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Emergence of Cognitive Deficits after Mild Traumatic Brain Injury due to Hyperthermia

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Abstract

Mild elevations in core temperature can occur in individuals involved in strenuous activities that are risky for potentially sustaining a mild traumatic brain injury (mTBI) or concussion. Recently, we have discovered that mild elevations in brain temperature can significantly aggravate the histopathological consequences of mTBI. However, whether this exacerbation of brain pathology translates into behavioral deficits is unknown. Therefore, we investigated the behavioral consequences of elevating brain temperature to mildly hyperthermic levels prior to mTBI. Adult male Sprague Dawley rats underwent mild fluid-percussion brain injury or sham surgery while normothermic (37 °C) or hyperthermic (39 °C) and were allowed to recover for 7 days. Animals were then assessed for cognition using the water maze and cue and contextual fear conditioning. We found that mTBI alone at normothermia had no effect on long-term cognitive measures whereas mTBI animals that were hyperthermic for 15 min prior to and for 4 h after brain injury were significantly impaired on long-term retention for both the water maze and fear conditioning. In contrast, hyperthermic mTBI animals cooled within 15 min to normothermia demonstrated no significant long-term cognitive deficits. Mild TBI irrespective of temperature manipulations resulted in significant short-term working memory deficits. Cortical atrophy and contusions were detected in all mTBI treatment groups and contusion volume was significantly less in hyperthermic mTBI animals that were cooled as compared to hyperthermic mTBI animals that remained hyperthermic. These results indicate that brain temperature is an important variable for mTBI outcome and that mildly elevated temperatures at the time of injury result in persistent cognitive deficits. Importantly, cooling to normothermia after mTBI prevents the development of long-term cognitive deficits caused by hyperthermia. Reducing temperature to normothermic levels soon after mTBI represents a rational approach to potentially mitigate the long-term consequences of mTBI.

Graphical abstract

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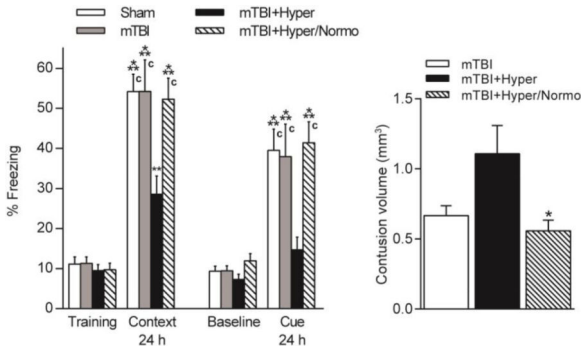

Keywords

Cognition; Concussion; Hyperthermia; Memory; Temperature; Traumatic Brain Injury

Introduction

With nearly 4 million concussions or mild traumatic brain injuries (mTBIs) occurring every year in the United States, understanding the variables that play a significant role in the successful management of a mTBI is an important area of research that could impact public health (Guerriero et al., 2012; Langlois et al., 2004; McMahon et al., 2014; West and Marion, 2014). Interventions initiated at the time of injury are a priority but largely untapped opportunity to potentially improve outcome. Thus, an active area of research includes determining what factors may adversely influence outcomes after a concussion or mTBI and the establishment of guidelines and treatment protocols (Giza and Difiori, 2011; Thurman et al., 1998; West and Marion, 2014). Both clinical and preclinical investigations have documented the sensitivity of white matter to mTBI (Povlishock et al., 1983; Yuh et al., 2014). Recently, environmental and genetic factors along with the interval between multiple insults have been reported to contribute to short-and long-term morbidities after mTBI (McAllister et al., 2005; Meehan et al., 2012; Prins et al., 2013).

Previous studies have emphasized the importance of targeted temperature management in the treatment of moderate to severe TBI and identified the pathomechanisms of how elevated temperatures worsen outcome after moderate to severe TBI (Jia et al., 2010; Kinoshita et al., 2002; Suh et al., 2006; Taylor et al., 2002; Thompson et al., 2005; Vitarbo et al., 2004). In an early experimental study, a brief period of mild hyperthermia induced 24 h after moderate TBI significantly increased mortality and contusion volume as compared to normothermic animals (Dietrich et al., 1996). The delayed period of induced hyperthermia increased vascular and axonal damage and inflammatory cell infiltration (Chatzipanteli et al., 2000; Dietrich et al., 1996). In clinical studies, the occurrence of secondary complications after severe TBI including fever and their potentially detrimental effects on outcome have also been reported (Bao et al., 2014; Hermstad and Adams, 2010; Polderman, 2008; Signorini et al., 1999). Fever is a common condition in patients with severe brain injuries (20–50%) and may increase time in the intensive care unit and hospital stay as well as mortality (Diringer et al., 2004; Kilpatrick et al., 2000; Signorini et al., 1999). Current treatment protocols of

targeted temperature management have been implemented in many intensive care units to prevent harmful temperature elevations.

In contrast to available information regarding the consequences of hyperthermia after severe TBI, limited data are available regarding whether a concussion or mTBI is aggravated by mild hyperthermia (Dietrich and Bramlett, 2007; Signorini et al., 1999). Physiological strain during exercise or work in hot climates can result in heat stress and increased core temperatures under a number of situations and occupations (Army, 2003; Cadarette et al., 2001). Body temperature can rise as high as 39–40 °C in athletes during periods of strenuous activity such as football and this temperature increase is more long-lasting in hot environments (Goosey-Tolfrey et al., 2008; Nybo, 2008; Özgünen et al., 2010). Thus, in individuals that are engaged in strenuous activities including exercise, competitive sports or under highly stressful conditions, core temperatures may reach mildly hyperthermic levels that could potentially alter traumatic consequences. In a recent study, our laboratory first reported that artificially elevating brain temperature to 39 °C before mTBI significantly increased patterns of neuronal death within cortical and subcortical regions as compared to normothermic mTBI (Sakurai et al., 2012).

Whether or not these pathological changes observed with hyperthermic mTBI also result in persistent behavioral abnormalities is unknown. A subset of patients with mTBI or concussion sustain prolonged alterations in cognitive function (Collins et al., 1999; De Beaumont et al., 2012; McAllister et al., 2006; McMahon et al., 2014). The goal of this study was therefore to determine if mild elevations in brain and core temperature at the time of mTBI could aggravate cognitive deficits. For this purpose we assessed animals on several distinct learning tasks, the water maze and cue and contextual fear conditioning. Since working memory is highly sensitive to brain trauma, we also utilized a working memory version of the water maze to evaluate this memory modality (McAllister et al., 2006). Elevations in brain and body temperature from strenuous physical exercise may be reduced by cessation of the activity and the use of cooling strategies (DeMartini et al., 2011; Nybo, 2012). Therefore, we also tested whether reducing brain and body temperature to normal physiological temperatures after hyperthermic mTBI would reduce or prevent potential cognitive problems.

Materials and methods

Fluid-percussion injury surgery

Experimental procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Miami Animal Care and Use Committee. Animals were maintained on a 12/12 h light/dark cycle and had free access to food and water. Surgical and temperature groups were prospectively randomized and animals were assessed by individuals that were blinded to the experimental groups. A power analysis was conducted to determine the animal numbers needed to detect a 10% difference in water maze probe trial performance between groups at 80% power and with a significance level of 0.05 (Titus et al., 2013). The estimated sample size was 12 animals per group. To determine if hyperthermia in combination with a mTBI worsens cognitive recovery, we utilized the parasagittal fluid-percussion injury (FPI) model in adult male Sprague Dawley rats (n=61,

2–4 mos, Charles Rivers Laboratories, Wilmington, MA, USA). Animals were anesthetized (70% nitrous oxide, 1–3% isoflurane, and 30% oxygen) 24 h prior to injury and surgically prepared for FPI as previously described (Sakurai et al., 2012; Titus et al., 2013). Briefly, anesthesia was induced with 3% isoflurane, 70% nitrous oxide and 30% oxygen, and animals were maintained at 1–2% isoflurane, 70% nitrous oxide and 30% oxygen. A 4.8 mm craniotomy (−3.8 mm bregma, 2.5 mm lateral) was prepared by trephination over the right parietal cortex and an 18 gauge syringe hub was secured to the craniotomy window with cyanoacrylate and dental cement. After recovery for 24 h during which the animals were fasted and provided water ad libitum, animals were anesthetized (70% nitrous oxide, 1–3% isoflurane and 30% oxygen), intubated and mechanically ventilated (Stoelting, Wood Dale, IL, USA). A catheter was placed in the tail artery to monitor mean arterial blood pressure and arterial blood samples were used to calculate blood gases (pO_2 and pCO_2) and blood pH. Physiological parameters were monitored for at least 15 min prior to the mTBI, within 15 min after the mTBI, and hourly for the surgery duration. After stabilization of physiological parameters, animals received mTBI (1.4–1.6 atmospheres) or sham injury. Penicillin/benzathine (20,000 IU/kg, intramuscular) was give once prior to the brain injury surgery, and buprenorphine (0.01 mg/kg, subcutaneously) and saline (5 ml, subcutaneously) were given once at the completion of the surgery.

Beginning 15 min prior to the brain injury, the brain and body temperature of the animals were elevated to a mildly hyperthermic level (39 °C) or kept at normothermic levels (37 °C) as previously described (Sakurai et al., 2012). A rectal thermistor was used to monitor core temperature and a thermistor placed into the temporalis muscle was used as an indirect measure of brain temperature (Jiang et al., 1991). Two automatic feedback heating lamps were placed over the animal's head and body to regulate brain and core temperature. Animals were maintained at normothermia or mild hyperthermia temperatures for 4 h post-injury. To determine if cooling the brain and body to normothermic levels (37 °C) after hyperthermic mTBI would prevent cognitive deficits, a subgroup of hyperthermic mTBI animals were cooled to normothermic levels at 15 min post-injury and maintained for 4 h post-injury at normothermia. Sham-operated animals received identical surgical and temperature manipulations as mTBI animals but were not injured. Sham animals were maintained for 4 h at normothermia or hyperthermia during the surgery. No significant differences in behavioral outcome on the water maze task or fear conditioning were observed between normothermic sham and hyperthermic sham groups and therefore, the groups were combined.

Criteria for exclusion from the study were: mortality (one normothermic sham, one normothermic TBI, and one hyperthermic TBI animal, 5% rate), >15% loss of body weight, non-resolving infection at the surgical site (one normothermic sham, one normothermic TBI, and three hyperthermic TBI animals), temperature not maintained at protocol levels for 4 h during surgery (two hyperthermic sham animals), inability to feed or drink, motor paralysis, listlessness (one normothermic sham and one hyperthermic sham animal), self-mutilation, excessive grooming leading to loss of dermal layers, spontaneous vocalization when touched, lack of completion through the behavioral experiments due to minor claw injuries (one normothermic sham and two hyperthermic TBI animals), or poor grooming habits. Animals were evaluated daily after surgery. Due to this exclusion criteria, 4 normothermic

sham animals, 3 hyperthermic sham animals, 2 normothermic mTBI animals, 6 hyperthermic mTBI animals, and 0 hyperthermic/normothermic mTBI animals were removed from the study. Final *n* values were: normothermic sham (*n*=5), hyperthermic sham (*n*=7), normothermic mTBI (*n*=11), hyperthermic mTBI (*n*=11), and hyperthermic/normothermic mTBI (*n*=12).

Cue and contextual fear conditioning

Animals were allowed to recover for 7 days, and then evaluated for cue and contextual fear conditioning as previously described (Titus et al., 2013). This time point was chosen since most concussive symptoms resolve within 7–14 days post-injury (McCrea et al., 2003; Vagnozzi et al., 2010). On the first day, the animals habituated to the fear conditioning apparatus for 10 min (30.5×24.1×21 cm, Coulbourn Instruments, Allentown, PA, USA). At 24 h following habituation, animals were placed in the apparatus for 180 s. A tone was given during the last 30 s (75 dB, 2.8 kHz). During the last 1 s of the tone, a 1 mA foot shock was delivered through the electrical grid floor. The apparatus was cleaned with 70% ethanol between animals. At 24 h and 3 weeks after training, contextual fear conditioning was assessed by placing the animals in the apparatus for 5 min and measuring freezing. Cue fear conditioning was assessed 1 h after contextual fear conditioning by placing animals in an altered apparatus with different ambient light, white background noise, changed textured chamber floor and walls, and a novel odorant. The animal's freezing behavior was measured for 3 min. The tone (75 dB, 2.8 kHz) was delivered for the last 1 min. Freezing behavior was measured using video-based analysis (FreezeFrame, Coulburn Instruments). Contextual fear conditioning was analyzed by comparing freezing levels on the training day prior to the tone and foot shock delivery versus the testing day. Cue fear conditioning was analyzed by comparing freezing levels in the novel context prior to the tone presentation versus freezing during the tone. Shock threshold was evaluated by determining the minimum shock intensity to elicit a flinch, jump, and vocalization. Foot shocks were delivered every 30 s in 0.02 mA increments beginning at 0.1 mA. This behavior test was performed at the end of all cognitive testing at 4 weeks post-surgery.

Water maze

The standard hidden platform version of the Morris water maze task was used to test spatial memory at 2 weeks post-surgery (Titus et al., 2013). Acquisition was conducted with 4 trials per day (60 s trial duration) and inter-trial intervals of 4 min. If the rat failed to locate the platform by the end of the trial, the rat was guided to the platform and remained on the platform for 10 s. Probe trial duration was 60 s and assessed 24 h after the last acquisition day. Escape latency, time spent in each quadrant, and swim speed were analyzed with EthoVision software (Noldus Information Technology, Leesburg, VA, USA).

At 3 weeks post-surgery, spatial working memory was assessed with a delayed match-to-place task utilizing the water maze (Hoskison et al., 2009). Four paired trials were given each day for 2 days with inter-trial intervals of 4 min. Trial duration was 60 s. The hidden platform remained invariant in location for each pair of trials. Upon reaching the platform, the animal remained on the platform for 10 s. After a 5 s delay, the animal was released into the water to again search for the hidden platform in the same location. Escape latency

differences between the first location trial and subsequent match trial were measured. Improvement in escape latency, (location trial-match trial)/location trial, was calculated. Data shown are from day 2.

Histopathological analysis

At 1 month post-surgery, animals were deeply anesthetized (3% isoflurane, 70% nitrous oxide and 30% oxygen) and transcardially perfused with saline (75 mL) and 4% paraformaldehyde (150 mL) in 0.1M phosphate buffer pH 7.4. Brains were sectioned coronally (10 μm thick) in a stereological series (150 μm apart). Tissue was stained with hematoxylin and eosin (H&E) plus Luxol fast blue. The ipsilateral and contralateral cortex and hippocampus were traced using Neurolucida 10.50.2 (MBF Bioscience, Williston, VT, USA) with an Olympus BX51TRF microscope (Olympus America, Center Valley, PA, USA) between bregma levels −3.8 to −5.8 mm. Cortical contusions in the external capsule between the parietal cortex and hippocampus were traced across the entire extent of the contusion and demarcated by infiltrating inflammatory cells, vacuoles, edema, hemorrhage, and disordered white matter. Percent atrophy was calculated as the difference between contralateral and ipsilateral volume, normalized to the contralateral volume to account for differences in tissue shrinkage between animals. Images were obtained with a 20× objective on an Olympus BX51TRF microscope (Olympus America) using Neurolucida 10.50.2 (MBF Bioscience).

Data analysis

Statistical comparisons were made using GraphPad Prism 6.05 (La Jolla, CA, USA) and SigmaPlot 12.0 (San Jose, CA, USA). Significance was designated at $p<0.05$. Results presented are mean±SEM. Physiological data were analyzed with repeated measures two-way ANOVA and Tukey's post-hoc analysis with factors surgery treatment x time. Cue and contextual fear conditioning data were analyzed with repeated measures two-way ANOVA and Tukey's post-hoc analysis with factors surgery treatment x time. Water maze data were analyzed with repeated measures two-way ANOVA (factors surgery treatment x training day) or one-way ANOVA where appropriate and Tukey's post-hoc analysis. Working memory data were analyzed with repeated measures two-way ANOVA and Tukey's post-hoc analysis with factors surgery treatment x trial or a one-way ANOVA where appropriate. Atrophy and contusion volume were analyzed with a one-way ANOVA and Tukey's post-hoc analysis.

Results

Physiological variables

Physiological variables of mean arterial blood pressure (MABP), blood pO_2, pCO_2, pH, and head and body temperature were controlled throughout the surgery and analyzed by repeated measures two-way ANOVA using the factors surgery treatment and time (Table 1). No significant differences were observed between surgery treatment groups for MABP, blood pO_2, pCO_2, and pH and none of these variables were outside normal physiological ranges. As expected, a significant interaction of surgery treatment and time was observed for both head temperature ($F_{(20,247)}=68.69$, $p<0.001$) and body temperature ($F_{(20,247)}=102.94$, $p<0.001$). Post-hoc analyses indicated that for hyperthermic/normothermic mTBI animals,

head and body temperatures at 15 min prior and 15 min post-surgery were significantly different than temperatures at 1 h, 2 h, 3 h, and 4 h post-surgery.

Fear conditioning

To assess cognitive deficits after mTBI and hyperthermia, animals were fear conditioned to associate a context and tone with a mild foot shock. Fear conditioning was assessed by measuring freezing behavior. No significant differences in freezing behavior were observed between treatment groups during training (Fig. 1A). Likewise, no significant differences in shock threshold were observed between animal groups (Fig. 1B). When animals were assessed 24 h after training for contextual fear conditioning, a significant interaction between surgery treatment and time was found ($F_{(3,91)}$=4.17, p=0.011). All animal groups froze significantly greater 24 h after conditioning as compared to during the training day prior to conditioning (Fig. 1C). However, hyperthermic mTBI animals demonstrated significantly less contextual fear conditioning as compared to sham or normothermic mTBI animals. Promisingly, hyperthermic animals that were cooled within 15 min after the mTBI had significantly greater contextual fear conditioning as compared to hyperthermic mTBI animals.

To assess cue fear conditioning, animals were assessed in a novel context 24 h after training. Within the novel context, a significant interaction of surgery treatment and time was observed ($F_{(3,91)}$=3.86, p=0.016). Baseline freezing was unaffected in any animal group (Fig. 1C). When freezing levels were assessed during the cue presentation, sham, normothermic mTBI, and hyperthermic/normothermic mTBI animals froze significantly greater as compared to baseline freezing levels. Hyperthermic mTBI animals froze significantly less than sham, non-injured animals or normothermic mTBI animals in response to the cue. Cooling hyperthermic animals to normothermia after mTBI rescued these impairments in cue fear conditioning.

To evaluate the persistence of these effects, animals were re-tested 3 weeks later for cue and contextual fear conditioning. Repeated measures two-way ANOVA analysis indicated a significant interaction of surgery treatment and time for both contextual ($F_{(3,89)}$=6.87, p<0.001) and cue fear conditioning ($F_{(3,89)}$=3.06, p=0.039). Sham, normothermic mTBI and hyperthermic/normothermic mTBI animals demonstrated long-term memory recall for both cue and contextual fear conditioning (Fig. 1D). In contrast, hyperthermic mTBI animals had significantly decreased long-term cue and contextual fear conditioning as compared to sham or normothermic mTBI animals. These results indicate that mTBI alone does not result in significant long-term deficits in cue and contextual fear conditioning, and that cooling hyperthermic mTBI animals to normothermia prevents both short- and long-term deficits in cue and contextual fear conditioning observed in hyperthermic mTBI animals.

Water maze

Next, to evaluate another hippocampal-dependent learning task and determine if these results generalized across similar cognitive domains, animals were trained at 2 weeks post-surgery to find a hidden platform in a water maze using spatial cues over 4 days of training. On the fifth day, animals were tested in a probe trial with the platform removed to determine

the search strategy employed by the animal. We found that normothermic mTBI animals had no significant acquisition deficits in the water maze as compared to sham, non-injured animals (Fig. 2A). In contrast, mTBI animals that were hyperthermic for 15 min prior to the brain injury and remained hyperthermic for 4 h demonstrated a slight acquisition impairment on day 4 (Fig. 2B). When hyperthermic mTBI animals were cooled to normothermia levels at 15 min after the mTBI, their acquisition in the water maze task was comparable to sham, non-injured animals. On day 5, animals were assessed for their search preference in the target quadrant in a probe trial (Fig. 2C). Similar to sham animals, normothermic mTBI animals demonstrated a significant increase in time spent in the target quadrant, whereas hyperthermic mTBI animals had no quadrant bias, indicating no spatial memory for the platform location. However, hyperthermic mTBI animals treated with normothermia significantly preferred the target quadrant. Swim speed was not significantly different between any animal groups (Fig. 2D). These results indicate that mTBI does not result in significant acquisition or retention deficits on the water maze task, but that hyperthermia at the time of mTBI induces a mild acquisition deficit in the water maze and significant retention deficits. Treatment with normothermia after a mTBI while hyperthermic prevents the development of spatial learning deficits in the water maze. These results indicate that the beneficial effects of cooling after a mTBI while hyperthermic can be generalized across several learning tasks.

Working memory

Clinical assessments of learning and memory ability have revealed that a single concussion or mTBI can result in significant impairments in working memory, which can significantly interfere with quality of life (Konrad et al., 2011; Malojcic et al., 2008; McAllister et al., 2001; Vanderploeg et al., 2005). To determine if mTBI results in working memory deficits that are exacerbated by hyperthermia, we utilized a version of the water maze where animals were trained on a location trial to find a hidden platform, and then were tested 5 s later on a match trial to re-find the hidden platform (Hoskison et al., 2009). A decrease in escape latency on the match trial is indicative of working memory ability. Repeated measures two-way ANOVA analysis indicated that there was no significant interaction of surgery treatment and trial (Fig. 3A). However, there was a main effect of surgery treatment ($F_{(3,91)}$=4.24, p=0.011) as well as trial ($F_{(1, 91)}$=57.32, p<0.001). Post-hoc analysis of the main effect of trial indicated that all animal groups demonstrated significant improvement in escape latency on the match trial as compared to the location trial. Post-hoc analysis of the main effect of surgery treatment indicated that normothermic mTBI and hyperthermic mTBI animal groups were significantly different from sham or hyperthermic/normothermic mTBI animals. To further analyze these differences, we calculated the percent improvement in escape latency on the match trial. Sham animals demonstrated significantly greater improvement on the match trial as compared to all other groups, normothermic mTBI, hyperthermic mTBI, or hyperthermic/normothermic mTBI animals. These results indicate that mTBI at either normothermic or hyperthermic conditions resulted in modest, but significant working memory deficits (Fig. 3B).

Pathology

In a previous study, we reported that hyperthermic mTBI significantly increased cortical contusion volume and neuronal loss (Sakurai et al., 2012). To extend these observations and determine if post-injury cooling can prevent these pathological changes, we evaluated cortical and hippocampal atrophy as well as cortical contusions at 1 month post-surgery (Fig. 4A). We found that mTBI resulted in a small, significant atrophy of the cortex for all temperature groups (Fig. 4B). There was no significant hippocampal atrophy in any of the mTBI treatment groups (data not shown). Cortical contusions were also observed in all mTBI animal groups, but were significantly smaller in cooled hyperthermic mTBI animals as compared to hyperthermic mTBI animals that remained hyperthermic throughout the entire surgery (Fig. 4C). These results indicate that in this model of complicated mTBI, a small degree of pathology is observed, and cooling after hyperthermic mTBI is partially protective for contusion development within the cortex.

Discussion

Previous clinical and experimental studies have emphasized the importance of elevated temperatures in potentially worsening outcomes after moderate and severe TBI (Dietrich and Bramlett, 2007; Signorini et al., 1999). In contrast, limited information is available regarding whether behavioral recovery after mTBI or concussion is aggravated by mild hyperthermia. Body temperatures can significantly rise in individuals during periods of strenuous activity such as exercise, participating in sporting events, as well military operations (Goosey-Tolfrey et al., 2008; Nybo, 2008; Özgünen et al., 2010). In preclinical animal studies, mTBI in conjunction with mild hyperthermia (39 °C) caused increased vulnerability of cortical and hippocampal neuronal populations and damage to white matter regions (Sakurai et al., 2012). In the present study, this model of complicated mTBI experienced in the context of hyperthermia also produced prolonged cognitive deficits in several different learning and memory tasks. Normalizing brain temperature after the hyperthermic insult attenuated most, but not all of these behavioral deficits. Working memory was impaired after mTBI regardless of the temperature manipulations. These preclinical results indicate that brain temperature can be a significant variable in concussion and mTBI outcome and that mild hyperthermia at the time of brain injury could potentially result in the development of persistent cognitive deficits.

Recent studies have reported that following even a single episode of mTBI produced by a concussive or blast injury device, micro-structural alterations including altered blood-brain barrier damage and white matter changes can be observed (Perez-Polo et al., 2013; Tomkins et al., 2011). Using high resolution MRI approaches, clinical data indicate that mTBI can cause structural changes that have not previously been routinely observed (Belanger et al., 2007; Inglese et al., 2005; Mayer et al., 2010; Yuh et al., 2014). Recently, the prospective TRACK-TBI study has reported that mTBI patients even with a negative head computed tomography report long-lasting symptoms and functional impairments for up to 1 year post-injury (McMahon et al., 2014). In particular, functional MRI studies have revealed abnormalities in brain activation during working memory tasks in concussed adults even in the absence of observable behavioral deficits (Chen et al., 2004; Dettwiler et al., 2014).

Concussions and mTBIs can result in significant long-term working memory deficits in children as well (Dettwiler et al., 2014; Keightley et al., 2014). Our results are relevant to young athletes at risk for a concussion or mTBI and indicate that strategies beyond cooling may be necessary to prevent the development of working memory deficits caused by mTBI or concussion.

Long-term memory in particular, was improved by temperature manipulations after hyperthermic mTBI. Specific cell signaling cascades associated with hippocampal-dependent learning and memory have been reported to be temperature sensitive (Atkins et al., 2007). Long-term spatial memory and fear conditioning involves initiation of CREB signaling mechanisms and CREB is actually activated by hypothermia treatment (Atkins et al., 2007; Bourtchuladze et al., 1994). Whether CREB and downstream signaling mechanisms involved in long-term memory formation are altered by hyperthermia remain to be determined. Hyperthermia is also known to increase extracellular glutamate levels, neurotoxic zinc translocation, and potentiate inflammatory signaling to increase neuronal death (Sharma, 2006; Suehiro et al., 1999; Suh et al., 2006; Takagi et al., 1994). In particular, infiltration of inflammatory cells is aggravated by hyperthermia (Chatzipanteli et al., 2000; Thompson et al., 2005; Whalen et al., 1997). The increase in inflammation may have contributed to neuronal death in structures involved in the formation of long-term memories for these particular learning tasks (Sakurai et al., 2012; Suzuki et al., 2004). In addition to increasing neuronal and vascular vulnerability, mild elevations in temperatures may increase the magnitude of traumatic axonal injury, demyelination, synaptic dysfunction, or alter lipid homeostasis (Cornelussen et al., 2001; Dietrich et al., 1996; Mayer et al., 2011; Suh et al., 2006). In this study, we found that this model is analogous to a complicated mTBI model, with very modest but measureable pathology. Cortical contusions, but not cortical atrophy were affected by cooling after hyperthermic mTBI. This result may be reflective of the recovery time point of 1 month. With this complicated mTBI model at 1 month post-injury, atrophy is beginning to develop whereas contusions are resolving. These results suggest that effects on atrophy may not be fully realized at this assessment time point. Alternatively, the contusion characterized by hemorrhage, extravasation of inflammatory cells, and white matter tract disruption may be most sensitive to hyperthermia. These results are in accordance with a study by Povlishock and colleagues which documented that axonal injury and blood-brain barrier changes are consistent features of mTBI (Povlishock et al., 1983). The TRACK-TBI study has confirmed these preclinical results and reported white matter injury as detected by diffusion tensor imaging in mTBI patients (Yuh et al., 2014). Thus, future investigations would be useful to evaluate the long-term white matter changes and circuitry alterations associated with hyperthermia and mTBI.

The exacerbation of injury mechanisms by hyperthermia is not unique to TBI and has been reported in several other CNS injuries. In models of global and focal ischemia, for example, induced periods of hyperthermia during or following the insult significantly aggravated histopathological damage and worsen behavioral outcomes (Chopp et al., 1988; Dietrich et al., 1990; Wang et al., 2009). In traumatic spinal cord injury, exacerbated structural damage and behavioral outcomes with hyperthermia has also been reported (Urdzikova and Vanicky, 2006; Yu et al., 2001). The underlying mechanisms responsible for the worsened histopathological and behavioral outcomes observed with hyperthermia remain to be

delineated in these various CNS injuries (Campos et al., 2013; Dietrich and Bramlett, 2007). However, evidence suggests that regardless of the underlying mechanisms, behavioral recovery is typically negatively impacted. Thus, despite a diverse array of underlying mechanisms, temperature manipulations to reduce the effects of hyperthermia on CNS injury outcome may be beneficial among several patient populations.

A caveat of the present study is the complicating factor of the surgery and anesthetic duration. Several animals were excluded due to surgery complications and attrition. These animals were present in both sham and mTBI animal groups and hyperthermia and normothermia treatments. Prolonged anesthesia in some circumstances impairs learning and memory ability (Lin and Zuo, 2011; Uchimoto et al., 2014). Comparing sham animals to mTBI animals facilitated identifying the specific effects of mTBI, but this prolonged anesthetic duration likely complicated the interpretation of these results.

Our model of mTBI alone did not result in measurable behavioral deficits with the exception of working memory. Likewise, clinical studies indicate that most people report a resolution of symptoms and recovery from concussion by 7–14 days (Lovell et al., 2003; McCrea et al., 2003). However, a subpopulation of people report long-term symptoms (Gagnon et al., 2009; McMahon et al., 2014; Meehan et al., 2013). Because of the significant numbers of individuals sustaining concussions each year in the United States due to athletic or military operations, understanding the variables that determine full or incomplete recovery from a concussion or mTBI is an important area of study (Langlois et al., 2004). Some of these variables are already appreciated, such as older age, repetitive injuries, environmental stressors, genetic polymorphisms, and pre-existing psychiatric conditions, yet others remain unknown (Lingsma et al., 2014; McAllister et al., 2005; Meehan et al., 2012; Prins et al., 2013). In the current study, temperature at the time of injury was a significant factor in behavioral recovery and further studies are needed to determine if this translates into the clinical population.

Within the sports literature, attempts to increase performance with cooling strategies have been successful (Siegel and Laursen, 2012; Tyler et al., 2013). A number of methods decrease core and brain temperature, including ingestion of cold liquids or ice, ice water immersion, cooling vests, and application of ice packs on high perfusion areas of the skin (DeMartini et al., 2011). An important finding of the present study was that by acutely reducing elevated brain temperatures to normothermic levels after mTBI, the cognitive deficits seen in hyperthermic mTBI animals were significantly reduced. These results indicate that therapeutic interventions initiated early after concussion including normalizing core temperature could be a simple, yet effective strategy for reducing the potentially detrimental effects of mTBI or concussion.

In summary, we report that a mildly hyperthermic state can promote persistent cognitive deficits after complicated mTBI. Excitingly, cooling to normothermia after the trauma rescued the cognitive deficits caused by the hyperthermia. Many athletes, civilians and military personnel may experience concussions or mTBI in the context of mild systemic hyperthermia (>38 °C). The current study emphasizes the use of temperature management

strategies following a concussive event as a promising avenue for future clinical investigation.

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Highlights

- Mild TBI alone did not result in significant, long-term cognitive deficits
- Mild TBI while hyperthermic resulted in long-term learning and memory deficits
- Cooling after hyperthermic mild TBI prevented development of cognitive deficits
- Cortical contusions were decreased by cooling after hyperthermic mild TBI

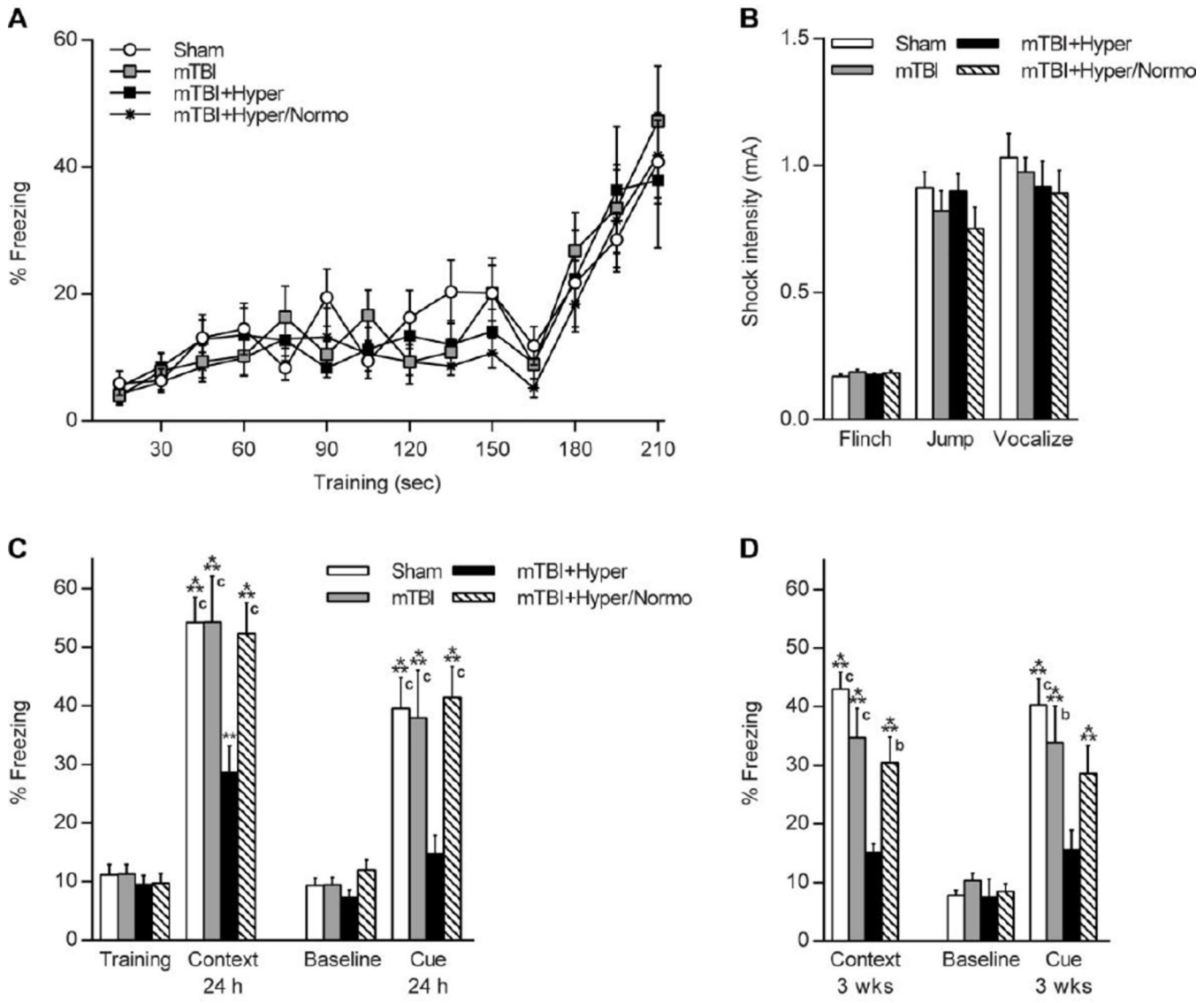

Fig. 1.

Improvement in cue and contextual fear conditioning by cooling after a mTBI. (A) Freezing responses during training were not significantly different between sham, normothermic mTBI (mTBI), hyperthermic mTBI (mTBI+Hyper), or hyperthermic/normothermic mTBI (mTBI+Hyper/Normo) animals. (B) Shock threshold sensitivity was similar between all animal groups. (C) Cue and contextual fear conditioning at 24 h after training. All animal groups demonstrated significant contextual fear conditioning. Hyperthermic mTBI animals froze significantly less than sham, normothermic mTBI, or hyperthermic/normothermic mTBI animals. Cue fear conditioning was significantly impaired in hyperthermic mTBI animals as compared to sham, normothermic mTBI, and hyperthermic/normothermic mTBI animals. (D) Cue and contextual fear conditioning at 3 weeks after training. Sham, normothermic mTBI, and hyperthermic/normothermic mTBI animals demonstrated significant cue and contextual fear conditioning. Hyperthermic mTBI animals demonstrated no significant retention of either cue or contextual fear conditioning at 3 weeks. ***$p<0.001$

vs training for contextual fear conditioning, ***$p<0.001$ vs baseline for cue fear conditioning, [b]$p<0.051$, [c]$p<0.001$ vs hyperthermic mTBI animals, repeated measures two-way ANVOA and Tukey's post-hoc analysis. $n=12$ sham animals, $n=11$ normothermic mTBI animals, $n=11$ hyperthermic mTBI animals, $n=12$ hyperthermic/normothermic mTBI animals.

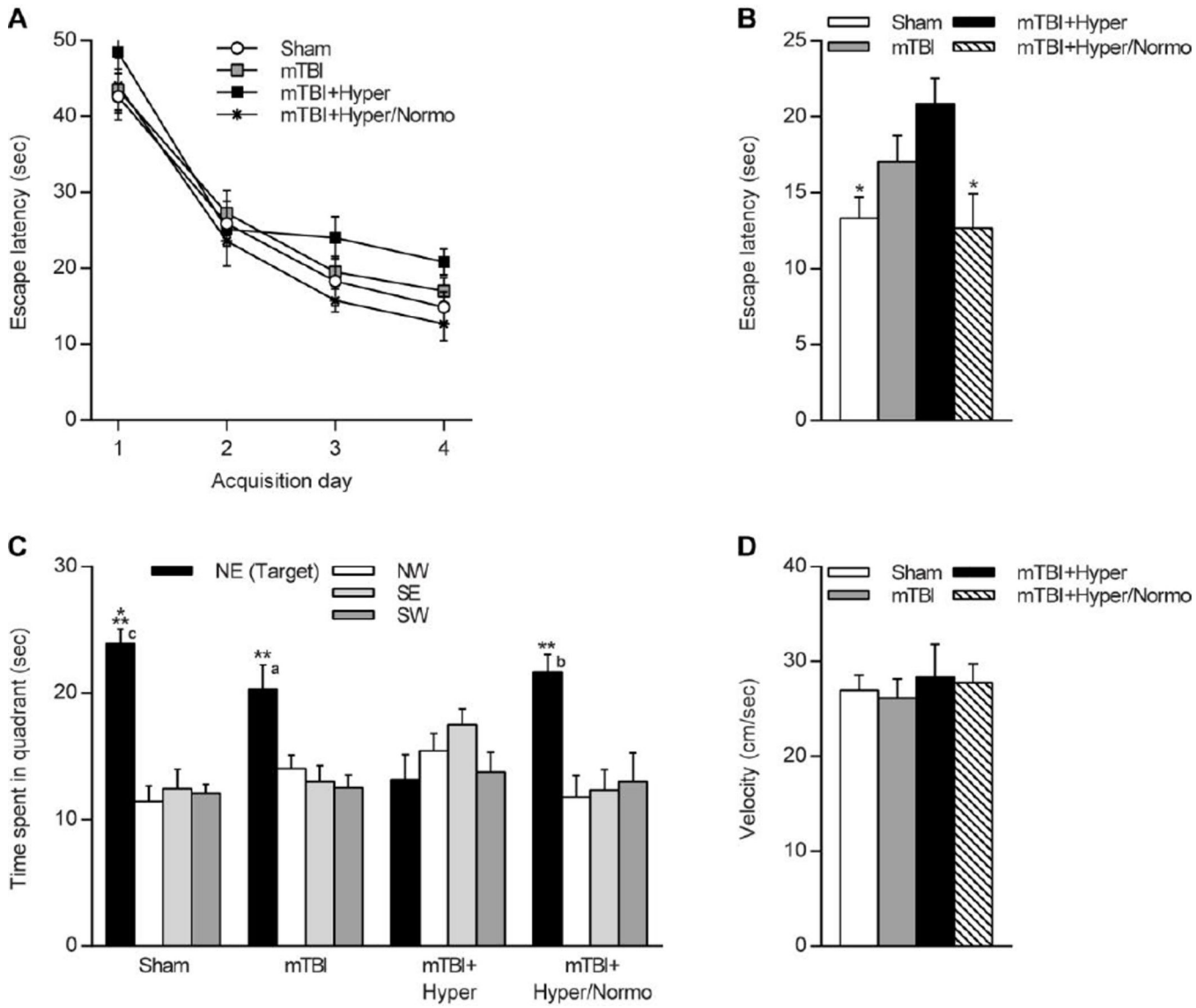

Fig. 2.

Effects of temperature manipulations on water maze performance after mTBI. (A) Escape latency to find the hidden platform across training days was not significantly different between sham, normothermic mTBI (mTBI), hyperthermic mTBI (mTBI+Hyper), or hyperthermic/normothermic mTBI (mTBI+Hyper/Normo) animals. (B) Analysis of escape latency on training day 4. Hyperthermic mTBI animals had significantly longer escape latencies as compared to sham animals or hyperthermic/normothermic mTBI animals. *p<0.05, one-way ANOVA and Tukey's post-hoc analysis. (C) Retention of the water maze task by assessing time spent in the trained quadrant 24 h after the last acquisition day. Sham, normothermic mTBI, and hyperthermic/normothermic mTBI animals spent significantly more time in the target quadrant as compared to other quadrants. Hyperthermic mTBI animals had no significant increased time in the target quadrant. **p<0.01, ***p<0.001 target quadrant vs other quadrants, one-way ANVOA and Tukey's post-hoc analysis. Comparison of time spent in the target quadrant indicated that hyperthermic mTBI animals

were significantly different than sham, normothermic mTBI, or hyperthermic/normothermic animals. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ target quadrant time for hyperthermic mTBI vs other animal groups, one-way ANVOA and Tukey's post-hoc analysis. (D) Swim speed was not significantly different between animal groups. $n=12$ sham animals, $n=11$ normothermic mTBI animals, $n=11$ hyperthermic mTBI animals, $n=12$ hyperthermic/normothermic mTBI animals.

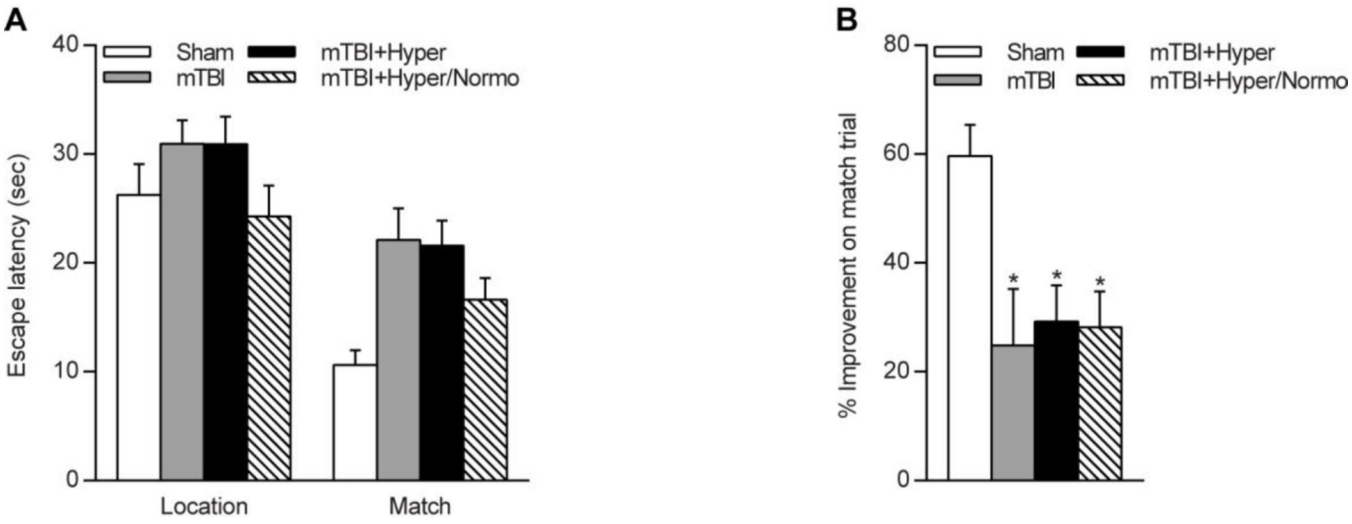

Fig. 3.

Working memory deficits after mTBI. (A) Escape latencies of the location and match trials. All animal groups demonstrated a significant improvement in escape latency on the match trial as compared to the location trial. Normothermic mTBI and hyperthermic mTBI animals performed significantly worse as compared to sham or hyperthermic/normothermic mTBI animals. (B) Percent improvement in escape latency. Normothermic mTBI, hyperthermic mTBI, and hyperthermic/normothermic mTBI animals all had significantly less improvement in escape latency on the match trial as compared to sham animals. *p<0.05 vs sham, one-way ANVOA and Tukey's post-hoc analysis. n=11 sham animals, n=11 normothermic mTBI animals, n=11 hyperthermic mTBI animals, n=12 hyperthermic/normothermic mTBI animals.

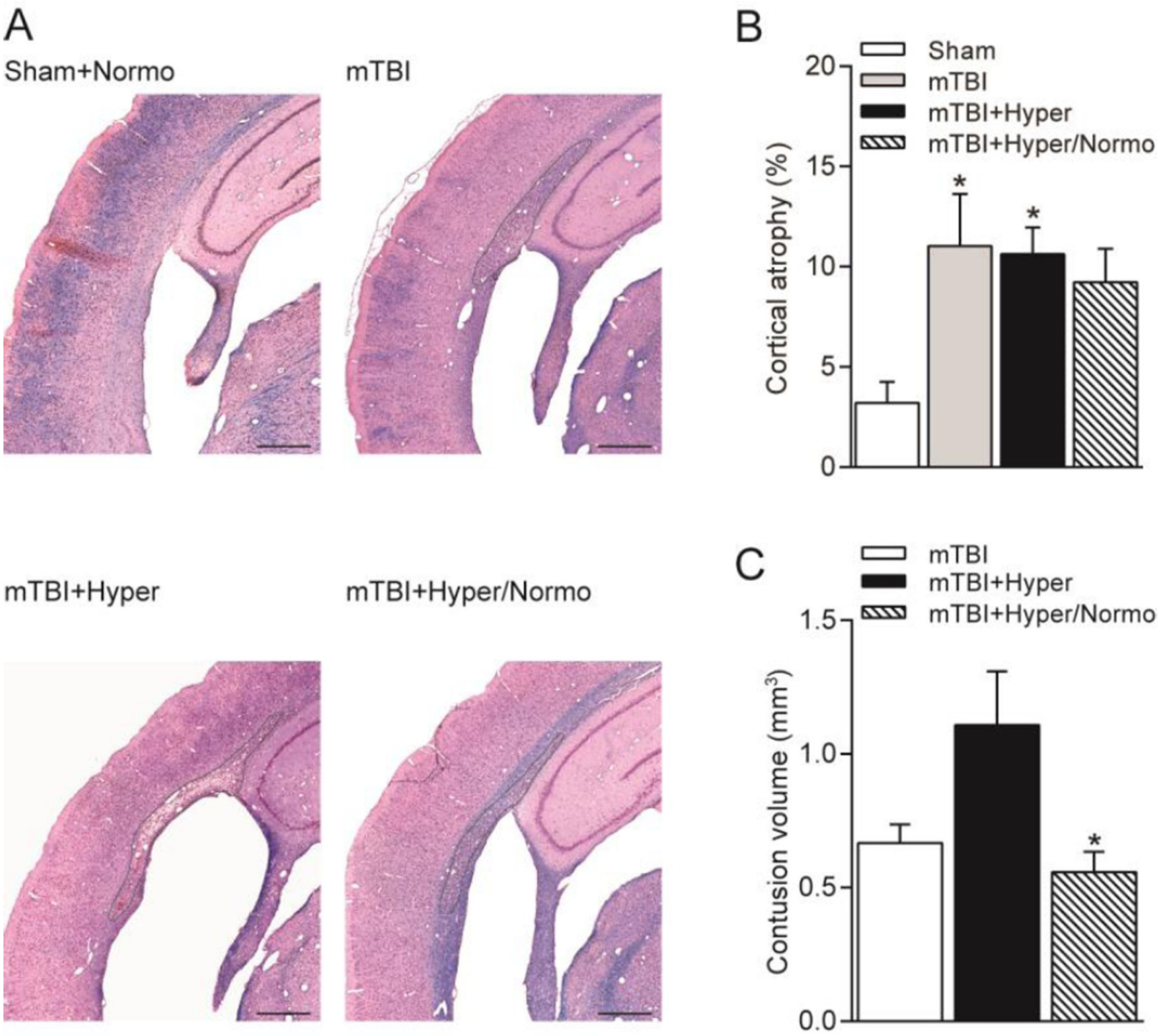

Fig. 4.

Histopathological analysis after mTBI and hyperthermia. (A) Representative sections at bregma level −3.8 mm stained with H&E and Luxol fast blue. Lines demarcate the contusional areas. Scale bars 500 μm. (B) Cortical atrophy was present in all mTBI groups and significantly increased from sham animals. (C) Contusions in the external capsule between the parietal cortex and hippocampus were observed in all mTBI animal groups. Contusion volume was significantly less in cooled hyperthermic mTBI animals as compared to hyperthermic mTBI animals. *$p<0.05$, one-way ANVOA and Tukey's post-hoc analysis. $n=11$ sham animals, $n=11$ normothermic mTBI animals, $n=11$ hyperthermic mTBI animals, $n=12$ hyperthermic/normothermic mTBI animals.

Table 1

Physiological Parameters

Parameter	Surgery groups	15 min prior	15 min post	1 hr post	2 hr post	3 hr post	4 hr post
Weight	Sham	372.2±15.1					
(g)	Normothermic mTBI	348.7±9.9					
	Hyperthermic mTBI	346.0±11.2					
	Hyperthermic/normothermic mTBI	347.9±9.2					
ATM	Normothermic mTBI	1.47±0.01					
	Hyperthermic mTBI	1.51±0.02					
	Hyperthermic/normothermic mTBI	1.51±0.01					
MABP	Sham	124.3±3.7	120.1±3.9	115.5±3.4	113.1±3.6	108.6±2.6	110.7±3.7
(mmHg)	Normothermic mTBI	117.8±4.4	117.2±3.4	111.0±2.4	108.3±3.6	106.5±1.9	108.6±1.9
	Hyperthermic mTBI	118.6±3.3	111.4±2.8	103.7±1.4	101.0±1.8	101.6±0.3	105.6±3.9
	Hyperthermic/normothermic mTBI	121.1±3.4	115.1±2.5	108.5±1.8	105.8±1.5	109.5±2.2	112.6±3.3
BloodpO_2	Sham	146.0±7.0	140.9±5.8	139.4±7.6	131.8±8.7	131.5±10.9	137.4±12.1
(mmHg)	Normothermic mTBI	165.6±7.4	148.8±7.8	145.0±6.6	145.6±7.9	149.0±5.0	135.8±5.6
	Hyperthermic mTBI	137.1±6.1	137.1±9.0	140.4±8.3	137.6±11.5	126.8±4.5	124.6±5.1
	Hyperthermic/normothermic mTBI	138.1±4.2	119.9±3.8	136.5±6.0	138.3±9.5	137.5±4.4	135.9±8.6
BloodpCO_2	Sham	38.4±0.9	38.6±0.7	37.4±0.7	38.5±0.9	38.1±1.1	37.7±0.7
(mmHg)	Normothermic mTBI	39.2±0.6	37.4±0.4	37.5±0.4	38.5±0.5	37.7±0.5	38.5±0.7
	Hyperthermic mTBI	39.1±0.8	37.6±0.4	37.3±0.6	37.7±0.4	37.2±0.4	37.3±0.5
	Hyperthermic/normothermic mTBI	38.1±0.7	38.0±0.6	37.3±0.5	38.4±0.6	37.1±0.5	37.1±0.4
Blood pH	Sham	7.47±0.01	7.47±0.01	7.45±0.02	7.45±0.01	7.45±0.01	7.45±0.01
	Normothermic mTBI	7.44±0.01	7.46±0.01	7.46±0.01	7.45±0.01	7.45±0.01	7.44±0.01
	Hyperthermic mTBI	7.44±0.01	7.44±0.01	7.44±0.01	7.44±0.01	7.45±0.01	7.45±0.01
	Hyperthermic/normothermic mTBI	7.45±0.01	7.45±0.01	7.45±0.01	7.45±0.01	7.45±0.00	7.45±0.01
Head	Normothermic Sham	36.7±0.1	36.7±0.1	36.9±0.1	36.9±0.1	36.9±0.0	36.7±0.1
Temperature	Hyperthermic Sham	38.6±0.1	38.6±0.0	38.6±0.0	38.7±0.1	38.8±0.0	38.7±0.0

Parameter	Surgery groups	15 min prior	15 min post	1 hr post	2 hr post	3 hr post	4 hr post
(°C)	Normothermic mTBI	36.8±0.0	36.7±0.1	36.8±0.1	36.9±0.1	37.0±0.1	36.9±0.1
	Hyperthermic mTBI	38.7±0.0	38.7±0.0	38.7±0.0	38.7±0.0	38.8±0.1	38.9±0.0
	Hyperthermic/normothermic mTBI	38.7±0.0	38.6±0.0	37.0±0.1[c]	37.0±0.1[c]	37.0±0.1[c]	36.9±0.0[c]
Body	Normothermic Sham	36.9±0.0	36.9±0.1	36.7±0.0	36.8±0.1	36.7±0.1	36.8±0.1
Temperature	Hyperthermic Sham	38.9±0.1	39.0±0.1	38.7±0.1	38.6±0.0	38.7±0.1	38.8±0.0
(°C)	Normothermic mTBI	36.9±0.0	36.9±0.1	36.8±0.0	36.9±0.1	36.9±0.0	36.9±0.0
	Hyperthermic mTBI	38.9±0.0	38.8±0.1	38.9±0.1	38.8±0.1	38.8±0.0	39.0±0.1
	Hyperthermic/normothermic mTBI	38.9±0.0	38.8±0.0	37.0±0.0[c]	36.8±0.0[c]	36.7±0.0[c]	36.9±0.1[c]

ATM, atmospheric pressure; MABP, mean arterial blood pressure

[c] $p<0.001$ vs 15 min prior or 15 min post, repeated measures two-way ANOVA and Tukey post-hoc analysis