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Report Title

Evaluation of Premarin in a Rat Model of Mild and Severe Hemorrhage

ABSTRACT

This report details a cumulative account of our research findings for the DARPA SBL program. Our rat model system enabled us to assess the effects of various soluble estrogens (cyclodextrin microencapsulate 17beta-estradiol [E2], sulfate conjugated E2, Premarin® and ethynylestrogen-3-sulfate [EE-3-SO4, the formulation of choice]). EE-3-SO4 has also been selected for IND application. For E2 we evaluated optimum doses and routes of entry, determining by pharmacokinetics that a 1 mg/kg dose delivered via intravenous or intraosseous routes was optimal. The high efficacy of E2-SO4 and EE-3-SO4, precluded combinatorial testing with other drugs as being difficult to evaluate and nonproductive. The mode of action for E2-SO4 with severe hemorrhage was evaluated with several techniques. MRI revealed that ATP production following E2 treatment was maintained at a level to sustain life, as was the prevention of lowered intracellular pH. SPECT-CT confirmed the enhancement of cardiac performance with E2 treatment, a finding corroborated by positive dP/dT measurements. Cardiovascular benefits from E2-SO4 and EE-3-SO4 treatment were also demonstrated in vitro with isolated vascular rings, confirming reduced vascular resistance. The rings were also used to confirm that the relaxation was mediated by nitric oxide. Finally, it appears that steroid sulfatases are not involved in “activating” E2.

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Number of Papers published in non peer-reviewed journals:

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Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Patents Submitted

Methods and Compositions for Treating Trauma-Hemorrhage Using Estrogen and Derivatives Thereof

Patents Awarded

Awards

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<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
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Total Number:	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Irshad H Chaudry	1.00	No
William J Hubbard	1.00	
FTE Equivalent:	2.00	
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Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PHDs

<u>NAME</u>
Total Number:

Names of other research staff

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Dharmalingam Prahadeeswaran	0.10
Joel Robertson	0.52
Harry Mahtani	0.10
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Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

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Statement of the Problem Studied

Our research is directed toward enabling survival from severe blood loss brought about by traumatic injury in the battlefield. Our model system in rats is based on soft-tissue trauma (midline laparotomy) in conjunction with 60% blood loss; conditions selected to replicate battlefield blood loss in a precise and reproducible fashion. The problem is additionally constrained to limit the volume of drug delivery to a level well below that which would constitute resuscitation, since resuscitation is not practical for self or buddy administration. This limitation was also placed on the therapy so as to not overly burden the warfighter's field pack's capacity of weight or space. Also of note for the problem is a requirement that the drug be administered as a single dose. Finally, the most significant aspect of the problem as specified by DARPA was that animals must be able to survive for 3 hours without fluid resuscitation following severe blood loss. This specification was extended to 6 hours in follow-on rounds of funding.

To these ends, we have addressed the problem through the administration of estrogen. Since it is requisite that the estrogen possess both maximum potency and be water soluble, we initially used soluble 17β -estradiol (cyclodextrin microencapsulated or sulfate-conjugated). The latest iteration of the drug is ethynylestradiol 3-sulfate (EE-3-SO₄), where the ethynyl moiety increases potency due to a longer half-life, and sulfate conjugation confers solubility. The drug can be delivered either intravenously or intraosseously, the former being made possible by the training of a field medic and the latter being deliverable with an autoinjector by the wounded warfighter or his or her buddy.

Introduction. The DARPA “Surviving Blood Loss” (SBL) program follows the agency’s proven paradigm of sponsoring and skillfully managing high-risk/high-payoff research. In this instance, there was a simple and unequivocal goal put forward, namely to produce a treatment that enables an experimental animal to survive for several hours after removal of 60% of its peripheral blood. Because this extent of blood loss would be almost universally fatal, the challenge was daunting. Furthermore, the therapeutic material must be delivered after blood loss in a small volume that would in no way be considered resuscitation. Thus while no complexity is required to describe and specify the goals of the program, its realization proved quite formidable. Finally, the program managers stated from the outset that the emphasis was single-mindedly on survival. Mechanistic details would be welcomed, but such experimental data was to be considered secondary to efforts directed toward attaining the maximum numbers of survivors. This point was made clear by the expectations put forth by DARPA. The first iteration of the research program called for a rat model, where the treated animal was expected to survive for 3 hours. For the funded research teams that survived this first round of trials, the follow-on Phase II round’s specifications called for 6-hour survival.

Body of Report. In this document we will detail accomplishments for our entire DARPA funding period in the following areas dealing with 60% blood loss.

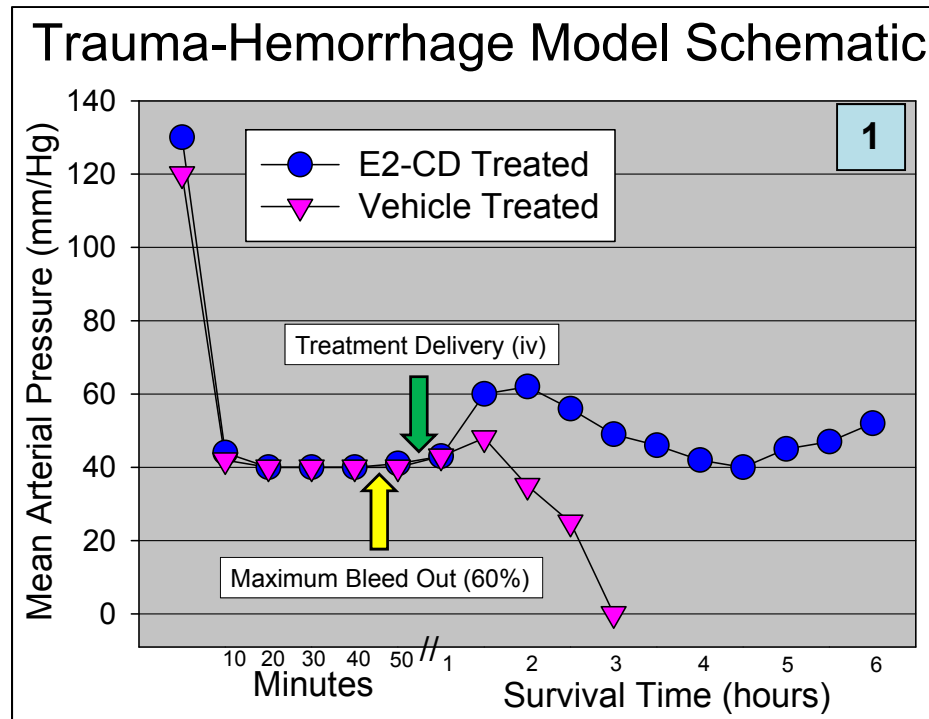
- Model system and 3-hour survival studies
- Combinatorial testing of 17β -estradiol (E2) with glucosamine, flutamide, DHEA, HBOC and ATP-MgCl₂
- 6-hour survival studies
- Efficacy of different soluble estrogens
- Determination of optimal estrogen dose based on survival
- Evaluation of Premarin[®] as an estrogen source
- Assessment of effectiveness for various routes of administration and their pharmacokinetics
- Testing of soluble estrogen in the pig
- Procurement of pharmaceutical grade EE-3-SO₄

In addition to survival data, we have explored features of basic science which offer insights on the mode of action of estrogen(s), and aspects that might enable greater optimization of the therapy. These include:

- Relevant physiological and biochemical markers for vehicle control and estrogen-treated rats
 - Magnetic nuclear spectroscopy measurement of ATP and intracellular pH in hemorrhage model rats
 - Improvement of cardiac performance with E2 following hemorrhage

- SPECT-CT ejection volume
- Positive dP/dt measurements of left ventricular performance
- Biochemical markers for hemorrhage in vehicle control and EE-3-SO₄-treated rats
- Rat pharmacokinetic studies for FDA preclinical support data
- *In vitro* correlates of the survival response using isolated vascular rings
 - Isolated vascular rings and relaxation responses to estrogen(s)
 - Vascular rings responding to estrogen via NO-mediated relaxation
 - Use of vascular rings to measure the activity of soluble and insoluble 17β-estradiol
- Insights into the role of steroid sulfatase in modification of EE-3-SO₄
- Estrogen treatment of hemorrhage is actually a triple therapy for injury and sepsis

Basis for the Model and Experimental System. The model system used to generate the data is shown in **Figure 1**. Briefly, it is the same system as reported for both Phase I and II studies. The model involves induction of hemorrhagic shock in male rats by rapid withdrawal of peripheral blood obtained through an arterial catheter, to a level calculated to be 60% of the circulating blood volume, based on body weight. At the time



point just after maximum bleedout (MBO) has been attained, the test agent is administered through a venous catheter. Blood pressure and heart rate are continuously monitored and recorded via a third (arterial) catheter, connected to a digital instrument and data logging workstation. The above figure reveals

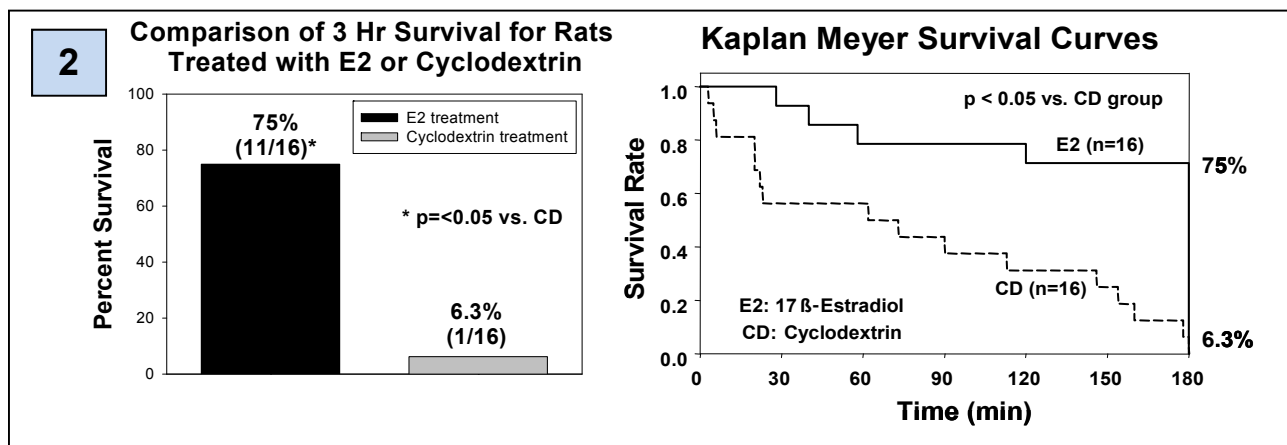
the pattern of survival for a rat treated with cyclodextrin-microencapsulated E2, and death with a control rat receiving cyclodextrin (vehicle only). As previously, we selected “empty” cyclodextrin, which is an otherwise innocuous cyclized glucose polymer, to be used as the vehicle control, since it is the molecule used to microencapsulate the E2.

This complex is formed when E2 is “docked” within the hydrophobic center of a cyclodextrin “cage.” The advantage of cyclodextrin microencapsulation is that it confers excellent water solubility, bioavailability and stability to its guest molecule. As previously noted, it should be stressed that the volume in which the E2 is delivered is too small (~150 μ l/rat) to function as resuscitation, and thus the rats are in a state of hypotension for the duration of the 3 or 6 hours of observation.

Finally, with this model we determined early on that if fluid resuscitation was provided after 3 or 6 hours following trauma-hemorrhage (T-H), the surviving animals observed for an additional week showed no signs of deficits or physiological impairment, so this observation was discontinued.

Results: Phases I and II

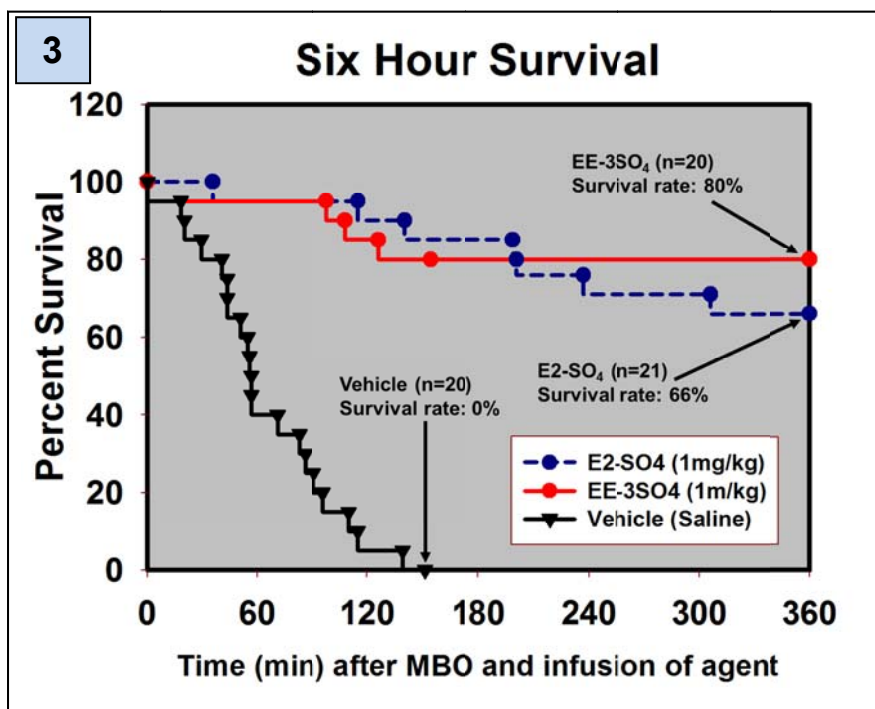
DARPA 3-hour Survival Goal and our Rat Model. The DARPA Phase I of the SBL program set as a target a goal of promoting survival of serious hemorrhage (i.e., 60% blood loss). While this was admittedly a difficult aim, we added additional stress in the



form of a midline laparotomy (soft tissue trauma), in order to simulate an injury that would precede and promote the loss of blood. We were able to satisfy the critical specification for the program, namely to enable a majority of rats to survive 3 hours subsequent to this soft tissue trauma plus 60% hemorrhage. We proposed to use E2, made soluble by microencapsulation in β -cyclodextrin. The rationale for this estrogen treatment approach was based on our laboratory’s initial observations of enhanced T-H survival in proestrus female mice, where levels of estrogen are at their highest. In addition, follow-up experiments confirmed the gender-independent benefit of E2 administration by promoting T-H survival in oophorectomized female and male rats. The results are shown in **Figure 2**, in the form of a bar graph and step survival plot. Based on these clear data, DARPA project managers deemed the remainder of the additional objectives from the original statement of work unnecessary, and advanced our project to Phase II.

Combinatorial Testing. As noted above, the DARPA managers found our results conclusive enough to waive further experiments we had proposed, namely to enhance survival using combinations of E2 with drugs or metabolic support. In addition, since our surviving rats were already at a high percentage, it was apparent that it would be difficult to demonstrate statistically significant improvements with E2 combinations. This likewise would require large numbers of test subjects, which would not have been a humane use of the rats. These bypassed combinations involved other hormones and antagonists (DHEA and flutamide) or metabolic support (HBOC-205 blood substitute, glucosamine and ATP-MgCl₂).

6-Hour Survival. As noted above, a major Phase II objective for rat studies was to double the survival evaluation period from 3 hours to 6 hours. The outcome of these experiments was quite positive, in that we saw a survival rate of 80% in animals treated with EE-3-SO₄, where vehicle controls showed a survival rate of 0%. Results for 6-hour survival are presented in the graph, **Figure 3**. Also presented is the survival



rate for rats treated with an alternate soluble form of estrogen, E2-SO₄ which produced a survival rate of 66%. Reasons for its inclusion are discussed below.

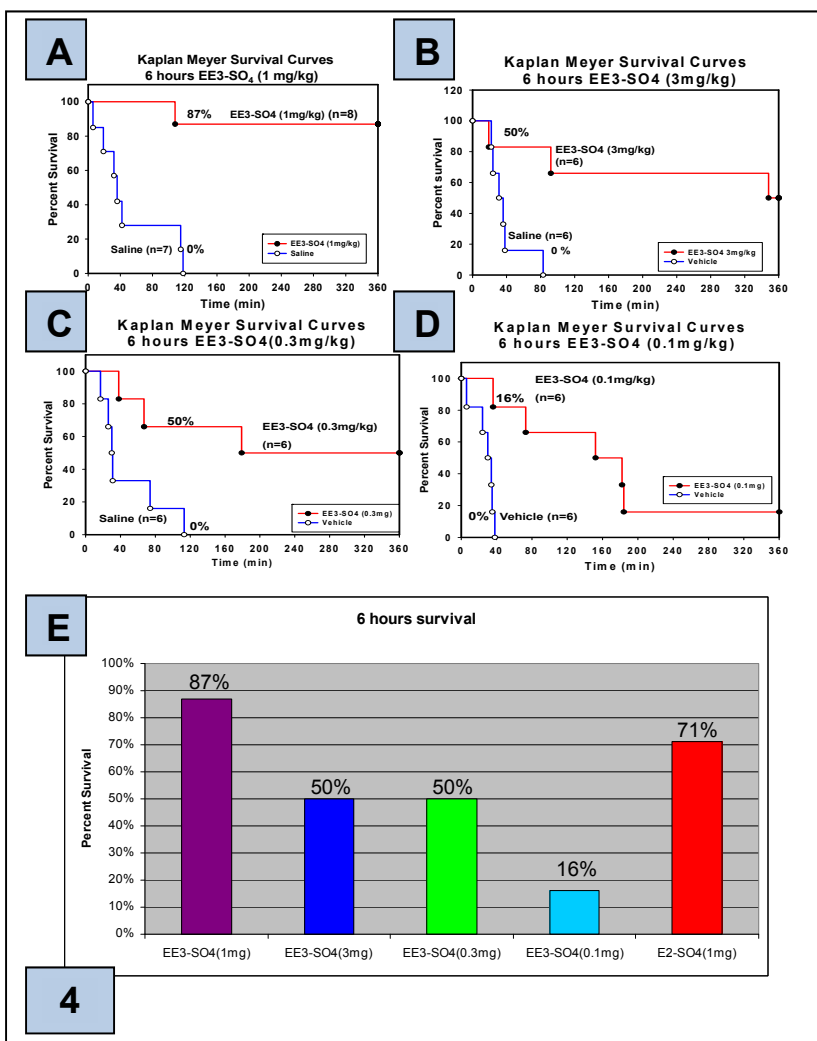
Efficacy of Different Soluble Estrogens. As can be seen, the presence of an ethinyl group on E2 improves the survival of test rats, most likely due to the longer half-life of ethinyl-estrogen-SO₄. The selection, evaluation and possible acquisition of various forms of soluble estrogen was an extensive and time-consuming process. It became apparent that availability of the drug in a GMP preparation was in fact the rate-limiting step. Thus, while it was clear that most soluble form of estrogens was effective in promoting survival, none were available “off the shelf” in a pharmaceutical grade. Those we attempted to acquire were E2-CD, E2-3-sulfate, E2-3,17-disulfate and E2-glucoside. All acquisition efforts failed. We also evaluated an alternative natural estrogen conjugate, E2-glucuronide, and found it to be lacking any survival-promoting activity.

Evaluation of Premarin® for use in the SBL program will be presented separately in a following section.

We were encouraged to evaluate EE-3-SO₄ at the suggestion of our consultants. Since the sulfated form was not available, we began tests with a custom synthesis. This preparation was indeed found to have superior activity. This has ultimately led to a path of custom synthesis of pharmaceutical grade material, which will be the basis for applying for investigational new drug (IND) status from the FDA.

Optimization of Estrogen Dose. The data in the composite graph below describes dose optimization based on 6-hour survival results. These results were also derived

from the most effective dose of EE-3-SO₄, i.e., 1 mg/kg (87%, **Figure 4A**), that being the dose which produced the greatest survival rate. Of note, in this set of data the 6-hour survival rate is higher than that presented in the 6-hour survival section preceding this study. The difference of 87% vs. 80% is most probably accounted for by sample size, which was n=6 for the latter vs. n=20 for the former. Overall, the superiority of this 1 mg/kg dose is evident when one examines survivals attained with the other doses selected for evaluation, namely 0.1 mg/kg (16%, **Figure 4B**), 0.3 mg/kg (50%, **Figure 4C**) and 3 mg/kg (50%, **Figure 4D**). It

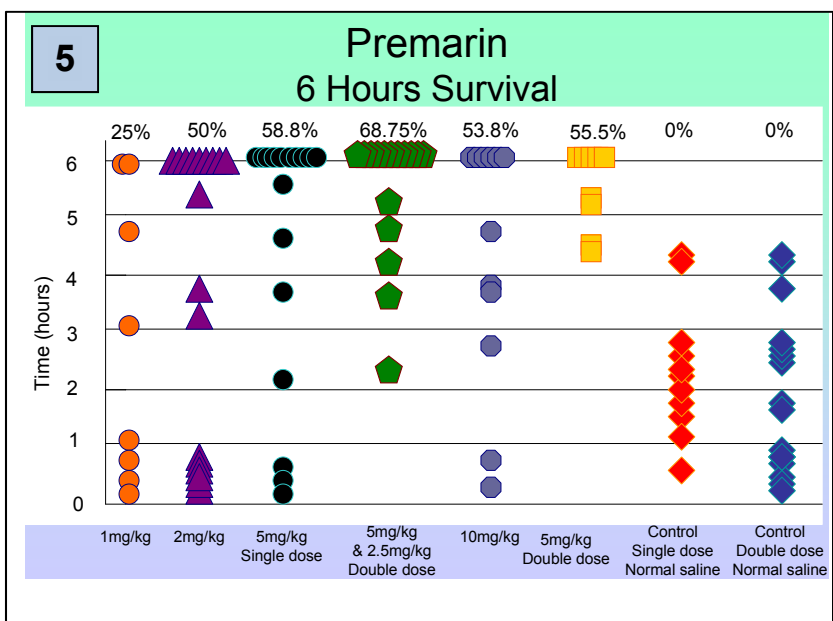


should be noted that all of the saline vehicle controls were comparable among the 4 groups. Finally, the apparent higher efficacy of EE-3-SO₄ as compared to E2-SO₄ is evident in a bar graph which compares the range of doses and formulations (EE-3-SO₄, 4 bars on left and E2-SO₄, single red bar, right (**Figure 4E**). While EE-3-SO₄ is clearly the best formulation, it is not conclusively known whether the difference observed

between EE-3-SO₄ and E2-SO₄ represents higher efficacy *per se*, or a difference in specific activity or purity between the different products, since these formulations were compared on a mol/mol basis. Analyses to resolve this question are beyond our capacities, and furthermore are no longer germane since the DARPA team has selected EE-3-SO₄ as the candidate formulation.

Evaluation of Premarin® as an Estrogen Source. To our knowledge, Premarin® is the only soluble estrogen approved for clinical use, including iv administration. As such, besides its long-standing use as hormone replacement therapy, it has frequently been used experimentally as an approved biological.

However, it has the problematic aspect of being a complex admixture of a large variety of sulfate-conjugated estrogens and estrones, the composition and proportions of which vary, and are not completely divulged by the manufacturer (Wyeth Pharmaceuticals, Madison, NJ), as it is closely held as a trade secret. These conjugated estrogens are



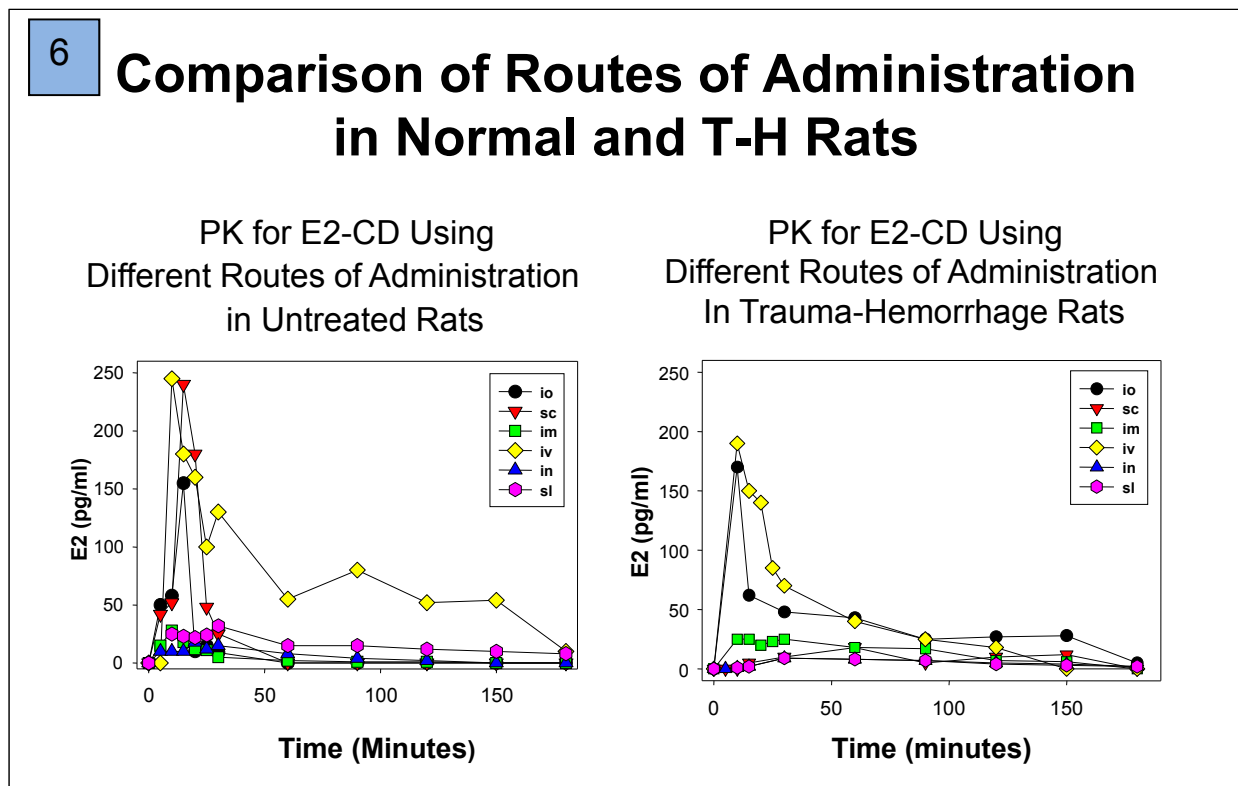
derived from the urine of pregnant mares, hence its name (pregnant mare's urine). Complexity notwithstanding, because it was a clinically approved biological, it was a candidate for use in the SBL program.

As mentioned above, Premarin® is a venerable hormone replacement therapy preparation that additionally has been approved for use to treat uterine bleeding¹, delivered as a high-dose iv injection. Interestingly, as detailed earlier, this same Premarin® injectable formulation has been used experimentally in humans, notably males, to demonstrate lowered vascular resistance, improved cardiac performance and rapid enhancement of microcirculation and following iv administration²⁻⁵, supporting a military application for treatment of severe blood loss in males with estrogen (Premarin® specifically and by inference, E2 in general).

It is important to reiterate that application of Premarin® to protect from severe blood loss has one problematic aspect, namely that Premarin® is a natural product comprised of a mixture of various conjugated estrogens and estrones. Thus, our comparison was between a single estrogen species (at that time, E2-CD) and an essentially unknown

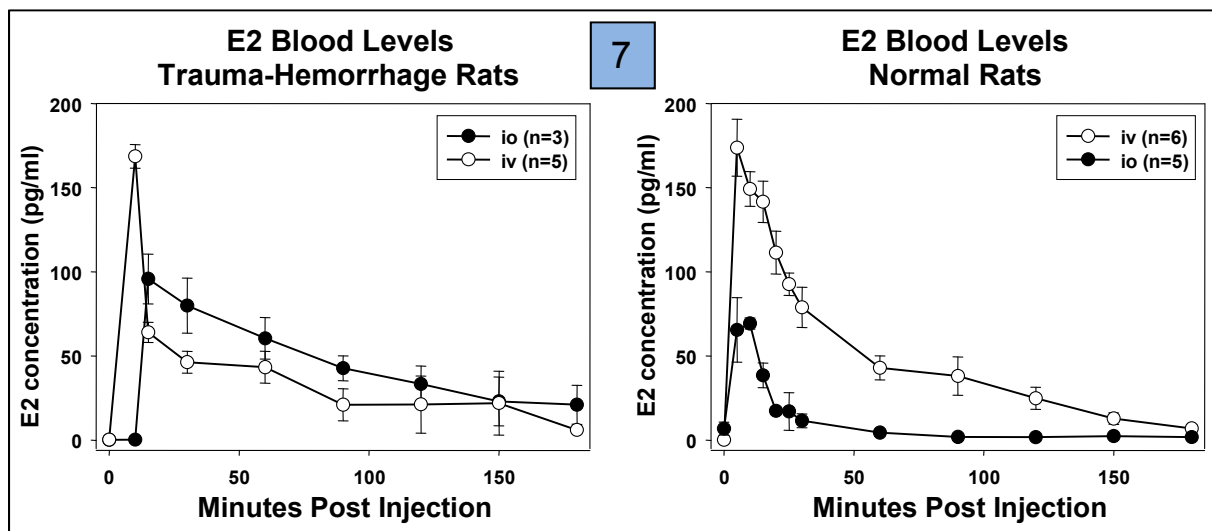
mixture. As such, in order to bracket the relatively unknown dose of estrogen(s) in Premarin[®] vis-à-vis our standard 1 mg/kg dose of E2, we evaluated Premarin[®] at single and multiple doses. Results are shown in **Figure 5** (previous page) for Premarin[®] (only) employing single doses (1, 2, 5 and 10 mg/kg) and double doses (5+5 mg/kg and 5 + 2.5 mg/kg). The second dose was delivered at 3 hours (after maximum bleedout is attained). It is clear that Premarin[®] has high efficacy in promoting 6-hour survival after 60% blood loss in rats (~69% vs. 50% for E2-CD). Thus, Premarin[®] specifically, or sulfated estrogens by inference, showed promise as agents to promote survival from severe blood loss. Premarin[®], as available, comes in a vial that contains only 25 mg of the test substance. Thus, in order to give 70 kg humans a dose of 5 mg/kg (highest single dose that was effective in improving survival), it would require 350 mg, i.e., 14 vials of Premarin[®]. Clearly it will not be possible for soldiers to carry this many vials with them in the far forward situation. In addition to the limitations of carrying so many vials per trauma victim, the practical aspects of using Premarin[®] were derailed because negotiations with Wyeth were fruitless. In particular, they were explicitly not interested in working with the SBL program, presumably because of the small niche market and liabilities that may arise.

Pharmacokinetics and Routes of Administration. Through a series of meetings between UAB investigators, DARPA staff and consultants, it was determined that we should conduct experiments in the rat to find the optimum route of administration for E2-



CD. As seen in **Figure 6**, the routes selected were intravenous (iv, yellow),

subcutaneous (sc, red), intramuscular (im, green), sublingual (sl, magenta), intranasal (in, blue) and intraosseous (io, black). Our first objective was to examine pharmacokinetics for the different routes. Blood samples were taken at regular intervals and the concentration of E2 was determined by ELISA. The results above clearly showed that in terms of either maximum concentration of E2 in the peripheral blood and the rapidity of delivery of E2 into the blood, iv and io routes were superior, as measured in either normal animals or T-H rats. In fact, as is seen in **Figure 6**, iv and io were indeed the only routes that delivered therapeutic levels of E2 to the bloodstream (normal rats in left panel, T-H rats in right panel). More detailed studies of the iv and io administered E2 in normal and T-H rats shows good efficacy for both routes, with iv providing the best delivery of E2 to the peripheral blood. The kinetics are essentially the same for both routes, either in normal or T-H rats. This is shown in **Figure 7**, which details blood levels of E2 in T-H rats (left panel) and normal rats (right panel).



Testing of Soluble Estrogen in the Pig. The phase II SBL specifications called for continued rat experimentation plus evaluation of survival from hemorrhage in a large animal model. Sinclair mini-pigs were selected as the test subject. Preliminary evaluations began at ISR in San Antonio and at our institution. While useful preliminary data was generated (data not shown), the DARPA advisory team deemed it most efficient and timesaving to conduct pig experiments in a Good Laboratory Practice (GLP) facility in order to have appropriate data for an FDA IND application. Thus all experimental swine testing was relocated to Texas A&M University (TAMU), which has established a contract research GLP facility. At this juncture, the SBL program was reconfigured with DARPA program management, consultants, and investigators from UAB and TAMU as a team focused on ultimately conducting clinical trials with hemorrhaged wounded warfighters to be treated with EE-3-SO₄. The obvious spinoff benefit to civilian application of this therapy for military trials was also factored into this

working group. Given this separation of efforts, the progress reporting for the pig and FDA applications will be done under separate cover.

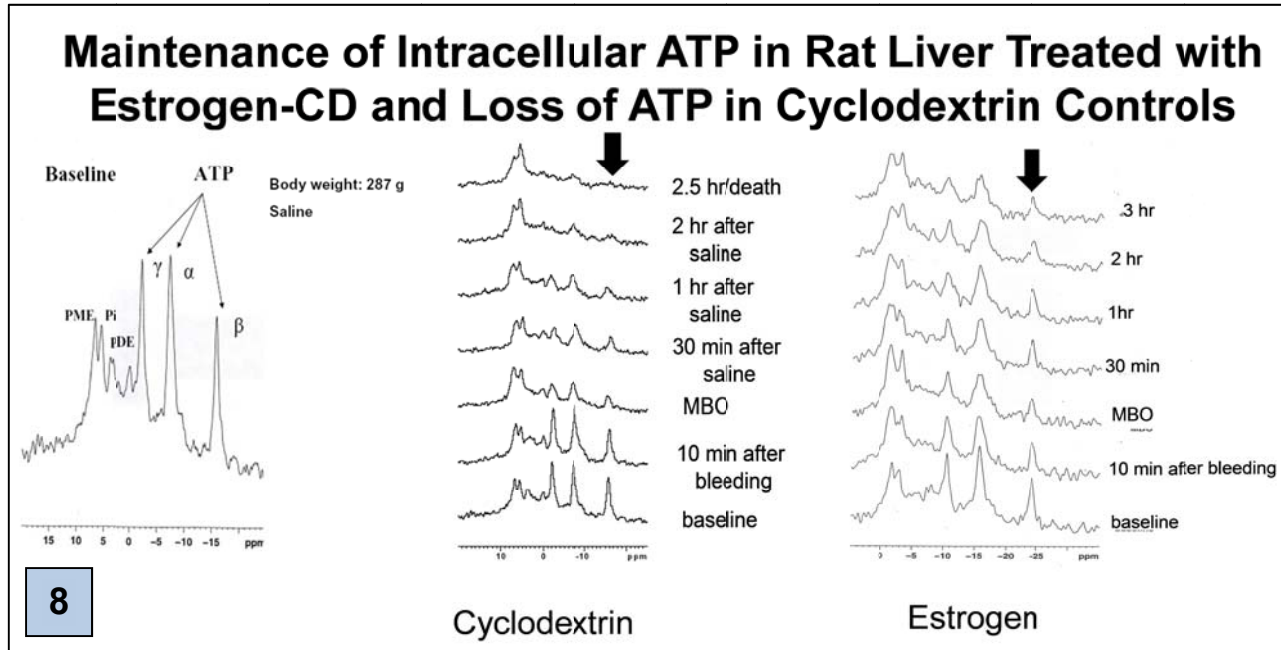
Procurement of Pharmaceutical Grade EE-3-SO₄. It should be noted that a significant non-experimental goal was met as a team effort between DARPA staff, consultants and UAB, namely the obtaining of a soluble estrogen which could satisfy the FDA requirements to be administered to patients. Failing this, we would have had a significant (and ultimately prohibitive) barrier to going forward with both animal studies and future clinical trials, since for obvious reasons animal testing must be performed on the drug of choice for clinical investigations. By way of background, our treatment for severe blood loss requires that a slow infusion or a bolus of estrogen be delivered iv in a minimum volume of vehicle, at a supraphysiologic concentration, at or about a dose of 1 mg/kg body weight. This delivery is only possible with a freely soluble estrogen, since the native (i.e., unconjugated) form of estrogen is highly hydrophobic, and cannot be delivered iv.

This process of first selecting an appropriate soluble formulation and subsequently locating a competent laboratory to synthesize the drug was accomplished in large part by the tireless efforts of Dr. Kaveh Zamani, who diligently explored avenues for obtaining a potential GMP (good manufacturing practice) formulation of estrogen through numerous contacts and businesses. Through multiple iterations of potential formulations, our team was able to settle on sulfate-conjugated ethinyl estradiol as the drug of choice. As noted, prior to obtaining this particular compound through a custom synthesis funded by DARPA, we have previously used off-the-shelf E2 sulfate or microencapsulated cyclodextrin E2 (E2-CD and E2-SO₄, respectively) obtained from Sigma Chemicals (St. Louis, MO). While efficacious, neither of these formulations would ever be a candidate for clinical use, owing to their lack of being available (i.e., off the shelf) as a GMP preparation.

Ethinyl estradiol is in widespread use as a drug for hormone replacement therapy in post-menopausal women, where the ethinyl moiety confers a longer half-life and thereby extends exposure to the hormone. Regarding sulfate conjugation as applied to this synthetic estrogen, it constitutes the utilization of the natural biochemical means of processing by the body to solubilize estrogen for either excretion or reutilization, and as such would be comparable to the conjugated estrogens present in such widely used hormone replacement preparations such as Premarin[®] (Wyeth Laboratories). Our test article was synthesized initially by Panslavia Chemicals (Milwaukee, WI) and has progressed to a GMP facility (Sigma Aldrich Chemicals, St. Louis, MO).

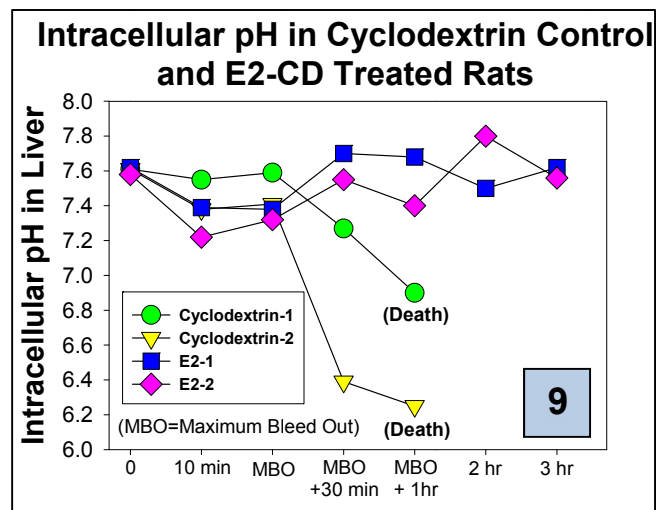
Rat Physiological and Biochemical Profile in Response to Estrogen-Treated Hemorrhagic Shock. In conjunction with the survival studies, we have undertaken monitoring of physiological performance in E2-SO₄- and EE-3-SO₄-treated rats with

paired vehicle-treated controls to shed light on the nature of protective effects of estrogen on severe blood loss. Previous experiments pointed toward several areas where E2-SO₄ and EE-3-SO₄ appear to exert their effects. Most notably, these are reduced vascular resistance (reported below in *in vitro* vascular ring studies), improved cardiac performance and enhanced mitochondrial function.



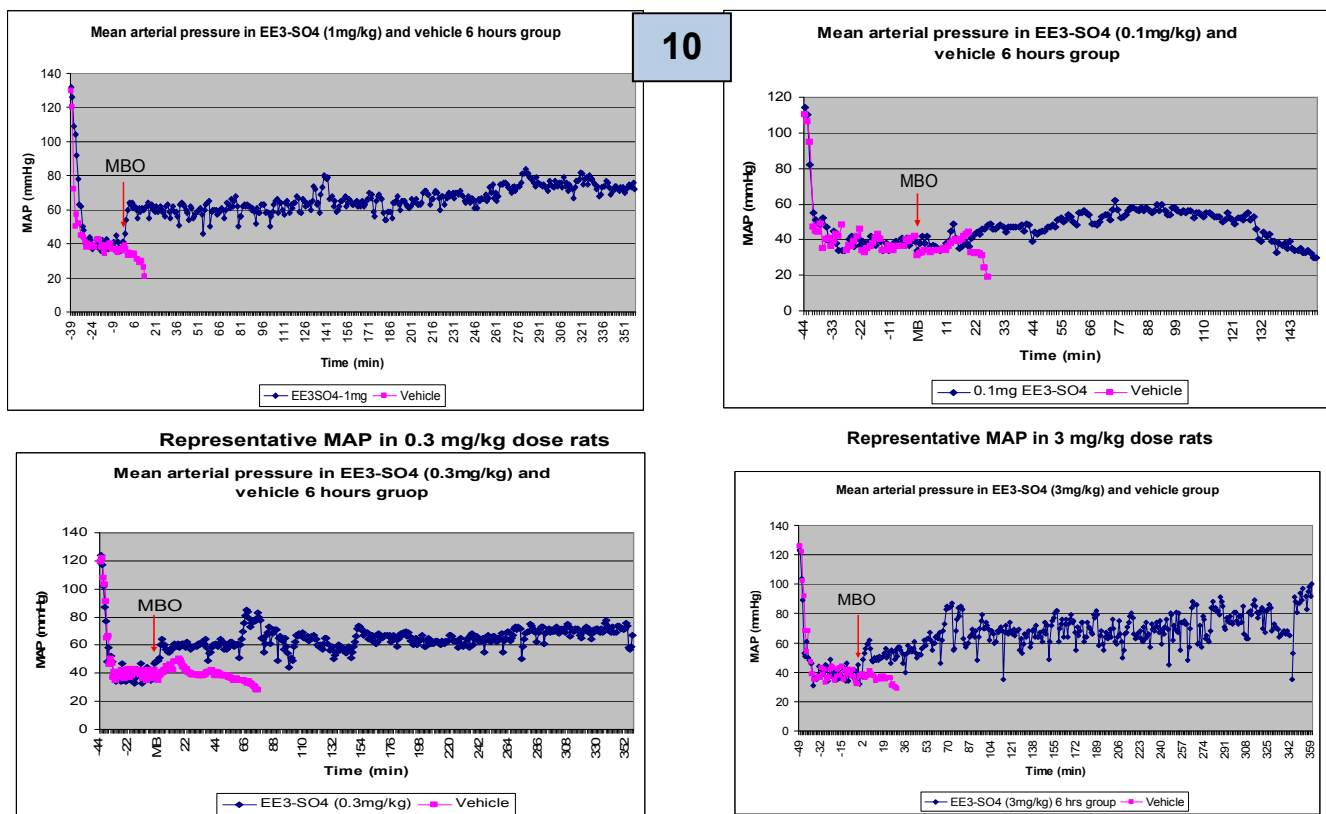
Magnetic Resonance Spectroscopy examining Liver ATP and Intracellular pH. Instrumentation for magnetic resonance imaging (MRI) can also be utilized for magnetic resonance spectroscopy (MRS), which is able to follow the presence of molecules of interest in a semi-quantitative fashion in real time. Utilizing this technology, we could assess the E2-CD-treated and untreated rat metabolic performance as ATP production and metabolic stability as intracellular pH, both being calculated from proton resonance signals, measured with a custom-fabricated coil placed atop the rat's liver. As seen in

Figure 8, there was sufficient amount of ATP production sufficient to maintain viability of rats following 60% blood loss with E2-CD treatment. The most reliable molecule for monitoring ATP is the β -phosphate, identified by bold arrows, in the figure above. It is clear that the vehicle cyclodextrin-treated rats continued to lose ATP unto the point of death, while the E2-CD-treated rats



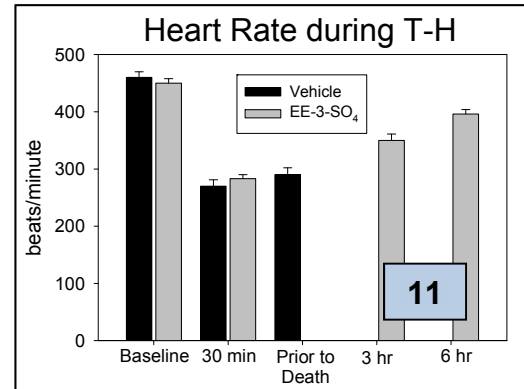
maintained adequate ATP to survive for 3 hours. In addition, because the signal followed was proton resonance, it also provided data for intracellular pH (Figure 9, previous page). Once again, it was obvious that the intracellular pH was maintained at a level compatible with viability in E2-CD-treated rats (blue and magenta symbols), while vehicle control rats exhibited an intracellular acidic pH that could not be corrected (green and yellow symbols).

Cardiovascular Performance. We have collected data for cardiovascular performance from several assays. Widely used are mean arterial pressure (MAP) and heart rate. Figure 10 shows the representative MAP for the optimum 1 mg/kg dose (upper left) and its corresponding vehicle control. It is clear that the hormone promotes



an immediate rise in MAP followed by a gradual but fairly robust recovery at a life-sustaining level. Once again, we have confirmed the optimum dose of estrogen (i.e., EE-3-SO₄) to produce survival. As with the direct measurements of survival *per se* shown previously, benefits to MAP and heart rate follow the same hyperbolic curve. Thus these effects were not so pronounced for surviving rats at the 0.1 mg/kg dose (upper right), 0.3 mg/kg dose (lower left) and 3.0 mg/kg dose (lower right), which was correlated in terms of survival. It can be seen that the treated rats (blue connected dots) all showed benefit for MAP, while all vehicle controls (magenta connected dots) died rapidly. Finally, the heart rate during T-H for EE-3-SO₄-treated groups and their vehicle

controls are shown in **Figure 11**. While an expected reduction in heart rate is displayed after induction of hemorrhagic shock, it is clear that EE-3-SO₄ promotes a return to a greatly improved heart rate by 6 hours, which is in strong contrast to the vehicle controls, which steadily decline in heart rate, leading to death.

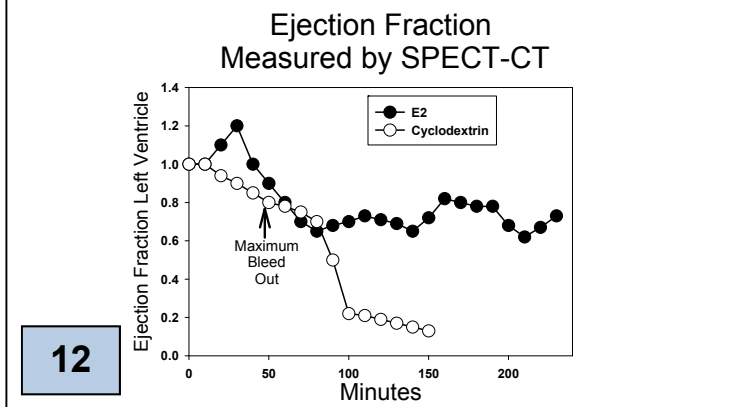


SPECT-CT Measurement of Heart Performance.

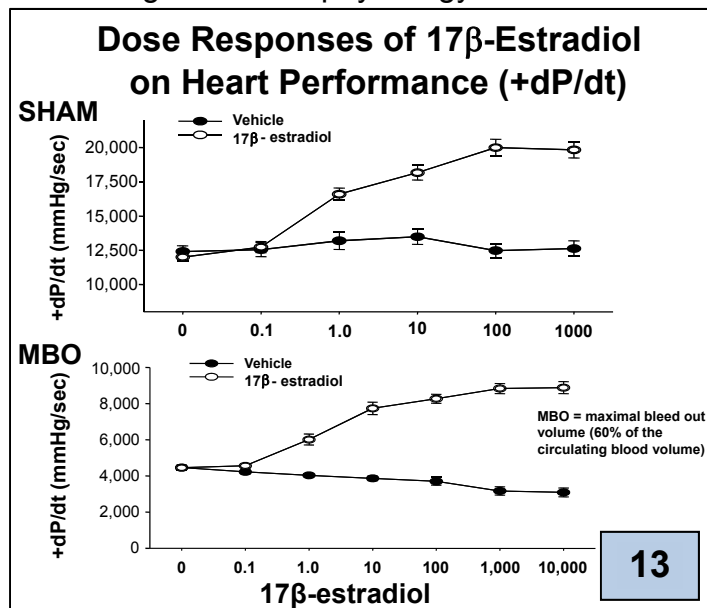
The clinical benchmark for cardiac performance determinations is single photon emission computed tomography-computed tomography (SPECT-CT),

which combines visualization of the heart's force (i.e., ejection fraction) via following a radionuclide tracer ⁹⁹Tc (SPECT) with tomographic X-ray-based imaging of anatomy (CT), which gives a volumetric parameter of heart contractions. In **Figure 12**, the preservation of heart function with E2-CD administration is contrasted with the failure of the heart and subsequent death in vehicle control rats. These studies have resulted in a publication detailing the cardiovascular benefits of E2-CD ⁶

SPECT-CT Imaging Shows Robust Cardiac Performance in E2 Treated T-H Rats



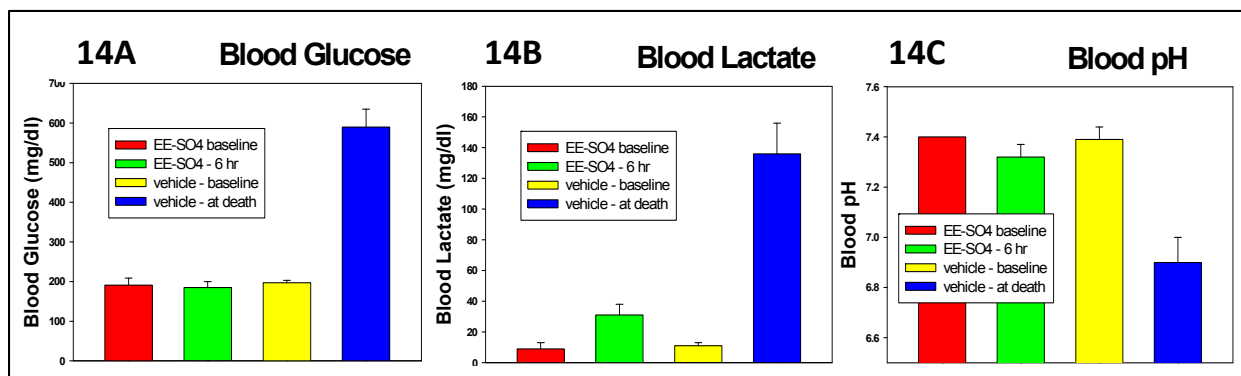
Left Ventricle Performance as Measured by Positive dP/dt. Our instrumentation for monitoring rat cardiophysiology allows us to determine contractile force of the left



ventricle (+dP/dt), and the re-filling of the left ventricle after ejection (-dP/dt). We designed experiments to using +dP/dt to assess the pharmacologic actions of estrogen on normovolemic and 60 % blood volume hemorrhaged rats. We titrated the dose upward with logarithmic increments for molarity of E2. In **Figure 13**, it is clearly demonstrated that escalating doses of E2-CD given to both normovolemic (SHAM) and

hemorrhaged (MBO) rats produce an increase in positive dP/DT. It can be seen that the +dP/dt values increase proportionally to the dose, regardless of whether the animal is normal or hemorrhaged. This gives credence to the fact that E2 has profound effects on the heart and circulatory system with acute administration.

Biochemical Markers for Hemorrhage. There are secondary effects driven by the enhancement or deficit in circulatory system performance seen with estrogen (1 mg/kg dose) or vehicle treatment, respectively. Examples of these changes are manifest in



clinical chemistry profiles of blood glucose, blood lactate and blood pH (seen in **Figures 14A, B and C**). Clearly the interrelated hyperglycemia, high lactate production and acidosis attendant with hemorrhagic shock in vehicle controls as compared to EE-3-SO₄-treated hemorrhaged rats are signs of a morbid status for the animals, which appear alleviated with EE-3-SO₄ treatment. The groups represented by bars are vehicle controls (baseline controls yellow, value at time of death, blue) and EE-3-SO₄-treated experimentals (baseline, red and 6 hours, green).

Rat Pharmacokinetic Studies for FDA Preclinical Support Data. An essential data set for the evaluation of any new drug by the FDA is pharmacokinetic (PK) studies. This line of research is problematic for a small animal such as the rat, owing to the fact that serial samples for conventional assays such as ELISA require large volumes (e.g., 0.5-1 ml) for replicates. While this would be problematic in an ordinary setting, withdrawal of any sample above low microliter ranges is prohibitive in small rodents already bled to 60% blood loss, in that it likely could trigger death in animals already stressed by severe blood loss. As a solution to this issue, the DARPA team proposed the technique which employs a micromethod using dried blood sampling. This was facilitated by collaboration with a pioneering laboratory devoted to the technology at WRAIR and headed by Captain Pribus. The liquid chromatography/mass spectrometry-based (LC/MS) technology enabled sample volumes of 20 μ l to be employed. We proposed that a twofold fluid replacement volume of Ringer's Lactate, administered immediately after sampling, would lessen the stress on our rats and allow for serial sampling at T₀, 30 min, 3 hours and 6 hours whenever possible. Furthermore, the study was in the form of a matrix, where besides multiple time points, treated animals would also be examined

at 3 doses (0.3, 1.0 and 3.0 mg/kg). We have saved our standard MAP and heart rate data for these PK study rats, should this information be called upon for more detailed analyses. The results for the pharmacokinetic studies were analyzed by the DARPA team experts, and were presented to the FDA in conjunction to the parallel studies being performed on the pigs from TAMU.

In Vitro Vascular Reactivity Studies. Owing to the surgically complex (see rat survival studies, above) and time consuming nature for monitoring of estrogen activity based on a readout determined as an animal's survival endpoint, it was clear that a surrogate or *in vitro* correlate analytical system would be advantageous for several reasons. It would reduce the use of animals and provide a less complex and time consuming system. It also enables dose responsive or serial testing on the same vascular rings. To that end, we have used a sensitive and reliable assay centered on modulation of contractility of rat aortic vascular rings. We have also adapted this assay to pig vascular rings to complement the pig survival studies being performed at TAMU, which will be described below. The results of this line of testing have two applications. First, it is a means to evaluate activity of estrogens as

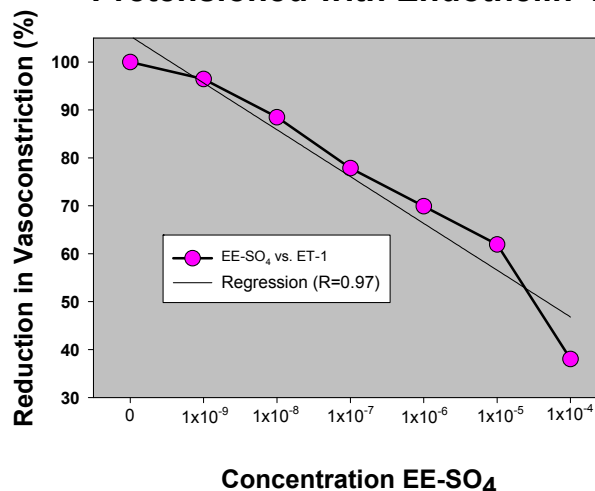
a substitute for intact rats in a sensitive and quantitatively accurate assay system. This involves the positioning of an excised 2-4 mm section of rat aorta which is immersed in a constant temperature bath containing Krebs's buffer. The ring is connected to a force displacement transducer (i.e., strain gauge) which translates muscle contraction into a proportional voltage signal. In order to facilitate more accurate recording and interpretation of

the vascular ring data, we have replaced the analog polygraph strip chart recorder on our Grass Technologies instrument (West Warwick, RI) with a computerized digital data logger, which also ports the data directly into Microsoft Excel.

The performance of the vascular ring assay can be seen in **Figure 15**. It shows a linear response to EE3-SO₄ as delivered via logarithmically increasing doses into the fluid bathing a rat aortic ring. The stepwise drop in tension corresponds to increasing doses of EE3-SO₄, ranging from 10⁻⁹ to 10⁻⁴ M. This validates the accuracy of the method, although it must be said that tuning the vascular ring assay to make it reproducible and

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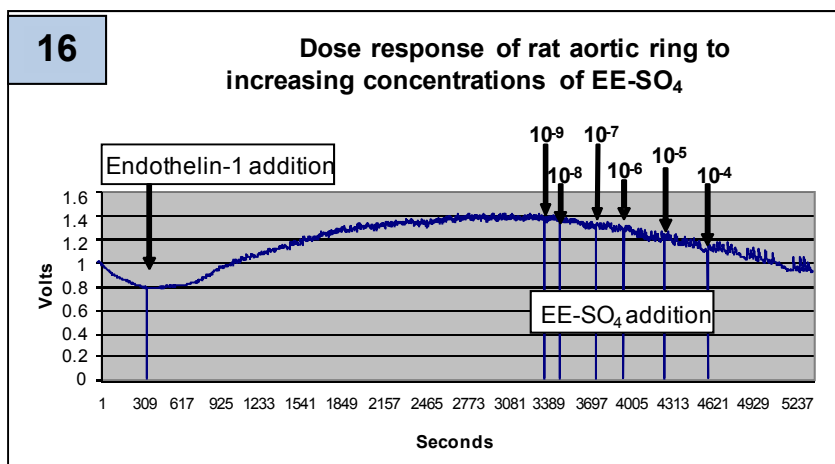
Rat Aortic Ring Relaxation By EE-SO₄ Pretensioned with Endothelin-1



robust presents its own set of challenges. That notwithstanding, we have built upon our experience with the rat system, and applied this knowledge to pig carotid artery rings. This transition was not without difficulty either, because the pig rings did not behave in a similar fashion as the rat rings, in that they require more mechanical pre-tensioning to establish a baseline and respond less well to endothelin-1, phenylephrine and norepinephrine as physiological contracting (i.e., vasoconstricting) agents as compared to the rat arterial rings.

The *in vitro* vascular ring technology offers an approach which allows for the direct testing of our hypothesis that a central action of estrogen as a treatment for severe blood loss is via a physiological response, mediated by the vascular endothelium, to lower peripheral resistance in the capillary bed. The vascular ring technique is ideally suited to examine whether EE3-SO₄ is acting through the endothelium's cell surface estrogen receptors. Parenthetically, we had reported in our progress report from year 1 of this grant that the rat endothelium expresses abundant cell surface estrogen receptors, as determined by immunohistochemistry. That the response to estrogen is driven by cell surface receptors is also supported circumstantially by the fact that the observed response to estrogen is rather rapid, such as that seen for mean arterial pressure detailed in **Figure 1**, page 3 and in **Figure 10**, page 12. This reaction to estradiol is quite distinct from the much slower genomic response via cytoplasmic and nuclear estrogen receptors which depends on gene transcription and protein synthesis. Indeed, this fact has been determined experimentally in a convincing fashion for estradiol on human endothelial cells⁷.

The steps in this determination involved following published protocols and establishing that: 1) EE3-SO₄ stimulates relaxation of the aortic rings; 2) the action is specific to EE3-SO₄ through the use of estrogen receptor-specific inhibitors; 3) determining that

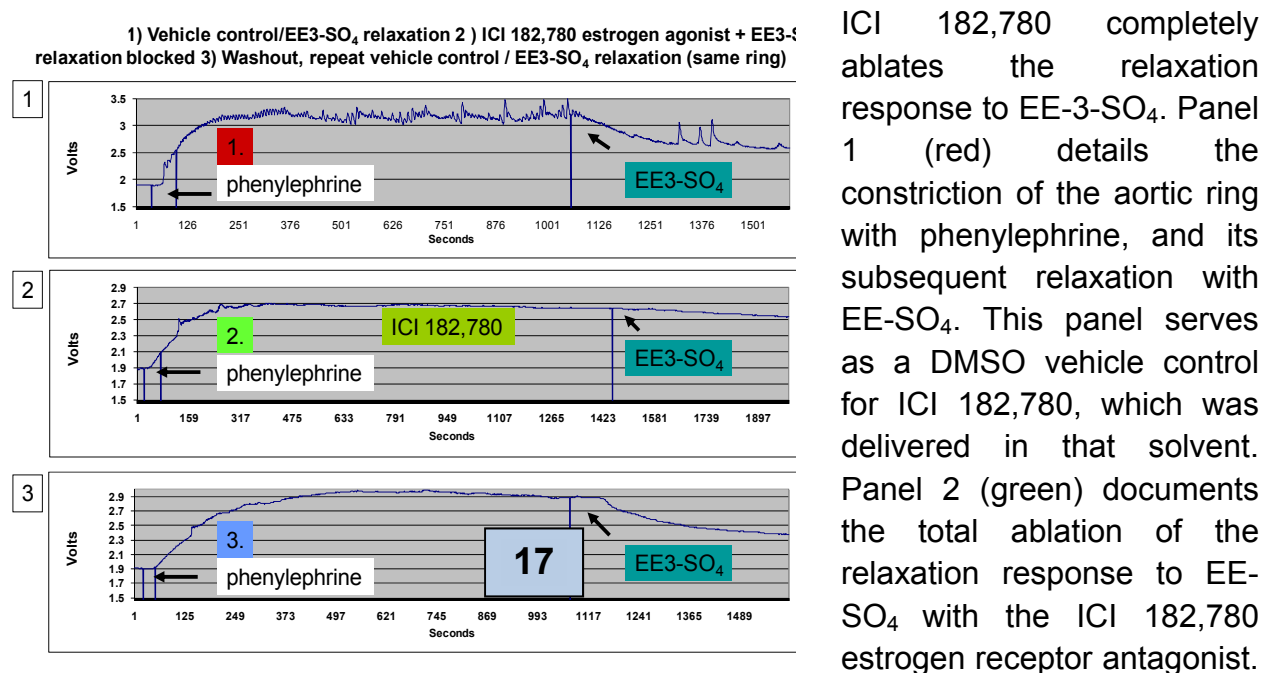


the mode of action for vascular relaxation is through the nitric oxide synthase (NOS) pathway using the NOS inhibitor L-nitrosyl arginine methyl ester (L-NAME); and 4) confirming the role of the endothelium by using aortic rings that are denuded of endothelial tissue and demonstrating a

failure to react to EE3-SO₄. These steps have all been satisfied, confirming that a significant mode of action for EE3-SO₄ is to lower vascular resistance through the action

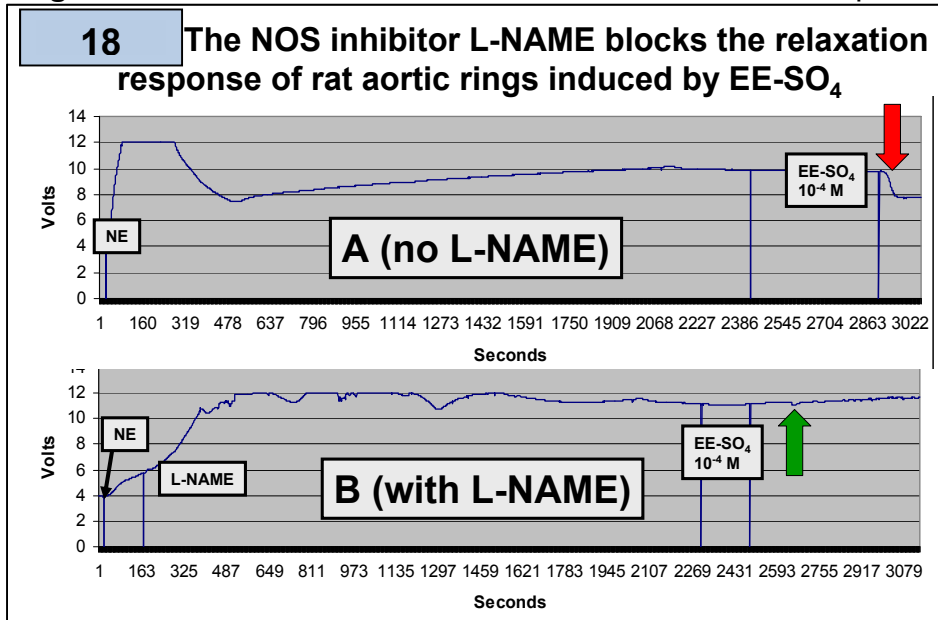
of nitric oxide. **Figure 16** (previous page) shows a digital recording of the aortic ring relaxation response to EE3-SO₄. This is another data set similar to that seen in **Figure 15** (page 15), only in the form of a digital recording. What it depicts is the initial tensioning or vasoconstriction being affected by endothelin-1, which is subsequently relaxed in a stepwise fashion by increasing concentrations of EE3-SO₄. We have elected to use the highest (1x10⁻⁴ M) dose employed in order to challenge the two inhibitors used for this test.

Next we confirmed that the vasorelaxation action is specific to estrogen through the addition of the estrogen receptor specific antagonist ICI 182,780. **Figure 17** verifies that



Panel 3 (blue) shows the restoration of responsiveness of the vascular ring to EE-3-SO₄ by washing out the ICI 182,780 with fresh Krebs buffer (3 times). It is likewise a repeat of the vehicle control.

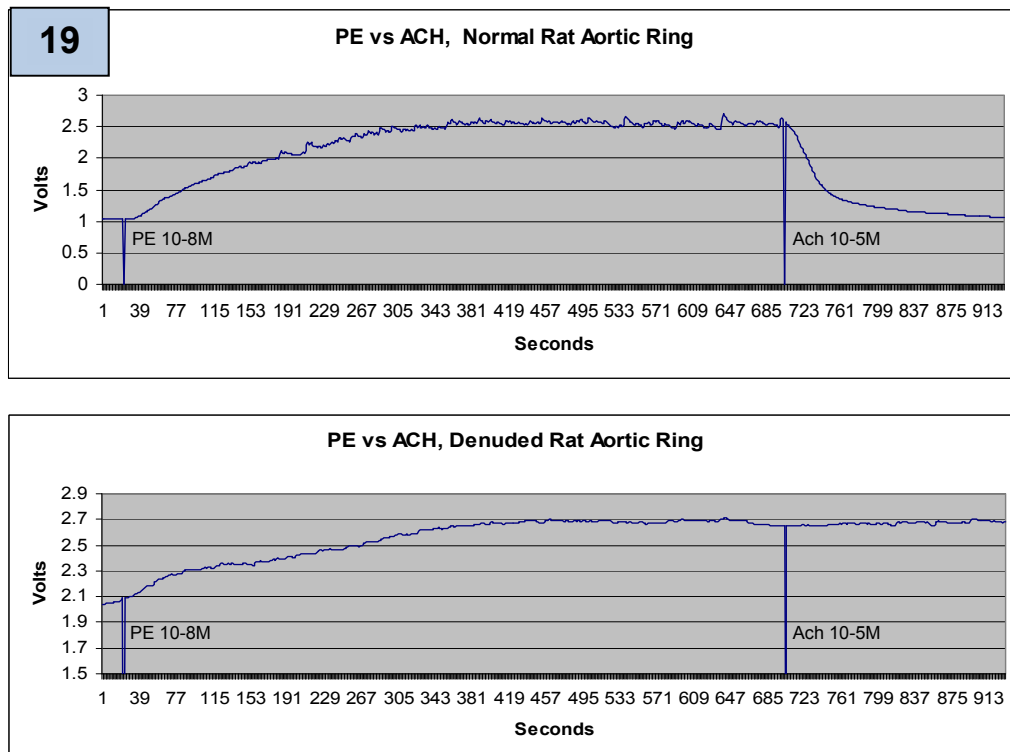
Figure 18 demonstrates the blockade of the relaxation response, where we once again performed the test on an aortic ring which serves as its own control through washout of the different reagents. In this instance, we examined inhibition of NOS (nitric oxide synthase) by L-NAME. The upper panel is a vehicle control with no L-NAME administered to the bath for the



vascular ring. The expected response of vasorelaxation is seen at the end of the chart, marked by the red arrow. The lower panel shows an L-NAME-induced blocked state of receptor-driven vasorelaxation, as marked by the green arrow. This constitutes a standard demonstration of vasorelaxation mediated by NOS-generated NO.

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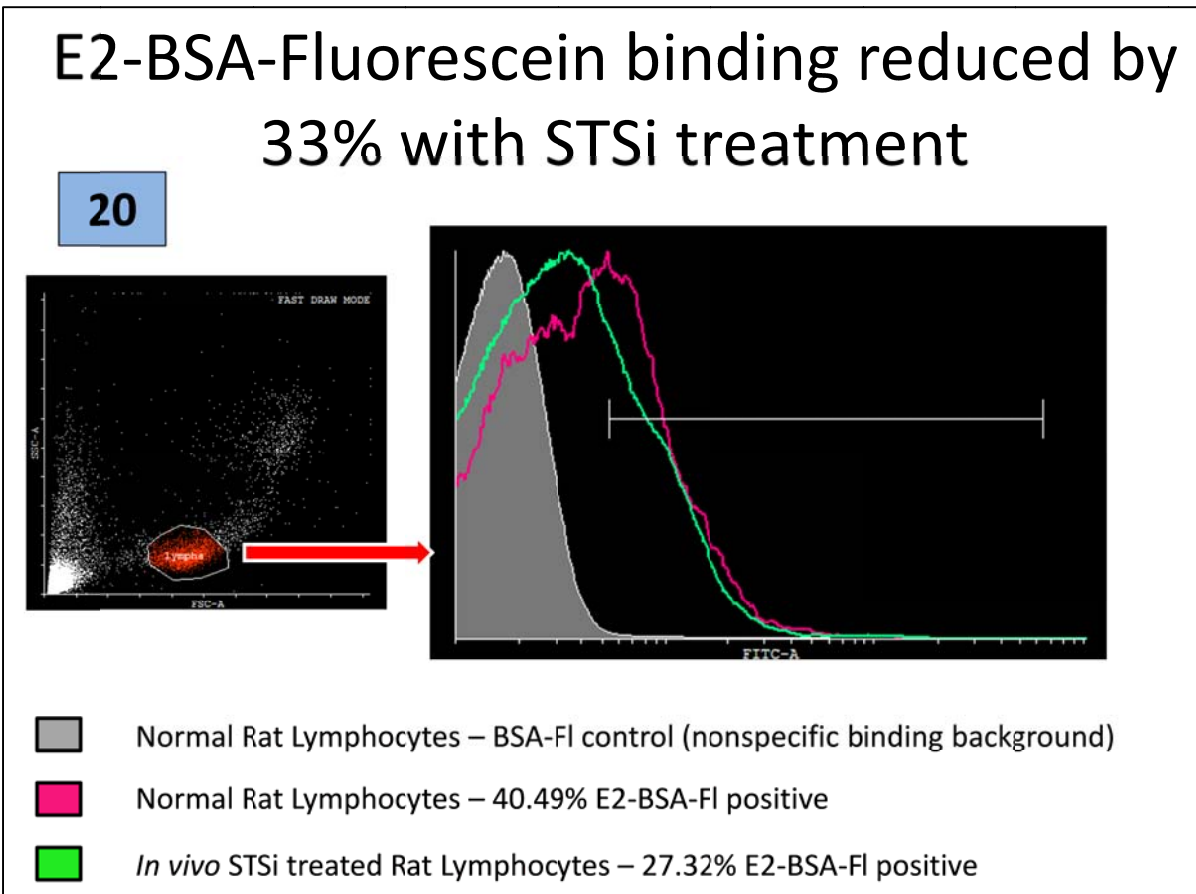
Figure 19 shows that vascular rings denuded of endothelium failed to respond to EE3-SO₄, yet showed the obverse vasotensioing response to acetylcholine, quite opposite the vasorelaxation response seen if the endothelium is intact. This result both confirms that vascular smooth muscle is unresponsive to EE3-SO₄ and likewise documents the



absence of functional endothelium.

This set of experiments strongly supports our hypothesis that estrogen in part exerts its beneficial effect following severe blood loss by lowering peripheral vascular resistance, mediated via cell surface estrogen receptors on the endothelium. In our previous reports, we have documented two additional major benefits to SBL by estrogen, namely the enhancement of cardiac ejection fraction⁶ and preservation of ATP levels at a life sustaining level⁸. We speculate that this triumvirate of protective benefits, all derived from estrogen administration, accounts in large part for its potential to save lives on the battlefield in situations of severe blood loss.

Role of Steroid Sulfatases as a Possible Mediator of EE-3-SO₄ Activity. An abiding question for the use of conjugated estrogens is whether the conjugated form possesses biological activity. The unequivocal rapid induction of biological activity by our soluble EE-3-SO₄ presents a conundrum, since it is widely assumed that the active form of



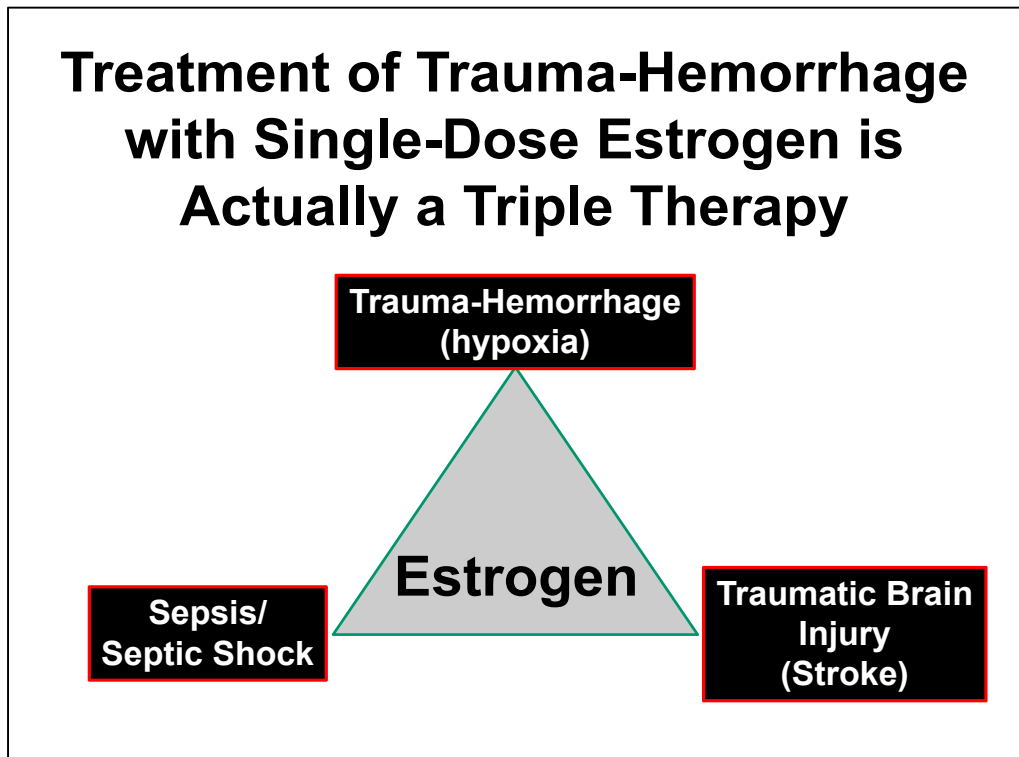
estrogen is unconjugated⁹, where a return to estrogenic activity would be mediated via the sulfate moiety's enzymatic cleavage by steroid sulfatases (STS). We hypothesized that, if sulfate cleavage activates EE-3-SO₄, then this activation could be reversed through blockade of STS by irosustat (667 coumate)¹⁰, a pharmacological STS inhibitor

(STSi)¹¹. However, our *in vivo* and *in vitro* testing using STSi proved inconclusive, owing to an unanticipated broad reactivity. In particular, this was manifest where STSi partially displaced the binding of E2-conjugated fluorescein-BSA (an extracellular-only ligand) on peripheral blood lymphocytes, as revealed in flow cytometric analysis. In **Figure 20** (previous page) it is clear that *in vivo* treatment with the STSi 667 coumate caused a reduction in binding for rat peripheral blood lymphocytes from 40% level without STSi treatment (magenta) which dropped to 27% with STSi pretreatment *in vivo*. This precluded accurate evaluation of EE-3-SO₄ as ligand for ER in the context of experimental STSi administration. In fact, this may be an intrinsic property of STSi to a variable extent, since newer generations of STSi have been synthesized to be dual purpose, inactivating both STS and ER¹².

Lack of experimental confirmation notwithstanding, an in-depth literature search revealed several facts concerning behavior of both steroid sulfatases and conjugated estrogens that would support EE-3-SO₄ having biological activity. First, STS are intracellular, and tethered to the endoplasmic reticulum¹³. Accordingly, since we introduce EE-3-SO₄ into the blood intravenously, it would be required to enter the cell to be a substrate for steroid sulfatases. We theorize that cell entry is unlikely, based on an elegant study by Revankar, *et al.*, who demonstrated that synthetic cell impermeable estrogen, (i.e., soluble, hydrophilic 17 β -estradiol constructs), were completely unable to enter the cell and engage the intracellular estrogen receptor GPER, where synthetic cell permeable (i.e., hydrophobic constructs) freely entered the cell and engaged GPER, efficiently transducing signals¹⁴. Accordingly we speculate that the targets for EE-3-SO₄ are extracellular, and thus are likely to be cell-surface but not cytoplasmic ER.

Summary of the Most Important Results. We have conducted experiments with EE-3-SO₄ to examine its salutary properties for both severe blood loss and, more recently for traumatic brain injury. In addition, our laboratory has applied estrogen therapy to sepsis and septic shock, using the experimental protocol of cecal ligation and puncture, which mimics real world polymicrobial infections arising from trauma. This concept of estrogen as a triple therapy is diagrammed on the next page. The results from all three areas of endeavor are highly encouraging, and lead us to speculate that estrogen administration will be therapeutically useful for either a single injury, or as is more probable in the battlefield, multiple injuries.

Treatment of Trauma-Hemorrhage with Single-Dose Estrogen is Actually a Triple Therapy



The findings of our SBL research efforts have led to several significant conclusions that offer a great deal of promise for application in trauma-hemorrhage and as noted, other injuries. They are:

- A single bolus of soluble estrogen, such as EE-3-SO₄, can extend the golden hour several folds and mitigate for survival from otherwise fatal hemorrhage.
- The quantities of estrogen must be supraphysiologic and delivered relatively rapidly into the bloodstream.
- Our SBL treatment does not involve resuscitation. This is a distinct advantage for several reasons:
 - Transport of large, heavy volumes of fluids into the theater of battle is not required for this therapy.
 - Nascent hemostasis is not threatened by a rapid rise in blood pressure and volume.
 - Because of the single shot and small volume, administration by field medics or even the warfighter him/herself or a buddy is possible with intraosseous auto-injectors.
- The therapy works equally well in males and females, owing to the presence of estrogen receptors on the cells of both genders.

- Tissue perfusion is enhanced by our estrogen-based therapy via higher cardiac output working against lowered vascular resistance, made so by reduced vasoconstriction.
- Energetics as measured by ATP levels are preserved with estrogen therapy.
- Biochemical profiles (blood and intracellular pH, blood lactate, blood glucose levels) stay in a range that is less precarious as compared to hemorrhaged sham animals.

Presentations

- Chen J, et al., *17 β -estradiol (E2) administration after major blood loss improves liver ATP, 3-hour survival and also long-term survival following prolonged hypotension (3 hour) and fluid resuscitation* at the Twenty-Ninth Annual Conference on Shock, Broomfield CO, June 3-6, 2006.
- Kim H, et al. *Single photon emission computed tomography demonstrated efficacy of 17 β -estradiol therapy in male rats following trauma-hemorrhage and extended hypotension* at The 54th annual meeting of Society of Nuclear Medicine, Washington DC, June 2-6, 2007.
- Hubbard WJ, et al. *Water soluble estrogens prolong permissive hypotension following major blood loss even without fluid resuscitation* at the Seventh Congress of the International Federation of Shock Societies and the Thirty-Fifth Annual Conference on Shock, Miami Beach FL, June 9-13, 2012.

Publications

- Kim H, Chen J, Zinn DR, Chaudry IH. Single photon emission computed tomography demonstrated efficacy of 17 β -estradiol therapy in male rats following trauma-hemorrhage and extended hypotension. *J Trauma* (69(5):1655-73, 2010. (PMID 20571453)
- Kozlov AV, Duvigneau JC, Hyatt TC, Raju R, Behling T, Romana T, Hartl RT, Staniek K, Miller I, Gergor W, Redl H, Chaudry IH. Effect of estrogen on mitochondrial function and intracellular stress markers in rat liver and kidney following trauma-hemorrhagic shock and prolonged hypotension. *J Mol Med* 16(7-8):254-61, 2010. (PMID 20379612; PMC 2896467)

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