

# Simvastatin Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

Andrea Mountney,<sup>1</sup> Helen M. Bramlett,<sup>2</sup> C. Edward Dixon,<sup>3</sup> Stefania Mondello,<sup>4</sup> W. Dalton Dietrich,<sup>2</sup> Kevin K.W. Wang,<sup>5</sup> Krista Caudle,<sup>1</sup> Philip E. Empey,<sup>6</sup> Samuel M. Poloyac,<sup>6</sup> Ronald L. Hayes,<sup>7</sup> John T. Povlishock,<sup>8</sup> Frank C. Tortella,<sup>1</sup> Patrick M. Kochanek,<sup>9</sup> and Deborah A. Shear<sup>1</sup>

## Abstract

Simvastatin, the fourth drug selected for testing by Operation Brain Trauma Therapy (OBTT), is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor used clinically to reduce serum cholesterol. In addition, simvastatin has demonstrated potent antineuroinflammatory and brain edema reducing effects and has shown promise in promoting functional recovery in pre-clinical models of traumatic brain injury (TBI). The purpose of this study was to assess the potential neuroprotective effects of oral administration of simvastatin on neurobehavioral, biomarker, and histopathological outcome measures compared across three pre-clinical TBI animal models. Adult male Sprague-Dawley rats were exposed to either moderate fluid percussion injury (FPI), controlled cortical impact injury (CCI), or penetrating ballistic-like brain injury (PBBi). Simvastatin (1 or 5 mg/kg) was delivered via oral gavage at 3 h post-injury and continued once daily out to 14 days post-injury. Results indicated an intermediate beneficial effect of simvastatin on motor performance on the gridwalk (FPI), balance beam (CCI), and rotarod tasks (PBBi). No significant therapeutic benefit was detected, however, on cognitive outcome across the OBTT TBI models. In fact, Morris water maze (MWM) performance was actually worsened by treatment in the FPI model and scored full negative points for low dose in the MWM latency and swim distance to locate the hidden platform. A detrimental effect on cortical tissue loss was also seen in the FPI model, and there were no benefits on histology across the other models. Simvastatin also produced negative effects on circulating glial fibrillary acidic protein biomarker outcomes that were evident in the FPI and PBBi models. Overall, the current findings do not support the beneficial effects of simvastatin administration over 2 weeks post-TBI using the oral route of administration and, as such, it will not be further pursued by OBTT.

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

## Introduction

STATINS ARE POTENT INHIBITORS of 3-hydroxy-3-methylglutaryl coenzyme A (HMG) reductase and are used clinically to reduce serum cholesterol. Recent studies have highlighted the pleiotropic properties of statins and their utility in promoting therapeutic benefit in pathologies other than hyperlipidemia. For neurological disorders, in particular, accumulating experimental evidence suggests that statins exert neuroprotective effects against

a variety of central nervous system (CNS) disorders including stroke,<sup>1</sup> subarachnoid hemorrhage (SAH),<sup>2,3</sup> intracerebral hemorrhage (ICH),<sup>4,5</sup> and traumatic brain injury (TBI).<sup>6–9</sup>

Simvastatin was selected for testing by the Operation Brain Trauma Therapy (OBTT) consortium based on evidence demonstrating significant therapeutic benefits of the compound after oral administration in animal models of severe TBI. Simvastatin is a lactone prodrug that is highly lipophilic and can readily cross the blood–brain barrier (BBB) and has been shown to reduce

<sup>1</sup>Brain Trauma Neuroprotection/Neurorestoration, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, Maryland.

<sup>2</sup>Department of Neurological Surgery, The Miami Project to Cure Paralysis, Miller School of Medicine, University of Miami, Miami, Florida; Bruce W. Carter Department of Veterans Affairs Medical Center, Miami, Florida.

<sup>3</sup>Department of Neurological Surgery, Brain Trauma Research Center, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

<sup>4</sup>Department of Neurosciences, University of Messina, Messina, Italy.

<sup>5</sup>Center of Neuroproteomics and Biomarkers Research, Department of Psychiatry and Neuroscience, University of Florida, Gainesville, Florida.

<sup>6</sup>University of Pittsburgh School of Pharmacy, Pittsburgh, Pennsylvania.

<sup>7</sup>Center for Innovative Research, Center for Neuroproteomics and Biomarkers Research, Banyan Biomarkers, Inc., Alachua, Florida.

<sup>8</sup>Department of Anatomy and Neurobiology, Virginia Commonwealth University, Richmond, Virginia.

<sup>9</sup>Department of Critical Care Medicine, Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

neurofibrillary tangles in a mouse model of Alzheimer disease.<sup>10</sup> After oral administration, simvastatin is reversibly converted to the active acid form (via hydrolysis by nonspecific carboxyesterases). Other statins, such as pravastatin and lovastatin, enter the brain by active transport mediated by the organic anion transporter polypeptide family, specifically OATP2 (located on the rat BBB and choroid plexus) and by the monocarboxylic acid transporter (MCT4), which has been identified in brain neurons and astrocytes.<sup>11–13</sup>

This may also be true for simvastatin, although it is not yet reported. Additional evidence suggests simvastatin exerts neuroprotection via multiple mechanisms that include reduction of pro-inflammatory cytokines,<sup>7,14</sup> promotion of angiogenesis and neurogenesis,<sup>1,15,16</sup> and reduction of vasospasm.<sup>17,18</sup> Simvastatin has also been implicated in altering levels of specific genes associated with apoptosis (c-fos, c-myc, H1.2, and Bcl-2) and there is evidence suggesting that chronic administration may lead to statin accumulation in the brain.<sup>19</sup>

While a number of experimental TBI studies have reported positive results after oral administration of simvastatin, not all studies have been positive.<sup>6,20</sup> Chen and associates<sup>20</sup> in 2008 reported some beneficial effects of simvastatin on edema in the FPI model but no therapeutic benefits were detected on recovery of motor deficits. Similarly, Indraswari and colleagues<sup>6</sup> reported in 2012 no beneficial effects of simvastatin (1 or 5 mg/kg) on rotarod performance in a mouse TBI model. Notably, more recent work has reported results showing that simvastatin (10 mg/kg/2×day) significantly increased levels of inflammatory genes upregulated by TBI at 72 h and 7 days post-injury, suggesting a possible deleterious effect of simvastatin at higher doses.<sup>21</sup>

The mixed results regarding the therapeutic potential of simvastatin in pre-clinical TBI may be partly because of wide variations in dose concentrations (0.5 to 100 mg/kg) and dosing durations. The most consistent beneficial results reported to date in pre-clinical models of TBI have come from a series of studies that used sustained administration of low doses ranging from 1–3 mg/kg delivered via oral (PO) gavage once daily for 14 consecutive days and reported beneficial effects on reduction of interleukin (IL)-expression,<sup>22</sup> angiogenesis,<sup>15,23</sup> improved cognitive outcome,<sup>15</sup> and increased neurite outgrowth.<sup>24</sup> PO doses as high as 37.5 mg/kg have shown benefit after TBI in multiple reports.<sup>20,25</sup>

Overall, simvastatin is generally well tolerated and has demonstrated a long clinical track record of safety/tolerability profiles in critically ill patients, with mild, easily monitored side effects. Thus, if shown to be beneficial, it could be moved forward readily into TBI clinical trials after evidence of significant therapeutic benefit.

The current study was designed to evaluate the therapeutic efficacy of simvastatin across three established pre-clinical models of TBI including (1) fluid percussion injury (FPI),<sup>26</sup> (2) controlled cortical impact (CCI) injury,<sup>27</sup> and (3) penetrating ballistic-like brain injury (PBBi).<sup>28</sup> The specific dose (1 mg/kg) and dosing duration (14 days) tested were selected based on multiple reports demonstrating efficacy in the TBI literature,<sup>29,30</sup> while the dose of 5 mg/kg provided an exploratory assessment of dose response.

## Methods

Methods will be briefly described given that this is the fourth in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma*. For additional detail on the individual models, please see the first therapy article published in this issue.<sup>31</sup>

Male Sprague-Dawley rats (270–320 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 NIH *Guide for the Care and Use of Laboratory Animals* (National Research Council; 2011 edition), and other federal statutes and regulations relating to animals and experiments involving 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 the methods.

## Animal models

**FPI model—Miami.** Rats were anesthetized (70% N<sub>2</sub>O/30% O<sub>2</sub>, 1–3% isoflurane) 24 h before injury and surgically prepared for parasagittal FPI as described previously.<sup>32</sup> A craniotomy was performed over the right hemisphere, 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, and the rat was intubated and subjected to a moderate FPI. Blood gas levels and physiological parameters were measured from arterial samples 15 min before and 30 min after FPI.

FPI served as our sentinel model for assessing the effects of TBI on acute physiological parameters including hemodynamics and blood gases, and in this study, the 30 min post-insults time point provided an assessment of the effect of TBI on acute physiology to ensure a stable animal post-insult. Blood gases and physiological parameters including pH, glucose, lactate, and electrolytes were assessed to ensure that injury had no acute adverse effects. 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*.

**CCI model—Pittsburgh.** Rats were anesthetized (2–4% isoflurane in 2:1 N<sub>2</sub>O/O<sub>2</sub>), 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.<sup>27</sup> The scalp was sutured closed, and rats were returned to their home cages. Sham rats underwent all procedures except for the CCI.

**PBBi model—Walter Reed Army Institute of Research (WRAIR).** PBBi was performed as described previously.<sup>33</sup> 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

Simvastatin powder (Sigma-Aldrich) was initially dissolved in 100% undenatured ethanol and formulated in a vehicle (VEH) solution containing 0.5% methylcellulose in water. Rats were dosed with VEH or simvastatin (1 and 5 mg/kg) by oral gavage starting at 3 h post-injury and daily thereafter for 14 days. Sham operated rats received no drug treatment. The drug was prepared at each site by a person 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 withdrawals for the FPI and PBBi models were taken from an indwelling jugular catheter at above specified time points after TBI

TABLE 1. SUMMARY OF EXPERIMENTAL GROUP SIZES FOR TRAUMATIC BRAIN INJURY/SIMVASTATIN STUDY

Group	Sham	TBI+ Vehicle	TBI + 1 mg/kg	TBI + 5 mg/kg	N
FPI - Miami	10	9	9	10	38
CCI - Pittsburgh	10	8	10	10	38
PBBI - WRAIR	14	13	12	13	52

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

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.<sup>31</sup> All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, LLC, for further analysis of biomarker levels. Details of the biomarker methods are provided in the companion article focused on biomarkers in this issue.<sup>34</sup>

### Primary outcome metrics

The overall approach to outcome testing, scoring, and details of the specific outcome methods and metrics are described in the first therapy article within this issue.<sup>31</sup> 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.<sup>35</sup> The gridwalk task was used as well to determine fore- 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.<sup>36</sup> 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.<sup>37</sup> Additional assessments of motor coordination and balance used the fixed-speed rotarod task on days 7 and 10 post-injury.<sup>33</sup>

### 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.<sup>31</sup>

### Histopathological assessments

After behavioral testing, rats were anesthetized and perfused with 4% (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 (H&E) for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.<sup>31</sup> Both lesion volume and tissue volume loss were expressed as a

percent of the contralateral (“non-injured”) 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.

### 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 4–24 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.<sup>38</sup>

### Statistical analysis

Normality was assessed and data are expressed as mean  $\pm$  standard error of the mean (SEM) 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 or Tukey test.

Biomarker variables were summarized with standard descriptive statistics (median and interquartile range). Delta 4–24 h biomarker levels in injured groups were calculated as the difference between 24 h and 4 h biomarker concentrations. Because the distribution of the biomarkers was strongly and positively skewed, the differences in biomarker concentration among the groups in each TBI model were analyzed using the Kruskal–Wallis test followed by *post hoc* comparisons applying Mann–Whitney *U* and Bonferroni correction. All statistical analyses were two-tailed and a *p* value <0.05 was considered significant. Data analysis was performed using SAS (SAS version [9.2] of the SAS System. Copyright © 2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

## Results

### Physiological parameters

Physiological parameters of mean arterial blood pressure (MABP), PaO<sub>2</sub>, PaCO<sub>2</sub>, and blood pH taken in the FPI model (Miami) are provided in Table 2. Physiological variables were taken before TBI and were also assessed at 30 min post-injury. All physiological values were within normal range, and there were no significant differences between the various experimental groups in terms of MABP, PaO<sub>2</sub>, PaCO<sub>2</sub>, and blood pH.

### Sensorimotor parameters

**FPI model.** Rats were assessed using the cylinder task for spontaneous forelimb usage (Fig. 1A). After injury, rats showed forelimb impairment and exhibited contralateral forelimb placing deficits (asymmetry index of <0.5). Rats treated with simvastatin at both doses trended toward improved asymmetry indices compared with TBI-VEH. One-way ANOVA, however, showed no significant difference between groups (*p*=0.42).

To assess sensorimotor function, rats were tested using the gridwalk test at 7 days post-injury in which independent footfalls for each fore- and hindlimb were counted and expressed as a percentage of total steps per limb (Fig. 1B). Rats treated with the low

TABLE 2. EFFECTS OF SIMVASTATIN ON FLUID PERCUSSION INJURY PHYSIOLOGY

Group	Sham	TBI + Vehicle	TBI + 1 mg/kg	TBI + 5 mg/kg
<b>Pre-TBI</b>				
pH	7.43 ± 0.01	7.42 ± 0.01	7.41 ± 0.01	7.44 ± 0.01
pO <sub>2</sub> (mm Hg)	158.4 ± 8.41	147.3 ± 8.95	142.5 ± 7.23	125.64 ± 7.11
pCO <sub>2</sub> (mm Hg)	40.82 ± 1.17	41.5 ± 0.90	42.78 ± 0.76	40.47 ± 0.68
MAP (mm Hg)	123.28 ± 3.29	118.54 ± 1.97	124.18 ± 3.97	121.48 ± 4.26
Brain temp (°C)	36.8 ± 0.05	36.7 ± 0.05	36.8 ± 0.06	36.7 ± 0.06
Body temp (°C)	36.9 ± 0.09	36.8 ± 0.08	36.7 ± 0.07	36.8 ± 0.08
<b>Post-TBI</b>				
pH	7.44 ± 0.01	7.44 ± 0.01	7.42 ± 0.01	7.44 ± 0.01
pO <sub>2</sub> (mm Hg)	161.9 ± 7.36	147.3 ± 7.48	142.6 ± 6.84	134.55 ± 8.96
pCO <sub>2</sub> (mm Hg)	39.88 ± 0.97	40.13 ± 0.94	40.41 ± 0.80	40.15 ± 1.02
MAP (mm Hg)	130.0 ± 3.84	121.92 ± 4.08	117.25 ± 3.44	116.9 ± 3.56
Brain temp (°C)	36.8 ± 0.06	36.7 ± 0.05	36.7 ± 0.05	36.6 ± 0.04
Body temp (°C)	36.8 ± 0.07	36.8 ± 0.07	36.8 ± 0.07	36.8 ± 0.09

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

dose of simvastatin (1 mg/kg) showed reduced footfalls; the most poignant effects were evident on the left and right hind limbs. These changes, however, were not significantly different from VEH treatment. One-way ANOVA revealed a significant injury effect on the left forelimb ( $p < 0.05$ ) and *post hoc* comparisons showed significant differences between TBI-VEH and sham, but not between simvastatin-treated groups and sham. This produced +1.0 points (half of the total point value for this outcome) for both doses on the OBTT scoring matrix. No between-group differences were detected on other limbs. Overall, simvastatin showed an intermediate effect on gridwalk.

**CCI model.** For the beam balance test, two-way repeated measures ANOVA revealed a significant group main effect for beam balance latencies over the 5 post-injury days ( $p = 0.005$ ) (Fig. 1C). Both CCI-injured rats treated with VEH or 1 mg/kg simvastatin performed significantly worse than sham. CCI-injured rats treated with 5 mg/kg simvastatin, however, did not perform significantly worse than sham indicating the presence of an intermediate beneficial effect of simvastatin on motor outcome and once again resulted in a +1.0 (half of the total point value for this outcome) for this dose on the OBTT scoring matrix.

In a separate assessment of latency to traverse the beam, 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, regardless of treatment, 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, 1G). Repeated measures ANOVA for mean motor scores (4 groups  $\times$  3

speeds) revealed a significant group main effect ( $p < 0.05$ ) and a significant time effect ( $p < 0.001$ ) with motor impairment evident within all groups.

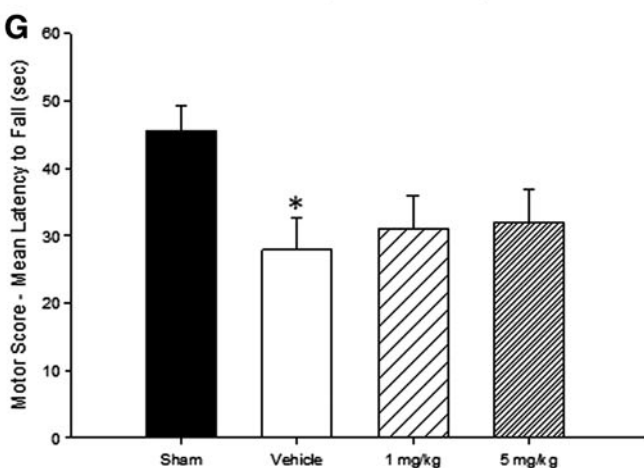
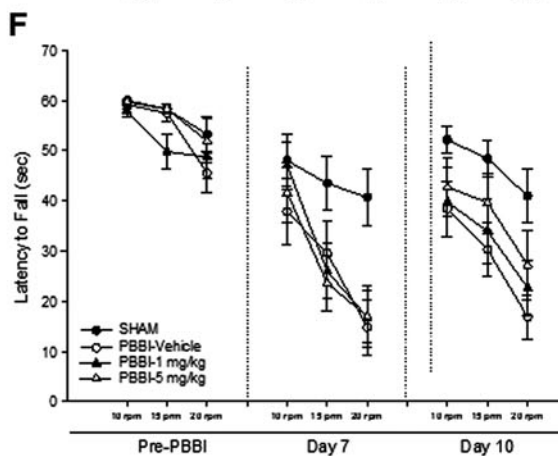
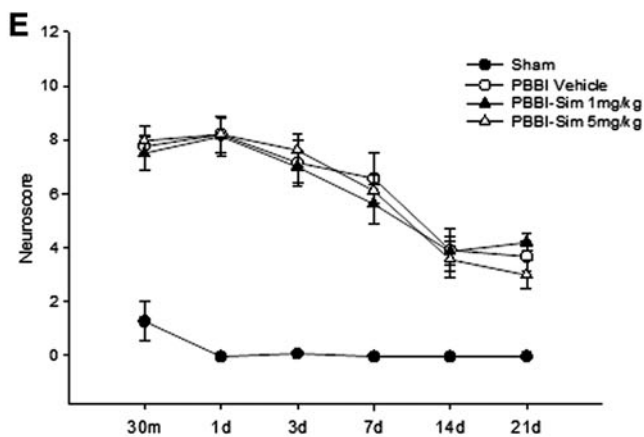
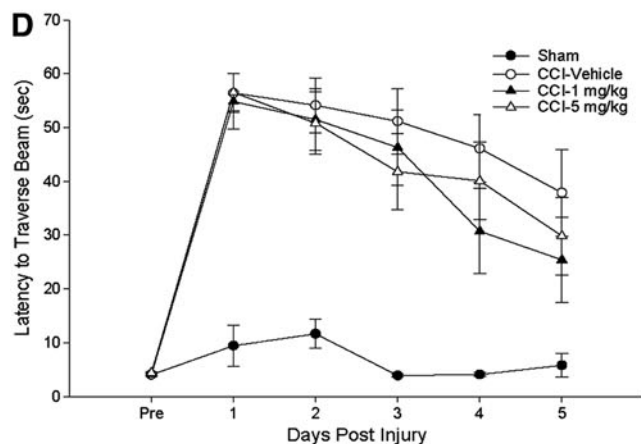
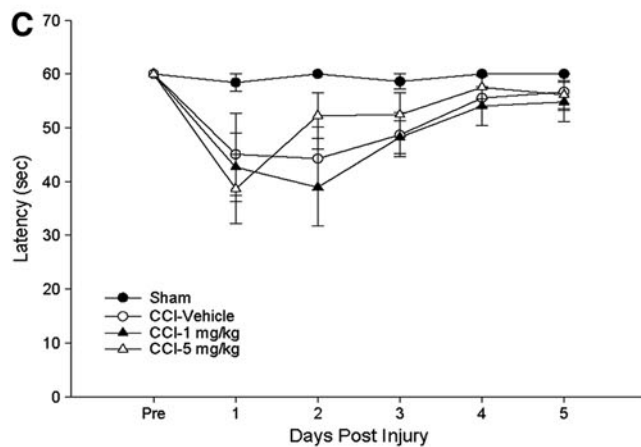
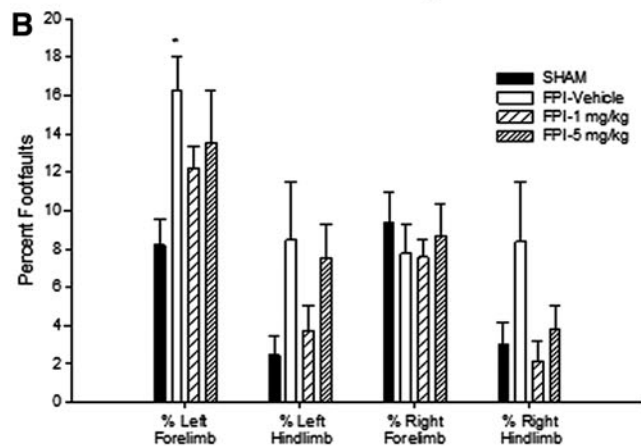
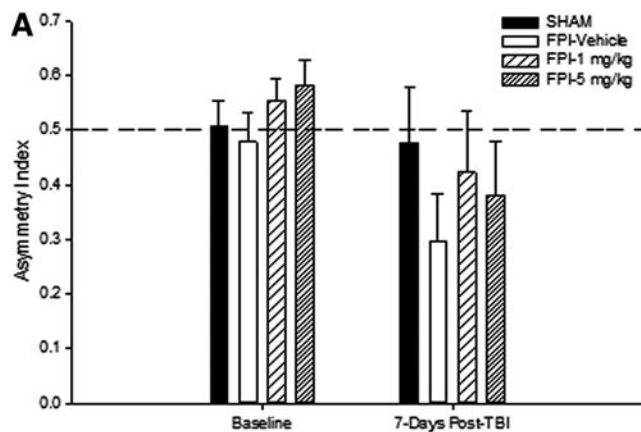
At 10 days post-injury, VEH-treated rats showed significant motor impairment; however, simvastatin treatment again showed an intermediate benefit because PBBI-injured rats treated with either the low or high dose of simvastatin were not significantly different from shams (Fig. 1G). In this case, this resulted in +1.5 points (half of the total point value for the rotarod task) for each dose. There was also a significant effect of speed (rpm) at 7 days ( $p < 0.001$ ) and at 10 days post-injury ( $p < 0.001$ ), but no significant interaction. Overall mean rotarod latency scores were reduced by 42 ± 10% (PBBI+VEH), 32 ± 11% (1 mg/kg), and 33 ± 10% (5 mg/kg) vs. sham ( $p < 0.005$ ).

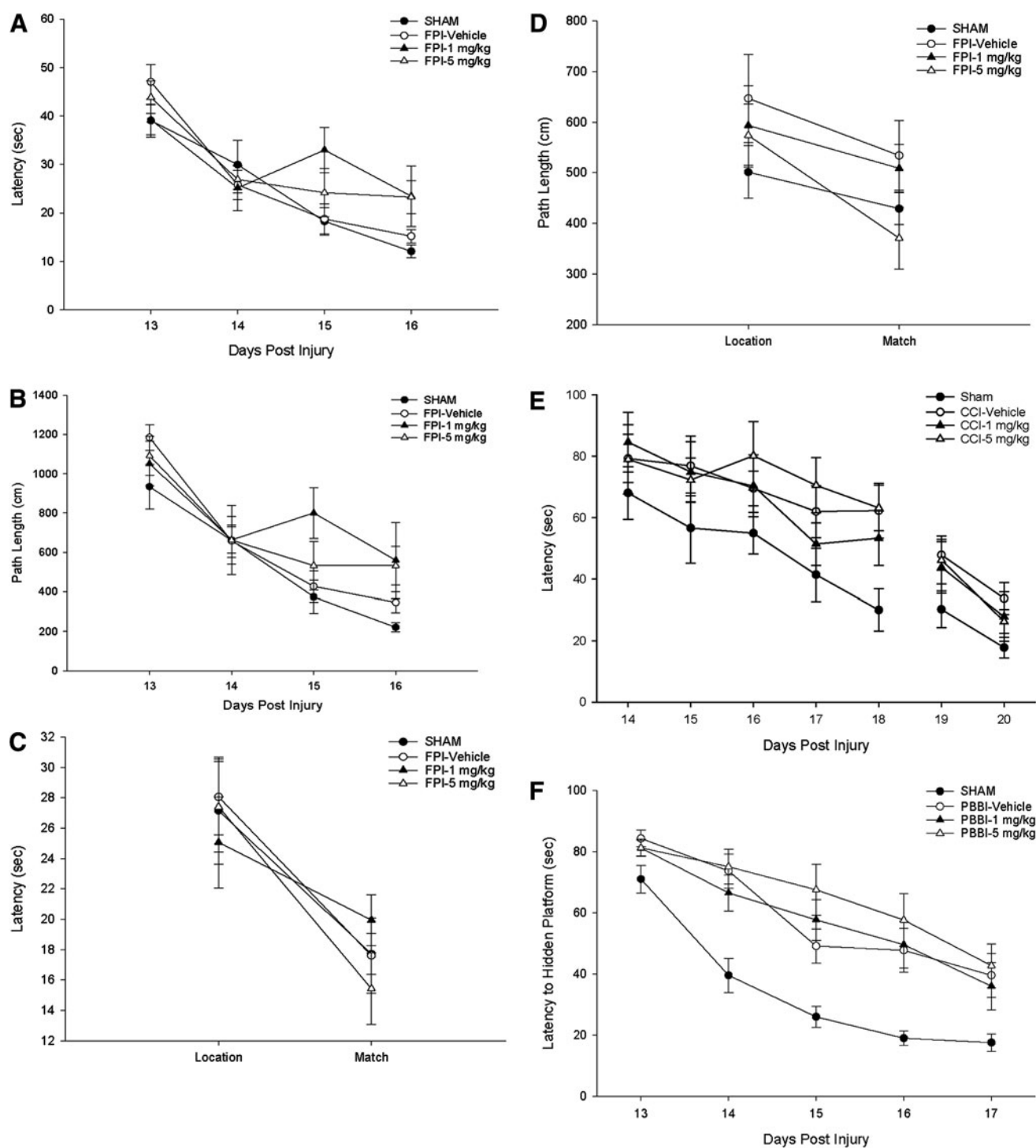
### Cognitive testing

**FPI model.** Cognitive function was assessed using a hidden platform task (Fig. 2A,B) over 4 days (days 13–16 post-injury) followed by a probe trial and subsequent working memory test (Fig. 2C, 2D). Although all rats showed decreased latencies over the 4-day testing period, TBI-injured rats showed higher latencies to hidden platform than sham. Two-way repeated measures ANOVA revealed significance for time ( $p < 0.001$ ) and a group  $\times$  time interaction ( $p = 0.014$ ) but was not significant for group ( $p = 0.445$ ). *Post hoc* analysis revealed that rats with TBI treated with low dose simvastatin (1 mg/kg) were significantly different from both sham animals and TBI + VEH-treated animals at day 3. This resulted in full negative points (-2.0) for this outcome in the OBTT scoring matrix. Clearly there was no effect of simvastatin on improving cognitive function on this task.

Analysis of path lengths (distance traveled) showed similar results to MWM latencies in which VEH and simvastatin (1 mg/kg) treated rats exhibited longer path lengths compared with sham. Once again this resulted in full negative points (-2.0) for this outcome in the OBTT scoring matrix. One-way ANOVA was not

**FIG. 1.** Sensorimotor outcome. Fluid percussion injury (FPI) model (A, B): bar graphs show the results of (A) spontaneous forelimb assessments and (B) the gridwalk task. Controlled cortical impact (CCI) model (C,D): line graphs show the results of the balance beam 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. Data represent group means  $\pm$  standard error of the mean; \* $p < 0.05$  compared with sham. See text for details.





**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 latency to the hidden platform over 5 days of MWM testing and mean swim latencies to the “visible” platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBi) model (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 showing (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. Data represent group means  $\pm$  standard error of the mean. \* $p < 0.05$  compared with sham, \*\* $p = 0.049$  for overall ANOVA; however, none of the injury groups reach significance vs sham on *post hoc* testing after correction from multiple comparisons. See text for details.

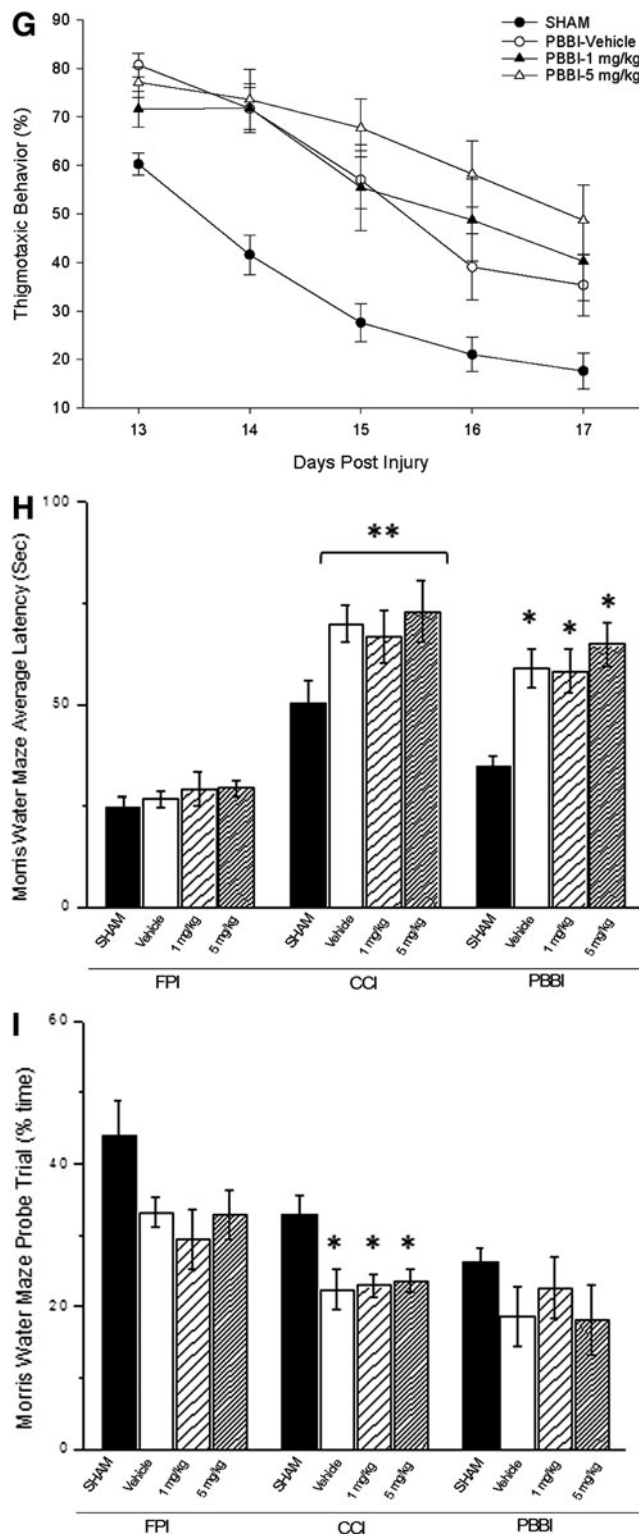


FIG. 2. (Continued)

significant for group ( $p=0.063$ ) in the probe trial. In the working memory task, all rats with TBI, regardless of treatment, showed poor cognitive performance on the short-term memory task. Two-way repeated measures ANOVA for working memory latency was not significant for group ( $p=0.962$ ) but was significant for trial ( $p<0.001$ ) because rats located the platform more quickly on the

second of the paired trials. Although not significant, rats treated with 5 mg/kg simvastatin trended toward improved performance.

Similar results were seen for working memory path length. Repeated measures ANOVA was significant for trial ( $p<0.003$ ) but not for group or group  $\times$  trial. Just as seen in the working memory latency, the 5 mg/kg dose showed a similar albeit insignificant trend toward the shortest distance traveled to locate the platform versus other groups.

**CCI model.** For the hidden platform MWM task (Fig. 2E), two-way repeated measures ANOVA for latency revealed a significant group main effect ( $p=0.049$ ). *Post hoc* analysis of swim latencies across days, however, did not differ between the injured groups regardless of treatment. The probe trial also showed no effect on improvement after simvastatin treatment (Fig. 2I). One-way ANOVA was significant for group ( $p=0.007$ ) in the probe trial with all injury groups performing significantly worse than shams, and simvastatin-treated rats were indistinguishable from VEH.

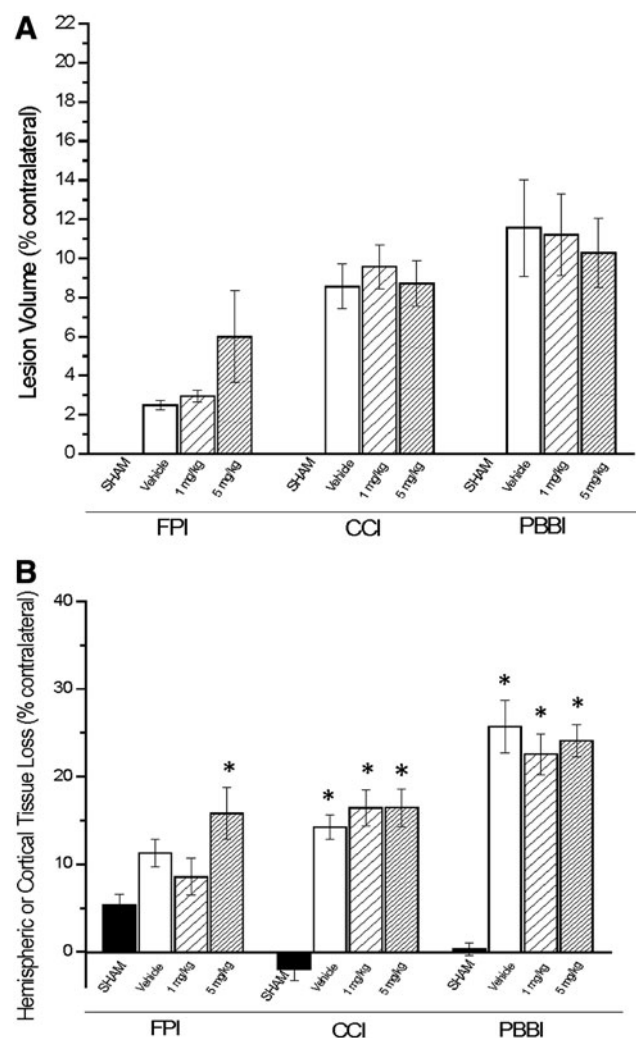
**PBBI model.** Assessments of spatial learning and thigmotaxic behavior after TBI and simvastatin treatment are represented in Figure 2F, 2G. Two-way repeated-measures ANOVA on latency to locate the hidden platform was significant for group ( $p<0.001$ ) and trial ( $p<0.001$ ) and showed a group  $\times$  trial interaction ( $p=0.009$ ). *Post hoc* analysis revealed significant differences between sham and PBBI-injured groups with average escape latency (across all testing days) increased by  $70 \pm 13\%$  (PBBI),  $68 \pm 16\%$  (simvastatin 1.0 mg/kg), and  $87 \pm 13\%$  (simvastatin 5.0 mg/kg) versus sham ( $p<0.05$ ). Swim pattern analysis showed that all injured groups displayed a thigmotaxic response, indicative of attention deficits.

Two-way repeated measures ANOVA on percent time spent circling the outer perimeter of the maze was significant for group ( $p<0.05$ ) and for trial ( $p<0.001$ ) and showed a group  $\times$  trial interaction ( $p=0.048$ ). *Post hoc* analysis revealed that all injured groups spent a significantly greater percentage of time circling the outer perimeter of the maze versus sham ( $p<0.05$ ) (Fig. 2G). One-way ANOVA results of the probe trial did not show a significant difference between any groups ( $p>0.05$ ) in time spent searching the target (missing platform) zone. No significant therapeutic benefits of simvastatin were detected on any MWM parameter.

#### Pooled analysis of therapeutic effects

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, 2I and 3A, 3B).

**Cognitive outcomes.** The effect of simvastatin treatment on the average latency collapsed across days, and the latency of the probe trial across all models in OBTT is represented in Fig. 2H,I. For MWM average latencies, both CCI and PBBI models exhibited significant deficits after injury compared with sham ( $p<0.05$ ). Both doses of simvastatin showed no therapeutic benefit in cognitive function versus TBI-VEH. Although there was no deficit in average latency across all testing days after FPI, simvastatin treatment did not appear to alter performance, because all groups were indistinguishable from sham levels. A somewhat more severe injury level may have been more optimal for the evaluation of therapeutic efficacy in FPI for this task. The MWM probe trial showed similar results across models: no cognitive benefit of simvastatin after TBI across models. Notably, CCI injured animals exhibited significant reductions in percent time in the target



**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, there was no drug effect on lesion volume in any of the three models, although there was a trend toward expansion of the lesion in the FPI model with high dose (5 mg/kg) simvastatin treatment. Consistent with this finding, high dose simvastatin significantly increased the lesion versus sham in the FPI model, but neither the TBI+VEH or low dose (1 mg/kg simvastatin) groups differed from sham. There were no treatment effects on hemispheric tissue loss in either CCI or PBBI. Data represent group means  $\pm$  standard error of the mean; \* $p < 0.05$  compared with sham.

quadrant on this task; however, once again there was no effect of simvastatin treatment.

**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).

In all three models, there was no therapeutic benefit of simvastatin treatment on reducing lesion volume. However, after FPI a significant increase in cortical tissue loss was detected in animals treated with the high dose of simvastatin, with  $p < 0.05$  versus sham, contrasting the lack of difference between either TBI-VEH or TBI-simvastatin (1 mg/kg) and sham (both  $p > 0.05$ ). Thus, negative half of the total point value for this task (i.e.,  $-1.0$ ) was given to the 5 mg/kg simvastatin group in the FPI model for this outcome.

In the CCI model, lesion volumes ranged between  $\sim 8$ –10% of the contralateral hemisphere regardless of treatment, and one-way ANOVA did not differ significantly between the injury groups. Similarly, hemispheric tissue loss (% of contralateral side) was not significantly reduced in CCI by simvastatin treatment. In the PBBI model, ANOVA revealed a significant injury effect, but no simvastatin treatment effect, on both metrics of lesion volume (PBBI =  $12 \pm 2$  mm<sup>3</sup>; simvastatin 1.0 mg/kg =  $10 \pm 2$  mm<sup>3</sup>; simvastatin 5.0 mg/kg =  $9 \pm 1$  mm<sup>3</sup>;  $p > 0.05$  vs. PBBI) and hemispheric tissue loss (PBBI =  $26 \pm 3\%$ ; simvastatin 1.0 =  $22 \pm 2\%$ ; and simvastatin 5.0 mg/kg =  $24 \pm 2\%$ ).

### Biomarker assessments

Biomarker data were available from 127 of the 132 rats. Missing data resulted from failed sample collection. Biomarker data from all three models at 4 h and 24 h post-injury are shown in Figure 4.

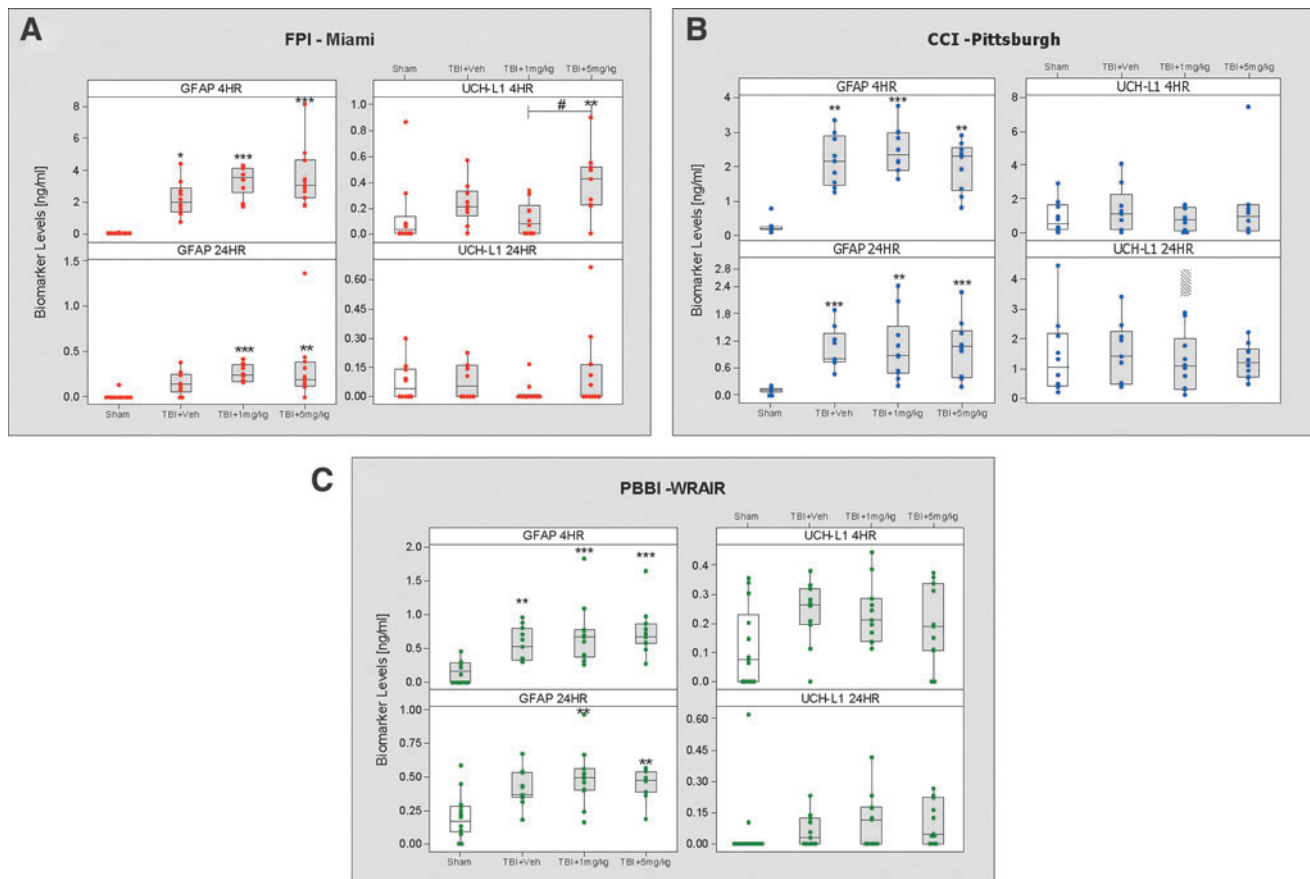
**FPI model.** Relative to sham, all injured groups demonstrated significant increases in glial fibrillary acidic protein (GFAP) levels at 4 h post-injury ( $p < 0.0001$ ), but only FPI + simvastatin at both high and low doses showed significant increases at 24 h ( $p < 0.0001$ ), indicating a potential injury exacerbation with treatment. This resulted in a  $-0.5$  point (intermediate effect; one half of the total point value) for both doses in the scoring matrix. No significant treatment effect on delta 24–4 h GFAP levels was found (Fig. 4A, 5A).

At 4 h post-injury, a Kruskal-Wallis test revealed a significant group effect as well as a treatment effect on ubiquitin-carboxyl-terminal-hydrolase (UCH-L1) levels ( $p = 0.007$  and  $p = 0.009$ , respectively). *Post hoc* analysis detected a significant increase in UCH-L1 levels between FPI rats treated with simvastatin 5 mg/kg and both the sham and the FPI + simvastatin 1 mg/kg groups (Fig. 4A). Recall, however, that only 24 h and delta 4–24 h data were determined *pre hoc* to contribute to scoring in the OBTT scoring matrix. There was no significant group effect or treatment effect on post-injury serum levels of UCH-L1 at 24 h. Delta 24–4 h UCH-L1 levels also showed no evidence of a treatment effect (Fig. 5A).

**CCI model.** Significant group effects on post-injury levels of GFAP were detected at 4 h ( $p < 0.0001$ ) and 24 h ( $p < 0.0001$ ) post-injury, with all three injured groups showing significantly elevated levels at both time points compared with shams; however, there appeared to be no treatment effect. The delta 4–24 h GFAP levels also did not show evidence of a treatment effect (Fig. 4B, 5B). Unlike GFAP, there were no significant group differences on either post-injury levels of UCH-L1 at 4 h, 24 h, or delta 4–24 h UCH-L1 levels (Fig. 4B, 5B).

**PBBI model.** Similar to the FPI model, all injured groups demonstrated significant increases of GFAP concentrations compared with sham at 4 h post-injury ( $p < 0.0001$ ), but only rats treated with simvastatin (both doses) showed significant increases in GFAP at 24 h ( $p = 0.001$ ). No significant group effect on delta 24–4 h GFAP levels were detected (Fig. 4C, 5C). Although UCH-L1 was higher in all





**FIG. 4.** Box plots illustrating circulating glial fibrillary acidic protein (GFAP) and ubiquitin-carboxyl-terminal-hydrolase (UCH-L) 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 (\* $p < 0.05$ ), \*\*( $p < 0.01$ ), or \*\*\*( $p < 0.001$ ) versus sham group. #( $p < 0.05$ ) high dose simvastatin group vs. low dose simvastatin group. Relevant to scoring in Operation Brain Trauma Therapy, treatment with simvastatin in both the FPI and PBBI models exacerbated injury as reflected by increased GFAP levels versus sham at 24 h after TBI. See text for details.

injured groups compared with sham at both 4 h and 24 h post-injury, there were no significant differences between these groups ( $p = 0.07$  and  $p = 0.06$ , respectively) (Fig. 4C). Delta 24–4 h GFAP levels also showed no evidence of a treatment effect (Fig. 5C).

#### OBTT outcome scoring matrix

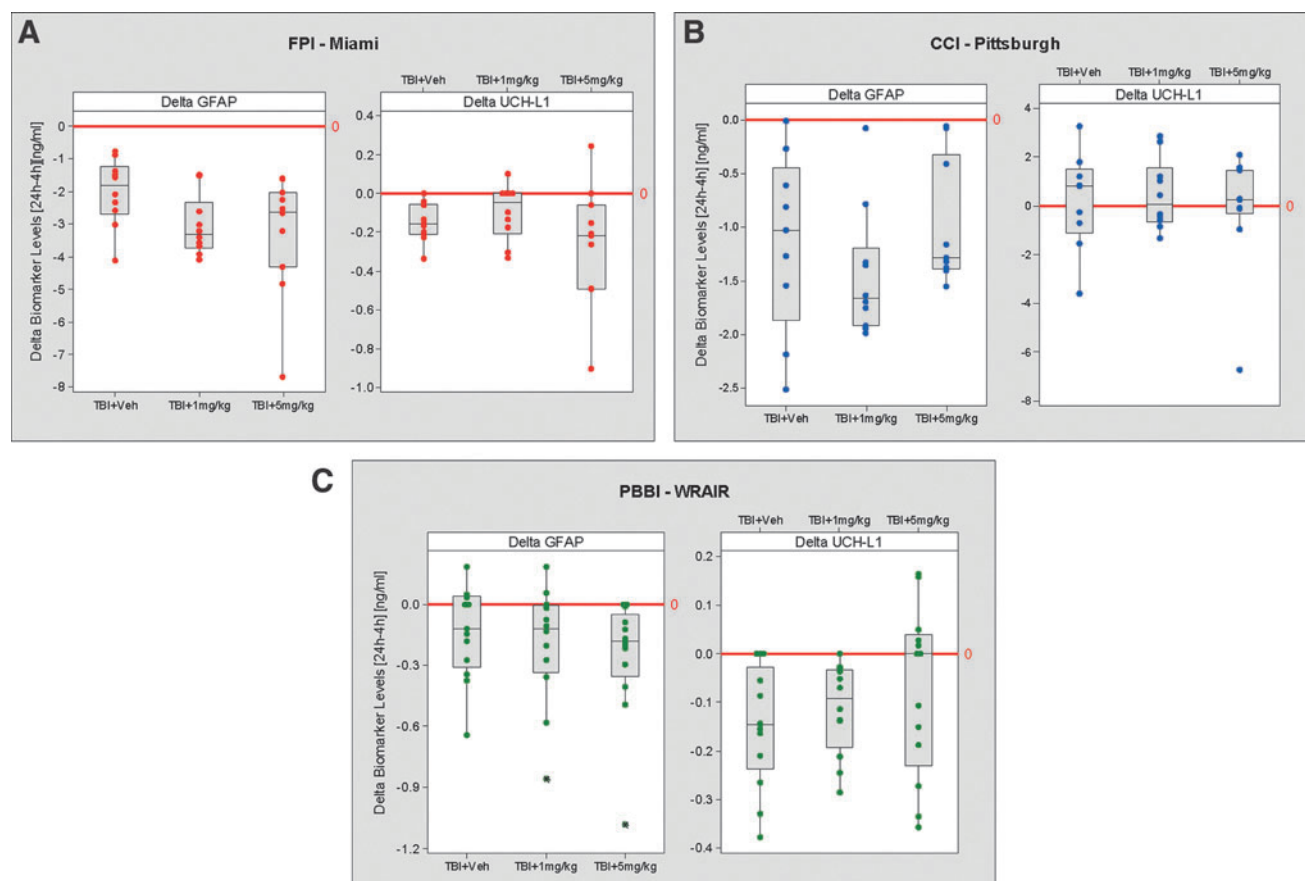
The overall scoring matrix is shown in Table 3 for the effect of simvastatin across all models. In general, simvastatin produced extremely small overall effects across OBTT. Low dose simvastatin across models generated a net overall  $-2.5$  point final score largely influenced by detrimental effects on two of the cognitive outcome parameters in the FPI model—which were mitigated somewhat by positive effects on motor function in FPI and PBBI. High dose simvastatin produced a small  $+1.5$  point overall effect across models, largely related to the fact it actually produced intermediate benefit across all models on at least one aspect of motor function.

#### Discussion

Pre-clinical data suggest that simvastatin is a potent anti-inflammatory with neuroprotective properties.<sup>8,23,29,30</sup> Therefore,

we sought to determine whether simvastatin treatment would be effective across three established TBI models that reproduce a range of injury severities and pathophysiological consequences. We noted a positive, albeit modest, intermediate effect on motor improvement across models in rats treated with simvastatin. By contrast, remarkably, no beneficial effects were noted on any of the other outcome measures that included histopathology, cognitive impairment, and biomarker assessments in any of the models in OBTT. Further, in FPI, simvastatin resulted in a detrimental effect on cognitive performance (low dose) and tissue loss (high dose) that was mirrored by the elevated 24 h GFAP levels, which suggests adverse effects of the drug in this model. This could be important given that FPI represents the mildest TBI model within OBTT.

Our findings appear in accord with the recent failure of the STASH (Simvastatin in Aneurysmal Subarachnoid Hemorrhage) multicenter Phase III trial, where patients with acute SAH were given either simvastatin (0.5 mg/kg) or placebo for up to 21 days. The study found that simvastatin afforded no significant benefit in clinical outcomes after acute SAH.<sup>39</sup> A similar trial has not been conducted with simvastatin in TBI; however, ClinicalTrials.gov identifies NCT0195228 as a trial of simvastatin in TBI that is



**FIG. 5.** Box plots illustrating delta (24–4 h) circulating glial fibrillary acidic protein (GFAP) and ubiquitin-carboxyl-terminal-hydrolase (UCH-L) 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. There were no significant differences between groups. See text for details.

currently recruiting patients. The trial is listed as an assessment of simvastatin on altering markers of neurodegeneration after mild TBI. Clinical trials of two other statins in TBI are also listed on ClinicalTrials.gov, although neither appeared to represent a large, multicenter outcome randomized controlled trial.

Early and more recent studies have used different dose ranges, routes of administration, and treatment periods in multiple experimental models of TBI. Wang and colleagues<sup>7</sup> reported that animals injected with the pharmacologically active form of simvastatin, simvastatin hydroxyl acid (20 mg/kg) for 14 days demonstrated improved rotarod performance that was sustained through 21 days post-injury. Animals treated with simvastatin showed reduced neuronal injury and improved cerebral blood flow in this mouse model.

In a similar study,<sup>30</sup> oral simvastatin (0.5 and 1 mg/kg over 14 days) was found to modestly reduce neurological impairment through 3 months post-injury. The authors noted that 0.5 mg/kg simvastatin seemed more effective than higher 1 mg/kg simvastatin, although the difference was not statistically significant. In a separate study, Wu and coworkers<sup>23</sup> in 2011 found that simvastatin treatment at the same dose (1 mg/kg, 14 d, oral) significantly reduced the incidence of footfalls on the gridwalk task at 4–14 days post-injury and increased angiogenesis in the brain hippocampus and cortex.

As previously stated, statins has shown varying degrees of sensorimotor function improvement in rodent TBI models. Within

the literature, changes in motor performance range from significant,<sup>7,23,30,40,41</sup> to variable,<sup>20</sup> to absent<sup>6,42,43</sup> after statin treatment. In our multicenter blinded study, we showed that simvastatin-treated rats trended toward improved motor performance after TBI. We found intermediate effects of simvastatin treatment on a variety of sensorimotor metrics across models, the most salient signals being evident at low dose simvastatin (1 mg/kg) in the gridwalk task of the FPI model; however, such changes were not significantly different from VEH treatment, indicating that simvastatin afforded no additional benefit over placebo.

A similar intermediate effect was seen at 7 and 10 days on the rotarod task after PBBI, which was more prominent at the lower dose. In the beam balance test, the CCI + VEH and CCI + simvastatin (1 mg/kg) performed significantly worse than sham while the CCI + simvastatin (5 mg/kg) did not differ from sham. Similar to the FPI and PBBI models also used in OBTT, modest benefit was limited to motor performance. Ultimately, in our cross-model comparison, simvastatin treatment failed to elicit significant improvements, affording only intermediate motor benefit after TBI at best. It is plausible that the lack of a robust effect may be because of variations within animal models and the associated pathophysiological heterogeneity of the injury.

Previously, others have reported data showing that dietary supplementation with simvastatin for 8 weeks after CCI significantly

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 pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (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,+1	–2, –2,0,0,0	0,0	–0.5,0,0,0	–3.5
Dose 2		0,+1	0,0,0,0,0	0,–1	–0.5,0,0,0	–0.5
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	0,0	0,0	0,0,0,0	0
Dose 2		+1,0	0,0	0,0	0,0,0,0	+1
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	+1.5	0,0,0	0,0	–0.5,0,0,0	+1
Dose 2	0	+1.5	0,0,0	0,0	–0.5,0,0,0	+1
Grand total						
Dose 1	0	+2.5	–4	0	–1,0,0,0	–2.5
Dose 2	0	+3.5	0	–1	–1,0,0,0	+1.5

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin-carboxyl-terminal-hydrolase; WRAIR, Walter Reed Army Institute of Research.

( ) = point value for each outcome within each model.

DRUG: Simvastatin; Dose 1 = 1 mg/kg; Dose 2 = 5 mg/kg.

improved spatial learning and memory in the MWM task and reduced perseverative responses in the Y maze.<sup>44</sup> Other work has indicated a beneficial effect of simvastatin that was primarily evident on the probe (memory retention) trial<sup>42</sup> as well as enhanced spatial memory concomitant with elevated expression of brain-derived neurotrophin factor (BDNF) in the dentate gyrus.<sup>9</sup> Across all three injury models in our study, simvastatin afforded no cognitive benefit in spatial learning, working memory, or memory retention testing paradigms. In fact, in the FPI model, simvastatin treatment appeared to worsen cognitive impairment, although this was not significant.

Given that the FPI is the least severe of our three OBTT injury models, it is plausible that the deleterious effects observed with simvastatin in the FPI model became evident because the PBBI and CCI cause far more severe neurological and functional damage that may indeed mask modest adverse drug toxicities vis-à-vis a ceiling effect. Overall, OBTT acknowledges that the lack of benefit of simvastatin on cognitive outcome in any model was indeed disappointing.

The ability of statins to reduce lesion volume after brain injury is also variable. Despite having been shown to be effective in reducing post-injury infarct size when administered within a 3 h window,<sup>45,46</sup> in our experiments, simvastatin treatment failed to reduce lesion volume across all three TBI models. In fact, in the FPI model, the high dose of simvastatin significantly increased hemispheric tissue loss. This was an unexpected finding and provides evidence that the effects of a treatment can be unpredictable and injury dependent. Taken together, these findings emphasize the need for better characterization of injury-specific variability and for targeted personalized approaches to therapeutic intervention.

Simvastatin did not induce a remarkable effect on either UCH-L1 across models or GFAP in CCI. Conversely, in PBBI and FPI, a significant increase of circulating GFAP concentrations was noted at both doses of simvastatin. In particular, increases in GFAP were associated with poor cognitive performance (low dose) and tissue loss (high dose) in FPI, suggesting that detrimental/side effects of a treatment can be detected and monitored by GFAP. Theranostic utility of GFAP to predict tissue sparing was seen in our studies of

levetiracetam therapy in the OBTT consortium.<sup>47</sup> Such observations concur with previous findings in our laboratory<sup>48</sup> and suggest that biomarkers have the potential to serve as either efficacy or safety markers to identify potential beneficial or detrimental effects—at least in pre-clinical investigations. Further study is needed.

Simvastatin undergoes extensive first-pass metabolism, resulting in extremely poor oral bioavailability (<5%). Studies in nonhuman primates have shown that an oral dose of 20 mg/kg simvastatin corresponded to maximal blood concentration (C<sub>max</sub>) of ~8 ng/mL and 3 ng/mL of the lactone and acid form, respectively. Peak levels for both forms are reached between 1.5 and 5 h after dosing and are rapidly reduced.<sup>49</sup> The majority of the drug is extensively metabolized in the liver and intestine, never reaching systemic circulation. Given the nature of TBI and the compartmentalization of the brain, a drug treatment should be expected to be able to reach the target injured tissue or at least be of high enough levels to elicit a systemic response.

Other work, evaluating CNS levels of simvastatin in naïve rats, has shown that after an initial increase, brain levels of simvastatin rapidly fall after oral administration. A high (50 mg/kg) dose of simvastatin resulted in brain levels of 600 pmol/g at 1 h post-administration that were reduced by 83% (~100 pmol/g) at 6 h post and were barely detectable at 24 h.<sup>50</sup> These results suggest that statins do not accumulate in the brain but instead are rapidly metabolized or effluxed by transporters. The oral route of administration of simvastatin that was used in OBTT, however, was based on a large number of successful pre-clinical trials in rat models of TBI (discussed previously) and the fact that this agent is given orally in routine clinical use and thus could be rapidly translated to patients.

Finally, we recognize that some other investigators have used much higher doses of simvastatin<sup>20,25</sup> such as 37.5 mg/kg and we cannot rule out the possibility that such an approach would be efficacious. It might have been wise to consider one of these much higher dosing regimens in our screening approach.

## Conclusion

The future role of statins as therapeutic interventions for TBI remains unclear. A number of variables, including optimal statin type, route of administration, and dosing regimen, have not been systematically defined. Although a number of pre-clinical studies have demonstrated as proof of principle that statins exert robust neuroprotective effects after acute brain injury, our results indicate that treatment with simvastatin at a dose previously reported to be effective in the published literature failed to provide significant protection and improve functional outcome across three models of TBI.

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## Author Disclosure Statement

Drs. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Drs. 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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Address correspondence to:  
*Patrick M. Kochanek, MD, MCCM*  
*Department of Critical Care Medicine*  
*Safar Center for Resuscitation Research*  
*University of Pittsburgh School of Medicine*  
*3434 Fifth Avenue*  
*Pittsburgh, PA 15260*  
*E-mail: kochanekpm@ccm.upmc.edu*