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TITLE: Treatment of TBI with Hormonal and Pharmacological Support, Preclinical Validation Using Diffuse and Mechanical TBI Animal Models

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14. ABSTRACT

We established the lateral fluid percussion model for induction of TBI, and applied it to a substantial panel of in vivo tests to evaluate the effects of E2-SO₃ (early years test article) or ethinyl estradiol-3-sulfate (EE-3-SO₃; later years test article) on ameliorating early events for TBI pathology. This panel of TBI tests included standard clinical evaluations for physiological deficits (i.e., intracranial pressure, brain O₂ tension, cranial perfusion pressure). In vivo testing also included behavioral and physical activity evaluations, including memory, cognition, vestibulomotor function, fear and anxiety. These behavioral tests were conducted under the direction of Dr. Thomas van Groen (Neurobiology Core Facility Director). In addition we examined general physical activity, using a custom-fabricated instrumental system of our design. This was applied to measuring generalized nocturnal activity, confirming that EE-3-SO₃ treated TBI rats have higher activity levels vs. vehicle-treated TBI rats.

Our in vivo tests also included MRI imaging, focusing on edema resolution and reduction of diffuse axonal damage (fractional anisotropy). Here we found that EE-3-SO₃ was protective if given within the first hour post injury.

For in vitro testing, we performed histochemical and immunohistochemical analyses for neuronal damage and integrity plus in vitro examinations of neuronal stretch damage mitigation by E2-SO₃. All assays showed a protective advantage for EE-3-SO₃. These data, which are published, confirm that EE-3-SO₃ is as effective as or better than the previously used drug, E₂-SO₃ whose results are also published.

We have applied for and received two patent awards; one for the use of EE-3-SO₃ for treating severe hemorrhage and the other for treating TBI. In addition, we have received a contract from DoD to move toward clinical trials with EE-3-SO₃ for severe hemorrhage. The goal is to establish safety and efficacy for EE-3-SO₃ which could constitute an important enabling step for the use of EE-3-SO₃ for treatment of TBI.

15. SUBJECT TERMS

Traumatic brain injury; Ethinyl Estradiol-3-SO₃; lateral fluid percussion; magnetic resonance imaging; memory and cognition; fear and anxiety; vestibulomotor function; intracranial pressure, brain O₂ tension; cranial perfusion pressure, histological testing.
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INTRODUCTION: Our efforts to administer soluble estrogen as a pharmaceutical drug to ameliorate the early damage for TBI span several years (2008-2016), and have produced promising results. Notable findings are that delivery of 17β-estradiol sulfate (E2-SO$_4$ or E2S) and later ethinyl estradiol 3-sulfate (EE-3-SO$_4$ or EES) within the first hour post injury, and at a dose of 1 mg/kg, resulted in reduced edema and fractional anisotropy at the lateral fluid percussion site. Invasive measurements revealed lowered intracranial pressure, and improved oxygen tension and cerebral perfusion pressure, all of which are clinically relevant. Longer term behavioral assessment shows reduced anxiety and fear, and improved cognition and memory. In addition, vestibulomotor performance was also enhanced with EES treatment.

KEYWORDS: Traumatic brain injury, TBI, estrogen, estradiol, ethinyl estradiol-3-sulfate, lateral fluid percussion, intracranial pressure, cerebral perfusion pressure, partial oxygen tension, golden hour, magnetic resonance imaging (MRI), fractional anisotropy, cerebral edema, cognition, memory, vestibulomotor function, fear and anxiety, physical activity.

OVERALL PROJECT SUMMARY: As per DoD instructions set forth in the Assistance Agreement and Program Announcements, we will align our data with the tasks set out in each SOW. This consists of the original SOW, followed two extensions without funding (EWOF, also called no-cost extensions - NCE) which were granted. Please note that “administrative” tasks (i.e., those that generate no data) will be assumed to be completed unless otherwise noted, and thus no detail will be provided, other than to note that it was accomplished (i.e., done).

Initial Award – Project Start Date 09/01/08, end date 08/15/12
1. Order critical supplies (09/01/08 - 11/01/08) done
2. Hire and train personnel for rat model and in vivo tests (09/01/08 - 01/01/09) done
3. LFP model generating data (01/01/09 - 04/01/11) done
4. In vivo and in vitro testing and sampling (01/01/09 - 06/01/12). See number 5, which follows.
5. E2 testing (01/01/09 - 11/01/11) Evaluation of soluble estrogens. In our research on severe hemorrhage, we had previously determined that iv administration of estrogen was most effective. As such, we have investigated efficacy for several soluble forms of estrogen, initially with microencapsulated 17 β-estradiol, in the form of E2-cyclodextrin (E2-CD), and followed by E2-sulfate (E2S). All were efficacious, but E2-cyclodextrin was ultimately abandoned because there were no assurances that a GMP source was available. In the interim, E2S was used as the test article. We eventually focused on ethinyl estradiol-3-sulfate (EES) as the drug of choice. Since this is explicitly listed in the later EOWF SOWs, we will report on the testing with E2S in this opening section and report data derived with EES in the last two EWOF sections.

Examples to follow deal with MRI estimates of edema, histological evidence for E2-mediated neuroprotection and effects of E2 treatment on TBI-induced deficits in memory and
learning. Please note that all figures and legends were taken from submitted annual reports, and in addition some of these data have been published.

Intracranial measurements. One of our most important objectives was to obtain instrumentation and develop methods for measurements in the rat that mirror those used clinically. One of our original collaborators, Dr. Anise Ardelt, was to bring her clinical and experimental expertise for such measurements to the team. Unfortunately, she decided to move to Chicago prior to activation of this grant. While that was a setback, through our own searches we were able to obtain and evaluate instrumentation that gave satisfactory performance for intracranial measurements. The targets were intracranial pressure, intracranial partial O₂, and intracranial perfusion pressure. Results obtained with sham, TBI vehicle controls and TBI with E₂S treatment are shown above in Figure 22. It is readily apparent that when E₂S is delivered 1 hr post injury, it is possible to significantly ameliorate the damaging effects on the brain induced by lateral fluid percussion, as seen above.

MRI results: E₂ lessens lesion edema and volume. Through the use of magnetic resonance imaging (MRI) we have discerned that the single treatment of 1 mg/kg E₂, given 1 hr after TBI, significantly reduces lesion edema and thus volume. Serial “slices” were made through the ipsilateral brain (as contrasted with the negative contralateral side), and volumes were calculated for the slices and summed. It can be seen from representative samples for each treatment group in the MRI tomographic sections in the on the next page, that there is a statistically significant reduction in edema (p=0.002), which is easily visualized. Because this is a proton MRI, the scale inserted in the images reflects increasing strength of the proton signals. Thus edema and cerebrospinal fluid by their “watery nature” would produce the strongest (i.e., dark reddish-brown) signals. The lesion margins (seen in left side of the series of inverted brain images, below) are outlined in green lines. Originally we gathered data from a 4.7 tesla instrument, which was replaced with a 9.4 tesla MRI used in the later studies.
Histological Studies. E2S greatly reduces the number of damaged neurons (Fluoro-Jade). Using the anionic fluorophore Fluoro-Jade which specifically binds to damaged neurons, it is possible to estimate the extent of neuronal injury with and without E2S treatment. The figure below (next page) shows the comparisons between E2S treated or vehicle control treated cortex and hippocampus. The differences between E2S-treated and vehicle control groups was statistically significant at the p=0.005% level.
In addition, the bar graph below provides another histological study result. This particular examination uses cresyl violet stain to visualize Nissl substance as a marker for surviving neurons. Here it is clear that E2S treatment significantly enhanced neuronal survival in the hippocampus, as determined 24 hr post-injury.
The last example of application of histological methods to evaluate E2S efficacy concerns the use of immunohistochemistry; in particular, antibodies vs. glial fibrillary acidic protein (GFAP) to visualize neuronal survival. This method determines the extent of gliosis. Thus the lessening of the GFAP signal when rats are treated with E2S attests to enhanced neuron survival. The figure below confirms the damage created by TBI, and the benefit of E2S treatment, which the bar graph labeled Figure 10 displays quantitatively.

Behavioral studies: E2 treatment effects a modest improvement in Morris Water Maze performance. The Morris Water Maze (MWM) is the de facto standard test for rodent cognitive and memory function. In the following figure (below) it is apparent that the single E2 treatment caused a modest improvement in MWM performance. Beginning 6 days after injury and treatment, it can be seen that there is a progressive (albeit slight) improvement in location of the hidden target. Furthermore, this difference is not attributable to a locomotor advantage (i.e., swimming speed), as sham, treated and untreated groups had similar performance. Finally, it is worth noting that the severity of injury had sufficient latitude to facilitate clear evaluation of improved performance between treatment groups, an important consideration if we added additional neuroprotective agents to the test matrix. (N.B. There is additional data for behavioral studies in the report for the final EWOF tasks, which follows).
6. Glucosamine testing (07/07/10 - 06/01/11) Testing of glucosamine in our TBI model showed little efficacy, and thus subsequent experiments were not deemed worthwhile. An example of glucosamine’s modest effect on neuroprotection in the hippocampus is seen in the figure below (maroon bar, with sham green, and TBI-vehicle, aqua).

7. Combinatorial testing (07/01/11 - 06/01/12) Combinatorial testing. Owing to the disappointing performance of glucosamine when used alone, we decided to abandon combinatorial testing with E2S and glucosamine. However, in the subsequent EWOF work, we revisited combinatorial testing with additional drugs.
8. Blast wave model at UAB (09/02/13 - 06/01/14). The blast wave device which was to be fabricated for us and tested by CFDRC (Huntsville, AL) was never delivered. This resulted in a severing of our contract with the company.

9. Transfer test data to CFDRC (01/01/09 - 07/01/12). We regularly transferred experimental data to CFDRC, which enabled them to deliver mathematical models or in silico TBI rats, and additionally a suite of software for pharmacokinetics. These goals were originally of interest to DARPA, and thus the collaboration with CFDRC was “piggybacked” onto our earlier DARPA-funded studies of severe hemorrhage, which carried over to this DoD-sponsored TBI research. However, our group is not oriented toward mathematical modeling, and the failure to deliver a blast wave device in the penultimate year of the initial period presented a reasonable cause and occasion to cease the subcontract-based interactions with CFDRC, and eliminated their participation in the follow-on EWOF research projects.

10. Begin clinical trial feasibility planning (06/01/12 - 08/15/12). Our planning for clinical trials was focused in large part on obtaining high quality EES for testing in TBI. With the expert guidance from one of our DARPA-associated collaborators, we were able to acquire a custom synthesis of EES, and applied it to TBI testing. During the latter two years, we additionally obtained (through DARPA’s offices) a cGMP-grade EES (current good manufacturing practice, per FDA guidelines) as part of the FDA-specified IND (investigational new drug) process and planning for Phase I trials.

11. Prepare final report (06/01/12 – 08/15/12). Done.

**TBI First No-Cost Extension Task List**

NCE Project Start Date: 09/01/13; Project End Date: 08/31/14.

12. Train new operators in rat TBI model system (06/03/13 - 07/26/13). Done.

13. Initiate combinatorial testing with EE-3-SO\(_4\) and G-1 (06/03/13 - 08/09/13). Results of testing G-1 and EES are found in a published paper by Day, et al., which is provided in the appendix. G-1 is a non-steroidal GPER agonist which is pleiotropic, and indeed sometimes conflictory in that it can, for example, both promote and arrest tumor growth. Add to that the fact that more recently GPER has come under question as to whether it is a bona fide estrogen receptor, largely because it lacks the ability to mediate endocrine and developmental functions exhibited by E2. Thus we decided that further pursuit of GPER testing was not warranted.

14. Initiate combinatorial testing with EE-3-SO\(_4\) and minocycline (08/12/13 - 10/11/13). We withheld the testing of minocycline for the second EWOF period to allow all testing to be done under the same conditions with the same operators as well. These results appear in the following (final year’s) results, SOW task 1, detailed for intracranial pressure. It can be seen that minocycline while effective as a single agent vs. vehicle controls, nonetheless did not appear to have promise for enhancing responses of EES in combination.

15. Initiate combinatorial testing with EE-3-SO\(_4\) and memantine (10/14/13 - 12/27/13) *Memantine*. The results with memantine are also shown in the Task 1 of the 2\(^{nd}\) EWOF SOW. The results for memantine, alone or in combination with EES were not encouraging.
Thus as with all instances of combinatorial testing, we were not persuaded to continue such evaluations, preferring to study EES as a single agent.

16. Monitor brain intracranial pressure, temperature and pO2 in subset of TBI rats (EE-3-SO4 treatment/no treatment) and control rats (06/07/13 - 06/01/14)

Intracranial pressure (ICP) is reduced in measurements taken 24 hr post injury (figures to left). In addition, there is improved delivery of O2 as seen with the values for partial brain oxygen pressure (pO2). Cerebral perfusion pressure is derived from ICP and pO2, so it would be anticipated to reflect the improvements to those values.

17. Conduct activity monitoring with longitudinal measurements (06/03/13 - 06/01/14)

Activity monitoring. As noted earlier, we wished to gain insight into the E2S treated rats. improved behavioral performance, as well as a consistent observation of improved weight gain (rats gain weight constantly over their lifespan). Our hypothesis was that they were able to resume normal activity faster after injury when treated with E2S. Since there was no instrumentation available to accomplish this task, we designed and fabricated our own
instruments. This consisted of a passive infrared sensor connected to an interface board, which was ported into a PC’s Excel spreadsheet. Activity was scored as movement events seen in 10 second intervals, running continuously from 6pm to 6am, which corresponds to the normal rat (nocturnal) circadian rhythm. The bar graph below shows that the rats did indeed have greater activity with treatment (starting at the first day post-treatment), which also suggests that whole-body physiology is improved, since movement is a complex process. However, the adjacent graph to the right, above, reveals an important aspect of the LFP model, which is the necessity of creating a trephine to allow for the percussion pulse to be delivered to the brain. This time interval begins a day before craniotomy, and it is evident that at day 2 (i.e., the first after craniotomy), rat activity is depressed in all but the normal animal which wasn’t subjected to craniotomy. Thus it is also impressive that EES administration allows for a recovery of activity comparable to the sham rats, while injury with only saline vehicle treatment shows activity still suppressed on day 3.

18. Extract and process brain tissue samples and blood plasma for subsequent batchwise analyses of cytokines with cytokine bead array (07/01/13 - 04/03/14). Done.
19. Conduct histological and biochemical examinations of brain tissues from drug combination trials (09/02/13 - 06/01/14). The histological examinations of sham, TBI vehicle controls and TBI EES treated brains used the same methodology as shown above for E2S, and produced nearly identical results (data not shown).
20. Collate and analyze data, prepare manuscripts (07/01/13 - 08/31/14). Done
21. Plan clinical trials using GMP EE-3-SO₄; all UAB investigators and clinical consultants (03/01/14-08/31/14). Done.

We were able to justify and receive an additional EWOF for the period from September 2014 through February 2016. The approved SOW for this extension follows below:

**Projected Period**  
09/01/2014-01/31/2016

**Task Number and Planned Experiments and Activities**

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<td>Prepare rats with lateral fluid percussion traumatic brain injury for various objectives (sham, vehicle, ethinyl estradiol-3 sulfate (EE-3-SO₄) plus/minus minocycline or memantine-treated rats). Test EES and memantine, minocycline in model system. We chose to evaluate the testing of memantine and minocycline (Mino), as well as combinations of memantine (Mem) and EES with intracranial pressure measurements. As can be seen in the composite graph (to the left), while all drugs and combinations showed efficacy vis-à-vis the TBI vehicle control, the results for single agent tests and...</td>
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the memantine + EES combination were essentially the same insofar as ameliorating elevated intracranial pressure. Thus once again, the value of combinations was not supported, and we deemed it not worthwhile of the considerable time and effort to pursue this line of research.

09/01/2014-01/15/2016 2. Conduct TBI studies and bank tissue specimens for histological analyses and perform analysis on specimens from TBI sham, vehicle and EE-3-SO₄-treated rats. Done, in part. We have created an extensive bank of tissue samples from the various groups of TBI rats under study. However, when coworkers are aware of the end of funding, they rightly seek other positions, which happened with us. Thus the loss of personnel with histology experience (and the inability to hire replacements for a very short time period) has prevented us from analyzing the frozen tissue stockpile. Thus the histological analyses are not done, but the tissue banks are filled.

10/01/2014-01/30/2016 3. Conduct physical activity monitoring of sham, injured vehicle control and EE-3-SO₄-treated rats to obtain groups of sufficient number for statistical analyses. Activity monitoring gave results similar to those seen for E2S (task 17 of previous EWOF). To reiterate, the measurement interval was for 12 hr, commencing at 6pm and extending to 6am. This corresponds to the rat’s normal nocturnal activity cycle. It is clear that EES is capable of restoring physical activity to the level seen in the normal rat controls by the third day-post injury. Regarding weight gain, we posit that the observations that EES treated rats, by virtue of their better mobility, are able to resume weight gain faster than their vehicle control counterparts. It was this observation of greater weight gain which rats continue to manifest throughout their lives (weight gain data not shown).

10/01/2014-02/01/2016 4. Evaluate sham, injured vehicle control and EE-3-SO₄-treated rats for cognition and memory function.

The apparatus for assessing memory and cognition (as well as curiosity) is shown in a photograph, to the right. It has been selected to be the method of choice for evaluating memory and cognition over the Morris Water Maze, as originally stated. The test involves first exposing the rat to an area containing two identical objects for an hour. After an hour’s rest away from the apparatus, rats are placed into the field again, but with one object different from the original pair. The response to the new object is tracked with video and results processed at a workstation. The apparatus has recently been updated to enable spatial recognition of the rat’s nose and base of its tail to reveal and record body orientation.
5. We will also attempt to evaluate sham, injured vehicle control and EE-3-SO₄ plus minocycline- or memantine-treated rats for cognition and memory function. Not done. Owing to the complexity of this particular task, it was anticipated that it should be held for the last undertaking. The assessment was correct, and we were could not initiate the experiments in the allotted time. Thus we were unable to provide combinatorial data. However, our prior experience with combinations was not positive, and it is anticipated that we would not have derived useful data regardless. This reasoning was part of the decision to give this task a lower priority.

6. Evaluate sham, injured vehicle control and EE-3-SO₄-treated rats for vestibulomotor performance. Vestibulomotor function was analyzed with an automated foot misplacement apparatus. The instrument scored both front and rear foot placement on a series of rods, which resembled a horizontal ladder with circular rungs. It was determined that EES indeed markedly improved foot placement as compared to vehicle treated control TBI rats, and even sham rats, which were subjected to a craniotomy. For these studies, the EES treated rats were comparable to normal rats, as seen in the figure above right. Also of note is the reinforcement of our finding that a craniotomy is not a trivial procedure and can affect the rat’s performance negatively.

7. Evaluate sham, injured vehicle control and EE-3-SO₄-treated rats for anxiety behavior. Anxiety is scored by whether the animal is aversive to entering the center region, which would constitute a threatening environment, which could subject the rat to
predation. From the data below, it is clear that the injured, vehicle control rats displayed considerable aversion to the center region, and that treatment with EES restored the behavior to “normal” vis-à-vis that seen with the sham rats.

We also employed the Elevated Plus Maze to measure fear, so named for it resemblance to a ‘plus’ character. As seen in the photograph of the device below, it consists of 4 convergent arms, 2 which are covered and thus “safe” and 2 that are open and “dangerous”, since the potential harm from falling is apparent to the rat. The 4 arms of the maze are seen in the upper right and lower left side of the figure, with the closed arms on the horizontal plane, and the open arms on the vertical plane. The lower right image is an example of the behavior trace. It is clear that the rat is strongly aversive to entry into the open arms. Data generated with the plus maze displayed essentially no significant differences among the 4 groups (normal, sham, TBI-vehicle and TBI-EE-3-\(\text{SO}_4\)) (data not shown). Rats spent roughly 5x more time in the covered arms as with the open arms, suggesting a possible interpretation that, regardless of whether injured, treated, or normal, the fear of falling is paramount and apparently preserved.

Key Methodology used during the reporting period:

Materials and Methods

Animals
Adult male Sprague-Dawley rats (2 months old, 300-350g; Charles River Laboratories International, Inc.) were housed two/cage on a 12 hour light/dark cycle in a temperature- (22°C) and humidity-controlled facility and allowed standard rat chow and water ad libitum. All animal care and experimental procedures complied with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. For generation of histology tissue, animals were divided into four groups. Uninjured control animals received craniectomy only. These subjects were treated with either vehicle (n=4) or E2 + Memantine (MEM) (n=4). As there were no significant differences observed between control groups treated with vehicle or E2 + MEM, they were pooled for all analyses (SHAM, n=8). The injured groups were 1) vehicle-treated TBI (TBI VEH, n=8); 2) 0.05mg/kg MEM-treated TBI (TBI MEM 0.05, n=8); 3) 0.5mg/kg MEM-treated TBI (TBI MEM 0.5, n=8); 4) 5.0mg/kg MEM-treated TBI (TBI MEM 5.0, n=8); 5) 1mg/kg E2 + 0.05mg/kg MEM-treated TBI (TBI E2
MEM 0.05, n=8); 6) 1mg/kg E2 + 0.5mg/kg MEM-treated TBI (TBI E2 MEM 0.5, n=8); and 7) 1mg/kg E2 + 5.0mg/kg MEM–treated TBI (TBI E2 MEM 5.0, n=8). For behavioral analyses, a separate cohort of animals was examined: 1) uninjured sham control (SHAM, n=10); 2) vehicle-treated TBI (TBI VEH, n=10); and 3) 0.5mg/kg MEM + 1mg/kg E2- treated TBI (TBI MEM E2, n=10). Animals were humanely euthanized 24 hours post-TBI (histology only) or following the completion of all behavioral testing, and tissue was extracted for histological evaluation.

**Surgical Procedure**

Animals were anesthetized with 4% isoflurane gas in an O2 carrier for 4 minutes, then anesthesia was continued via ventilation with 3.0% isoflurane gas for the duration of surgery. Normothermia was maintained by keeping the animal on a water-jacketed heating pad. After securing the animal in a stereotaxic frame, a midline scalp incision was made and the skin and fascia reflected to expose the bregma, lambda, and sagittal sutures as well as the lateral ridges. A 4.8-mm craniectomy was trephined over the right parietal cortex, midway between bregma and lambda, tangential to the sagittal suture as previously described (Floyd et al., 2002). A rigid plastic injury tube (modified female Luer-lock 20G needle hub) was bonded to the skull with cyanoacrylate adhesive over the open craniectomy with the dura intact and a stabilizing screw was placed in a burr hole drilled rostral to bregma on the ipsilateral side. The injury tube and stabilizing screw were secured with dental acrylic. Then the scalp was sutured, and the animal was returned to a warmed recovery cage.

**Induction of lateral fluid percussion traumatic brain injury**

Experimental TBI was induced following craniectomy using a fluid percussion device (VCU Biomedical Engineering, Richmond, VA) as previously described (Dixon et al., 1987). The device consists of a Plexiglas cylinder (60 cm in length and 4.5 cm in diameter) filled with sterile water. A piston is mounted on O-rings at one end and an extracranial pressure transducer (Entran Devices, Inc., EPN-0300A, Fairfield, NJ) connected to a storage oscilloscope (Tektronix, TDS 310, Beaverton, OR) is attached to the opposite end. A 5-mm tube (internal diameter 2.6 mm) ending in a male Luer-lock is fitted at the end. The animal was anaesthetized with 4% isoflurane gas for 4 minutes, and moderate TBI was induced by rapidly injecting a small volume of sterile saline into the closed cranial cavity over the right ipsilateral hemisphere with the fluid percussion device. Immediately after the impact the animal was removed from the device, monitored for duration of apnea and unconsciousness, and re-sutured while receiving supplemental oxygen ventilation. The magnitude of the pressure pulse was measured by a pressure transducer, stored on an oscilloscope, and later converted to atmospheres (ATM). The pressure pulse was monitored and controlled in order to deliver an equivalent impact to each animal.

**Administration of drug**

At 45 minutes post-TBI, animals were anesthetized with 4% isoflurane gas for 4 minutes and then maintained on 2% isoflurane ventilation for the duration of drug delivery. An incision was made in the right inner flank and the femoral vein was isolated and cannulated using polyethylene tubing (PE50). At 1 hour post-TBI, either E2 + MEM (1mg/kg EE-3-SO4 + 0.05, 0.5, or 5.0mg/kg of Memantine in a carrier of sterile saline, Sigma-Aldrich Co., St. Louis, MO) or vehicle (sterile saline) was administered via the femoral vein. The femoral vein was then ligated with braided silk thread and the incision closed, and the animal was returned to a warmed recovery cage.
**Behavioral Analysis**

*Beam Walk*

Vestibulomotor skills were assessed with the Beam Walk task as previously described (Floyd et al., 2002). Briefly, animals were subjected to a bright light and white noise which they evaded by navigating a 2.5 x 100 cm elevated beam to enter a darkened goal box on the opposite end. Latency to cross the beam was recorded for three consecutive trials per subject, and pre-injury mastery criterion was set at traversing the beam in ≤4 sec. Animals were pre-trained three days before induction of injury, pre-assessed immediately prior to surgery, then tested one day post-TBI. The mean latency to cross the beam was calculated from three trials per rat. In addition, numbers of subjects that fell off the beam during the attempt to cross were noted.

*Elevated Plus Maze*

Anxiety-like behavior was evaluated with testing in the Elevated Plus Maze (Walf and Frye, 2007). Briefly, seven days post-injury, animals were introduced into a raised, plus-shaped maze consisting of two open and two closed arms. Subjects were allowed to freely explore the maze for 4 minutes and a computerized video camera tracking system (EthoVision XT, Noldus Information Technology) recorded their location and distance traveled. The amount of time spent in the open arms and the distance traveled were analyzed for all animals.

*Morris Water Maze*

The Morris water maze is a test of hippocampal-dependent spatial learning and memory in rodents (Morris, 1984). The standard acquisition protocol was followed to evaluate cognitive function. Briefly, animals were introduced into an arena comprised of a circular pool divided in 4 quadrants containing a platform submerged 2 cm below the water surface, with distal visual cues placed around the perimeter. The platform was kept in the same location for the duration of testing. Subjects were lowered into the pool at one of four start locations and swam until they located the hidden platform or until 120 sec elapsed, whichever occurred first. If animals were unable to find the escape platform in 120 sec, they were manually placed on it. Rats remained on the platform for 30 sec following each trial, then were placed in a heated cage during the inter-trial interval. Beginning 7 days post-surgery, each subject was tested over five days in 4 trials per day, with each trial beginning at a different start location in the pool. On day 5, rats were additionally tested in the Probe Trial, in which the escape platform was removed and the subjects were allowed to search the pool for 120 sec. Latency to find the hidden platform and swim speed were recorded for all trials by a computerized video tracking system (EthoVision XT, Noldus Information Technology).

*Open Field*

In order to assess anxiety levels, animals were tested in the Open Field (Walsh and Cummins, 1976). One day post-injury, subjects were placed in a square Plexiglas box divided into spatial arenas and their movement was recorded by a computerized tracking system (EthoVision XT, Noldus Information Technology) for 4 minutes. The numbers of entries into the designated center quadrant of the box, as well as the duration of time spent in the center, were analyzed for all subjects.
**Rotorod**

The Rotorod task was utilized to evaluate vestibulomotor function as previously described (Hamm, 2001). Animals were placed on a rotating rod that accelerated gradually using a pre-selected ramp profile. Trials were terminated when either the rat fell off the rotating rod or 120 sec elapsed. Subjects were pretrained until they reached mastery criterion of remaining on the rod for at least 30 seconds over 3 separate trials. The day prior to surgery, rats were pre-assessed over 3 trials for latency to fall off the rod. Testing was conducted on days 2 and 4 post-injury and the latency to fall was recorded during 3 separate trials and averaged for each subject.

**Histological analysis**

**Tissue preparation**

At 24 hours post-TBI, animals were humanely euthanized with Fatal Plus (100mg/kg i.p.; Vortech Pharmaceuticals, Dearborn, MI) and perfused intracardially with ice-cold 0.1M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4% paraformaldehyde (PFA) for 20 minutes. Brains were harvested and post-fixed for 24 hours at 4°C in 4% PFA, then subsequently cryoprotected in an increasing gradient of 10%–30% sucrose for 24 hours at 4°C. The brains were marked with tissue dye over the right hemisphere, blocked and trimmed at 5 mm rostrally and 8 mm caudally, then embedded in OCT™ Compound (Tissue-Tek; Fisher Scientific, Pittsburg, PA) and frozen in ice-cold 2-methylbutane. Tissue was stored at -80°C until serial random sectioning. Serial 50-µm slices were sectioned on a cryostat (Leica Instruments, Nusloch, Germany) and collected from bregma -0.8mm to -4.8mm, encompassing the cortical region at the injury epicenter as well as the entire hippocampal formation. The sections were mounted on 1% gelatin-coated slides and stored at -20°C until further histological analysis.

**Cresyl violet histochemistry**

Cresyl violet histological processing of tissue demarks Nissl substance, which is composed mostly of rough endoplasmic reticulum and is lost after neuronal injury or axonal degeneration (Carson, 1990). For cresyl violet histochemistry, tissue was rinsed and dried overnight. Sections were dehydrated through graded alcohol to xylene for two changes of 5 minutes each, and then rehydrated through graded alcohol to water. Sections were then submerged in 0.1% aqueous cresyl fast violet (EM Science, Gibbstown, NJ) in a sodium acetate buffer for 4 minutes, followed by differentiation in 95% ethanol with 0.2% HCl for 5 minutes. Differentiation was timed such that both Nissl substance and cell nuclei were clearly visible. Slides were washed in graded alcohol and xylene and coverslipped with Permount mounting media (Fisher Scientific, Pittsburgh, PA).

**Fluoro-Jade B histochemistry**

Fluoro-Jade B is an anionic fluorescein derivative that binds to degenerating neurons (Schmued and Hopkins, 2000). Briefly, sections were rehydrated through graded ethanol to distilled water, then incubated in 0.06% potassium permanganate for 15 minutes to reduce non-specific fluorescence. Tissue was rinsed in distilled water and processed 0.006% Fluoro-Jade B in 0.1% acetic acid for 30 minutes at room temperature, then sections were washed with distilled water (1 minute x 3) and dried for 30 minutes at 37°C, followed by drying at room temperature overnight. Finally, sections were rinsed in xylene (5 minutes x 2) and coverslipped with DPX mounting media (Electron Microscopy Sciences Inc., Hatfield, PA).
Glial fibrillary acidic protein (GFAP) immunohistochemistry to evaluate gliosis

Reactive glial response was determined by measuring the luminance intensity of GFAP immunoreactivity. Slide-adhered sections were washed in 0.1M PB (10 minutes x 3) and then blocked in an endogenous peroxidase treatment (0.5% hydrogen peroxide in 0.1M PB) for 30 minutes. Following washes in 0.1M PB and PBS (5 minutes x 3), non-specific background was blocked with a solution of 3% normal goat serum, 3% BSA, 0.3% Triton X, and 0.1M PBS. Tissue was rinsed in 0.1M PBS and incubated in a diluent mixture (1% normal goat serum + 2% BSA + 0.3% Triton X + 0.1M PBS) containing anti-GFAP (Dako, Carpinteria, CA) at a 1:400,000 titre for 30 minutes at 37°C, then overnight at 4°C. Next, tissue was washed in 0.1M PBS (10 minutes x 9), then incubated for 24 hours at 4°C in the diluent mixture (described above) containing secondary Ab serum at a 1:400 titre (goat anti-rabbit Alexa Fluor 488; Invitrogen, Grand Island, NY). Sections were rinsed in 0.1M PB (10 minutes x 3) and 0.1M PBS (10 minutes x 6), then slides were coverslipped with DPX mount (Electron Microscopy Sciences Inc., Hatfield, PA).

Unbiased stereology and quantification of histological markers

Beginning at a randomly chosen first section near bregma -0.8mm, measurements were obtained in every 10th section throughout the rostral-caudal extent of the lesion, ending approximately at bregma -4.8mm (~4mm total tissue). All assessments were performed by investigators naïve to the treatment of the animal. Stereological counting was conducted on an Olympus BX-51 microscope linked to a MicroFire® true color CCD digital camera (Optronics, Goleta, CA) using StereoInvestigator software (Microbrightfield Inc., Williston, VT) at 200X-400X magnification. In the regions of interest, the optical fractionator probe was used to quantify the total number of neurons. For analysis of cresyl violet histochemistry, only neurons possessing a soma diameter greater than 9µm and a clearly defined nucleus and/or nucleolus were counted. For assessment of Fluoro-Jade B histochemistry, only cells with fluorescence intensity twice that of background were counted. GFAP–positive cells were quantified using relative luminance intensity. This was calculated from the fluorescence intensity in three 50×50µm sampling boxes which were randomly placed in the regions of interest. Micrographs were taken with a 20x objective. All intensity values presented in the figures are raw data obtained from captured images. These pixel intensities were kept within the camera's dynamic range (0–4095) and pixel saturation was avoided by manipulating the imaging parameters of gain, offset, and exposure time to ensure that intensity values fell within the middle of the dynamic range. All images were captured with identical settings. Three intensity values per region were averaged.

Statistical analysis

All data were analyzed with SigmaPlot® (v11; Systat Software Inc., San Jose, CA) and are presented as mean ± SEM. Kruskal-Wallis one-way analysis of variance (ANOVA) on Ranks was performed, followed by Tukey post-hoc analysis, or ANOVA with Holm-Sidak post-hoc analysis, for all pairwise multiple comparisons. Statistical significance was set at p<0.05.
MRI and PET-CT evaluation of edema, axonal integrity and metabolism:

MR Imaging. MR images were acquired with a 9.4T MR imaging system (Bruker BioSpin Corp., Billerica, MA) with a surface coil as receiver. The animal was placed in an animal bed equipped with circulating warm water to regulate body temperature, and was anesthetized using isoflurane (2 - 2.5%) during imaging. Anatomic imaging was acquired with a T2-weighted turbo spin-echo sequence (rapid acquisition with relaxation enhancement: RARE). The detail parameters were as follows: Repetition time (TR) = 3000 ms; echo time (TE) = 34 ms; RARE factor = 4; field of view (FOV) = 30 x 30 mm; matrix size = 128 x 128; 30 slices with 1-mm thickness. Diffusion tensor imaging (DTI) was performed using a modified Stejskal and Tanner spin-echo diffusion-weighted sequence with the following parameters: TR = 3001 ms; TE = 32 ms; matrix size = 128 x 128; FOV = 30 x 30 mm; 6 slices with 1-mm thickness. Image with b = 0 s/mm^2 was acquired first, and then diffusion sensitizing gradients were applied along six directions as follows: \([G_x, G_y, G_z] = [1, 1, 0], [1, 0, 1], [0, 1, 1], [-1, 1, 0], [0, -1, 1], and [1, 0, -1] with b = 800 s/mm^2. The diffusion tensor was represented by a 3 × 3 matrix with three eigenvalues \(\lambda_1, \lambda_2, \text{ and } \lambda_3\). The apparent diffusion coefficient (ADC) was calculated by

\[ADC = \frac{\lambda_1 + \lambda_2 + \lambda_3}{3},\]

and fractional anisotropy (FA) was calculated by

\[FA = \sqrt{3[(\lambda_1 - ADC)^2 + (\lambda_2 - ADC)^2 + (\lambda_3 - ADC)^2]} \sqrt{2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}\]

In ADC map, the edema region was determined as the area in the ipsilateral side having ADC values larger than the mean plus two standard deviations of ADC values in the contralateral side. The white matter was determined in FA maps as the region presenting higher FA values than the surrounding tissue. The relative FA value was calculated as the ratio of mean FA value in the region of white matter in ipsilateral side to that in contralateral side in each image slice, and the relative FA values of all 6 slices were averaged. ADC and FA values were calculated using computer software written with MATLAB (7.11.0, MathWorks, Inc.), and the region of interest (ROI) was segmented using conventional software, ImageJ, version 1.44p (National Institutes of Health, Bethesda, MD).

PET/CT Imaging. PET/CT imaging was performed using Triumph, a PET/CT dual-modality imaging system (GE, Northridge, CA) right after completing MR imaging. X-ray CT (computed tomography) imaging was applied together with PET imaging to identify the injured area. \(^{18}\)F labeled fludeoxyglucose (FDG) (65±1 MBq (mean ± SE) in 300 µl phosphate buffer saline (PBS)) was administered intravenously, and a 10-min scan was performed at 43±2 minutes after injection. Animals were under isoflurane anesthesia (2~2.5%) during dosing and imaging. The temperature of the animal bed in the Triumph scanner was maintained to 37°C during imaging. PET images were reconstructed with maximum likelihood expectation maximization algorithm (10 iterations) in high-resolution mode. The axial FOV of PET images was set to 37.5 mm, and the axial spatial resolution and sensitivity at the center of FOV were 2.2 mm and 5.9%, respectively (4). In CT imaging, the X-ray tube voltage and anode current were set to 75 kVp and 0.11 mA, respectively, and the axial FOV was 78.9 mm. 256 projections were acquired in fly gantry-motion mode for 1.07 minutes. The co-registration of PET and CT images was performed using ImageJ version 1.44p (National Institutes of Health, Bethesda, MD). In PET images, the standardized uptake value (SUV) was calculated by \(SUV = (C \times W) / D\), where \(C\) is
tissue activity concentration (MBq/ml), $W$ is animal body weight (g), and $D$ is the administered dose (MBq). The relative SUV was determined by the ratio of mean SUV in the upper half brain to that of central region (about 10 mm$^2$) per each slice showing skull opening, and the relative SUVs of 4 images (slice thickness: 1.175 mm) were averaged. The upper half brain region was manually determined based on the rat brain anatomy in CT images, while the central region was determined using shell analysis technique introduced in our previous study (5). The segmentation of central brain region and SUV quantification were implemented using computer software written with Labview 2010 (National Instruments Co., Austin, TX). The upper half brain region was segmented using conventional software, ImageJ, version 1.44p (National Institutes of Health, Bethesda, MD).

KEY RESEARCH ACCOMPLISHMENTS: Using E2-SO$_4$ and EE-3-SO$_4$ (E2S and EES, respectively) we have been able to:

- Confirm that early administration of E2S/EES (i.e., 1 hr after TBI) will lessen TBI damage and potentially mitigate for extending the “Golden Hour”.
- Reduce clinically-important pathological conditions associated with TBI, such as intracranial pressure, partial oxygen tension and cerebral perfusion pressure.
- Lessen the extent of cerebral edema and diffuse axonal injury, as revealed by MRI.
- Demonstrated improvement in behavioral and physical performance for memory, cognition, fear, anxiety This was correlated with physical activity measurements using an automated instrument of our own design.
- *In vitro* testing with histological methods such as Fluoro Jade B, creosyl violet and immunohistological determinations with glial fibrillary acidic protein (GFAP) all demonstrated significant preservation of E2S treated neurological tissues in the brain as compared to vehicle treated, LFP-injured rats.

CONCLUSION: We have determined that when EES is administered 1 hour after TBI, several highly relevant associated pathologies are diminished. Among these are intracranial pressure, cerebral edema, neuronal death (assayed by multiple histological tests), axonal integrity and programmed cell death. We have also found that metabolic activity in the brain injury site is enhanced with EES treatment (determined by PET-CT).

The same reduction in pathology appears to be true for the intact organism. For this we have measured multiple biomarkers for behavior, including cognition and memory, fear and anxiety, vestibulomotor performance and generalized physical activity. These behavioral enhancements are important because they extend the benefits of the single dose of EES given in the first hour past the first week post-injury.

Taken together, we feel that our hypothesis that early administration of EES post TBI, preferably within the “golden hour”, is valid. Thus EES has potential to significantly reduce the damage and pathologies associated with TBI, and given its relative ease of delivery and portability, can be a potential TBI treatment in the far forward battlefield or for delivered by civilian first responders at the scene of injury.

- **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

  - Peer-Reviewed Scientific Journals: Published journal articles are listed below, with full text as PDF documents.
- Lay Press: Nothing to report

- Invited Articles: Nothing to report from journals, invited book chapter below.

- Abstracts:
  - **2010** No presentations
  - A second presentation was made at the ATACCC Military Medicine Meeting in August 2011. The title of the presentation was “Estrogen Sulfate Administration Post-TBI Decreases ICP, Increases Partial Brain O2 and Facilitates a Return to Normothermic Brain Temperature.”*
  - **2012** We again presented our work at two different meetings. First was at the MHSRS meeting in Ft. Lauderdale, FL, August 14, 2012 titled “Salutary effects of estrogen sulfate (E2-SO4) following traumatic brain injury (TBI)”*. The authors were A Ayub, H Kim, G Zhai, WJ Hubbard, CL Floyd, NL Day, KR Zinn and IH Chaudry. The second presentation was at the Shock Society meeting, June, 2012, in Miami, FL titled “Estrogen sulfate (E2-SO4) administration one hour post-traumatic brain injury (TBI) helps to lessen edema, improve metabolism and physiological conditions in the rat brain.” Shock 37 (supplement 1): 44, 2012. The authors were A Ayub, H Kim, G Zhai, WJ Hubbard, CL Floyd, NL Day, KR Zinn and IH Chaudry.*
  - **2013** There was a presentation by Guihua Zhai, et al., at the 2013 World Molecular Imaging Society meeting held in Savannah, GA. This poster presentation titled “Evaluation of ethynylestradiol-3-SO4 (EE-3-SO4) in a rat model of traumatic brain injury using MRI and PET/CT”. The work was found to be exceptional and was nominated for a poster award.
  - **2014 - 2016** no presentations.

- **INVENTIONS, PATENTS AND LICENSES:** We have been awarded two patents. The inventors are Irshad H. Chaudry, William J. Hubbard and Feng Ba. One patent pertains to trauma-hemorrhage (9,301,970, which was filed May 22, 2006 and issued April 5, 2016) and the other pertains to TBI (9,359,451), filed March 15, 2013 and issued June 7, 2016. This patent is accompanied by an international patent (bearing the same grant number 9,359,451) which was filed March 15, 2013 and issued June 7, 2016. Both domestic patent issues and the international grant bear the title: “Methods and composition for treating trauma-hemorrhage using estrogen and derivatives thereof.”

- **REPORTABLE OUTCOMES:** Our list of reportable outcomes is as follows:
  - Using our rat LFP model of TBI, we have found convincing physiological, behavioral, histological, biochemical, and imaging evidence that EES, when administered one hour after injury, reduces pathology and hastens recovery from TBI.
Our data would favor the use of EES as a drug administered alone, rather than as a combinatorial treatment (minocycline, memantine, glucosamine), which greatly simplifies packaging and delivery.

- Patents have been issued which cover TBI, thus enabling the negotiations for pharmaceutical company partners for development and commercialization.
- Our findings validate the use of EES for treating a variety of injuries, which would be shared for both warfighter and civilian use.
- On a parallel track, we have DoD funding to pursue clinical trials for EES in treatment for severe blood loss. Thus if we successfully obtain FDA permission including IND status for EES, it would put the use of EES on a greatly simplified path toward TBI applications.

**OTHER ACHIEVEMENTS:** Nothing to report

**REFERENCES:** All important references are contained in the four publications provided (below). Furthermore they are in a more relevant and expansive context. Thus we would respectfully request that these sources provide the requisite references for scientific annotation and methodology. Furthermore, our invited chapter from a book dealing with TBI is a review, and thus contains a broad and useful compilation of references.

**APPENDICES:**

Peer-reviewed publications:


**QUAD CHARTS:**

**Study Aim(s):**
- Establish lateral fluid percussion model. Apply model to evaluate 17ß-estradiol sulfate (E2) for efficacy in TBI. Criteria will be amelioration of cellular pathology and preservation of behavior.
- Perform invasive and non-invasive testing of pathological and physiological parameters with E2 and EE (ethinyl estradiol sulfate).
- Evaluate combination of EE and memantine or minocycline for enhanced TBI treatment.
- Apply E2 to in vitro correlates and in silico modeling
- Assess impact of EE on ameliorating physiological deficits plus weight gain and physical activity, as well metabolic activity.

**Approach**
Our hypothesis is that early administration of soluble estrogen post-TBI will lessen the severity of brain damage. Hypothesis tested by measuring edema (MRI), neuronal integrity (DTI), and metabolic activity (PET-CT). Histology, intracranial pressure, perfusion pressure and partial O2 pressure, weight gain/physical activity.

**Goals/Milestones**

**CY 08-10 Goal**
- Establish LFP. Test E2 benefits for pathology and behavioral deficits

**CY 10-12**
- EE testing pathology, behavior. In silico modeling.
- MRI & PET/CT: Physical activity, weight gain. Manuscript prep.

**CY 12-13 13-15**
- MRI & PET/CT: Physical activity, weight gain. Manuscript prep.

**Actual/Estimated Budget ($K)**
- Estimated: $1,568,251
- CY 10-12: $1,585,785
- CY 12-13: $438,300
- CY 13-15: $208,853
- Total: $550,423

**Updated:** February 4, 2015

**MARKING OF PROPRIETARY INFORMATION N/A**