Comprehensive Evaluation of Neuroprotection Achieved by Extended Selective Brain Cooling Therapy in a Rat Model of Penetrating Ballistic-Like Brain Injury

Xi-Chun May Lu, PhD, Deborah A. Shear, PhD, Ying Deng-Bryant, PhD, Lai Yee Leung, PhD, Guo Wei, PhD, Zhiyong Chen, PhD, and Frank C. Tortella, PhD

Brain hypothermia has been considered as a promising alternative to whole-body hypothermia in treating acute neurological disease, for example, traumatic brain injury. Previously, we demonstrated that 2-hours selective brain cooling (SBC) effectively mitigated acute (≤ 24 hours postinjury) neurophysiological dysfunction induced by a penetrating ballistic-like brain injury (PBBi) in rats. This study evaluated neuroprotective effects of extended SBC (4 or 8 hours in duration) on sub-acute secondary injuries between 3 and 21 days postinjury (DPI). SBC (34°C) was achieved via extraluminal cooling of rats' bilateral common carotid arteries (CCA). Depending on the experimental design, SBC was introduced either immediately or with a 2- or 4-hour delay after PBBi and maintained for 4 or 8 hours. Neuroprotective effects of SBC were evaluated by measuring brain lesion volume, axonal injury, neuroinflammation, motor and cognitive functions, and post-traumatic seizures. Compared to untreated PBBi animals, 4 or 8 hours SBC treatment initiated immediately following PBBi produced comparable neuroprotective benefits against PBBi-induced early histopathology at 3 DPI as evidenced by significant reductions in brain lesion volume, axonal pathology (beta-amyloid precursor protein staining), neuroinflammation (glial fibrillary acidic protein stained-activated astrocytes and rat major histocompatibility complex class I stained activated microglial cell), and post-traumatic nonconvulsive seizures. In the later phase of the injury (7–21 DPI), significant improvement on motor function (rotarod test) was observed under most SBC protocols, including the 2-hour delay in SBC initiation. However, SBC treatment failed to improve cognitive performance (Morris water maze test) measured 13–17 DPI. The protective effects of SBC on delayed axonal injury (silver staining) were evident out to 14 DPI. In conclusion, the CCA cooling method of SBC produced neuroprotection measured across multiple domains that were evident days/weeks beyond the cooling duration and in the absence of overt adverse effects. These “proof-of-concept” results suggest that SBC may provide an attractive neuroprotective approach for clinical considerations.

Introduction

PRECLINICAL RESEARCH HAS consistently demonstrated that therapeutic hypothermia is a promising neuroprotective strategy for treating traumatic brain injury (TBI) by effectively reducing injury-induced increases in intracranial pressure, cellular damage, and improving neurological outcomes (Fingas *et al.*, 2007; Doll *et al.*, 2009; Dietrich and Bramlett, 2010; Wei *et al.*, 2011). In contrast, clinical studies have yielded less consistent results and failed to establish a standardized hypothermia strategy for treating severe TBI patients (McIntyre *et al.*, 2003; Hutchison *et al.*, 2008; Grande *et al.*, 2009; Adelson *et al.*, 2013).

In clinical settings, whole-body hypothermia is usually induced by skin surface cooling or intravascular cooling (Hemmen and Lyden, 2007; Holzer, 2008). Potential adverse effects of whole-body cooling include increased risk of coagulopathy (Watts *et al.*, 1998; Polderman and Herold, 2009), hypotension (Shiozaki *et al.*, 2001; Milhaud *et al.*, 2005), and infectious pneumonia (Shiozaki *et al.*, 2001; Alderson *et al.*, 2004). Critically, whole-body hypothermia would be contraindicated for treating TBI casualties who suffer polytrauma (Cobb and Pridgen, 2008; O'Connell *et al.*, 2012) as it causes increased platelet dysfunction and inhibits the coagulation cascade, which promote coagulopathy as it may exacerbate the coagulation cascade and contribute to the

lethal triad (Hess and Lawson, 2006; Misgav and Martinowitz, 2011).

To harness the potential therapeutic benefits of induced hypothermia while mitigating adverse effects, more recent pre-clinical research efforts have been directed toward techniques designed to selectively cool the brain while maintaining the normal body temperature. In keeping with this, we have developed a selective brain cooling (SBC) method in rats achieved by extraluminal cooling of the bilateral common carotid arteries (CCA) that can effectively reduce rat's brain temperature within 30 minutes without causing detectable pathology of the CCA or coagulopathy measured by simple tail bleeding time (Wei *et al.*, 2008, 2011). Previously, we demonstrated that when initiated immediately following a penetrating ballistic-like brain injury (PBBI) in rats, SBC (2-hours duration) effectively mitigated PBBI-induced acute (≤ 24 hours postinjury) increases in intracranial pressure, neuropathological, and neurofunctional deficits (Wei *et al.*, 2011; Yao *et al.*, 2011).

The focus of this study was to evaluate the sub-acute neuroprotective effects of extended SBC duration (e.g., 4 or 8 hours), initiated either immediately or after a 2–4 hours delay, using comprehensive outcome measures performed between 3 and 21 days postinjury (DPI) in the rat PBBI model.

Materials and Methods

Animals

Experiments were conducted using male Sprague Dawley rats (275–325 g; Charles River Laboratories, Raleigh, VA). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Walter Reed Army Institute of Research (WRAIR) and conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the "Guide for the Care and Use of Laboratory Animals." The animals housing facility was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Surgical procedures

All surgical procedures were conducted in anesthetized rats using aseptic techniques approved by WRAIR IACUC. The anesthesia was induced with 5% isoflurane delivered in a mixture of oxygen and air, and then maintained at ~ 1.5 –2%.

Penetrating ballistic-like brain injury. PBBI was performed as previously described (Shear *et al.*, 2010). Briefly, the rat was placed in a stereotaxic device. A craniotomy was made over the right frontal cortex (AP: 4.5 mm and ML: +2.0 mm to bregma). A PBBI probe was inserted at a fixed angle through the right frontal cortex to a depth of 12 mm. A computer-controlled pulse generator was then activated to rapidly inflate the silastic tubing on the PBBI probe to an elliptical balloon with a volume equal to $\sim 10\%$ of the total brain volume. Upon deflation of the balloon, the probe was withdrawn and the cranial wound was closed. Sham control rats received only craniotomy without the insertion of the PBBI probe and matching duration of anesthesia.

Selective brain cooling. Both CCAs were exposed and isolated. A custom-made cooling cuff was placed around the exposed segment of each CCA (~ 0.5 cm below the bifurca-

tion of internal and external carotid artery) and secured by a piece of silk suture. The SBC (34°C) was induced by continuously pumping ice-cold water through the cooling cuffs to cool the arterial blood as it entered the brain. Rewarming was achieved by terminating cold-water circulation to allow the brain temperature to normalize spontaneously. Brain temperature was monitored via a temperature probe positioned into the left cerebral hemisphere. Core body temperature was maintained at $\sim 37^{\circ}\text{C}$ using a heating blanket and monitored via a rectal temperature probe. Fraction of inspired oxygen (FiO_2) was maintained at 0.26–0.27 (Murakami *et al.*, 2012).

EEG electrode implantation. Four epidural electroencephalogram (EEG) electrodes (a piece of Nichrome wire soldered to a 080 stainless steel screw) were symmetrically implanted on the rat skull over the bilateral frontal and parietal regions of the brain (AP: +1 and -4 mm; ML: ± 3.5 mm to bregma). A reference electrode (*R*) was placed posterior to the lambda. The free end of each electrode wire was then soldered to a multi-pin connector (March Electronics, West Hempstead, NY) in the sequence of *a* (left frontal), *b* (left parietal), *c* (right frontal), and *d* (right parietal), which was individually referenced to the electrode *R* to create a four-channel mono-referential EEG recording montage (Lu *et al.*, 2013). The screw electrodes were secured on the rat skull with dental acrylic. All rats were allowed at least 5 days of recovery before receiving the PBBI surgery.

Histopathology

At the termination of respective experiments (defined below), rats were anesthetized with ketamine/xylazine (70/6 mg/kg) mixture and perfused with cold saline followed by 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Serial coronal sections ($40\ \mu\text{m}$) were cut through the whole cerebrum.

Hematoxylin and eosin (H&E) staining was used for quantifying brain lesion volume. Immunohistochemical staining of glial fibrillary acetic protein (GFAP) and rat major histocompatibility complex class I (OX-18) were used to detect activated astrocytes and microglia, respectively, in selected brain sections in the peri-lesional regions as a measure of neuroinflammation. Axonal injury was detected by human beta-amyloid precursor protein (β -APP) immunoreactivity in the peri-lesional regions and silver staining in the thalamus.

Acute postinjury lesion volume (3 DPI) was measured by tracing the perimeter of the lesion area on each affected brain section. Delayed brain damage (21 DPI) was quantified as the % tissue loss in the injured versus the contralateral hemispheres. The lesion volume or % tissue volume loss was calculated using the Cavalieri formula applied to the brain areas of the sequential brain sections (ImageJ software, Version 1.44; NIH, Bethesda, MD).

Immunoreactivity of GFAP, OX-18, and β -APP was quantified by threshold analysis within the regions of interest using the ImageJ software (Version 1.44; NIH) at $20\times$ magnification as described previously (Cunningham *et al.*, 2014). The threshold value of immunoreactivity (i.e., grey level index) for each stain was set to consistently detect maximal positive staining with minimal artifacts. All pixel measurements were converted into area measurements (mm^2) according to the resolution scale (pixels/mm) and expressed as the percent area (% area) for a given image. The threshold

analysis was also applied to the silver stained images at 40× magnification.

Neurobehavioral tests

Neurological test. Neurological deficits were evaluated at 3 DPI using a modified clinical examination. Neurological scores (neuroscore) were calculated based on a 12-point scale comprised of four neurological exams: (1) contralateral forelimb flexion during tail suspension, (2) shoulder adduction (body upward curling) during tail suspension, (3) impaired resistance to lateral push, and (4) abnormal circling behavior. Each exam was scored from 0 (normal) to 3 (severely impaired).

Rotarod task. The Rotamex-5 rotarod apparatus (Columbus Instruments, Columbus, OH) was used to measure animals' motor coordination and balance (Shear *et al.*, 2010). Before PBBI surgery, rats were trained to criteria on a fixed-speed version of the rotarod task. Preinjury baseline measures were taken 1 day before PBBI. Animals that failed to achieve preinjury baseline criteria (i.e., unable to maintain their balance on the rotarod for ≥ 50 seconds at 10rpm) were excluded from the subsequent experiments. After PBBI, the animals' ability to remain balanced on the rotating rod was assessed at fixed-speed increments of 10, 15, and 20 rpm for a maximum of 60 s/trial and 2 trials/speed with a 60-seconds inter-trial interval (ITI) between 7 and 21 DPI. Average latency to fall off the rotating rod was used to evaluate the treatment effects of SBC.

Morris water maze task. The Morris water maze (MWM) with a video-tracking system (Noldus EthoVision XT) was used to assess spatial learning performance (Shear *et al.*, 2010, 2011). The MWM apparatus consisted of a circular pool (75 cm deep, 175 cm diameter) filled with clear water ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$) to a depth of 60 cm. A clear Plexiglas platform was submerged 1 inch below the water surface and located in the center of the northwest quadrant. Animals were tested for a maximum 90 s/trial, 4 trials/day (30 minutes ITI) for 5 consecutive days between 13 and 17 DPI. Latency to locate the hidden platform was used to evaluate the treatment effect of SBC.

EEG detection of nonconvulsive seizures

Video EEG recordings were collected continuously in unanesthetized rats housed individually in EEG recording chambers via flexible shield cables connected to swivel commutators, which were interfaced with an EEG amplifier and a digital signal acquisition system (Stellate Harmonie software; Natus Medical Incorporated, San Carlos, CA). The primary seizure outcome measures included the following: (1) nonconvulsive seizures (NCS) incidence (the number of animals displaying NCS events, expressed as the percentage of the respective group), (2) NCS frequency (the number of NCS events experienced by a given animal), (3) NCS episode duration (seconds), (4) NCS cumulative duration (the sum of NCS episode duration for a given animal) (seconds), and (5) NCS onset latency (hours).

Experimental design

A total of 302 rats were used in the analysis of this study, not including those dropped due to mortality ($\sim 20\%$) and

justified exclusions ($\sim 15\%$), for example, statistical outliers, failure to meet preinjury behavioral training criteria, and so on. All animals were randomly assigned to the experimental groups described below.

Experiment 1. Physiological responses to prolonged isoflurane exposure in naïve rats. Before conducting experiments using an extended SBC treatment protocol, the safety profiles of prolonged isoflurane exposure were examined in naïve rats without mechanical ventilation. For this purpose, rats ($n=4$) were anesthetized with 1.5–2% isoflurane for 9 hours (the maximum anesthesia duration required for PBBI+SBC procedures). FiO_2 was maintained at 0.26–0.27. The body and brain temperatures were maintained at $\sim 37^{\circ}\text{C}$. Regional cerebral blood flow (rCBF) was continuously monitored using a laser Doppler probe (Moor Instruments, Devon, England) placed on the parietal cortex (AP: -6 mm and ML: 4 mm to the bregma). Mean arterial blood pressure (MABP) was measured via a femoral artery catheter connected to a pressure transducer (Harvard Apparatus, Holliston, MA). Arterial blood gases were measured at 0.5, 2.5, 5, 7, and 9 hours after the onset of anesthesia using a blood gas analyzer (Radiometer America, Inc., Brea, CA).

Experiment 2. Effects of 4 hours SBC treatment initiated immediately after PBBI. In this experiment, the experimental conditions included sham control (craniotomy followed by sham SBC procedures, that is, cooling cuff insertion and 4 hours isoflurane anesthesia), PBBI alone (PBBI followed by sham SBC), and PBBI+SBC (PBBI followed by 4 hours SBC). The SBC or sham SBC procedures were initiated immediately (within 30 minutes) after PBBI or craniotomy.

Before examining the effects of SBC on axonal injury, the time course of PBBI-induced axonal injury was determined in PBBI alone rats using β -APP staining at 2, 6 hours, 1, 3, 7, and 21 DPI and silver staining at 1, 3, 7, and 21 DPI ($N=6$ rats/group/time point). Subsequently, the effects of 4 hours SBC on early axonal pathology was examined at 3 DPI using β -APP staining (Sham: $N=6$; PBBI alone: $N=12$; PBBI+SBC: $N=13$), whereas the effects of SBC on delayed axonal pathology was examined at 21 DPI using silver staining (on the brain tissues generated from neurobehavioral studies described below).

The effects of SBC on H&E stained brain lesion in addition to GFAP and OX-18 stained activation of astrocytes and microglia, respectively, were also evaluated at 3 DPI using different series of brain sections from the respective groups of animals used for β -APP staining. In addition, the effects of SBC on delayed brain tissue loss were examined at 21 DPI on the brain tissues generated from neurobehavioral studies described below.

The neurofunctional measures (neurological, rotarod, and MWM tests) were performed in a separate cohort of animals, which were randomly assigned to sham control ($N=6$), PBBI alone ($N=16$), and PBBI+SBC ($N=17$) conditions. The neurological functions were assessed at 3 DPI, the rotarod performance was assessed at 7, 10, 14, and 21 DPI, and the MWM performance was assessed from 13 to 17 DPI.

Experiment 3. Effects of 8 hours SBC treatment initiated immediately after PBBI. In this experiment, the procedures of Experiment 2 were replicated in a separate cohort of animals except that the SBC duration and the corresponding

anesthesia duration in the control animals were extended to 8 hours. These animals were randomly divided into three groups for the 3 DPI histo- and immunohistopathology studies (Sham $N=6$; PBBI $N=14$; PBBI+SBC $N=13$) and three groups for the neurofunctional studies (Sham $N=11$; PBBI $N=8$; PBBI+SBC $N=13$). The brains of these animals were also used for H&E and silver staining at the end of the study (21 DPI).

Experiment 4. Effects of 4 hours SBC treatment initiated 2 or 4 hours after PBBI. In this experiment, the initiation of SBC treatment (4 hours in duration) was delayed until either 2 hours ($N=9$) or 4 hours ($N=12$) after PBBI. Matched sham control groups ($n=8$ /condition) and PBBI alone groups ($N=14$ /condition) were also included. Neuroprotective efficacy was measured on the fixed-speed rotarod task to evaluate motor coordination and balance from 7 to 14 DPI. Lesion volume (H&E staining) and axonal pathology (silver staining) were assessed at 14 DPI.

Experiment 5. Effects of SBC treatment (4 hours) on post-traumatic NCS. In this experiment, rats received either PBBI alone ($N=16$) or PBBI+SBC ($N=22$) as described above. Continuous video EEG recordings were collected for 72 hours initiated 4 hours post-PBBI (immediately after the termination of anesthesia in each group). The NCS were identified from offline review of EEG records and verified by synchronized video recordings.

Data analysis

One-way ANOVA, repeated measures ANOVA followed by Student–Newman–Keuls multiple comparison test and Student's t -test, or nonparametric Mann–Whitney test were used when appropriate. A $p \leq 0.05$ was considered statistically significant. Data are presented as mean $\pm$ standard error of the mean. The analyses of histopathology, neurobehavior, and NCS were conducted by experienced personnel who were blinded to the treatment conditions of individual animals.

Results

Experiment 1. Physiological responses to prolonged isoflurane exposure in naïve rats

Prolonged (9 hours) isoflurane anesthesia did not have significant effects on MABP and rCBF of naïve animals. The pH, partial pressure of carbon dioxide (PaCO_2), and partial pressure of oxygen (PaO_2) in arterial blood were also normal throughout the entire procedure (Table 1).

Experiments 2 and 3. Effects of 4 or 8 hours SBC treatment initiated immediately after PBBI

Brain temperature profile. Similar to our previous report, bilateral CCA cooling reduced brain temperature by $\sim 3^\circ\text{C}$ within 30 minutes after the initiation of SBC. Throughout the 4 hours SBC duration, the brain temperature was maintained at $34.5^\circ\text{C} \pm 0.3^\circ\text{C}$ and body temperature was maintained at $37.2^\circ\text{C} \pm 0.03^\circ\text{C}$ via use of a warming blanket (Harvard Apparatus). At the end of each cooling period, spontaneous rewarming allowed the brain temperature to return to baseline levels within 30 minutes. Similar body ($37.1^\circ\text{C} \pm 0.03^\circ\text{C}$, Fig. 1A) and brain ($34.1^\circ\text{C} \pm 0.17^\circ\text{C}$, Fig. 1B) temperatures were also achieved/maintained during 8 hours SBC durations.

Brain tissue damage. The analysis of histopathology at 3 DPI showed that 4 or 8 hours SBC treatment significantly reduced PBBI-induced lesion size by 24% ($p < 0.01$) and 25% ($p < 0.05$), respectively (Fig. 1C). However, by 21 DPI the brain tissue damage was manifested as an enlarged cavity owing to the significant loss of injurious tissue. Under such conditions, the early protective effects on brain lesion size were diminished in both 4 hours ($p > 0.05$) and 8 hours ($p > 0.05$) SBC treated animals (Fig. 1D).

Neuroinflammation. At 3 DPI, significant increases in GFAP and OX-18 positive staining were evident in the perilesion regions in the PBBI alone animals. SBC treatment reduced PBBI-induced activation of GFAP by 23% (4 hours SBC, $p < 0.01$) and 26% (8 hours SBC, $p < 0.05$) (Fig. 1E). Likewise, SBC treatment resulted in significant reductions in OX-18 positive staining by 58% (4 hours SBC, $p < 0.01$) and 33% (8 hours SBC, $p < 0.05$) in the perilesional regions (Fig. 1F).

Axonal injury. The majority of PBBI-induced axonal injury labeled by β -APP immunohistochemistry staining was detected in the anterior corpus callosum and caudate putamen adjacent to the injury core, showing typical “bead-like” or “bulb-like” features at $20\times$ magnification (Fig. 2Aa, the insert). The time course analysis showed that β -APP immunoreactivity significantly increased between 1 and 3 DPI, but was resolved by 7 DPI (Fig. 3A). In contrast, axonal degeneration indicated by silver staining was detected in areas more distal from the injury core, for example, posterior corpus callosum and the thalamus (Fig. 2B) and increased between 7 and 21 DPI, concomitantly with the decrease in β -APP staining (Fig. 3B).

TABLE 1. PHYSIOLOGICAL PARAMETERS IN ISOFLURANE-ANESTHETIZED NAÏVE RATS

	Baseline	2.5 h	5 h	7 h	9 h
MABP (mmHg)	90.5 $\pm$ 0.9	87.4 $\pm$ 3.7	95.1 $\pm$ 3.7	95.6 $\pm$ 9.4	99.2 $\pm$ 11.1
rCBF (flux)	323.2 $\pm$ 60.8	328.9 $\pm$ 69.4	317.7 $\pm$ 61.5	305.5 $\pm$ 47.8	281.6 $\pm$ 52.5
pH	7.41 $\pm$ 0.02	7.42 $\pm$ 0.01	7.42 $\pm$ 0.01	7.43 $\pm$ 0.01	7.43 $\pm$ 0.01
PaCO_2	44.0 $\pm$ 0.91	41.0 $\pm$ 1.82	40.5 $\pm$ 1.32	38.5 $\pm$ 1.25	37.3 $\pm$ 1.31
PaO_2 (mmHg)	110.2 $\pm$ 9.8	102.0 $\pm$ 10.8	105.8 $\pm$ 0.4	106.5 $\pm$ 1.4	104.1 $\pm$ 10.4

Data are expressed as mean $\pm$ SEM; $n=4$.

MABP, mean arterial blood pressure; PaCO_2 , partial pressure of carbon dioxide; PaO_2 , partial pressure of oxygen; rCBF, regional cerebral blood flow.

FIG. 1. (A, B) Time course profiles of body (A) and brain (B) temperature during an 8-hour selective brain cooling (SBC) period followed by spontaneous re-warming in anesthetized rats. (C) SBC significantly reduced brain lesion volume (mm^3) measured at 3 days postinjury (DPI). (D) SBC had no effect on delayed lesion cavity measured at 21 DPI (expressed as % tissue loss relative to the contralateral hemisphere). (E) SBC significantly reduced glial fibrillary acetic protein (GFAP) stained activation of astrocytes measured at 3 DPI. (F) SBC significantly reduced OX-18 stained activation of microglial cells measured at 3 DPI. * indicates $p < 0.05$ (penetrating ballistic-like brain injury [PBBI] vs. PBBI+SBC).

Treatment with SBC significantly reduced β -APP detected axonal injury at 3 DPI by 53% (4 hours SBC, $p < 0.01$) and 41% (8 hours SBC, $p < 0.01$) (Fig. 3C). The effects of SBC on the delayed axonal injury measured by silver staining at 21 DPI were also reduced, although not significantly, yielding 6% (4 hours SBC, $p = 0.54$) and 29% (8 hours SBC, $p = 0.21$) (Fig. 3D) reductions in quantified silver staining.

Neurobehavioral deficits. Compared to sham control animals, all PBBI alone animals displayed comparable impairment on neurological (neuroscore), motor (rotarod test), and cognitive (MWM test) functions regardless of 4 or 8 hours exposure of anesthesia. Thus, the sham control animals with 4 and 8 hours anesthesia were pooled together for motor and cognitive data analysis. This same approach was applied to the 4 and 8 hours PBBI alone animals (Fig. 4B, C).

Compared with PBBI alone group, SBC treatment produced significant, 41% (4 hours SBC, $p < 0.05$) and 44% (8 hours SBC, $p < 0.005$), improvement in neurological deficits measured by the neuroscore at 3 DPI (Fig. 4A). Significant improvement in motor function measured by the rotarod task

was apparent 7 DPI following 4 hours SBC treatment (PBBI vs. PBBI+SBC(4 hours): $p < 0.05$, average of three speeds) and 10 DPI following 8 hours SBC treatment, ($p < 0.005$, Fig. 4B). Such improvement was maintained throughout 21 DPI ($p < 0.05$ at 14 and 21 DPI for both SBC treatment protocols).

SBC treatment failed to improve cognitive outcome on the MWM task regardless of the treatment duration. While there appeared to be a trend toward improved spatial learning performance on the last day of MWM testing, this was not significant following either 4 hours ($p = 0.11$) or 8 hours ($p = 0.24$) of SBC exposure (Fig. 4C).

Experiment 4. Effects of SBC (4 hours) initiated 2 or 4 hours after PBBI

Both 4 and 8 hours SBC initiated immediately after PBBI produced comparable benefits. Thus, the effects of delayed SBC treatment was only evaluated using the 4 hours SBC duration protocol. Results showed that a 2-hour delay in SBC onset still provided significant improvement on motor function measured by the rotarod test at 7, 10, and 14 DPI ($p < 0.01$,

FIG. 2. (A) Representative images of a β -amyloid precursor protein (β -APP) stained brain section associated with brain lesions. The left image (a) was taken at 4 $\times$ magnification. The right image was the enlargement (10 $\times$ magnification) of the area marked by the rectangle in (a). The insert in image (b) was taken at 20 $\times$. (B) Representative images of a silver stained brain section posterior to brain lesions. The left image was taken at 4 $\times$ magnification and right image was the enlargement (10 $\times$ magnification) of the area marked by the rectangle in the image to the left.

$p < 0.005$, and $p < 0.05$, vs. PBBI, respectively), but a 4-hour delay was not effective ($p > 0.05$ vs. PBBI; Fig. 5A). At 14 DPI both the 2- and 4-hour delayed SBC treatment significantly reduced silver stained axonal pathology by 60% ($p < 0.05$ vs. PBBI) and 55% ($p < 0.05$ vs. PBBI), respectively (Fig. 5B).

Experiment 5. Effects of SBC treatment (4 hours) on post-traumatic NCS

Following PBBI, 56% of PBBI alone animals experienced NCS with an onset latency of 24 hours, whereas 41% of PBBI+SBC animals experienced NCS and their onset latency was delayed to 38 hours postinjury. Neither of these measures reached statistical significance ($p = 0.51$ and $p = 0.021$ vs. PBBI for NCS incidence and latency, respectively, Fig. 6B). Importantly, compared to PBBI alone animals, SBC treatment significantly reduced NCS frequency by 65% ($p < 0.05$, Fig. 6C), yielding a 59% reduction in cumulative NCS duration ($p < 0.05$, Fig. 6D), although the NCS episode duration was not affected (PBBI = 33.39 ± 6.01 s/episode; PBBI+SBC = 28.73 ± 7.44 s/episode, $p > 0.05$, data not shown).

Discussion

This study demonstrated comprehensive neuroprotective benefits of SBC (34°C) via the CCA cooling method in amelioration of brain tissue damage, axonal injury, neuroinflammation, and post-traumatic NCS during the sub-acute postinjury period (3 DPI). The protective effects of SBC on improving animals' motor functions persisted throughout 14–21 DPI, even when the SBC treatment was delayed by 2 hours after PBBI. However, SBC failed to protect against cognitive dysfunction or mitigate brain tissue damage out to 21 DPI, suggesting that enduring protection may require more prolonged exposure to SBC. In keeping with this, studies of ischemic brain injury have shown that an even 12-hours brain cooling duration failed to mitigate behavioral impairment and infarct size measured at 1 month after permanent middle cerebral artery occlusion, whereas significant beneficial effects were achieved by 48 hours brain cooling (Clark *et al.*, 2009).

FIG. 3. The time course analysis of PBBI-induced axonal injury stained by β -APP (A) or silver (B) showed different temporal profiles. SBC treatment (either 4 or 8 hours duration) significantly reduced β -APP-stained early axonal injury at 3 DPI (C), but not silver stained delayed axonal injury at 21 DPI (D). * indicates $p < 0.05$ (PBBI vs. PBBI+SBC).

FIG. 4. SBC treatment significantly improved neurological (A) and motor (B) functions measured by neuroscores at 3 DPI and rotarod test between 7 and 21 DPI, respectively. SBC treatment failed to improve cognitive performance measured by Morris water maze (MWM) test between 13 and 17 DPI (C). # indicates $p < 0.05$, Sham vs. PBBI (average three speeds in (C)). ^ indicates $p < 0.05$, PBBI vs. 4 hours SBC. * indicates $p < 0.05$, PBBI vs. both 4 and 8 hours PBBI+SBC.

The use of β -APP and silver staining in this study demonstrated a temporal profile of axonal injury following PBBI, which appeared to respond to the immediate SBC treatments differently. The early axonal injury near the injury core was likely caused by the acute axonal cytoskeletal disruption, which inhibited axoplasmic flow and resulted in β -APP accumulation (Maxwell *et al.*, 1997, 1999). The protective effects of the immediate SBC on reducing PBBI-induced early axonal injury were likely associated with hypothermic protection against axonal cytoskeletal damage via inhibiting calpain-mediated spectrin proteolysis (Buki *et al.*, 1999; Buki and Povlishock, 2006) and ameliorating the loss of axonal microtubules and compaction of neurofilaments (Maxwell *et al.*, 1999) as evidenced in other studies using different traumatic axonal injury models (Buki *et al.*, 1999; Maxwell *et al.*, 1999).

On the other hand, the silver stained axonal injury was detected in areas remote to the injury core, for example, thalamus, in the late phase of PBBI. SBC treatment, even

FIG. 5. (A) The neuroprotective effects of SBC on motor function were maintained when the initiation of the treatment was delayed to 2 hours but not 4 hours postinjury. (B) Delayed SBC treatment (either by 2 or 4 hours) significantly reduced silver stained axonal injury measured at 14 DPI. # indicates $p < 0.05$ (Sham vs. PBBI). ^ indicates $p < 0.05$ PBBI vs. PBBI+SBC (2-hours delay only), * indicates a $p < 0.05$ (PBBI vs. PBBI+SBC).

delayed by 2–4 hours postinjury, reduced silver stained axonal injury at 14 DPI, but such effects were diminished by 21 DPI. Admittedly, silver stain is considered less specific for axonal degeneration because it also stains neuronal damage. Thus, the intense silver staining in regions more distant from the injury core may not solely represent axonal injury, but could be indicative of neurodegeneration as a result of the loss of connections between thalamus and its input and output areas of the brain. In fact, by 21 DPI a large lesion cavity developed in the PBBI core region, which became unsalvageable by the SBC treatment. This could be responsible for the lack of SBC effects on silver stained axonal and neuronal injury at 21 DPI.

In contrast to the benefits of SBC on acute versus chronic injury-induced histopathology, SBC improvement of motor function (rotarod performance) manifested by 7–10 DPI was sustained through 21 DPI. This discrepancy in the time course of SBC neuroprotection measured for histopathology versus neurobehavioral deficits is intriguing, but not necessarily counterintuitive. Studies of brain trauma and ischemia have repeatedly shown that neurofunctional recovery may involve integrated compensatory abilities of the brain in the absence of focal neuronal survival (Dixon *et al.*, 1998; Lu *et al.*, 2009). In this study, the protective effects of SBC on acute postinjury

FIG. 6. (A) Representative EEG detected nonconvulsive seizure (NCS). SBC treatment (initiated immediately post-PBTBI and maintained for 4 hours) reduced NCS incidence (B) and significantly attenuated NCS frequency (C), and total duration (D) compared with untreated PBTBI group. * indicates a $p < 0.05$.

histopathology, neuroinflammation, and post-traumatic seizures might have played important roles in facilitating the brain's compensatory mechanisms to promote motor recovery.

However, limitations of our study are recognized. For example, protective effects measured may have indeed been greater if not for our method of spontaneous rewarming, which returns brain temperature back to 37°C within 30 minutes. This more rapid rewarming might potentially counteract even greater benefits of hypothermia therapy as have been described in other studies (Suehiro and Povlishock, 2001; Suehiro *et al.*, 2003). We should note, however, that by using an identical rewarming method we previously demonstrated acute neuroprotection in both ischemic and TBI without producing overt adverse effects on CCA or other cerebral physiological and histological parameters (Wei *et al.*, 2008, 2011). Consequently, to achieve long-term protective effects across the spectrum of outcome metrics, including cognitive function where only trends were observed in this study, slower rewarming may be required to maximize the measured therapeutic benefits of SBC. These studies are currently being addressed in our SBC model.

Another recognized limitation of this study was the use of male rats only. Considering the gender differences in response to brain injuries and hypothermia therapy, female rats should be evaluated in future studies.

Acute post-traumatic NCSs represent a debilitating consequence of severe TBI. However, treatments of these seizures are particularly difficult due to their sporadic nature, random

occurrence, and intractability to standard antiepileptic drugs. Ideal management of post-traumatic seizure would rely on safe and effective method for seizure prophylaxis.

The anticonvulsant property of hypothermia has been recognized for decades in clinical and preclinical studies of refractory status epilepticus (Schmitt *et al.*, 2006; Corry *et al.*, 2008) and brain injury-induced seizures (Clifton *et al.*, 1993; Hayes, 2009). In most clinical cases, whole-body hypothermia therapy requires a prolonged period of maintenance, for example, 24–48 hours, to cover the high-risk period of seizure occurrence (McIntyre *et al.*, 2003; Bernard and Rosalio, 2008; Diller and Zhu, 2009). During the whole-body cooling therapy, sedatives used to restrain patients may confound the anticonvulsant effects of sustained hypothermia. By contrast, our CCA cooling method significantly shortened cooling duration to achieve antiseizure effects without the complications of medications. More importantly, since the majority (~90%) of NCS occurred during 18–48 hours post-PBTBI (Lu *et al.*, 2011, 2013), many hours beyond the cessation of SBC treatment, the short-term SBC therapy has in fact achieved seizure prophylaxis.

In general, the mechanisms by which hypothermia is able to blunt seizure events are considered multifactorial with evidence centered in areas that hypothermia counteracts seizure-provoked increases in cerebral metabolic demand (Maeda *et al.*, 1999) and excessive release and accumulation of excitatory neurotransmitters (Busto *et al.*, 1989; Globus *et al.*, 1995). These mechanisms are plausible for situations

where sustained hypothermia has direct influence on seizure occurrence during the treatment. But in this study, the effects of seizure protection appeared to extend beyond the treatment duration; thus, other mechanistic actions of SBC might play an important role in its antiseizure activities.

Recently, we demonstrated that following PBBI, there was a positive correlation between PBBI-induced NCS activities and neuroinflammation mediated by activation of astrocytes (Lu *et al.*, 2015). The involvement of brain inflammation in seizures and epilepsy has been well documented in human and animals studies (Choi and Koh, 2008; Vezzani *et al.*, 2013a, 2013b). Activation of astrocytes and microglia has also been shown to facilitate the development of seizures and epilepsy by increasing brain excitability (Devinsky *et al.*, 2013) or promoting induction of major proinflammatory pathways (Vezzani *et al.*, 2013a, 2013b). In this regard, the attenuation of PBBI-induced astrocytes and microglia by SBC treatment measured by the reduction in GFAP and OX-18 immunoreactivity might, in part, account for its anticonvulsant effects.

In summary, the findings of this study extended our previous knowledge on the acute neuroprotective effects of SBC on neurophysiological outcomes achieved by 2 hours CCA cooling and demonstrated a broad spectrum of neuroprotection in the sub-acute phase (3–21 DPI) of TBI following more prolonged SBC durations (4–8 hours). The SBC protocols used in this study were safe and sufficient to achieve multiple beneficial effects days and weeks beyond the cooling duration, which can be a promising therapeutic approach for clinical consideration. Although at this stage the clinical feasibility of CCA cuff method of SBC remains to be evaluated for TBI patients, the results of this study provide a proof-of-principle for the usefulness of SBC as an alternative “hypothermia” therapy for mitigating the post-traumatic sequelae especially encountered in the TBI/polytrauma casualty (Tortella and Leung, 2015).

Acknowledgments

This work was supported by funding provided by the U.S. Army Medical Research and Materiel Command, Combat Casualty Care Research Program. We would like to thank Ms. Xiaofang Yang for her excellent technical support on these experiments.

Author Disclosure Statement

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 2011 edition.

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Address correspondence to:
Xi-Chun May Lu, PhD

Branch of Brain Trauma Neuroprotection
and Neurorestoration
Center for Military Psychiatry and Neuroscience
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring, MD 20910

E-mail: xi-chun.m.lu.civ@mail.mil