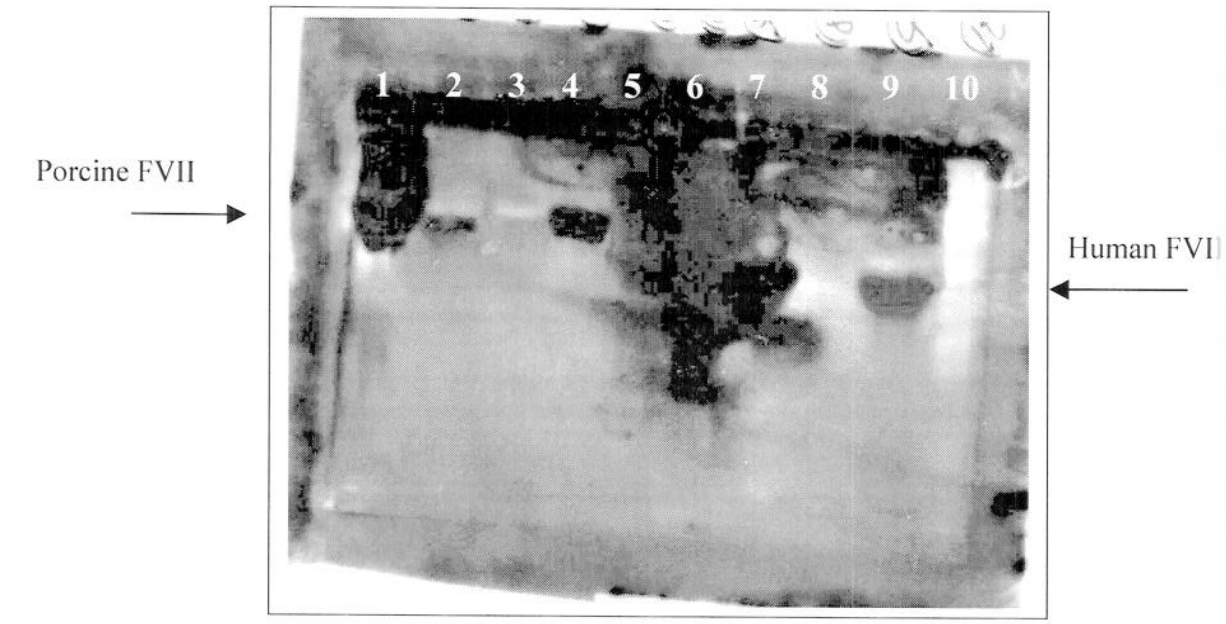
Figure 5. DEAE-Sepharose chromatography of porcine barium absorbate. The sample is obtained from barium citrate absorption and is applied in pH 7.4. Elution is accomplished with a linear gradient from 0.1 M to 0.6 M NaCl in the above buffer. Fractions of 15 ml were collected and subsequently assayed. **Corrected**, Absorbance at 280 nm corrected for scatter; **A280**, Absorbance at 280 nm; **A320**, Absorbance at 320 nm; **20 Fold**, Absorbance at 280 nm fraction samples diluted 20 fold; **FX-RVV**, Factor X activity evidenced by RVV-X assay; **FX-Control**, RVV-X assay components without RVV-X; **PC-Protac**, Protein C activity as evidenced by Protac assay ; **PC-Control**, Protac assay components without Protac; **FVII-multiplied**, Factor VII activity by utilizing a two-stage coupled amidolytic assay; **FactorVII-C1**, Control 1 of the two-stage coupled amidolytic assay no TF present ; **FVII-C2**, Control 2 of the two-stage coupled amidolytic assay no TF and no PCPS (lipid) present.

SDS-PAGE) between 0.17 A_{280}/ml to 0.84 A_{280}/ml and having factor VII activity equivalent to ~1.6 Units/A_{280} and ~6.0 Units/A_{280}. Activity units represent the assumption that porcine factor VII behaves somewhat equivalent to human factor VII in a standard factor VII deficient clotting assay and that the concentration of porcine factor VII is similar to human at 0.5 μg/ml. Therefore, for the purpose of this study 1 unit of porcine factor VII is equivalent to the amount of factor VII found in 1 ml of NPP (0.5 μg). As we further pursued the purification of these minimal quantities of factor VII we recovered only trivial amounts of activity and corresponding A_{280}. Attempts were made with starting quantities of porcine plasma ranging from 5 to 15 liters with theoretically

expected yields of 2.5 mg to7.5 mg. Based on our experience with human and other species of factor VII realistic yields should have been around 750 μg to 2.3 mg.

In preparation for affinity purification we screened a number of antibodies for cross reactivity towards porcine factor VII. Utilizing semi-purified preparations of porcine factor VII from conventional attempts we performed western blot analysis and determined inhibitory potential of these antibodies using standard clotting assays. The porcine samples tested include: 1. Porcine Vitamin K Eluate at 8.77 A_{280}/mL, 2. Q-Sepharose Fractions- Porcine VII enriched at 0.84 A_{280}/mL, 3. Porcine Factor X-some VII activity at 0.2 A_{280}/mL, 4. Sample thought to be porcine factor VII (N-terminal sequencing did not confirm Factor VII) at 0.17 A280/mL. All samples contained factor VII activity. Two identical western blots with corresponding SDS-PAGE analysis (Figures 6 and 7) were performed on the samples with the exception of the primary antibody used in the blots. The primary antibody used in blot #1 was a monoclonal antibody specific to human FVII Gla-domain (monoclonal antibody was from the laboratory of Dr. Paul Bajaj, UCLA), whereas a polyclonal antibody (PAHVII-S, HTI) to human FVII was used as the primary antibody in blot #2 (30 uL of the protein sample was loaded in each lane). Samples number 1 and 2 responded to both monoclonal and polyclonal antibodies but sample 4 appears to be only cross-reacting to the monoclonal antibody to human FVII Gla-domain. Interestingly, blotted samples of porcine factor VII appear to migrate slightly higher that human factor VII. This could indicate that porcine factor VII is of a higher molecular weight than its human counterpart probably due to differences in posttranslational modification or sequence dissimilarity or both.

Blot 1: Monoclonal Antibody



Blot 2: Polyclonal Antibody

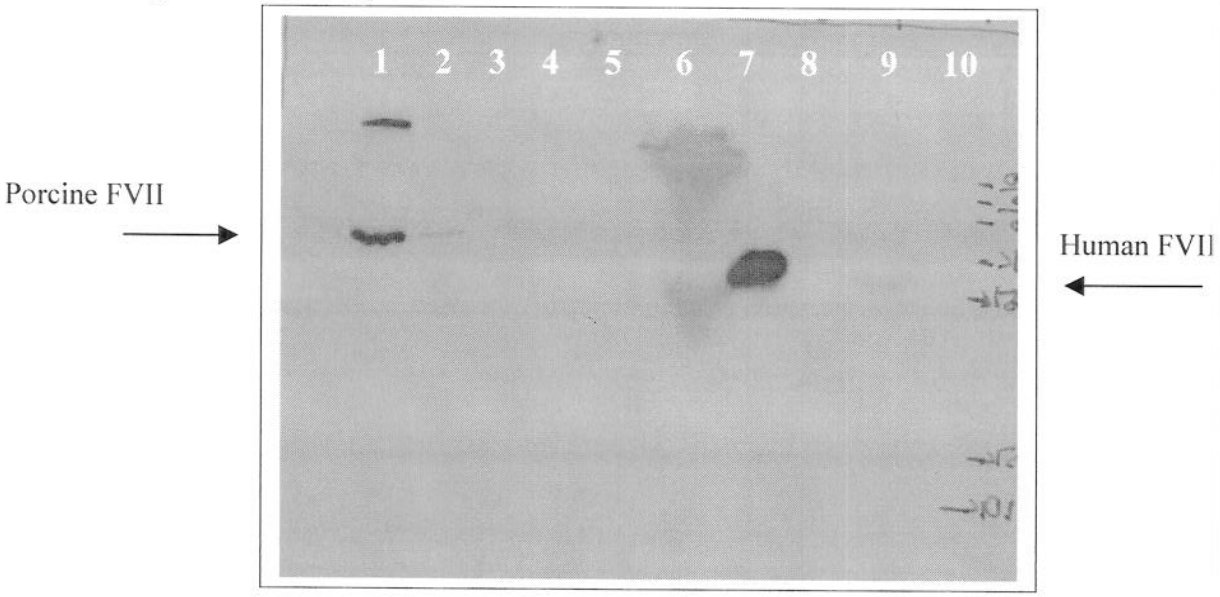


Figure 6. Western Blot Analysis of the Porcine VII Samples. The two identical western blots performed were identical with the exception of the primary antibody used in the blots. Lane 1. Porcine Vitamin K Eluate at 8.77 A_{280}/mL, Lane 2. Q-Sepharose Fractions- Porcine VII enriched at 0.84 A_{280}/mL, Lane 3. Porcine Factor X-some VII activity at 0.2 A_{280}/mL, Lane 4. Sample thought to be porcine factor VII (N-terminal sequencing did not confirm Factor VII) at 0.17 A280/mL, Lane 5. Blank, Lane 6. Porcine Normal Pooled Plasma (NPP), Lane 7. Human Factor VII, Lane 8. Blank, Lane 9. Recombinant Human Factor VII, Lane 10. Molecular weight marker.

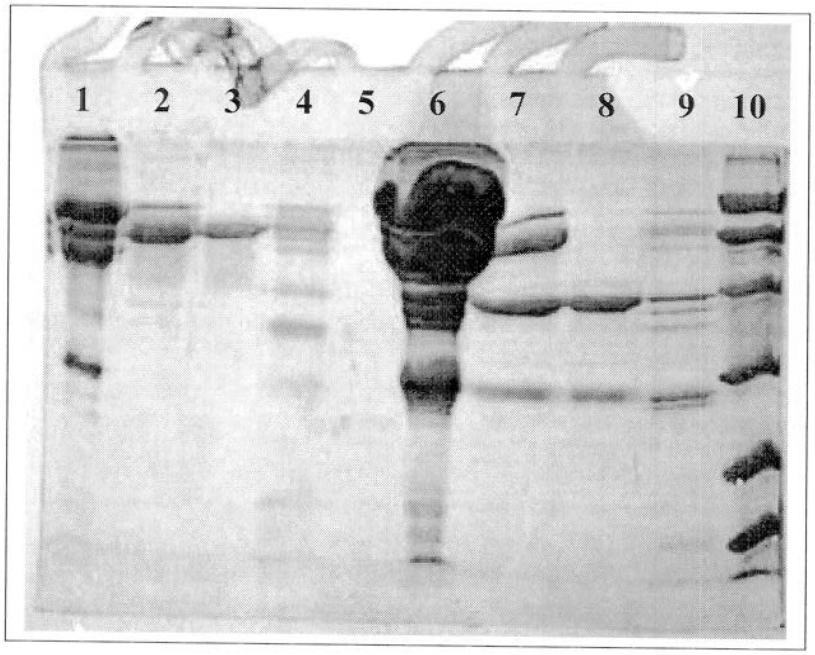


Figure 7. SDS PAGE Analysis of the Porcine VII Samples. Lane 1. Porcine Vitamin K Eluate (non-reduced), Lane 2. Q-Sepharose Fractions- Porcine VII enriched (non-reduced), Lane 3. Porcine Factor X-some VII activity (non-reduced), Lane 4. Sample thought to be porcine factor (non-reduced), Lane 5. Blank, Lane 6. Porcine Vitamin K Eluate (reduced), Lane 7. Q-Sepharose Fractions- Porcine VII enriched (reduced), Lane 8. Porcine Factor X-some VII activity (reduced), Lane 9. Sample thought to be porcine factor (reduced), Lane 10. Molecular weight marker.

We next tested the clot forming activity of the semi-purified porcine samples relative to a porcine normal pooled plasma standard curve as well as the inhibition of porcine factor VII fractions by the anti-Human FVII monoclonal antibody. Samples 1,2, and 4 all contained porcine FVII, with clotting activity of 1.6 U/A_{280}, 6 U/A_{280}, and 3.0 U/A_{280} respectively, relative to porcine NPP. Porcine samples 1, 2, and 4 were then incubated with several different concentrations of monoclonal anti-human FVII (monoclonal antibody was from the laboratory of Dr. Paul Bajaj) in presence of 10 mM Ca^{2+}. Human FVII samples were treated the same way as a control. Residual activity was measured in a clotting assay using a modification of the prothrombin time (PT) test. Serial dilutions (1:5 to 1:160) are made of pooled normal human plasma. These dilutions are then mixed

1:1 with factor-deficient plasma depleted of factor VII. Thromboplastin reagent (Hemoliance® RecombiPlasTin, Instrumentation Laboratory) is added to the mixture. The reaction is timed until clot formation occurs. Results are calculated by comparing clot times of various dilutions of the semi-purified porcine proteins to that of the standard curve prepared using the NPP dilutions versus clot time. Specific activity is defined where one unit is equivalent to the factor activity in one milliliter of normal plasma based on a human standard curve. Porcine samples were 50%-60% less inhibited by the antibody relative to human FVII. This result may suggest that the binding affinity of the anti-human for porcine FVII is ~ 50% less than that of human FVII. This result may also indicate a difference between porcine and human factor VII at the level of posttranslational modification or sequence dissimilarity or both.

Two attempts were made at purification using affinity purification as the primary technique with the cross reacting polyclonal antibody (PAHFVII-S). Starting plasma volumes used in these preparations were 9.0 liters and 15.0 liters with theoretical yields of 4.5 mg and 7.5 mg respectively. We utilized a 1.5 x 10cm column with 50 mg of PAHVII-S coupled to CNBr activated-Sepharose at 5 mg/ml. Plasma was prepared by barium citrate precipitation as described previously. The eluate from this precipitation was passed over the column followed by a wash step with TBS, pH 7.4 and subsequently followed by a 1M NaCl, TBS, pH 7.4 wash step. Elution was carried out with 3 M NaSCN. The protein was then dialyzed, concentrated via an Amicon Centriprep concentrator (10 MWCO) and assayed for activity. Yields from the 9.0 liter and 15.0 liter preps are as follows respectively: Volume = 16.0 ml at 0.02 A_{280} / ml and Volume = 0.65 ml at 0.23 A_{280} / ml. Both preparations at this point in the purification appear as

mixtures by SDS-PAGE analysis. The samples were then chromatographed on Q-sepharose (anion exchange chromatography) with linear gradient from 0 to 40 mM $CaCl_2$. Fractions containing factor VII activity were pooled and concentrated to yield the following amounts of impure proteins: 9.0 Liter sample, 0.65 ml at 0.23 A_{280} / ml and 15.0 Liter sample, 3.0 ml at 0.019 A_{280} / ml. Similar to the findings with conventional chromatography we were able to purify only trivial quantities of factor VII activity and corresponding A_{280}.

Functional Assays to Determine Bioequivalence

Human Xa versus Porcine Xa. To compare these enzymes directly in our "bioequivalence" assays we first had to accurately assess the protein concentration of the porcine and human factor Xa stock solutions. These two parameters would provide a guarantee that we are adding equivalent concentrations of protein that they are equivalent functionally. We followed a number of approaches including absorbance at 280 nm, Pierce Bradford assay, and active site titration. We made our initial assessment of protein concentration by measuring the absorbance at 280 nm using the extinction coefficient for human Xa that has been reported in the literature ($E^{1\%}_{1cm\ at\ 280\ nm} = 1.16$) [36]. Information regarding sequence homology is not currently available for porcine factor X so we assumed that the extinction coefficient is similar to that described for human. Using stock concentrations of factor Xa determined by absorbance at 280 nm a total protein determination was accomplished by the Pierce Coomassie® Protein Assay (modified form of the Bradford assay method). Quantitation of total protein was based on a comparison of serially diluted sample versus a standard curve generated using both human factor Xa and BSA as a standard. Total protein determination revealed

concentration values of the stock solutions within ± 2.9 % and ± 3.9 % as compared to the human factor Xa standard curve and the BSA standard curve respectively. We next compared the functional status of the two stock solutions. *p*-Nitrophenyl-*p'*-guanidinobenzoate (p-NPGB) was used as a substrate for the titration of purified factor Xa [37]. The substrate *p*-NPGB reacts stoichiometrically with activated factor X in a reaction in which the active site of the enzyme is acylated by the *p*-guanidinobenzoate group, and an amount of p-nitrophenol equivalent to the concentration of active sites present is released. The liberation of *p*-Nitrophenol can be conveniently monitored due to its absorbance in the range of 400-410 nm [37]. Titration of the enzymes revealed the stock solution contained 97.9% (Human Xa) to 104 % (Porcine Xa) of the active sites expected using the stock concentrations determined by absorbance at 280 nm. Based on cleavage of a small molecular substrate functional active sites between porcine Xa and human Xa are within 6.1% of each other and appear to be functionally similar in concentration based on the previously determined concentration by absorbance at 280 nm.

Taken as a whole, the results of this analysis allow us to make the assumption that the extinction coefficient reported in the literature for human factor Xa is a close representation to the actual extinction coefficient for porcine factor Xa. We therefore were able to use the reported extinction coefficient value to determine the concentrations of our factor Xa stock solutions. We then proceeded with reasonable confidence that we could measure equivalent amounts of both porcine and human Xa in our "bioequivalence" assays.

Rate of Thrombin Generation. Thrombin generation assays were used to compare the cross-species effectiveness of human factor Xa in a purified porcine system and porcine factor Xa in a purified human system. The activation of prothrombin to thrombin by prothrombinase (Factor Xa, Factor Va, PCPS, and calcium ions) was studied in the presence of dansylarginine-N- (3- ethyl-1, 5-pentanediyl) amide (DAPA). DAPA is a specific and potent inhibitor of thrombin that binds to thrombin at a stoichiometry of 1 mol of inhibitor per mol of thrombin [14]. DAPA provides a convenient continuous monitor of the progress of the prothrombinase reaction through enhanced fluorescence intensity of the thrombin DAPA complex [14-16]. Using this probe for the study of thrombin generation allowed us to compare the initial rates of thrombin generation between the human and porcine system. Essentially, we asked: if the same amount of human factor Xa could produce porcine thrombin at the same rate at which porcine factor Xa does? Using a purified porcine prothrombinase reaction including substrate prothrombin (porcine factor Xa, porcine factor Va, porcine prothrombin, PCPS, and calcium ions) we substituted equivalent amounts of human factor Xa and compared the rates of thrombin generation (Figure 8, panel A). We also conducted the opposite experiment were we used a purified human prothrombinase (human factor Xa, human factor Va, human prothrombin, PCPS, and calcium ions) and substituted equivalent amounts of porcine factor Xa and compared the rates of thrombin generation (Figure 8, panel B). A summary of the reaction conditions can be found in the legend of figure 2. For the porcine prothrombinase reaction we observed a rate equal to 9.46 dA/min and 10.08 dA/min for human factor Xa and porcine Xa respectively. For the human prothrombinase reaction we observed a rate equal to 3.37 dA/min and 3.30 dA/min for

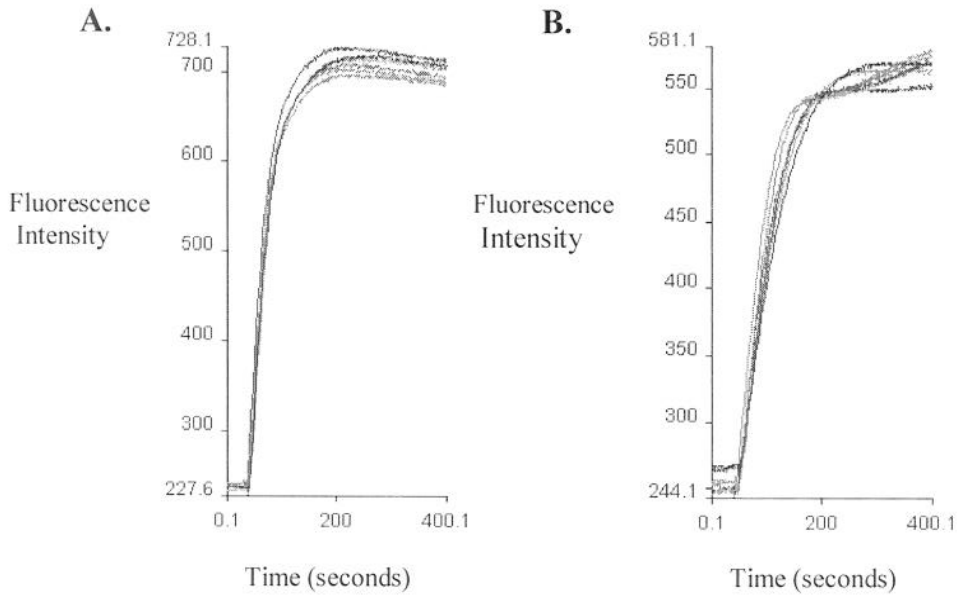


Figure 8. Human and Porcine Prothrombinase catalyzed activation of prothrombin to thrombin. A. Porcine Prothrombinase B. Human Prothrombinase. Measurements of initial rates were determined with reactions containing either porcine or human components at 1.4 μM prothrombin, 3.0 μM DAPA, 20 μM PCPS, 2.5 nM Factor Va, 2.0 mM $Ca2^{+}$ in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4. The reaction was initiated by the addition of factor Xa to a final concentration of 1.0 nM. Fluorescence intensity was monitored at 565 nm with an excitation wavelength of 280 nm. Data was recorded every 0.1 seconds.

human factor Xa and porcine Xa respectively. The enhancement or change in fluorescence intensity represented by dA per minute (rate) is synonymous with the conversion of prothrombin to thrombin. The rates appear to be quite similar for porcine factor Xa and human factor Xa indicating interchangeability in either species system with regard to the rate at which thrombin is generated. Comparing the two systems as a whole to one another we observed rates of porcine thrombin generation as compared to human generation that are about three fold faster. This would possibly indicate a difference between the two systems not at the factor Xa level. There may exist differences in prothrombin as a substrate where porcine prothrombin is more rapidly converted than human. Porcine factor Va may be a more potent cofactor than human factor Va in this reaction. Without teasing apart these intricacies it is difficult to make a conclusion about

these rate differences. Keep in mind that this should not present a barrier with regards to the interpretation of results in the planned animal experiments since we are only swapping human Xa for porcine Xa. However, when transitioning from the porcine to human system this difference should be taken into consideration.

Km Determination for Porcine Prothrombin. We determined and compared the Km values for porcine prothrombin for both factor Xa species in the purified porcine prothrombinase reaction. Analogous to the experiments mentioned previously, all components of the prothrombinase complex were kept constant while varying prothrombin (substrate) from 0.088 μM to 1.4 μM. Initial rates of prothrombin activation were measured by the fluorescence intensity of the DAPA-thrombin complex. Initial rates (vertical axis) are plotted against the reciprocals of the initial concentrations of prothrombin (horizontal axis). The data was fit using the Michaelis-Menten equation by least squares analysis (Figure 9). The affinity of both species factor Xa are essentially equivalent with respect to their ability to bind prothrombin associated with an assembled prothrombinase complex. Porcine Prothrombinase with porcine Xa had a Km value of 0.34 μM ± 0.14. Porcine prothrombinase with human Xa had a Km value of 0.26 μM ± 0.04. The Km values are very similar between both the human and the porcine enzymes (factor Xa). Km values reported in the literature for bovine prothrombin analyzed in a purified bovine system equals 0.6 μM which is similar to our results for human and porcine [38]. These findings suggest an additional level of "bioequivalence" between the two species of factor Xa based on their similar affinities for porcine prothrombin.

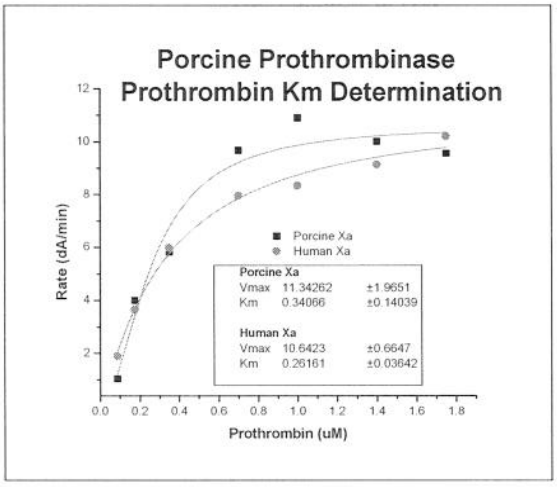
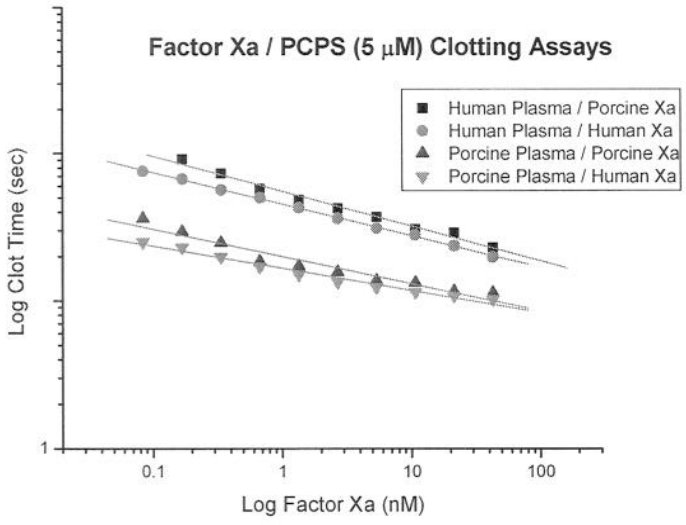


Figure 9. Km values for the conversion of prothrombin to thrombin. Measurements of initial rates were determined with reactions containing 0.088 μM to 1.4 μM prothrombin, 3.0 μM DAPA, 20 μM PCPS, 2.5 nM Factor Va, 2.0 mM $Ca2^{+}$ in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4 was initiated by the addition of factor Xa to a final concentration of 1.0 nM. Fluoresence intensity was monitored at 565 nm with an excitation wavelength of 280 nm. Data was recorded every 0.1 seconds.

Plasma-based Clotting Assays. The "bioequivalence" of porcine and human factor Xa was further tested in a plasma-based clotting assay. Using a modified clotting assay where 100 μl of either porcine or human normal pooled plasma (NPP) is incubated at 37°C with 100 μl of 5 μM PCPS in 20 mM Tris-HCl, 150 mM NaCl, 0.01% BSA, pH 7.4. Clotting was then initiated with 200 μl of varying concentrations of either porcine or human factor Xa in 25 mM $CaCl_2$. Clot times were measured and the results were plotted in figure 4. We tested both species of factor Xa in both porcine and human plasma systems. Human factor Xa in porcine plasma in comparison to porcine Xa in porcine plasma generated nearly an identical response to varying concentrations of factor Xa. The same response was seen for porcine Xa in human plasma in comparison to human Xa in human plasma. Once again we obtained results demonstrating equivalent behavior between the two species enzymes. However, we did observe differences when

comparing the two species systems as a whole. At all the concentrations of factor Xa tested porcine plasma clotted faster than the human plasma. These results concur with our previous interpretation of data where the rate of thrombin generation appears to be faster in the porcine system than in the human system. A faster rate of thrombin generation would account for the faster clot times observed with porcine plasma. These results are similar to other studies, where a short APTT in pigs was observed, compared with humans. This has been postulated to indicate an accelerated intrinsic cascade activity in pigs [39-41]. In contrast, it has been reported that human and porcine PT values are approximately equal [39-41].



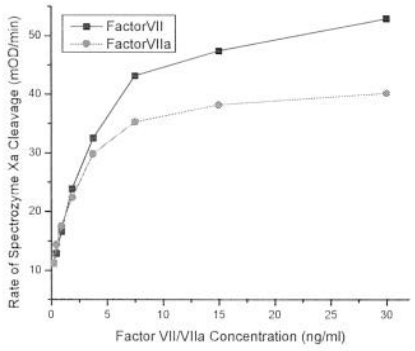
Figue 10. Porcine and Human Plasma Based Clotting Assays. Log of clot times are plotted against the log of the factor Xa concentration. The factor Xa concentration was varied from 0.084 nM to 86 nM. The phospholipid (PCPS) concentration was fixed at 5 μM.

Porcine VIIa and Human VIIa. Similar to the experimentation conducted for porcine and human Xa we sought to determine the effectiveness of human VIIa function in a

purified porcine system compared to porcine VII. Without the ability to use porcine factor VII we first established the assay conditions and proceeded to test human factor VII in the purified human system. Utilizing a two-stage coupled amidolytic assay we looked at the ability of human factor VII and VIIa to generate factor Xa in the presence of tissue factor (Figure 11). The assay is conducted where purified pig brain thromboplastin (source of tissue factor), human VIIa, and the chromogenic substrate Spectrozyme® fXa (American Diagnostica, Inc.) are mixed and incubated. Factor X conversion is initiated by the addition of porcine factor X. Factor Xa generation is quantitated by monitoring the increase in absorbance at 405 nm resulting from the factor Xa mediated hydrolysis of Spectrozyme® fXa. The rate of increase in absorbance at 405 nm is directly proportional to the presence of Xa. By comparison to a factor Xa standard curve we can determine the amount of (μg/ml) factor Xa generation and compare the effectiveness of both human and porcine VIIa to generate Xa (Figure 11). For example, based on the curves represented in figure 5 we can determine that at a concentration of 15 ng/ml human factor VII can generate 3 nmol/min human factor Xa (Figure 11). We have demonstrated the effectiveness of our assay to measure the ability of human factor VII to generate factor Xa. Once we obtain porcine factor VII we will be able to directly comparing the functional ability of both species factor VII to generate factor Xa. We will then be able to determine the level of "bioequivalence" between the two species.

Development of a Porcine Specific FDP Immunoassay. An ELISA was developed for measuring porcine FDP from serum samples collected during animal experimentation. Optimal concentrations of capture antibody, detection antibody, and enzyme-conjugate

A.



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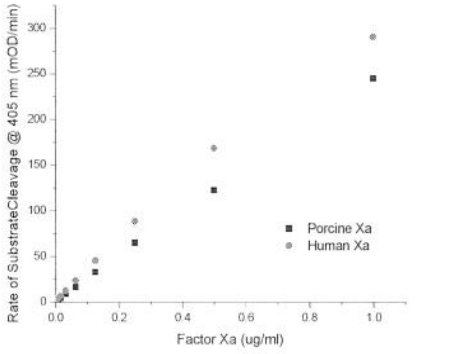
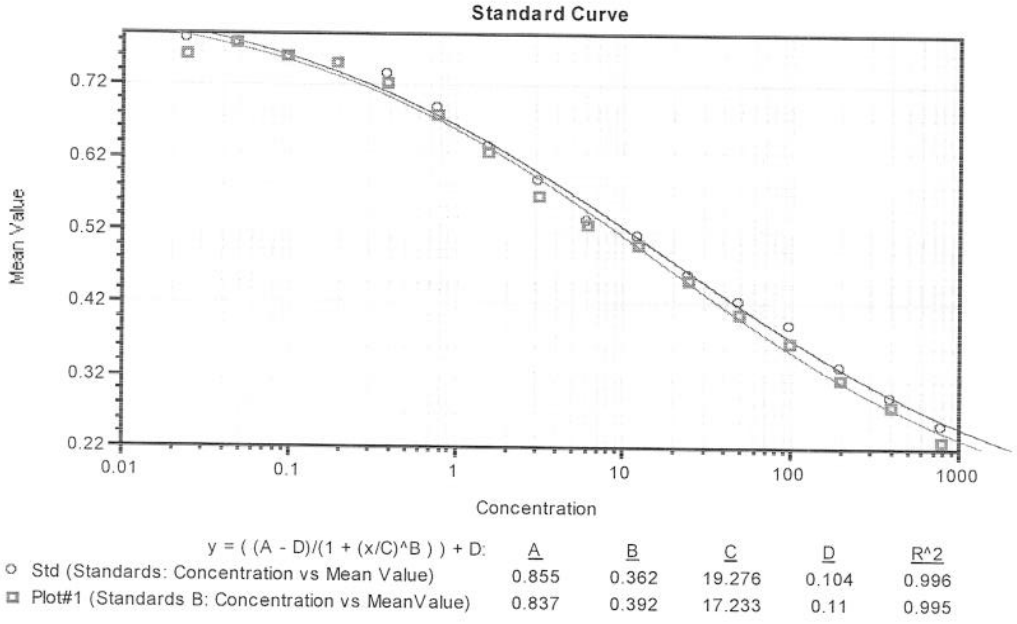


Figure 11. (A) Factor VII/VIIa Two-Stage Coupled Amidolytic Assay. Factor Xa generation was monitored after titration of factor VII/VIIa. Measurements of initial rates were determined with reactions containing either human factor VII or VIIa at a range of 0.23 to 30 ng/ml, 1:300 dilution of Simplastin® Excel PT reagent, 10 mM $CaCl_2$, 160 nM Factor X. Amidolytic activity was monitored at 405 nm using 200 μM Spectrozyme™ Xa substrate. (B)Human and Porcine Factor Xa Standard Curves. Measurements of initial rates were determined with reactions containing either human factor Xa or porcine Xa at a range of 7.8 to 1000 ng/ml. Amidolytic activity was monitored at 405 nm with using Spectrozyme™ Xa substrate.

were determined with serial twofold dilutions of plasmin-digested porcine fibrinogen diluted in assay buffer. This assay was used to determine the ideal sample dilutions needed for reliable quantitation of FDP in porcine serum. Serial dilutions of FDP resulted in a sigmoid titration curve with a maximal sensitivity of 0.1 μg and a dynamic quantitative range from 0.5 μg/ml to 200 μg/ml (Figure 12). The standard curves appear to be consistent between multiple lots of standard as well as under varying conditions. Standard curve values consistently generate CV values below 5% for all dilutions. Spike and recovery experiments revealed a range of recovery from roughly 50 to 150 %, suggesting that our systems could detect spiked FDP in serum samples. However, our confidence in these reported values is low. Samples exhibited a high degree of non-parallelism with rather large CV's (≥ 30 %). We were also able to quantify FDP in



y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Std (Standards: Concentration vs Mean Value)	0.855	0.362	19.276	0.104	0.996
Plot#1 (Standards B: Concentration vs MeanValue)	0.837	0.392	17.233	0.11	0.995

Figure 12. FDP Standard Curve. Concentration µg/ml mean value Y-axis absorbance at 490 nm Two different FDP standards Lot# S1027 and Lot# S0721.

porcine serum alone suggesting a baseline endogenous level of FDP (25-30 ug /ml) of multiple "normal" serum samples tested (CV <15%). Serum samples required roughly 1:10-fold dilution to alleviate the serum matrix effect. Beyond the 10-fold dilution we observed some degree of parallelism between the diluted samples. Overall the FDP assay development still remains a work in progress. Despite our setbacks we are committed to bringing this assay to market.

Work performed at US Army Institute of Surgical Research (ISR)

RESULTS

Development of Model

To determine whether mesenteric BT reflected coagulation status, BT was measured in 3 pigs after: 1) no treatment; 2) 50, 75, and 100 IU heparin/kg body weight; and, 3) three administrations of protamine sulfate (0.5 mg/kg). *In vivo* BT increased with increasing doses of heparin, and then decreased after multiple administrations of protamine ($p=0.042$; Figure 13). ACT varied in a similar manner ($p=0.06$; Figure 13). There was a highly significant correlation between in vivo BT and ACT ($r=0.88$; $p=0.003$).

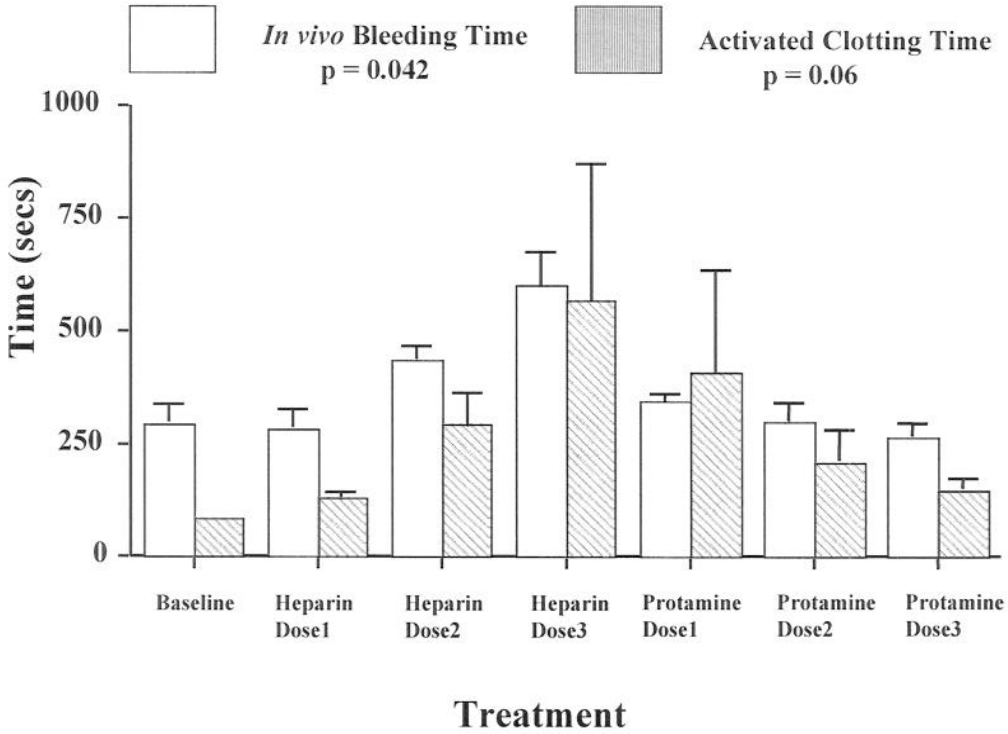


Figure 13. In vivo bleeding time (BT) and activated clotting time (ACT) following doses of heparin and protamine (n=3).

Multiple BT determinations were also made in 18 pigs with saline administration prior to each BT. BT did not differ with section of mesentery ($p>0.05$), nor with time after laparotomy ($p>0.05$; Figure 14). Determination of mesenteric BT is therefore highly reproducible and not dependent on position or time after laparotomy, indicating that multiple BT determinations using this method are valid.

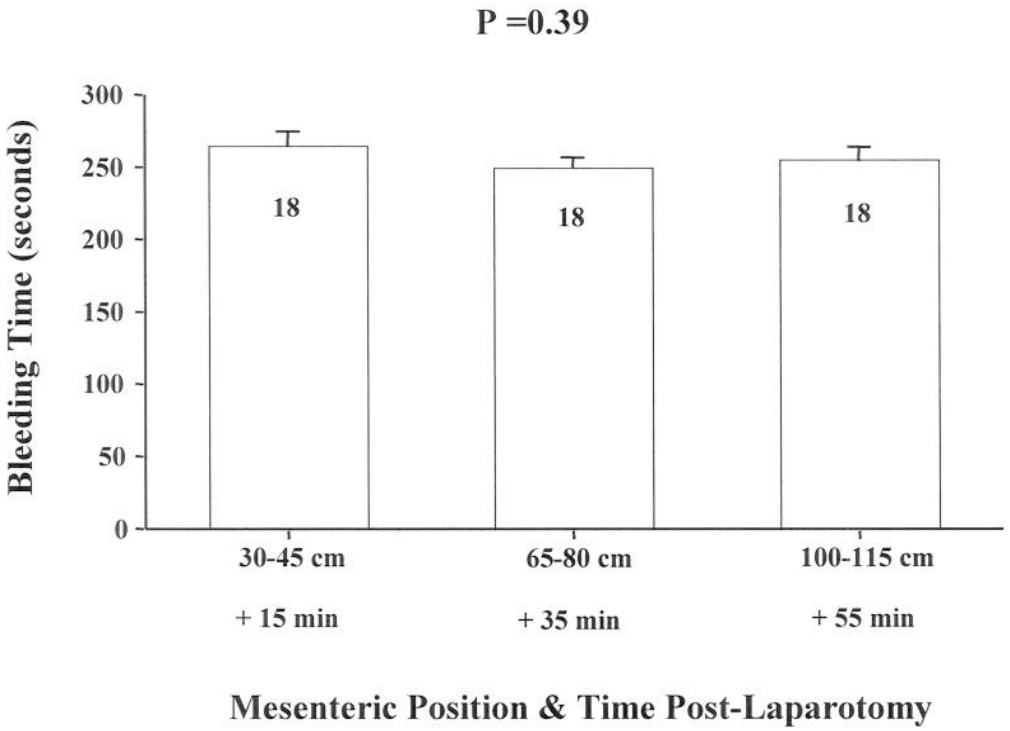


Figure 14. BT did not vary with mesenteric position. Distances are measured from the ileo-cecal junction.

Following determination of BT, a severe liver injury was performed. Through a series of range-finding experiments, we determined that excision of approximately 40-45% of the left medial and left lateral lobes was likely to produce hemorrhage of a severity that resulted in death within a 2 hour observation period in approximately 50%

of animals. Table 1 reports observations from a series of 10 pigs which received this degree of liver injury. This liver injury resulted in death of 60% of the pigs during the 2 hour observation period. Those pigs that survived did not differ from non-survivors in either the volume of blood lost or in the number or size of veins cut. Non-survivors did have a greater percent of their left lateral liver lobe removed ($p=0.009$). When all pigs were considered, 62% of the blood vessels cut were in the predefined small category, and this percentage differed ($p<0.001$) from those in the intermediate and large categories (23% and 16%, respectively).

Table 2. Characteristics of liver injury model in anesthetized swine.

Parameter	Survivors	Non-Survivors	Statistical Evaluation
N	4	6	
% Survival	40	60	P=0.53
Survival time (min)	120	61.7±19.9	P=0.004
Blood loss (gms)	824±106	884±103	P=0.71
% Lt. medial lobe cut	39.0±18.0	42.9±2.3	P=0.24
% Lt. lateral lobe cut	37.9±2.0	54.7±3.7	P=0.009
# Small veins cut/%**	26	36	P=0.61 (Chi-Square)
# Intermediate veins cut/%	12	11	
# Large veins cut/%	6	10	

**The total number of veins cut was 101. Therefore, the number of veins and the percent of the total veins are almost identical. In one of the 10 pigs (a non-survivor), the number of veins was not evaluated. Numbers of veins are absolute values across 9 pigs.

Drug Treatments

FVIIa-PCPS. Because *in vitro* studies had shown a profound magnification of the efficacy of FVIIa by the addition of PCPS [24,42], we initially sought to determine whether this same amplification of the FVIIa effect could be observed *in vivo*. Initial doses of FVIIa used in combination with the first two pigs (#24 and #55) were therefore minute (0.45 and 1.27 μg/kg for low and high doses, respectively) compared with previous doses

of rFVIIa administered to pigs (≥ 90 μg/kg). Subsequently, the largest doses of FVIIa available from Haematologic Technologies (17.5 and 35 μg/kg) were administered to a third pig (#53). Selected raw hematological and BT data are presented in Table 3. Administration of FVIIa with PCPS at concentrations previously found to be efficacious in *in vitro* studies [24] did not seem to decrease BT or enhance coagulation function in these three animals. Furthermore, administration of FVIIa-PCPS did not produce alterations in MAP. Following liver injury, blood loss and survival times were: 897.3 g and 120 min for pig #24; 1372.5 g and 29 min for pig #55; and, 864.2 g and 112 min for pig #53. There was no evidence of DIC on later histological examination.

Table 3. BT and coagulation parameters following infusion of various doses of FVIIa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 2. BT not measured at T4 (post-liver injury).

Pig #	Time Point	FVIIa (%)	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (μg/l)	Plt (10^3/mm^3)	BT (sec)
24	T1	89.3	105	11.3	16.2	119.8	13.64	205	255
24	T2	136.1	102	10.5	16.7	114.3	14.40	190	312
24	T3	181.5	106	9.8	16	114.1	19.44	241	188
24	T4	116.6	110	10.3	16	122.7	45.62	331	---
55	T1	102.3	108	11.3	15.5	130.2	4.97	354	319
55	T2	125.4	125	9.9	15.6	134.1	5.12	335	261
55	T3	183.1	108	9.6	15.8	134.3	9.07	348	231
55	T4	120.8	107	10	14.9	122.3	33.52	303	---
53	T1	96.3	106	10.9	16	120.4	7.2	185	268
53	T2	594.9	104	9.4	15.9	123.4	15.3	181	292
53	T3	596.9	96	9.4	16.3	117.1	16.6	139	333
53	T4	406.3	114	9.6	16.2	115.7	83.2	118	---

Although we wished to increase the dose of FVIIa to levels closer to those previously shown to alter coagulation function in pigs [24], this was not possible due to the inability of Haematologic Technologies to purify and supply the required quantities of FVIIa. We also explored the possibility of obtaining a donation of recombinant FVIIa

from NovoNordisk for testing purposes; such a donation was declined by NovoNordisk. At this point, the decision was made to drop continued testing of FVIIa in this study as we had no source for the drug at the levels required.

PCPS alone. In one pig (#25), PCPS was given by itself. Raw hematological and BT data are presented in Table 4. Infusion of PCPS did not seem to alter either coagulation function or BT. Furthermore, PCPS infusion did not alter MAP. There were no remarkable pathological findings in this pig.

Table 4. BT and coagulation parameters following infusion of two doses of PCPS (14 and 40 nmoles). Experimental time points according to timeline shown in Figure 2. BT not measured at T4 (post-liver injury).

Pig #	Time Point	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
25	T1	99	10.7	15.4	174.6	21.62	237	263
25	T2	96	10.7	15.1	170.8	22.9	206	292
25	T3	91	10.6	16.2	135.5	18.46	190	333
25	T4	99	10.3	15.5	153.1	50.15	266	---

FXa-PCPS. In the first series of FXa-PCPS experiments, the drug was administered according to the timeline depicted in Figure 2. Raw hematological and BT data are presented in Table 5. Pig #20 received doses of FXa-PCPS in a total volume of 8 ml; this infusion resulted in profound hypotension following the first dose and death immediately following the second. Following consultation with Dr. Michael Nesheim, it was then decided that the drug must be infused in larger volumes (1 ml/kg body weight); this was done in all subsequent pigs. Infusion of FXa-PCPS in these doses did not seem to enhance coagulation function or BT; in fact, BT and several coagulation factors (e.g., ACT, PT) were prolonged following administration of these doses.

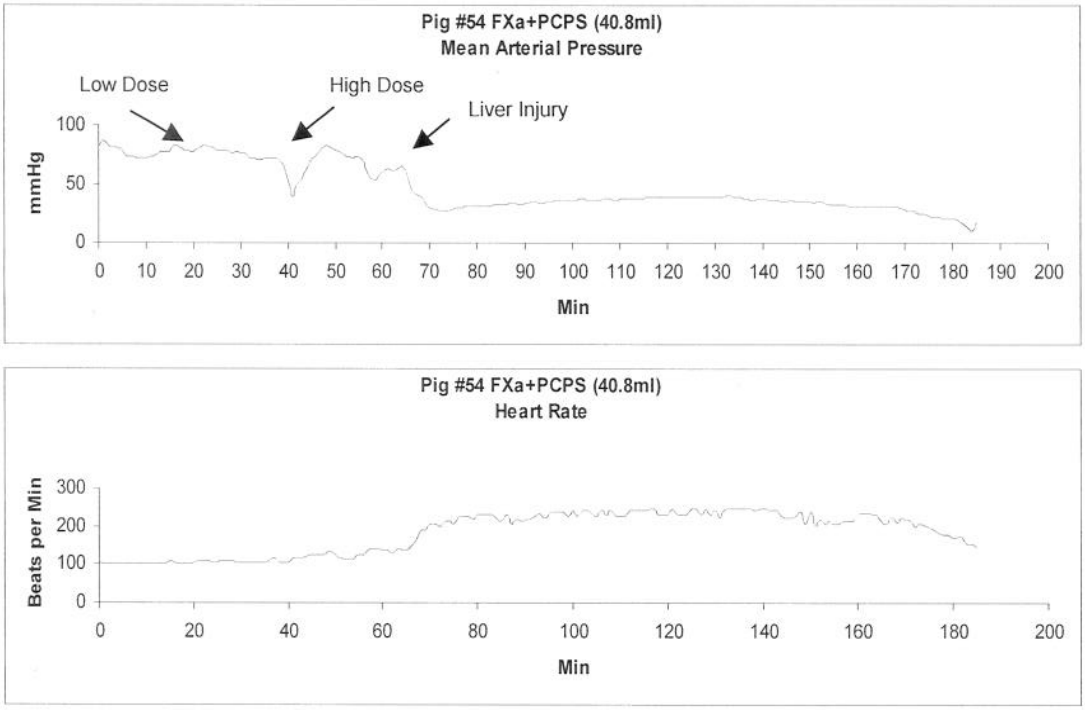
Table 5. BT and coagulation parameters following infusion of various doses of FXa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 2. BT not measured at T4 (post-liver injury). *No data collected due to death.

Pig #	Time Point	FXa (%)	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
20	T1	151.8	94	10.0	15.4	124.4	10.35	255	208
20	T2	18.6	170	14.3	17.0	No clot	1253.42	190	>600
20*	T3	---	---	---	---	---	---	---	---
20*	T4	---	---	---	---	---	---	---	---
21	T1	103.2	103	10.1	15.9	124.4	13.99	274	217
21	T2	103.6	101	10.2	15.2	129.1	28.35	290	227
21	T3	100.8	138	10.8	15.3	102.7	415.39	173	521
21	T4	86.0	107	11.3	16	103.6	105.37	206	---
54	T1	130.4	94	10.5	14.9	120.4	8.69	392	416.7
54	T2	132.6	93	10.5	14.8	124.2	17.39	408	526
54	T3	126.3	105	10.3	14.8	127.6	130.57	441	220.5
54	T4	104.4	92	10.7	14.9	154.7	115.66	402	---
62	T1	130.5	96	10.4	15.8	142.0	8.187	256	204
62	T2	113.4	103	10.4	15.4	127.0	11.411	299	505
62	T3	115.4	95	10.5	15.6	126.2	16.896	252	236
62	T4	105.0	108	10.1	15.5	153.2	54.730	258	---

Administration of these doses of FXa-PCPS also produced dose-dependent hypotensive responses, as shown in Figure 15. In performing BT measurements, we noticed that bleeding from the severed mesenteric vessels would decrease as MAP decreased and would increase markedly as MAP increased as a result of compensatory cardiovascular reflexes. In several of these experiments, bleeding would subsequently continue unabated until our 10-min maximum BT.

Following liver injury, blood loss and survival times were: 624.2 g and 120 min for pig #21; 864.2 g and 112 min for pig #53; and, 1089.6 g and 120 min for pig #62. There were no remarkable pathological findings in these pigs.

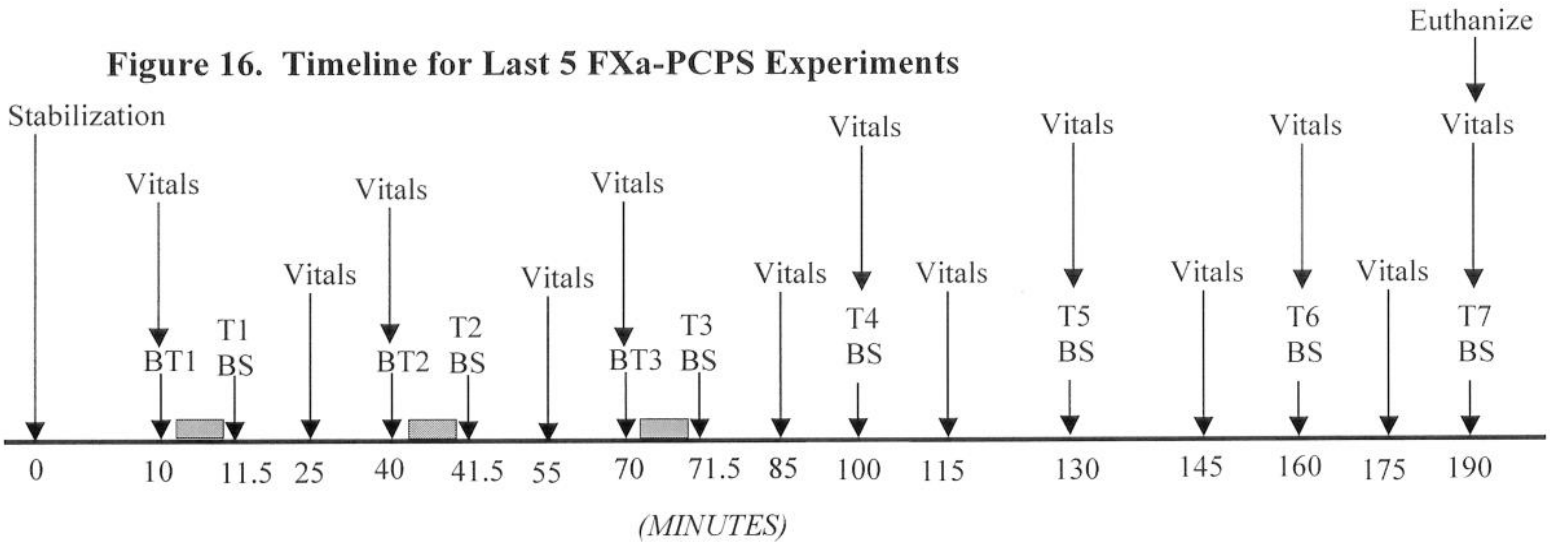
Figure 15. MAP and HR traces for a representative experiment. Following administration of the high dose in this pig, MAP decreased by 37 mm Hg.

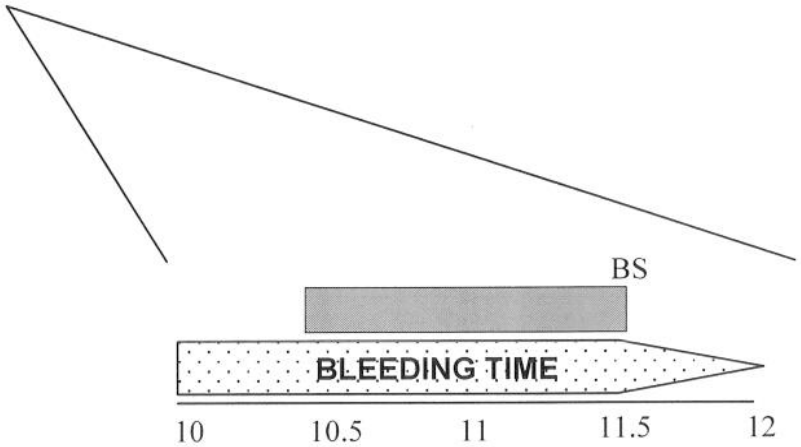


Following completion of these experiments, the decision was made to alter the timeline of subsequent experiments to maximize the chances of observing a beneficial effect on BT, as well as to allow collection of blood samples at a number of time points following drug infusion (instead of performance of a liver injury). In the remaining 5 pigs which received FXa-PCPS, the timeline depicted in Figure 16 was used. One feature of this timeline was the beginning of BT determination 30 sec before drug infusion, to take into account the extremely short half-life of FXa (<15 sec). In two of these pigs (#102 and #103), the low dose of drug (2.5 pmoles FXa/4.0 nmoles PCPS) was given first and was followed by the high dose (25.5 pmoles FXa/40 nmoles PCPS); this order was reversed in the remaining three pigs (#101, #118 and #121). This was done to ensure that administration of the low dose did not consume substrate (i.e., fibrinogen) to a point

which would not allow full expression of any beneficial hemostatic effect from the high dose. Again, this was to maximize the possibility of observing any reduction in BT.

Figure 16. Timeline for Last 5 FXa-PCPS Experiments





Drug Infusion
BT Bleeding Time
BS Arterial Blood Sample
* Taken before death (MAP≤20)

Raw hematological and BT data from this second group of five pigs treated with FXa-PCPS are found in Table 6. In each of the three pigs for which a complete set of BT's was collected, FXa-PCPS markedly increased BT. Pigs #118 and 121 died approximately 5 minutes after infusion of the high dose of FXa-PCPS, an occurrence which falsely lowered their BT's following FXa-PCPS (due to pronounced hypotension; see below). In each of the 5 pigs, infusion of the high dose of FXa-PCPS produced increases in PT, aPTT, and TAT, while decreasing Fib-C and PLT levels.

TEG was also measured in these 5 pigs (Table 7). In pigs #102 and 103, the low dose (administered first) did not seem to profoundly alter TEG parameters from baseline (T2 vs. T1). Subsequent administration of the high dose of FXA-PCPS did not seem to immediately (T3) alter R values, although all other parameters were profoundly changed, such that the MaxVel of clot formation and MA (a measure of clot strength) were both decreased. Thirty minutes after drug infusion (T4), R and K were both lengthened while MA, tMA, and MaxVel were all still depressed. In the only pig that survived administration of the high dose first (i.e., #101), R value was immediately depressed upon infusion (T2) but MaxVel was increased. Subsequently, R, K and tMA were lengthened while α-angle, MA, and MaxVel were lengthened. From these preliminary data and the BT, PT, and aPTT data, we concluded that administration of FXa-PCPS at doses sufficient to produce alterations in coagulation function negatively impacted the ability to clot.

Table 6. BT and coagulation parameters following infusion of various doses of FXa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 7.

Pig #	Time Point	FXa (%)	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
102	T1	128.5	104	10.4	15.5	165.1	5.64	159	296
102	T2	120.7	95	10.5	15.5	164.2	11.24	154	536
102	T3	105.9	90	11.4	17.1	90.5	266.45	46	>600
102	T4	104.8	143	13.3	18.1	83.0	199.23	136	---
102	T5	98.0	125	13.9	18.1	83.0	141.41	132	---
102	T6	101.0	120	13.6	18.1	83.6	114.48	139	---
102	T7	98.7	121	13.2	17.9	88.1	3.47	130	---
103	T1	149.9	97	9.6	15.8	178.6	11.99	431	480
103	T2	148.1	93	9.5	15.6	178.5	20.62	402	413
103	T3	128.0	96	12.5	16.5	83.0	194.16	230	>600
103	T4	140.2	123	12.1	17.5	83.00	176.35	360	---
103	T5	133.7	111	11.5	17.6	83.00	162.23	332	---
103	T6	128.6	113	11.4	17.0	93.10	132.85	344	---
103	T7	133.6	108	11.3	17.6	93.40	115.13	366	---
101	T1	105.9	95	10.9	15.8	122.8	4.15	297	320
101	T2	108.7	88	12.1	15.7	83.0	239.6	372	372
101	T3	97.6	101	13.3	16.6	83.0	154.0	275	477
101	T4	98.0	116	13.3	16.9	83.0	85.9	288	---
101	T5	83.5	108	13.2	17.7	83.0	28.0	291	---
101	T6	95.3	107	12.7	16.7	83.0	21.1	294	---
101	T7	99.8	103	12.5	16.7	86.9	17.9	276	---
118	T1	149.5	84	9.9	15.7	112.7	6.8	188	246
118	T2	150.7	75	11.8	15.8	83.0	180.2	79	145
118	T3	---	---	---	---	---	---	---	---
118	T4	---	---	---	---	---	---	---	---
118	T5	---	---	---	---	---	---	---	---
118	T6	---	---	---	---	---	---	---	---
118	T7	---	---	---	---	---	---	---	---
121	T1	119.0	105	10.7	15.3	96.7	4.7	350	230
121	T2	108.4	242	35.5	28.4	no clot	267.1	68	136
121	T3	---	---	---	---	---	---	---	---
121	T4	---	---	---	---	---	---	---	---
121	T5	---	---	---	---	---	---	---	---
121	T6	---	---	---	---	---	---	---	---
121	T7	---	---	---	---	---	---	---	---

Table 7. TEG parameters following infusion of various doses of FXa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 7.

Pig #	Time Point	R (min)	K (min)	α angle (degrees)	MA (mm)	tMA (min)	MaxVel (mm/min)	tMaxVel (min)
102	T1	2.4	0.9	78.1	65.4	12.4	23.08	3.33
102	T2	1.4	0.8	79.1	67.8	11.7	26.8	2.22
102	T3	2.2		20.3	9.6	10.6	1.76	1.13
102	T4	10.8	2.7	54.3	50.5	25.9	7.74	13.17
102	T5	5.2	1.7	65.5	52.8	18.9	12.28	6.53
102	T6	4.3	1.6	67.6	54.1	16.4	13.66	5.61
102	T7	4	1.4	70.2	54.8	14.8	14.55	5.17
103	T1	2.1	0.9	77.3	67.7	12.8	25.44	3.17
103	T2	1.6	0.8	79.2	70.8	11.4	28.95	2.42
103	T3	1.7	2.1	59.9	35.1	7.7	11.22	3.39
103	T4	6.4	1.7	63.2	53.7	18.6	13.85	7.92
103	T5	3.7	1.4	70.1	57.6	17	15.28	4.78
103	T6	3.7	1.4	68.1	59	17.8	16.22	4.97
103	T7	3.2	1.2	71.6	57.4	16.1	16.69	4.25
101	T1	1.8	0.8	79.5	67.5	12.9	31.03	2.47
101	T2	0.9	1.6	70.4	65.7	13.4	18.12	2.81
101	T3	2.7	1.6	67.1	59.9	17.8	12.58	3.92
101	T4	3.2	1.3	69.8	54.4	15.6	15.44	4.11
101	T5	3	1.3	68.6	58.1	17.4	15.71	4.25
101	T6	2.5	1.1	73.5	59.1	15.7	18.55	3.39
101	T7	2.8	1.2	73.3	61.1	14.2	18.11	3.69
118	T1	2.3	1.1	75.4	66.5	15.2	22.48	3.31
118	T2	1.8	2.1	60.6	57.4	12.8	11.28	3.42
118	T3	---	---	---	---	---	---	---
118	T4	---	---	---	---	---	---	---
118	T5	---	---	---	---	---	---	---
118	T6	---	---	---	---	---	---	---
118	T7	---	---	---	---	---	---	---
121	T1	2.3	0.8	78.8	66.4	12.9	28.67	3.06
121	T2	39.7	9.8	12.8	16.4	57.7	1.32	43.72
121	T3	---	---	---	---	---	---	---
121	T4	---	---	---	---	---	---	---
121	T5	---	---	---	---	---	---	---
121	T6	---	---	---	---	---	---	---
121	T7	---	---	---	---	---	---	---

Figure 17 depicts MAP and HR from a representative experiment in which the low dose of FXa-PCPS was administered first. Figure 18 shows MAP and HR from the only pig that survived infusion of the high dose of FXa-PCPS first (pig #101). In all 3 pigs that survived infusion of the high dose of FXa-PCPS, a profound hypotensive response was observed with this dose, with maximal decreases in MAP of 43 (pig #102), 41 (pig #103), and 42 mm Hg (pig #101). In these pigs, infusion of the lower dose of FXa-PCPS resulted in decreases in MAP of 17, 10, and 14 mm Hg (for pigs 102, 103, and 101, respectively). Figure 19 depicts MAP and HR during an experiment in which administration of the high dose of FXa-PCPS resulted in lethal hypotension.

Again, there was no evidence of DIC or microthrombi formation in these animals.

Figure 17. MAP and HR from a representative experiment in which the low dose of FXa-PCPS was administered first, followed by the high dose.

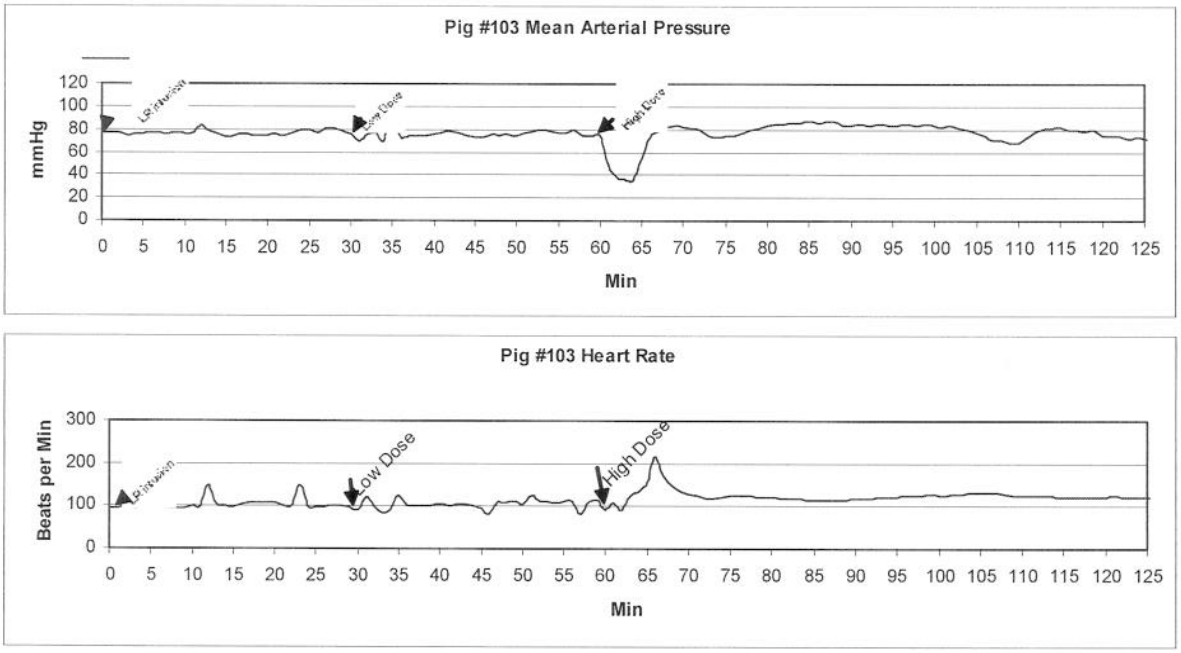


Figure 18. MAP and HR from pig #101, in which the high dose of FXa-PCPS was administered first, followed by the low dose.

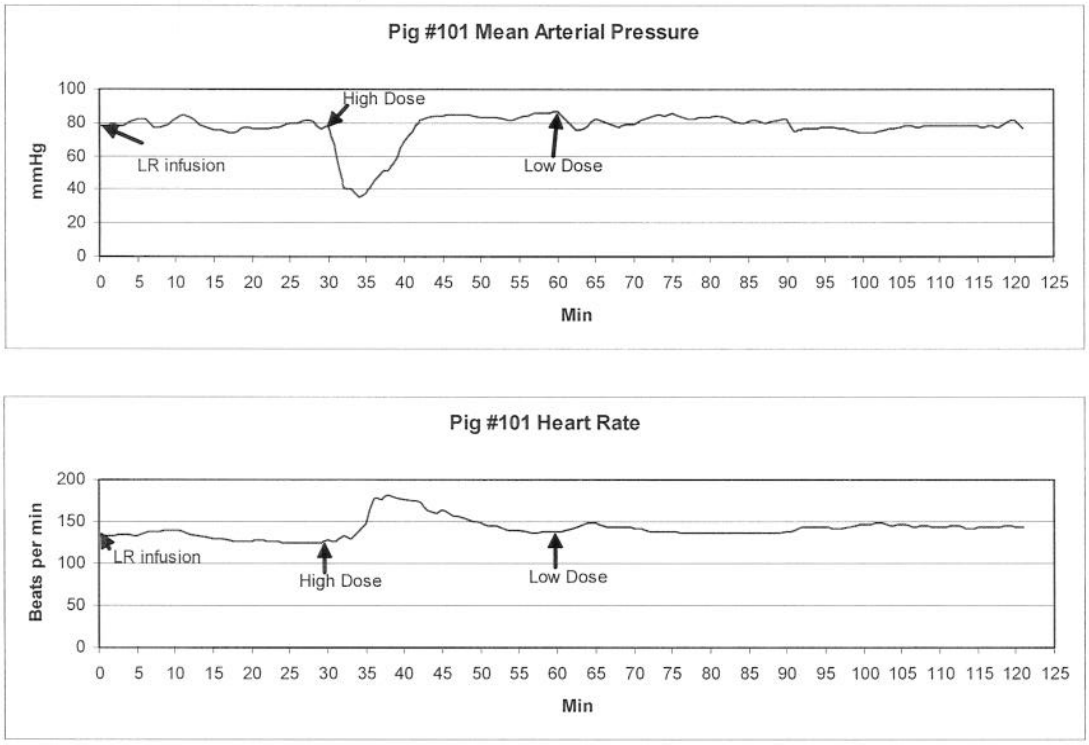
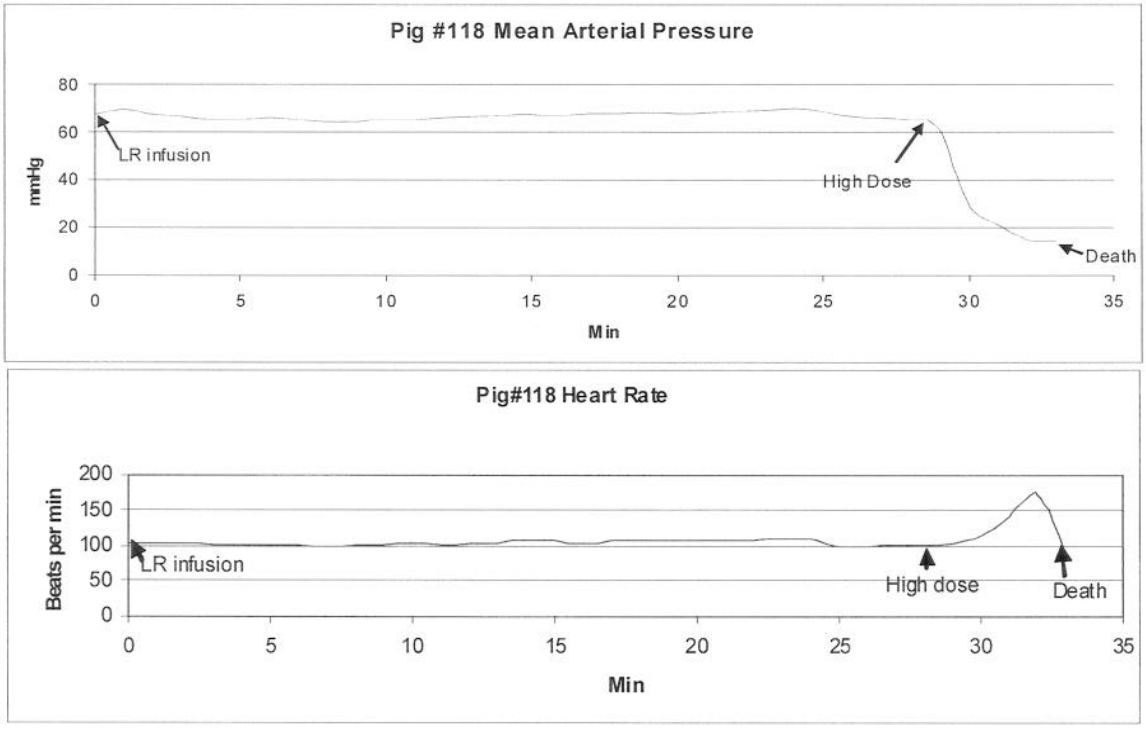


Figure 19. MAP and HR from a representative experiment in which administration of the high dose of FXa-PCPS produced lethal hypotension.



DISCUSSION

Work performed at Haematologic Technologies, Inc.

Porcine models are a valuable resource to test new strategies in the areas of trauma, therapeutic, preventive, early detection, and diagnostic imaging medicine. While the information that has been collected seems to universally depict events as they occur *in-vitro*, there is often a discrepancy with regard to the clinical relevance of the differences found between humans and pigs. This shortcoming however is related to the lack of reagents and assays that are required to perform an extensive analysis and gauge the balance thrombosis and hemostasis. To enhance the utility of porcine coagulation models, a critical evaluation of the important characteristics of porcine models versus that of the human system must be considered in the selection of appropriate models for the study of various research topics. The primary purpose of developing these species comparisons is to stimulate research using porcine models and to develop appropriate standards for characterization; validation and screening of porcine model systems. This will allow researchers to capitalize rapidly on discoveries in all facets of porcine model research and to accelerate the pace of these discoveries using innovative approaches or new technologies. We have provided a solid foundation that will allow us to substantiate the pig as a model for the study of non-compressible hemorrhage.

We have aptly demonstrated that human and porcine Xa are essentially bioequivalent and are therefore interchangeable in our experimentation using the pig as a model to study non-compressible hemorrhage. From this study we can conclude that both porcine and human factor Xa have a similar affinity for the substrate prothrombin in

a purified prothrombinase reaction. We can also conclude that they function similarly with regard to the rate at which they are able to generate thrombin in either the human or porcine system. Likewise they function equivalently in a plasma-based clotting system. Overall we can deduce there appears to be a high degree of cross-species reactivity between the two coagulation systems at the level of factor Xa. Comparing the two systems to one another as a whole we observed rates of porcine thrombin generation as compared to human generation to be faster. The difference in the rates of thrombin generation could have implications in the distant future with the transition from pigs to humans

There has been some difficulty in our ability purify porcine factor VII/VIIa which in turn has hampered our ability to examine the functionality of human VIIa in the porcine system. In the process of developing a purification strategy we have learned of potential structural differences at the level of amino acid sequence or post translational modification. These differences were revealed by SDS-PAGE and western blot analysis of semi-purified samples. Based on the prevalent use of recombinant human factor VIIa for treatment for a variety of bleeding abnormalities that are first evaluated in animal model systems it would behoove researchers to ultimately determine the functional characteristics of this molecule in comparison to other species.

The presence of fibrinogen degradation products (FDPs) in plasma can provide important information for the diagnosis of hemostatic disorders. Increased levels of FDPs are seen in fibrin clot breakdown as well as fibrinogenolysis. Elevated FDP levels are characteristic in disseminated intravascular coagulation (DIC). FDP levels also are seen in acute thrombotic episodes following surgery or trauma. The development of a

specific porcine FDP assay would allow us to further substantiate the pig as a model for use in hemorrhage control experimentation. Additionally, we can study an important parameter to monitor disseminated clotting or micro thrombi formation following treatment with our novel hemostatic drugs. We have made substantial progress towards developing such an assay. An obstacle in setting up and validating this assay was the lack of commercially available antibodies specific to porcine fibrinogen. We have solved this problem by generating our own polyclonal antibody project. Even after the funding period it is our intention to keep pursuing the development of this assay. A highly specific and simple assay system is required to measure the extent of fibrinolysis in the porcine model system. Therefore, the development of a suitable assay system is strongly anticipated.

The primary innovation for the proposed study was to develop of a novel approach for the treatment of non-compressible hemorrhage in battlefield casualties utilizing a mixture of highly purified blood coagulation factor(s) and phospholipid vesicles (PCPS). In this study, we purified and characterized the reagents and drug formulations to ascertain the ability of VIIa/PCPS and Xa/PCPS to enhance hemostatic function in porcine models of uncontrolled hemorrhage. If successful this application would have been well suited for the treatment of casualties in the combat arena. The long-term goal of this proposal was to ultimately develop a pharmaceutical composition for the treatment of acute non-compressible hemorrhage that would save lives and reduce battlefield and civilian mortality.

In conclusion, we have successfully purified protein reagents: Human Factor X/Xa, Human Factor VII/VIIa, Human Factor V/Va, Human Prothrombin, Porcine Factor X/Xa,

Porcine Factor V/Va, Porcine Prothrombin, Porcine Fibrinogen, and a sheep anti-porcine fibrinogen polyclonal antibody. We characterized and assayed porcine and human Factor Xa and established that these protein behave functionally equivalent. Although unsuccessful in purifying porcine factor VII we have established bioequivalence assays for human and porcine VIIa. We have development a porcine specific FDP immunoassay for use in future animal experimentation. Lastly, we have successfully formulated and delivered hemostatic drugs Xa/PCPS and VIIa/PCPS to ISR for the testing of intravenous hemostatic agents in a novel swine model of bleeding.

Work performed at US Army Institute of Surgical Research (ISR)

In this preliminary series of experiments, we were successful in developing a novel animal model in which intravascular hemostatic agents could be screened to determine suitability for further testing for possible treatment of traumatic injury. This model allows the determination of hemostatic responses to escalating doses of drug, an approach that has been used previously in this laboratory [24]. In this earlier study, BT was determined by injury to either the ear or the spleen of swine. Both methods have been associated with logistical problems, including the requirement to maintain ear temperature constant, the inability to perform multiple (>2) BT due to the low surface area available for injury sites, the inability to perform more than 2 replicates at each BT measurement, and the contractile nature of the spleen. The use of the mesenteric BT seems to obviate many of these problems and appears to be more reproducible than previous methods. Because of the evident reproducibility between anatomical locations in the gut, it is apparent that one can make BT determinations with multiple doses of

hemostatic drug. In further support of this contention, blood flow has been shown to be comparable among the porcine duodenum, jejunum, and ileum [43].

The primary blood supply to the porcine intestine is via the cranial mesenteric artery [44]. Within the mesenteries of the small intestine the arteries and veins lie adjacent to each other with a small amount of connective tissue between them [44]. Hence, BT determination using the current technique differs from other methods inasmuch as the mesenteric BT relies on bleeding from small arteries and veins rather than capillary bleeding. Despite this caveat, it is clear that the mesenteric BT performed herein was sensitive to alterations in coagulation function as BT was correlated with alterations in ACT produced by infusion of heparin and protamine. Furthermore, inhibition of coagulation function induced by FXa-PCPS infusion and reflected in alterations in TEG and other coagulation parameters were associated with prolongations of BT. It should be noted that this laboratory is currently performing experiments that will simultaneously determine both mesenteric and splenic BT for validation purposes.

Another feature of this animal model is that it allows, within the same animal, an initial determination of how pretreatment with a drug of interest affects a traumatic uncontrolled hemorrhage. In previous work, intravenous agents have been tested in a swine model (originally developed for testing the efficacy of hemostatic dressings) that included complete transection of major veins (>10 mm in diameter) of the liver [29,45-47]. While entirely appropriate for testing hemostatic dressings, it is questionable whether such a model is appropriate for testing intravenous hemostatic drugs, as it is improbable that such drugs could be highly effective in such a situation (i.e., big holes in big vessels). Additionally, this previously-used model required aggressive resuscitation to produce

exsanguinating hemorrhage leading to death. Again, such resuscitation is not entirely appropriate for testing intravascular agents as it leads to dilution of the intravascular agent under study. Because of these considerations, we sought to develop a severe liver injury model that 1) was exsanguinating without either heparinization or aggressive resuscitation; and, 2) did not involve transection of the major veins of the liver, but instead produced more diffuse bleeding from smaller vessels. Additionally, we desired an injury that produced a mortality rate of approximately 50% in order to discern whether the drug under study had either beneficial or detrimental effects on survival. The selective excision of approximately 45% of both the left medial and lateral lobes described herein achieved these goals as it produced 60% mortality within 2 hours via transection of predominantly small ($\leq$ 4 mm diameter) hepatic veins. Furthermore, this mortality rate was achieved without using either aggressive resuscitation or heparinization. We therefore believe that this model is a reasonable one for testing of intravenous hemostatic agents.

Having developed a model, we then sought to determine the efficacy of either FVIIa-PCPS or FXa-PCPS to reduce blood loss and enhance coagulation function. The available doses of FVIIa-PCPS did not seem to alter either hemostatic function or BT in these limited trials, despite the fact that such concentrations were effective in enhancing coagulation function *in vitro* [24]. These doses of FVIIa, however, were much lower than those previously shown to be efficacious for recombinant FVIIa in swine [29]. Whether the addition of PCPS to higher doses of FVIIa would shift the dose-response curve for FVIIa to the left (demonstrating increased potency) is a question that remains unanswered due to lack of the drug in these amounts.

The question of whether FXa-PCPS might be useful in traumatic indications has been answered to our satisfaction by these pilot experiments. Administration of FXa-PCPS produced detrimental effects on coagulation function (as assessed by TEG and standard coagulation tests) and on BT. The range of doses used in these preliminary experiments had previously been found to be efficacious in both normal and hemophilic dogs with cuticular bleeding [26,27,48,49]. Furthermore, similar doses had also been used in rabbits [25,49] and nonhuman primates [48,49]. In these experiments in pigs, however, these doses appeared to produce a consumptive coagulopathy, as suggested by the observed decreases in fibrinogen and platelets and associated detriments in coagulation function.

Additionally, we observed that administration of doses required to alter coagulation function also produced profound hypotension; although the decrease of blood pressure appeared to be dependent on dose, we were unable to find a dose in these pilot experiments that provided beneficial effects on coagulation function without producing unacceptable levels of hypotension or coagulopathy. During experiments, we observed that bleeding from the mesenteric vessels used for BT determination seemed to decrease as blood pressure decreased, but then increased again as blood pressure increased. It is therefore possible that cessation of cuticular bleeding in dogs could have also been associated with a decrease in blood pressure, although this was not measured [26,27]. Dr. Michael Nesheim also stated that a hypotensive response was often seen in nonhuman primates, although this has not been published (personal communication). Perhaps this profound hypotension should have been expected on infusion of FXa-PCPS, as elevation of FXa levels should increase systemic levels of thrombin (as suggested by the observed increases in TAT), which has previously been shown to produce vasodilation and

profound hypotension [50]. Regardless, the profound hypotension induced by FXa-PCPS is not acceptable for treatment of patients who have suffered traumatic hemorrhage and may already be hypotensive.

To conclude, we have successfully developed a novel swine model for preliminary screening of intravenous hemostatic drugs. Using this model, we tested the ability of FXa-PCPS to decrease BT and enhance coagulation function. Rather than providing beneficial effects, FXa-PCPS actually induced coagulopathy and unacceptable decreases in blood pressure. Based on these preliminary results, we conclude that further testing of this drug for the indication of traumatic bleeding is unwarranted.

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