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Erythropoietin Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Experimental studies targeting traumatic brain injury (TBI) have reported that erythropoietin (EPO) is an endogenous neuroprotectant in multiple models. In addition to its neuroprotective effects, it has also been shown to enhance reparative processes including angiogenesis and neurogenesis. Based on compelling pre-clinical data, EPO was tested by the Operation Brain Trauma Therapy (OBTT) consortium to evaluate therapeutic potential in multiple TBI models along with biomarker assessments. Based on the pre-clinical TBI literature, two doses of EPO (5000 and 10,000 IU/kg) were tested given at 15 min after moderate fluid percussion brain injury (FPI), controlled cortical impact (CCI), or penetrating ballistic-like brain injury (PBBI) with subsequent behavioral, histopathological, and biomarker outcome assessments. There was a significant benefit on beam walk with the 5000 IU dose in CCI, but no benefit on any other motor task across models in OBTT. Also, no benefit of EPO treatment across the three TBI models was noted using the Morris water maze to assess cognitive deficits. Lesion volume analysis showed no treatment effects after either FPI or CCI; however, with the 5000 IU/kg dose of EPO, a paradoxical increase in lesion volume and percent hemispheric tissue loss was seen after PBBI. Biomarker assessments included measurements of glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) in blood at 4 or 24 h after injury. No treatment effects were seen on biomarker levels after FPI, whereas treatment at either dose exacerbated the increase in GFAP at 24 h in PBBI but attenuated 24–4 h delta UCH-L1 levels at high dose in CCI. Our data indicate a surprising lack of efficacy of EPO across three established TBI models in terms of behavioral, histopathological, and biomarker assessments. Although we cannot rule out the possibility that other doses or more prolonged treatment could show different effects, the lack of efficacy of EPO reduced enthusiasm for its further investigation in OBTT.

Key words: biomarker; controlled cortical impact; fluid percussion; neuroprotection; penetrating ballistic-like brain injury; rat; therapy

Introduction

Traumatic brain injury (TBI) affects up to 2% of the population per year and is a serious clinical and common public health problem worldwide.[1–4] TBI is a major cause of death and disability throughout the world, and recently there has been an increase in the prevalence of TBI in the elderly because of falls and other traumatic insults. TBI is also the signature injury of modern warfare with ~20% of US soldiers returning from Afghanistan and Iraq with evidence of mild TBI.

The consequences of TBI are multifactorial and can include long-term cognitive, behavioral, or physical deficits including post-concussion syndromes. Although much research has been conducted to clarify the complex pathophysiology of TBI, neuroprotective

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treatments have not been successfully translated to the clinic.[5] There are various proposed reasons for this failure, including the complexity and heterogeneous nature of clinical TBI, as well as other limitations regarding adequate pre-clinical data to support the translation of therapies into the clinic.

A review of the experimental TBI literature suggests that erythropoietin (EPO) is a promising future therapy.[6–15] Subsequent to the selection and testing of EPO in Operation Brain Trauma Therapy (OBTT), however, a recent single-center clinical trial in EPO in severe TBI was completed that did not show efficacy.[16] Thus, controversy exists with regard to the potential efficacy of EPO, including effects across type and/or severity of injury.

EPO is a member of the type 1 cytokine superfamily consisting of a 165-amino acid sequence.[17] This hormone is produced by the kidneys and leads to production of erythrocytes.[17] It has been identified in brain astrocytes, and expression has been shown to increase under certain pathological conditions including hypoxia.[18] EPO expression has been documented in biopsies of the human hippocampus, amygdala, and temporal cortex with hypoxia inducible factor-2 being the major regulator of EPO expression during hypoxia.[19]

EPO has been shown to be neuroprotective in multiple pathological conditions including ischemia, hypoxia, neurotoxic and excitotoxic stress in the nervous system.[20] Also, EPO receptors have been identified in the brain, and their activation can mediate several potentially protective effects after brain injury.[21] In models of TBI, EPO doses of 5000 IU/kg have produced recovery of neurological function and tissue preservation after TBI.[21] In addition to dose response studies, the beneficial effects of EPO administration appear to be optimal within 6 h after injury, although some preservation has been seen in EPO given as late as 24 h after injury.[6,7,9,22]

In addition to preserving tissue integrity, EPO has also been shown to promote reparative events including angiogenesis and neurogenesis after TBI.[6] Subsequent studies have clarified that the effect of EPO on regenerative processes occurs through metalloproteinase 2 and 9 and/or other specific cell signaling cascades.[23–26]

The fact that EPO has been shown to be beneficial in multiple TBI models and produce long-term improvements in behavioral outcome makes it potentially promising for additional clinical testing—even with a negative clinical trial in severe TBI. Testing of EPO would also reflect on the ability of OBTT to predict efficacy of a therapy in a clinical trial of severe TBI.

Thus, the purpose of this study was to use the OBTT platform to test this therapy across three injury models for efficacy. We tested a dose of EPO (5000 IU/kg) that has shown efficacy in new treatment pre-clinical studies and a high dose (10,000 IU/kg) to explore whether there is a dose response in the protective efficacy. We assessed clinically relevant behavioral outcome measures including motor and cognitive function as well as circulating blood biomarker levels. Our studies provide novel data regarding the treatment effects of EPO across the various injury models as well as unique biomarker signatures for EPO treatment. Consistent with the clinical trial but not the pre-clinical literature, our studies with EPO did not show a positive effect in improving behavioral, histopathological, or biomarker outcomes across the OBTT consortium.

Methods

This treatment article is the third in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma*; thus, the methodology will only be briefly stated. Readers are referred back to the first therapy manuscript in this issue—namely, the article assessing the effects of nicotinamide—for more detailed methods.[27]

Male Sprague-Dawley rats (300–350 g) were used for all experiments. Animal care was in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee, the United States Army, and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*. Rats were housed in a temperature-controlled room (22°C) with a 12-h light/dark cycle. All animals had access to food and water *ad libitum,* except where noted in Methods.

Animal models

Fluid percussion brain injury (FPI) model—Miami. Rats were anesthetized (70% N_2O/30% O_2, 1–3% isoflurane) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[28] A right craniotomy was performed, and a plastic injury tube was placed over the exposed dura. The scalp was sutured closed, and the rats returned to their home cage. After fasting overnight, the rats were anesthetized, tail artery and jugular vein catheters were placed, the rat was intubated and underwent a moderate FPI. Blood gases were obtained while the animals were intubated, and levels were measured from arterial samples 15 min before and 30 min after moderate FPI.

FPI served as our sentinel model for assessing the effects of therapies on acute physiological parameters including hemodynamics and blood gases, and the 30 min time point provided an assessment of the effect of TBI and treatment at 15 min after drug administration. Sham rats underwent all procedures except for the FPI. After TBI, the rats were returned to their home cages with food and water *ad libitum.*

Controlled cortical impact (CCI) model—Pittsburgh. Rats were anesthetized (2–4% isoflurane in 2:1 N_2O/O_2), intubated, and placed in a stereotaxic frame. A parasagittal craniotomy was performed, and rats were impacted with the CCI device (Pittsburgh Precision Instruments, Inc.) at a depth of 2.6 mm at 4 m/sec.[29] The scalp was sutured closed, and rats were returned to their home cages. Sham rats underwent all procedures except for the CCI.

Penetrating ballistic-like brain injury (PBBI) model—Walter Reed Army Institute of Research (WRAIR). PBBI was performed as described previously.[30] Briefly, anesthetized (isoflurane) rats were placed in a stereotaxic device for insertion of the PBBI probe into the right frontal cortex at a depth of 1.2 cm. The pulse generator was activated, and the elliptical balloon was inflated to produce a temporary cavity in volume equal to 10% of the total brain volume. After probe withdrawal, the craniotomy was sealed with sterile bone wax, and wounds were closed. Sham rats underwent all procedures except for the PBBI probe insertion.

Drug administration

EPO was purchased at each site's pharmacy (Procrit, Amgen) and kept refrigerated until use. A new vial of EPO was used each day, because the drug is preservative free. Rats received one of two intravenous (IV) doses—5000 IU/kg or 10,000 IU/kg 15 min after injury over a 5 min period. The 5000 IU/kg dosing regimen was selected based on previous pre-clinical investigations.[6] Physiologic saline was administered as a control. Sham operated rats received no treatment. The drug was prepared at each site by an individual who did not perform the injury, behavioral testing, or histopathological analysis. Group numbers for each study site are summarized in Table 1.

Biomarker serum sample preparation

Blood samples (0.7 mL) were collected at 4 h and 24 h post-injury as well as before perfusion for histological analysis. Blood

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Erythropoietin Study

Group	Sham	TBI-Vehicle	TBI-5000 IU/kg	TBI-10,000 IU/kg	N
FPI - Miami	9	10	10	10	39
CCI - Pittsburgh	12	12	12	12	48
PBBI - WRAIR	9	15	14	15	53

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury; WRAIR, Walter Reed Army Institute of Research.

withdrawals for the FPI and PBBI models were taken from an indwelling jugular catheter at 4 h and 24 h after TBI and via tail vein at identical time points after CCI. Blood samples at the terminal end-point were taken via cardiac puncture for all models. Blood was prepared as described previously for serum in FPI and PBBI and plasma in CCI.[31] All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, Inc., for further analysis of biomarker levels.

Primary outcome metrics

The overall approach to outcome testing, scoring, and details of the specific outcome methods and metrics are described in detail in the first therapy article within this issue.[27] These outcomes include (1) sensorimotor, (2) cognition, (3) neuropathology, and (4) biomarkers.

Sensorimotor methods.

FPI model. The spontaneous forelimb or cylinder test was used to determine forelimb asymmetry as described previously.[32] The gridwalk task was used as well to determine forelimb and hindlimb sensorimotor integration. Rats were assessed at 7 days post-injury.

CCI model. Two sensorimotor tests were used—the beam balance task and the beam walking task, as described previously.[33] Rats were assessed during the initial 5 consecutive days post-CCI.

PBBI model. A modified neuroexamination was used to evaluate rats at 1, 7, 14, and 21 days post-injury.[34] Additional assessments of motor coordination and balance used the fixed-speed rotarod task on days 7 and 10 post-injury.[30]

Cognitive testing.

All sites used the Morris water maze (MWM) for cognitive testing. Spatial learning was assessed over ~13–18 days post-injury depending on the site. Primary outcomes included path latency (all sites), swim distance (only FPI), and thigmotaxis (only PBBI). All three sites also included a probe trial to determine retention of the platform location after removal. In addition, the Miami site tested the rats for working memory on days 20 and 21, and both the Pittsburgh and WRAIR sites used a visible platform task on days 19–20. Detailed descriptions of cognitive testing are described elsewhere.[27,35]

Histopathological assessments.

After behavioral testing, rats were anesthetized and perfused with 4% paraformaldehyde (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding or frozen sectioning. Coronal slices were stained with hematoxylin and eosin for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.[27] Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (''noninjured'') hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). In FPI, lesion volume and tissue volume loss were expressed as a percent of the contralateral cortex rather than the entire hemisphere given the small lesion size and established standard protocol in Miami.

Biomarker assessments.

Blood levels of neuronal and glial biomarkers, namely ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) were measured by enzyme-linked immunosorbent assay (ELISA) at 4 h and 24 h post-injury. Please see Mondello and associates[31] and Shear and colleagues[27] for a more detailed description of the ELISA and other biomarker-related methods used in these studies.

Primary outcome metrics for the biomarkers consisted of (1) evaluating the effect of drug treatment on blood biomarker levels at 24 h post-injury and (2) the effect of drug treatment on the difference between 24 h and 4 h (delta 24–4 h) levels. We chose these two primary outcomes for different reasons: 24 h post-injury represents an optimal time window for evaluating any substantial effects of a drug on biomarker levels. On the other hand, delta 24–4 h has a great appeal because assessment of drug effect will account for the initial severity of the injury while allowing each rat to serve as its own control.

For the sake of completeness, GFAP and UCH-L1 levels at 4 h post-injury were also reported. This information helps to characterize the release pattern of biomarkers in the acute phase and the relation to injury severity and may have potential clinical implications regarding the assessment of the temporal window of biomarkers for detecting a drug effect.

OBTT outcome scoring matrix

To determine therapeutic efficacy across all models, a scoring matrix summarizing all of the primary outcome metrics (sensorimotor, cognition, neuropathology [lesion volume, cortical volume]), and biomarker (24 h and delta 24–4 h) assessments was developed. A maximum of 22 points at each site can be achieved. Details of the OBTT Scoring Matrix are provided in the initial companion article in this issue.[35]

Statistical analysis

Normality was assessed, and data are expressed as mean ± standard error of the mean or median (interquartile range), as appropriate. Physiological data, contusion and tissue volumes, and probe trial were analyzed using a one-way analysis of variance (ANOVA). One-way ANOVA or repeated measures ANOVA was used to analyze motor tasks as appropriate, depending on the specifics of the data collection. Repeated measures ANOVA was also used to analyze data for the hidden platform and working memory tasks.

Post hoc analysis, when appropriate, used the Student-Newman Keuls (SNK) or Tukey test. The differences in biomarker concentration among the groups in each TBI model were analyzed with the Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction.

All statistical tests were two-tailed and a p value <0.05 was considered significant. Statistical analyses were conducted using SAS (SAS version [9.2] of the SAS System. © 2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Physiological parameters

Physiological parameters, including mean arterial blood pressure (MABP), PaO_2, $PaCO_2$, and blood pH, taken in the FPI model (Miami) are provided in Table 2. Physiological variables were taken before and after TBI. All physiological values were within normal range, and there were no significant differences between the various experimental groups in terms of MABP, PaO_2, $PaCO_2$, and blood pH. There was no effect of treatment on acute physiology or blood gases.

Sensorimotor parameters

FPI model. Rats were assessed using the cylinder task for spontaneous forelimb use (Fig. 1A). One-way ANOVA was not significant between groups ($p = 0.89$). All injured rats exhibited contralateral forelimb placing deficits with an asymmetry index of <0.5. There was no improvement on this task versus vehicle (VEH) treatment with either dose of EPO.

Sensorimotor integration was analyzed using the gridwalk test (Fig. 1B). Each forelimb and hindlimb is assessed independently for foot faults. Data are expressed as a percent of total steps for each limb. One-way ANOVA for both contralateral forelimb and hindlimb were not different between groups ($p = 0.658$ and $p = 0.715$, respectively). Similar findings were found for ipsilateral forelimb and hindlimb placement. One-way ANOVA for ipsilateral forelimb and hindlimb were not significant for group ($p = 0.933$ and $p = 0.886$, respectively). EPO treatment did not improve sensorimotor function as assessed by the gridwalk task.

CCI model. For the beam balance test, a two-way repeated measures ANOVA revealed a significant group main effect for beam balance latencies over 5 days post-injury ($p = 0.002$) (Fig. 1C). None of the injured groups differed from each other, however. While the CCI + VEH and CCI + low dose EPO treatment significantly differed from the sham group, the CCI + high dose EPO group did not differ from sham—indicating an intermediate motor benefit of high dose EPO in CCI on beam balance testing. This resulted in half of the possible points (+1) for this outcome for the high dose EPO group in the OBTT scoring matrix. A two-way repeated measures ANOVA revealed a significant group main effect ($p = 0.001$) for beam walking latencies over 5 days post-injury (Fig. 1D). All injury groups performed significantly worse after CCI versus sham. There were no significant differences between any of the treated and untreated injury groups.

PBBI model. *Post hoc* analysis of neuroscore assessments revealed significant abnormalities in all injured groups (vs. sham) that were sustained out to 3 weeks post-PBBI ($p < 0.05$) regardless of treatment (Fig. 1E).

Motor and balance coordination were assessed on fixed-speed version of the rotarod task (Fig. 1F, G). Repeated measures ANOVA (four groups × three speeds) revealed significant between-group effects at 7 days ($p < 0.001$) and 10 days post-injury $p < 0.001$) with significant motor impairment evident across all injured groups. There was also a significant effect of speed (rpm) at 7 days ($p < 0.001$) and at 10 days post-injury ($p < 0.05$) but no significant interaction. Overall mean rotarod latency scores were reduced by 51 ± 7% (PBBI + VEH), 38 ± 7% (EPO low dose), and 46 ± 8% (EPO high dose) versus sham ($p < 0.005$). Although PBBI rats treated with the low dose of EPO showed a positive (45 ± 13%) trend toward improved performance at 10 days post-injury on the rotarod task, it was not significant, and the trend was modest ($p = 0.217$ vs. PBBI).

Cognitive testing

FPI model. Cognitive function was assessed using a simple place task (Fig. 2A, B) over 4 days followed by a probe trial (see pooled analysis data later in the text), then a working memory test (Fig. 2C, D). For the simple place task or hidden platform task, sham rats showed decreased latencies over the 4 day testing period. Both TBI treatment groups had higher latencies than sham and TBI-VEH treated rats. Repeated measures ANOVA, however, was not significant for day ($p = 0.174$), group ($p = 0.239$), or group × day ($p = 0.373$). Similar findings were seen in the path length analysis. Repeated measures ANOVA for path length was significant for day ($p < 0.001$) but not for group ($p = 0.716$) and group × day ($p = 0.230$).

Drug treatment did not improve learning and memory using this paradigm. In fact, EPO treated rats performed numerically worse on this task than untreated or VEH treated TBI rats. There was also no effect on probe trial with EPO. Note that the probe trial is part of

Table 2. Effects of Erythropoietin on Fluid Percussion Injury Physiology

Group	*Sham*	*TBI-Vehicle*	*TBI-5000 IU/kg*	*TBI-10,000 IU/kg*
Pre-TBI				
pH	7.43 ± 0.01	7.45 ± 0.01	7.43 ± 0.01	7.41 ± 0.01
pO_2 (mm Hg)	152.2 ± 8.62	156.8 ± 8.95	157.4 ± 4.29	158.0 ± 8.92
pCO_2 (mm Hg)	40.51 ± 0.97	40.3 ± 1.21	40.96 ± 1.00	43.48 ± 0.59
MAP (mm Hg)	123.73 ± 3.75	129.1 ± 4.11	125.65 ± 2.94	131.8 ± 3.56
Brain temp (°C)	36.6 ± 0.06	36.0 ± 0.04	36.7 ± 0.05	36.7 ± 0.05
Body temp (°C)	36.8 ± 0.07	36.7 ± 0.07	36.8 ± 0.07	36.8 ± 0.08
Post-TBI				
pH	7.44 ± 0.01	7.46 ± 0.01	7.44 ± 0.01	7.43 ± 0.01
pO_2 (mm Hg)	147.3 ± 10.25	140.2 ± 6.98	131.1 ± 7.02	148.8 ± 7.16
pCO_2 (mm Hg)	40.1 ± 0.69	38.33 ± 0.68	40.19 ± 1.05	41.52 ± 0.71
MAP (mm Hg)	121.76 ± 2.48	125.47 ± 3.11	123.59 ± 2.88	124.22 ± 1.36
Brain temp (°C)	36.7 ± 0.04	36.7 ± 0.05	36.7 ± 0.05	36.7 ± 0.03
Body temp (°C)	36.8 ± 0.06	36.8 ± 0.06	36.8 ± 0.07	36.9 ± 0.07

TBI, traumatic brain injury; MAP, mean arterial pressure.

the pooled analysis data and is presented for all sites in Figure 2I. One-way ANOVA was not significant for group ($p = 0.810$) in the probe trial. In the working memory task, similar poor cognitive behavior on a short-term memory task was observed. Repeated measures ANOVA for working memory latency was significant for trial ($p < 0.001$) and group ($p = 0.037$) but not for group × trial.

Student-Newman-Keuls *post hoc* analysis was significant ($p < 0.05$) when comparing location to match trials collapsed across groups because the rats showed improvement in locating the hidden platform during the second paired trial. There were no significant differences between groups, however. EPO treated rats showed a trend toward worse performance on this task versus sham or TBI-VEH groups. Similar results were seen for the working memory path length analysis. Repeated measures ANOVA for working memory path length was significant for trial ($p < 0.003$) but not for group or group × trial. Student-Newman-Keuls *post hoc* analysis was significant ($p < 0.05$) for path length between the location to match trial collapsed across all groups. This only indicates that the rats were performing better from the first trial to the second trial in the pairing.

CCI model. For the hidden platform MWM task (Fig. 2E), two-way repeated measures ANOVA for latency revealed a significant group main effect ($p = 0.004$). Swim latencies across days, however, did not differ between the injured groups regardless of treatment. While swim latencies between the sham and TBI + VEH group only came close to reaching statistical significance ($p = 0.052$), there were significant differences between the sham and both EPO-treated groups—indicating an intermediate detrimental effect of EPO on this outcome. This intermediate detrimental effect resulted in negative half (-2.5) of the total points that could be awarded for this task for both EPO doses in the OBTT scoring matrix. The probe trial also showed no effect on improvement with EPO (Fig. 2I). One-way ANOVA was significant for group ($p = 0.001$) in the probe trial with all injury groups significantly worse than sham.

PBBI model. Spatial learning performance and thigmotaxic behavior (% time spent circling the outer perimeter of the maze) are represented in Figure 2 F, G, respectively. Repeated-measures ANOVA on latency to locate the hidden platform was significant

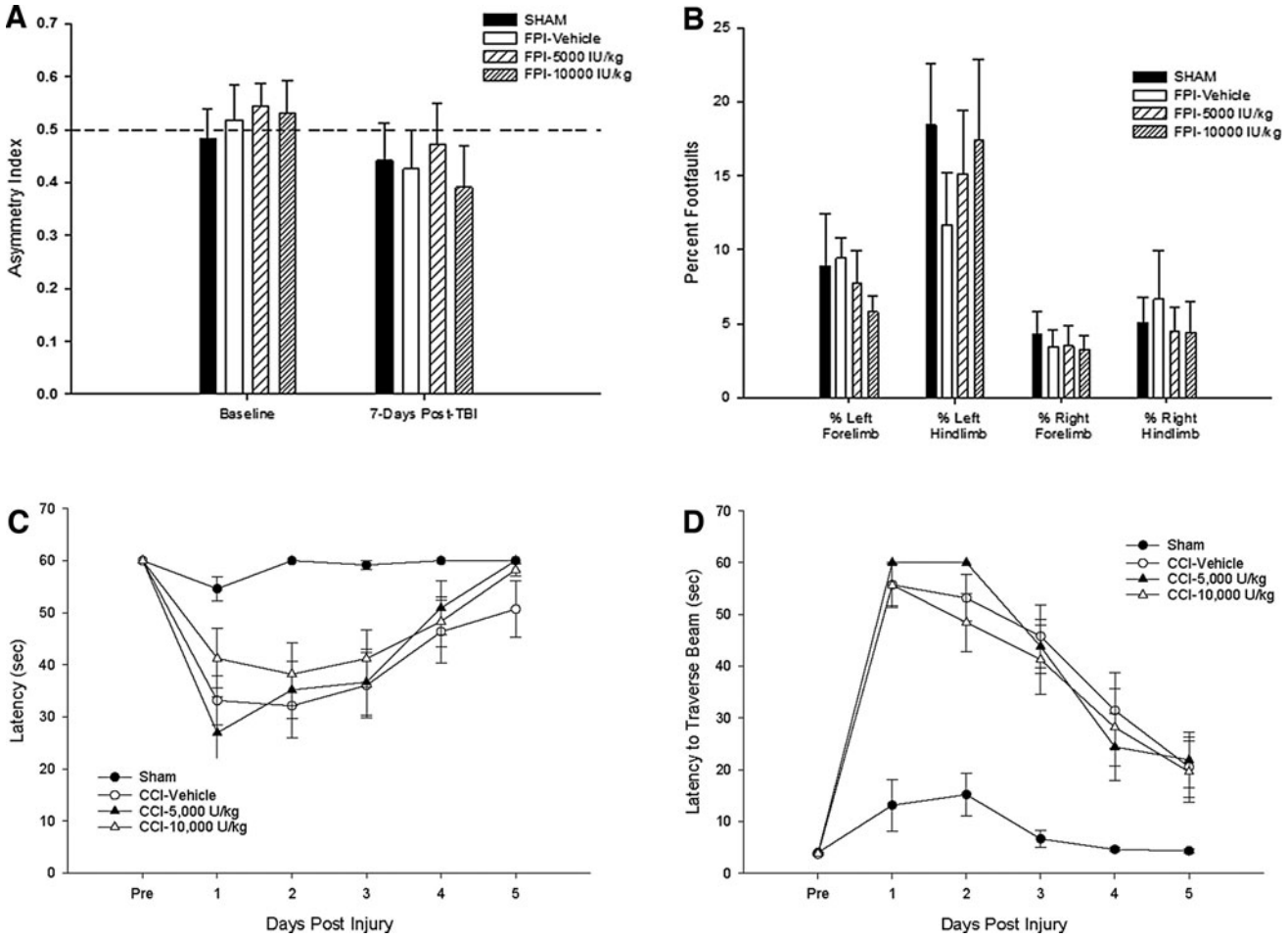

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A,B**): Bar graphs show the results of (A) spontaneous forelimb assessment and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): Line graphs show the results of the beam balance and walking task: (C) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E–G**): Graphs showing results from (E) neuroscore evaluations and (F,G) the fixed-speed rotarod task. Overall, high dose EPO treatment showed only modest benefit on beam balance in the CCI model. Please see text for details. Data represent group means ± standard error of the mean.

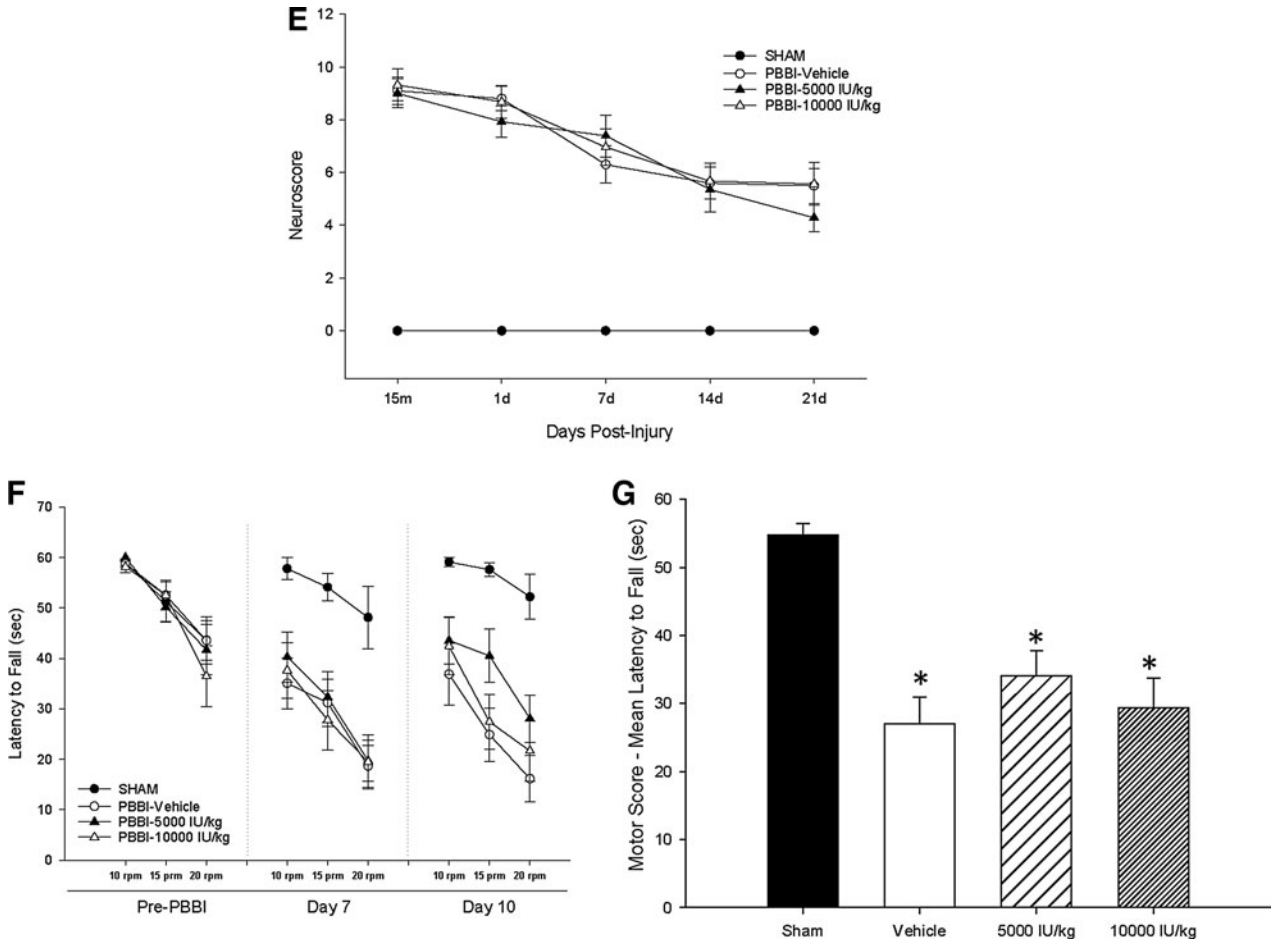

FIG. 1. (Continued).

for group ($p < 0.001$) and for trial ($p < 0.001$) but not for group × trial interaction ($p = 0.054$). *Post hoc* analysis revealed significant abnormalities in all injured groups with average escape latency (across all testing days) increased by 147 ± 13% (PBBI + VEH), 124 ± 15% (EPO-5000 IU/kg), and 115 ± 11% (EPO-10,000 IU/kg) versus sham (Fig. 2F; $p < 0.05$). Although both EPO-treated groups tended to perform better than PBBI + VEH, no significant benefits of EPO were detected on any MWM parameter. Repeated-measures ANOVA on percent time spent circling the outer perimeter of the maze was significant for group ($p < 0.001$) and for trial ($p < 0.001$) but not for group × trial interaction ($p = 0.46$). *Post hoc* analysis showed that all injured groups spent a significantly greater percentage of time circling the outer perimeter of the maze versus sham (Fig. 2G; $p < 0.05$).

ANOVA results on the probe trial were significant ($p < 0.001$) again, with all injured groups spending significantly less time searching the target (missing platform) zone versus sham (Fig. 2I; $p < 0.05$). Although drug-treated PBBI rats (both doses) tended to perform better than PBBI-VEH across all parameters, no significant therapeutic benefits of EPO were detected on any MWM parameter.

Pooled analysis of therapeutic effects across OBTT

For ease of comparison of the major findings, we present a pooled analysis of four key outcomes in OBTT—namely, average latency to find the hidden platform, probe trial, lesion volume, and tissue loss (Fig. 2H, I and 3A, B).

Cognitive outcomes. Figures 2H, I show the effect of EPO treatment across all models in OBTT for average latency across days and probe trial, respectively. For MWM average latencies, both CCI and PBBI models exhibited significant deficits after injury compared with sham ($p < 0.05$). In addition, as anticipated from the previous analyses, both doses of EPO showed no improvement in cognitive function versus TBI - VEH. Average latency across all testing days for FPI did not show a deficit in the TBI-VEH rats; thus, a somewhat more severe injury level may have been more optimal in FPI for this task.

The MWM probe trial followed a similar pattern with no benefit of EPO after TBI across models. Specifically, both CCI and PBBI models exhibited significant reductions in percent time in the target quadrant on this task; however, once again there was no effect of EPO treatment—although there was a trend toward benefit of EPO in the PBBI model. Once again, FPI did not show a deficit on this task, suggesting the need for a more severe injury level.

Histological outcomes. Cross model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figure 3A, B. Lesion volume was analyzed using one-way ANOVA as a percentage of the contralateral hemisphere in CCI and PBBI and as a percentage of the contralateral cortex in FPI (Fig. 3A). Similarly, hemispheric volume loss was analyzed as a percentage of tissue loss in the injured versus noninjured hemisphere in CCI and PBBI and as a percentage of contralateral cortex in FPI (Fig. 3B).

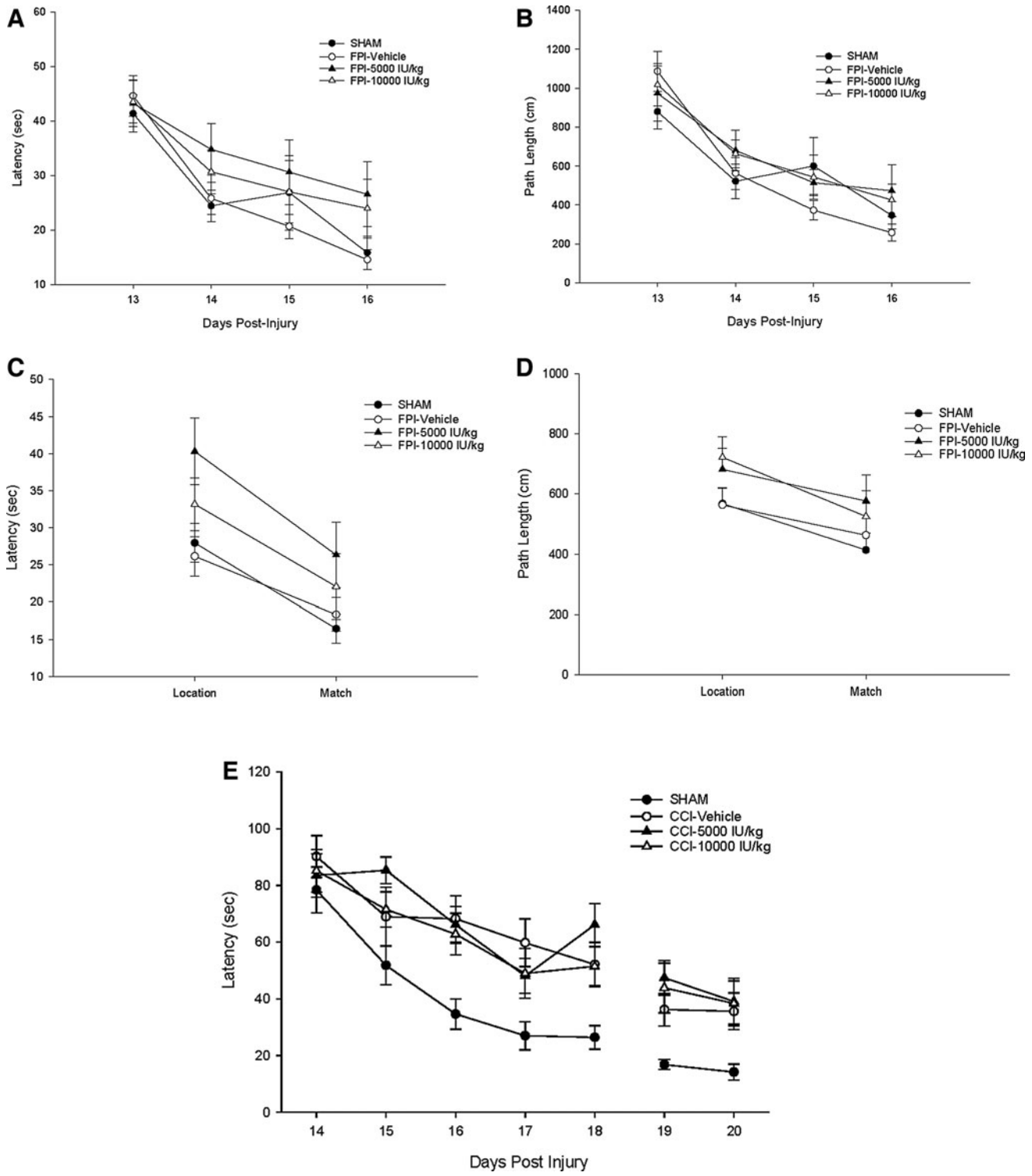

FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A–D**): Graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): Line graph showing the (E) latency to the hidden platform over 5 days of MWM testing and (F) mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) (**F, G**): Graphs showing (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H, I**): Graphs show (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. Overall, both doses of EPO showed modest detrimental effects on MWM performance in the CCI model. Please see text for details. Data represent group means ± standard error of the mean; * $p < 0.05$ compared with sham.

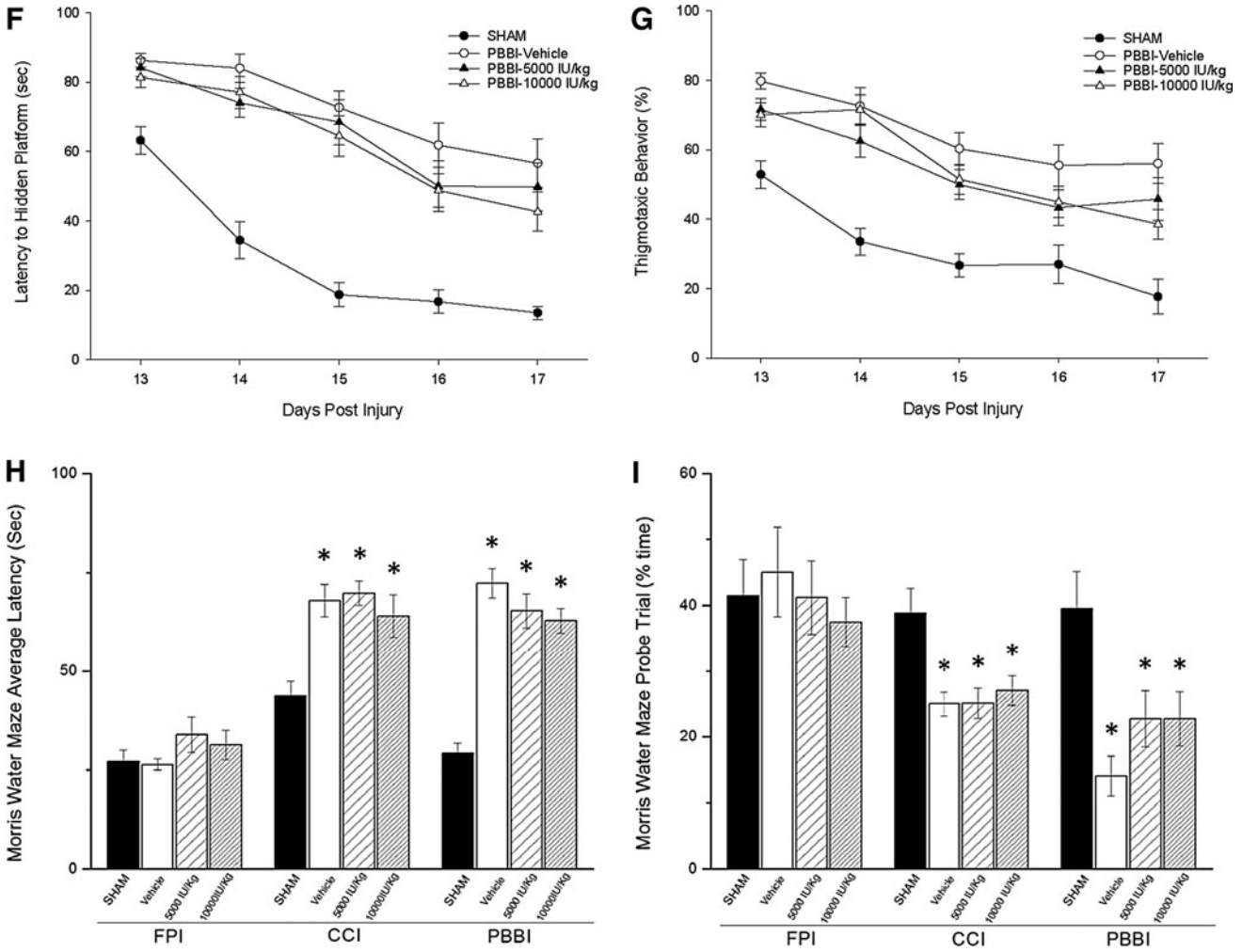

FIG. 2. (Continued).

FPI model. In the FPI model, there were no significant differences in lesion volume or cortical volume loss (a % of contralateral cortex), despite the fact that there was more cortical tissue loss ipsilateral to injury in all TBI groups (treated or VEH) versus sham.

CCI model. In the CCI model, lesion volumes ranged between ~8–9% of the contralateral hemisphere across all three TBI groups, and ANOVA did not differ significantly between groups. Similarly, hemispheric tissue loss was remarkably consistent at ~21–22% of the contralateral hemisphere in all three injury groups, which was in each case (ANOVA and Student-Newman-Keuls test) significantly different versus sham ($p < 0.05$). No treatment effect was seen in CCI.

PBBI model. In the PBBI model, ANOVA revealed a significant between-group difference on measures of lesion volume ($p < 0.05$) with post-injury administration of EPO (low dose only; 0.50 mL/kg) producing a significant and marked (>2X) increase in mean lesion volume versus PBBI + VEH (PBBI = $32 \pm 4\,mm^3$; *EPO low dose = $67 \pm 14\,mm^3$; EPO high dose = $42 \pm 7\,mm^3$; *$p < 0.05$ vs. PBBI). This deleterious effect resulted in a full −2.0 points for low dose EPO in the OBTT scoring matrix. One-way ANOVA conducted on percent hemispheric tissue loss also revealed a significant main effect ($p < 0.001$) with all injured groups showing significant hemispheric tissue loss compared with sham (*PBBI + VEH = 24 ± 1%; *EPO low dose = 32 ± 4%; *EPO high dose = 25 ± 2%; *$p < 0.05$ compared with sham). Despite a similar trend toward greater tissue loss with low dose EPO, however, neither dose had a significant effect on hemispheric tissue loss versus VEH.

Biomarker assessments

Circulating biomarker level assessments in rats from the study of the effect of EPO in OBTT were made with blood samples successfully collected from 135 of the 140 rats in this study. Effects of EPO on post-injury TBI circulating biomarker (UCH-L1 and GFAP) levels are shown in Figures 4A-C and 5A-C.

FPI model. A Kruskal-Wallis test revealed a significant main effect on GFAP levels at both 4 h and 24 h post-injury ($p = 0.0001$ and $p = 0.002$, respectively), with all injured groups showing significant increases in GFAP versus sham but no evidence of an EPO treatment effect (Fig. 4A). Consistently, delta 24–4 h GFAP levels, which measure the decay of serum GFAP levels from 4 h to 24 h, did not differ between TBI-VEH and TBI treatment groups for either dose (Fig. 5A). Unlike GFAP, no significant between-group effects for any TBI group versus sham were seen for post-injury

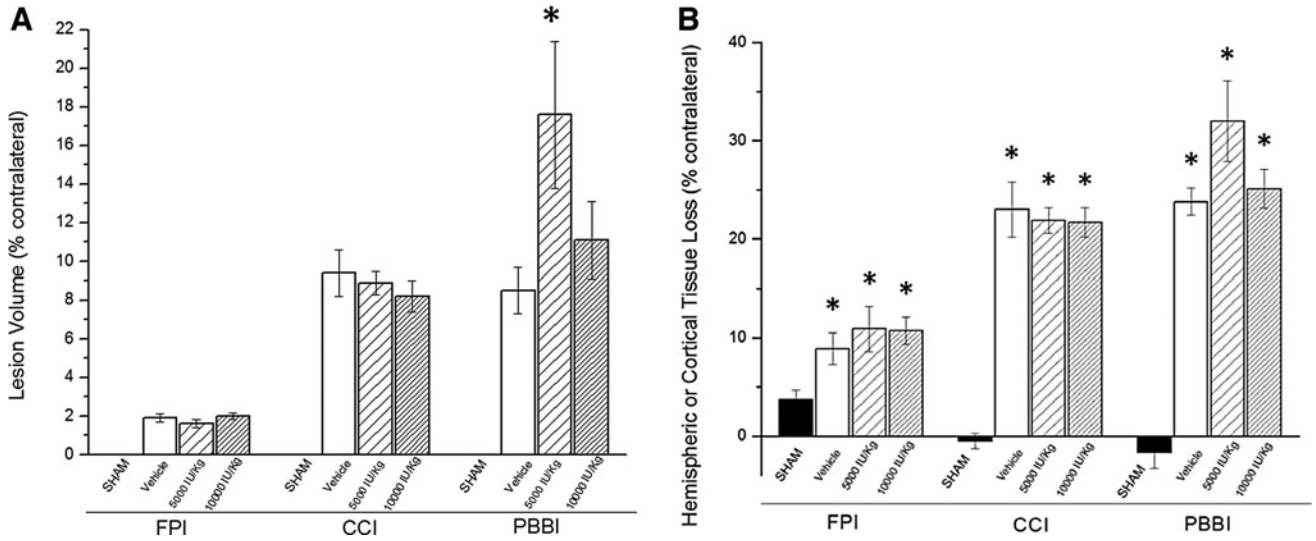

FIG. 3. Histopathology. Bar graphs showing cross-model pooled comparisons of (**A**) lesion volume as a percent of the contralateral cortex in fluid percussion injury (FPI) and hemisphere in controlled cortical impact (CCI) and penetrating ballistic-like brain injury (PBBI), and (**B**) tissue loss; cortical tissue loss in FPI (as a percent of contralateral cortex) and hemispheric tissue loss in CCI and PBBI (as a percent of contralateral hemisphere). Overall, low dose EPO showed a statistically significant detrimental effect on lesion volume in the PBBI model. Please see text for details. Data represent group means ± standard error of the mean; * $p < 0.05$ compared with sham.

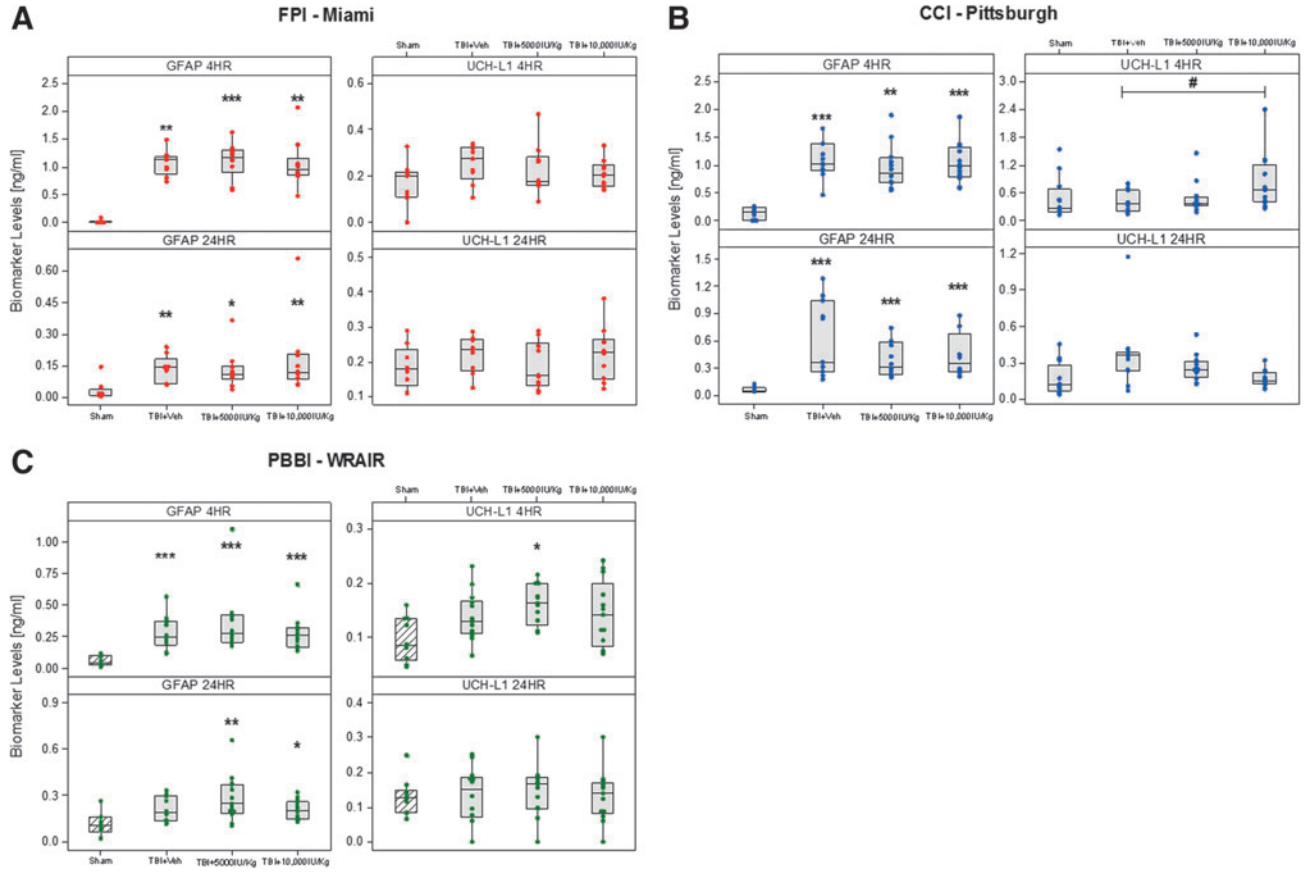

FIG. 4. Box plots illustrating glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels at 4 h and 24 h post-injury. GFAP and UCH-L1 concentrations at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, at 4 h, high dose EPO increased UCH-L1 levels versus VEH in CCI, and at 24 h both doses of EPO exacerbated the increase in GFAP levels in PBBI. *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$) vs. sham group. #(p0.05) TBI + VEH group vs. high dose EPO group. Please see text for details.

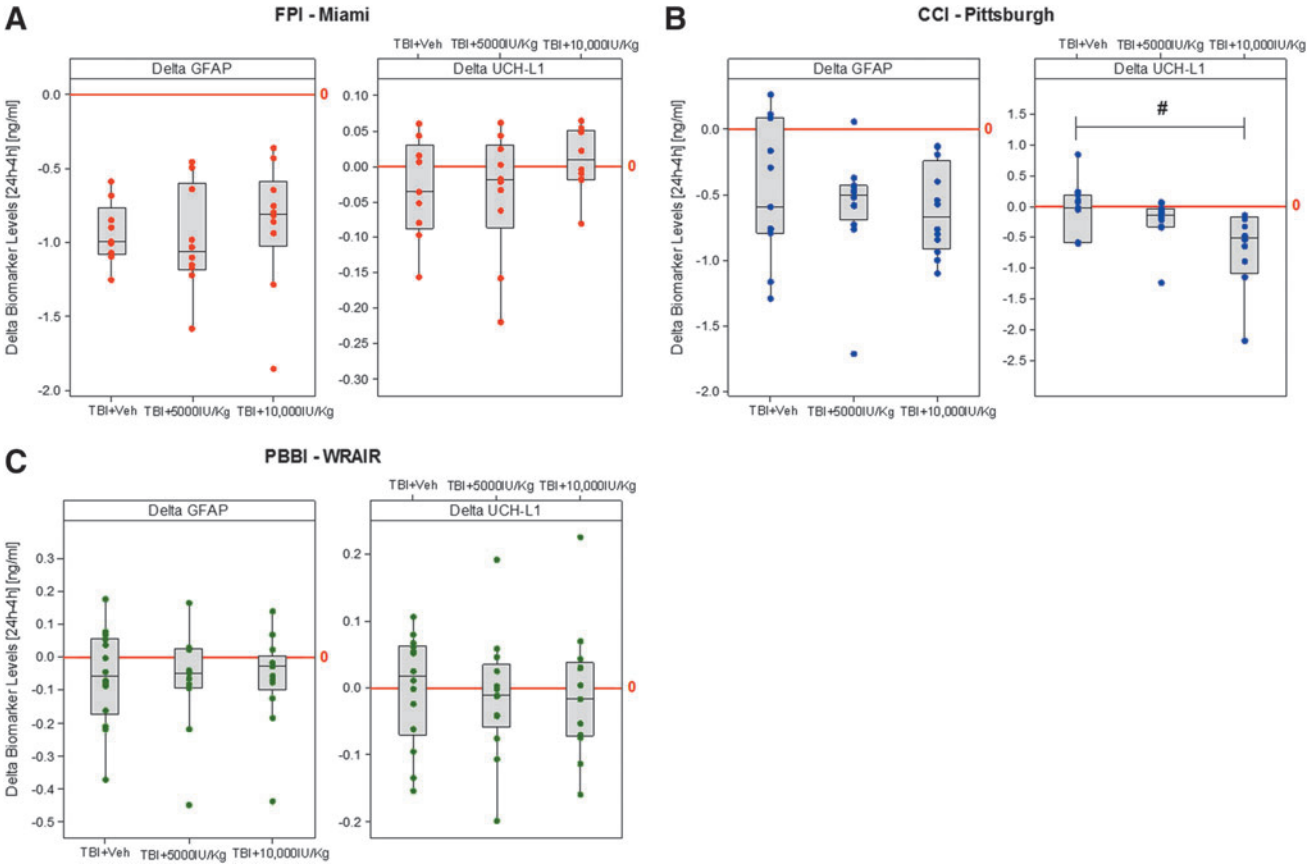

FIG. 5. Box plots illustrating delta (24–4 h) glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) biomarker levels. Delta 24–4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, high dose EPO significantly reduced delta 24–4 UCH-L1 levels in the CCI model indicating improved net UCH-L1 clearance. #($p < 0.05$). TBI + VEH group vs. high dose EPO group. Please see text for details.

levels of UCH-L1 at 4 h or 24 h (Fig. 4A). Delta 24–4 h UCH-L1 levels also showed no evidence of a treatment effect (Fig. 5A).

CCI model. Similar to FPI, significant between-group effects on post-injury levels of GFAP were detected at 4 h ($p < 0.0001$) and 24 h ($p < 0.0001$), with all three injured groups showing significantly elevated levels at both time points versus sham, but again no treatment effect (Fig. 4B). The overall analysis of delta 24–4 h GFAP levels comparing the injured groups also revealed no significant effect of group. While all three injured groups were not significantly different from sham groups for post-injury serum levels of UCH-L1 at 4 h or 24 h, surprisingly, levels of UCH-L1 at 4 h were significantly higher in the high dose EPO group versus the CCI + VEH group. In contrast, levels of UCH-L1 at 24 h were lower in the high dose EPO group versus the CCI + VEH group (median 0.15 vs. 0.36 ng/mL), although it did not reach statistical significance (Fig. 4B). As a result, delta 24–4 h UCH-L1 levels in the high dose EPO group differed significantly versus the CCI + VEH group ($p = 0.007$). This suggests a beneficial effect of EPO on UCH-L1 net clearance between 4 and 24 h after CCI and thus a full positive point for high dose EPO on this parameter in the OBTT scoring matrix (Fig. 5B).

PBBI model. The overall analysis revealed a significant main effect on GFAP levels at 4 h post-injury ($p < 0.0001$), with all injured groups showing significant increases in GFAP versus sham but no evidence of a treatment effect. Significant between-group effects on post-injury levels of GFAP were also detected at 24 h ($p = 0.003$), but only low and high dose EPO treatment groups showed significant increases versus sham ($p = 0.001$ and $p = 0.047$, respectively, Fig. 4C). This produced negative 0.5 point values for this parameter for both doses of EPO in this model in the OBTT scoring matrix. No significant between-group effects on delta 24–4 h GFAP levels were found (Fig. 5C). There was a significant between-group effect on post-injury levels of UCH-L1 at 4 h ($p = 0.023$), with a significantly higher value in the low dose treatment versus sham ($p = 0.013$, Fig. 4C). There were no significant group differences on either post-injury levels of UCH-L1 at 24 h or delta 24–4 h UCH-L1 levels (Fig. 4C and 5C).

OBTT outcome scoring matrix

The overall scoring matrix is shown in Table 3 for the effect of EPO across all models. Overall low dose EPO was deleterious, receiving a net negative 5.0 points, which was the result of negative points in the CCI and PBBI models, notably lesion volume in the PBBI model. High dose EPO received a net negative overall 1.0 points for efficacy across models. Surprisingly, no model showed a positive overall effect for EPO at either dose.

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overall total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,0	0,0,0,0,0	0,0	0,0,0,0	0
Dose 2		0,0	0,0,0,0,0	0,0	0,0,0,0	0
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		0,0	−2.5,0	0,0	0,0,0,0	−2.5
Dose 2		+1,0	−2.5,0	0,0	0,0,0,+1	−0.5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	0	0,0,0	−2,0	−0.5,0,0,0	−2.5
Dose 2	0	0	0,0,0	0,0	−0.5,0,0,0	−0.5
Grand total						
Dose 1	0	0	−2.5	−2	−0.5	−5.0
Dose 2	0	+1	−2.5	0	+0.5	−1

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; MWM, Morris water maze; WRAIR, Walter Reed Army Institute of Research
( ) = point value for each outcome within each model
Drug: EPO; Dose 1 = 5000 IU/kg; Dose 2 = 10,000 IU/kg

Discussion

The OBTT consortium tested the effects of EPO in three established rat models of TBI. Based on substantial pre-clinical data indicating that EPO is an endogenous and/or pharmacologic mediator of neuroprotection and using a similar dosing regimen to that used in some of these previous studies,[6,11,36] we sought to determine whether this treatment would be effective across three injury models that produce a range of injury severities and pathophysiological consequences. Unfortunately, EPO did not demonstrate significant effects on the outcome measures that were assessed, which included histopathology, behavioral monitoring, and biomarker assessments—and was thus remarkably disappointing in this regard.

Our findings, however, are consistent with the failure of EPO in a recently published high quality single center randomized controlled trial (RCT) in adults with severe TBI.[16] This work thus actually represents the first therapy in OBTT that has been evaluated in a clinical trial of severe TBI—and our findings are consistent with the results of that trial.

EPO has a long history of being tested in various models of TBI. In 2005, Yatsiv and colleagues[15] reported that recombinant human EPO (rhEPO) injected 1 and 24 h after TBI improved motor and cognitive function. Tissue inflammation, axonal degeneration, and apoptosis were also reduced with rhEPO (5000 IU/kg) treatment using this mouse model. Lu and associates[7] reported in rats that treatment with EPO daily for 14 days starting 1 day after CCI produced improvement in spatial memory and increases in the number of newly formed neurons.[7] These data suggested that in addition to neuroprotection, EPO treatment also had the capacity of being a neurorestorative therapy. Both of those putative properties made EPO a drug for testing by the OBTT consortium.

In another study, again using CCI injury in rats, Cherian and coworkers[6] reported that EPO (5000 IU/kg) treatments initiated at 5 min after injury led to reduced contusion volume and increased neuronal density in the CA1 and CA3 regions of the hippocampus. Therapeutic window was carefully evaluated with the beneficial effects of EPO being optimal when given within a 6 h post-traumatic time window. Using the lateral FPI model, Hartley and colleagues[36] reported that EPO (5000 IU/kg) treatment at 30 min after injury improved energy metabolism and reduced early indicators of histopathological damage.

In another supportive study, Xiong and associates[9] showed that EPO treatment at 6 h and at 3 and 7 days post-TBI (5000 IU/kg) reduced contusion volume and cell loss in the dentate gyrus and improved sensorimotor function and spatial learning performance.[9] EPO treatment also enhanced neurogenesis in the injury cortex and dentate gyrus after CCI injury. Thus, these studies suggest beneficial effects of EPO in two models of TBI commonly used in the field and also in OBTT.

We also tested EPO in a model of CCI injury and surprisingly did not observe any significant effect on either behavioral or histopathological outcomes. As suggested previously, the dose that we selected was based on the published literature. Thus, we evaluated both a low dose (5000 IU/kg) and a high dose (10,000 IU/kg) given 15 min after injury. One question with EPO that remains controversial is the optimal duration of therapy. With sustained therapy, there has been concern related to the development of polycythemia and hyperviscosity, which is a well-known limiting side effect, particularly in stroke trials.[37] Recent studies, however, have indicated that delayed treatment with EPO up to 24 h provides dose-dependent neurorestoration and improvement in functional recovery.[7,21]

In the present study, although we provided the drug within the established therapeutic window, significant effects were not seen. Whether or not multiple doses would have mediated a more robust or beneficial effect is unclear. In a study by Xiong and coworkers,[11] the beneficial effects of a single dose compared with a triple dose of EPO were examined in a rat model of CCI injury. EPO 5000 IU/kg in saline was therefore administered on day 1 or on days 1, 2, and 3. Although, histopathological improvement was seen in both treatment paradigms, the triple dose delayed EPO treatment showed better histopathological and functional outcomes in rats with TBI.

Indeed, some suggest that EPO's beneficial effect is greatest seen commonly in experiments where multiple treatments of EPO are given.[20] Given the potential controversy with prolonged therapy for clinical translation and the demonstrated efficacy of even single dose therapy, we tested whether a single dose of EPO would be effective across three different TBI models.

Many experiments have been conducted to elucidate mechanisms by which EPO may be protective against TBI. Bian and colleagues[38] evaluated the effects of EPO in a modified Feeney model and reported that EPO treatment reduced S100B and interleukin 6 levels. They suggested that one mechanism by which EPO was improving outcome was by decreasing the inflammatory response in the brain. EPO has also been shown to affect apoptotic neuronal death. In a study by Liao and colleagues,[24] EPO treatment, again in the Feeney model, reduced Bax mRNA and protein levels versus VEH treated rats. Also, the number of TUNEL positive cells was less in the EPO treated animals versus controls. These authors suggested that a mechanism by which EPO could have various antiapoptotic effects was with the differential regulation of genes involved in apoptotic processes. We did not assess hippocampal neuron counts given that it is not part of the outcome matrix in OBTT. We thus cannot rule out effects on that outcome parameter.

Other studies have assessed various cell signaling cascades that may be sensitive to EPO treatment. In a study by Valable and associates,[23] phosphorylation of two protein kinases including extracellular regulated kinase (ERK-1/-2 and AKT) was measured along with water content in animals given 5000 IU/kg recombinant human EPO. EPO treatment decreased the TBI-induced upregulation of ERK phosphorylation, although increased AKT phosphorylation was seen at 2 h after the insult. A reduction in brain edema was also seen, indicating that the antiedema effect of EPO could be mediated through early inhibition of ERK phosphorylation.

EPO treatment has also been shown to increase expression of growth factors including vascular endothelial growth factor (VEGF). In CCI, Xiong and colleagues[14] reported that delayed EPO treatment (5000 IU/kg) at 1, 2, and 3 days after injury improved sensorimotor and cognitive functional recovery as well as increased brain VEGF expression and phosphorylation of VEGF receptor-2. This suggested EPO mediated neurological recovery and vascular remodeling after TBI by engaging VEGF/VEGFR2.

Xiong and coworkers[12] also showed that EPO treatment reduced cortical tissue damage and hippocampal cell loss as well as improving spatial learning in mice that lack the EPO receptor (EPOR) in both neural and nonneural cells in the brain. EPO treatment was also shown in the EPOR-null mouse to upregulate antiapoptotic proteins (p-AKT and Bcl-XL), thus suggesting that EPO may provide neuroprotection after TBI via vascular events. We did not assess brain edema, cerebral blood flow (CBF), or the other molecular mechanisms in our studies, given the mandate in OBTT

drug screening to focus on key behavioral and histological outcomes rather than mechanism.

An interesting characteristic of EPO administration is that it increases angiogenesis and neurogenesis.[11] Several studies have reported that EPO treatment promotes cellular proliferation in the hippocampus associated with increased NeuN positive cells, indicating evidence of neurogenesis.[7] In addition, EPO has been reported to increase blood vessel formation after TBI that may improve CBF.

EPO has not been translated to benefit in clinical TBI, however, as evidenced in the aforementioned recent RCT. In addition to that trial, EPO has been tested in some other TBI clinical studies. In a study by Nirula and associates[39] in which EPO was tested in a randomized trial of patients with TBI, baseline and daily serum S100B and neuron-specific enolase (NSE) levels were measured. Compared with placebo treated patients, EPO treatment did not alter NSE or S100B levels.

Another clinical trial has suggested that EPO may reduce mortality in severely injured medical or surgical patients.[40] In that study, epoetin alfa (40,000 IU) was administered weekly for a maximum of 3 weeks with the primary end-point being a percentage of patients who received red blood cell transfusion, mortality, and change of hemoglobin concentration. Mortality rate was lower by day 28 among patients who received epoetin alfa versus placebo. That study did not focus on TBI, however.

Previous studies have shown that the beneficial effects of EPO can be separated from the erythropoietic characteristics.[41–43] In this regard, Robertson and and associates[44] recently provided new data for the use of an EPO mimetic peptide in the CCI model in rats. In that study targeting mild TBI, pyroglutamate helix B surface peptide improved performance on MWM and reduced inflammatory cell activation by cells labeled with CD68. Ongoing studies continue to test novel compounds that may be protective but do not necessarily stimulate erythropoiesis and may therefore be safer for disorders such as TBI.[44]

There are some limitations to our study. First, as suggested, we tested only single early post-TBI administration. Several studies, however, including those with Chopp and colleagues, gave multiple doses of EPO providing a longer drug exposure compared with the present OBTT studies.[7–9,11,13,14,21] In the recent negative clinical trial, EPO was administered only once in the majority of subjects because of Food and Drug Administration concerns.[16] Nevertheless, some studies have shown EPO efficacy with single administration.[6,11,36,45–48]

Second, in the FPI model in this study, the injury level was insufficient to provide a good target for all aspects of cognitive outcome. Higher injury levels in FPI can produce unacceptable mortality from apnea, so these were avoided. Given the importance of cognitive outcome scoring in OBTT, that may have limited the chances to show efficacy in FPI.

Another potentially important factor is route of EPO administration. While some studies have shown beneficial effects of EPO given IV, other studies report that intraperitoneal (IP) treatments are also neuroprotective. The method of injecting EPO could affect the temporal profile of blood levels that could potentially lead to both beneficial as well as detrimental effects. For example, IV administration could produce potentially toxic levels of a drug early on that could overshadow more appropriate therapeutic doses in terms of protecting cells from dying. The method of EPO administration in the present study differed from those produced with Chopp and colleagues and by Robertson and coworkers who used IP administration.[7–14,16]

Conclusion

Our results indicate that treatment with EPO at two doses previously reported to be effective in the published literature failed to provide significant protection and improve functional outcome across three models of TBI. Treatment was based on published data where a single treatment dose of EPO given early after TBI had been shown to be effective in improving outcome. Although we cannot rule out the possibility that other dosing regimens or more prolonged treatment could have shown different effects, the general lack of efficacy of EPO coupled with the recent results of the clinical RCT of this therapy in severe TBI reduced enthusiasm for further investigation of this agent within the OBTT mechanism.

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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