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Citation for published version:

Friess, SH, Ichord, RN, Ralston, J, Ryall, K, Helfaer, MA, Smith, C & Margulies, SS 2009, 'Repeated traumatic brain injury affects composite cognitive function in piglets' *Journal of Neurotrauma*, vol 26, no. 7, pp. 1111-21. DOI: 10.1089/neu.2008-0845

Digital Object Identifier (DOI):

[10.1089/neu.2008-0845](https://doi.org/10.1089/neu.2008-0845)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Neurotrauma

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Repeated Traumatic Brain Injury Affects Composite Cognitive Function in Piglets

Stuart H. Friess,¹ Rebecca N. Ichord,² Jill Ralston,³ Karen Ryall,³ Mark A. Helfaer,¹ Colin Smith,⁴ and Susan S. Margulies³

Abstract

Cumulative effects of repetitive mild head injury in the pediatric population are unknown. We have developed a cognitive composite dysfunction score that correlates white matter injury severity in neonatal piglets with neurobehavioral assessments of executive function, memory, learning, and problem solving. Anesthetized 3- to 5-day-old piglets were subjected to single ($n = 7$), double one day apart ($n = 7$), and double one week apart ($n = 7$) moderate (190 rad/s) rapid non-impact axial rotations of the head and compared to instrumented shams ($n = 7$). Animals experiencing two head rotations one day apart had a significantly higher mortality rate (43%) compared to the other groups and had higher failures rates in visual-based problem solving compared to instrumented shams. White matter injury, assessed by β -APP staining, was significantly higher in the double one week apart group compared to that with single injury and sham. Worsening performance on cognitive composite score correlated well with increasing severity of white matter axonal injury. In our immature large animal model of TBI, two head rotations produced poorer outcome as assessed by neuropathology and neurobehavioral functional outcomes compared to that with single rotations. More importantly, we have observed an increase in injury severity and mortality when the head rotations occur 24 h apart compared to 7 days apart. These observations have important clinical translation to infants subjected to repeated inflicted head trauma.

Key words: axonal injury; neurobehavioral assessment; pediatric brain injury; traumatic brain injury

Introduction

HEAD INJURY IS ONE OF THE LEADING CAUSES of death and disability in the pediatric population (Hoyert et al., 2006). Currently there is a paucity of age-specific data about mechanisms for primary traumatic brain injury in children. Furthermore, clinical studies and observations, supported by data from mice and rat models, have suggested cumulative effects of repeated mild head injury (Laurer et al., 2001; De-Ross et al., 2002; Guskiewicz et al., 2003, 2005; McCrea et al., 2003; Longhi et al., 2005; Huh et al., 2007). Recent evidence supports that neuropsychological deficits following single and repeated mild traumatic brain injury is a common problem in adults and children (De Monte et al., 2005; Barrow et al., 2006; Iverson et al., 2006; Wall et al., 2006). It has been demonstrated that following experimental concussion, the brain undergoes metabolic and ionic changes in calcium, potassium, cerebral blood flow and oxidative glucose metabolism

that can last up to 96 h after injury (Giza and Hovda, 2001); however their effect on how the brain responds to further injuries during this time period is unknown.

Inflicted head injury is the most common cause of death in infants (Duhaime et al., 1998). Victims of inflicted head trauma have delayed presentation to medical attention; the histories are often vague and unreliable. Radiologic evaluation of these patients typically reveals acute and chronic subdural hematomas and fractures in various stages of healing, implying repetitive episodes of inflicted trauma (Duhaime et al., 1998; Ewing-Cobbs et al., 2000). The effect of temporal intervals between repeated injuries is poorly understood.

Clinical guidelines for return to play following mild head injury have generated much discussion and controversy (Biasca and Maxwell, 2007; Schnadower et al., 2007; Standaert et al., 2007). A panel of international sports concussion experts recommend no athlete should return to play while still symptomatic physically, cognitively, or behaviorally (McCroory

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et al., 2005). Pediatric data and guidelines on this issue are lacking; there is some evidence to push for stricter return to play guidelines compared to that for adults (Lovell et al., 2003, 2004; Kirkwood, et al., 2006). The paucity of pediatric data on the effects and duration of mild repeated brain injury is certainly one of the underlying causes for this controversy. The impact of multiple injuries as well as the duration of the interval between injuries has not been clearly addressed in the literature. Do multiple injuries produce more severe and sustained neurologic deficits compared to single injuries, and does injury interval influence the degree of prolonged neurologic dysfunction?

Previous experimental studies in small animal models have examined the effect of repetitive head injury on functional outcomes. DeRoss and colleagues (2002) studied repeated concussions in adult male rats and demonstrated a return baseline performance in Morris water maze by 10 to 14 days after injury. Unexpectedly, they observed a decrease in the number of trials needed to return to baseline latency in the Morris water maze with multiple concussions (DeRoss et al., 2002), perhaps attributable to a training effect. Laurer and colleagues (2001) examined a shorter inter-injury interval and demonstrated increased deficits in the adult mice that received two injuries 24 h apart compared to those with a single injury. Longhi and colleagues (2005) investigated the temporal window during which a concussed adult rodent brain was vulnerable to a second injury. They observed that rodents receiving a second injury within 72 h resulted in more pronounced transient motor deficits, increased latency in a Morris water maze, and increased traumatic axonal injury by histopathology compared to rodents who received two injuries greater than 72 h apart or those with a single injury. More recently, Huh et al. (2007) utilized an immature rodent model of mild TBI and observed that young rats experiencing two or three successive impacts minutes apart had more extensive neuropathology at 7 days post-injury, but at 14 days post-injury no differences in the Morris water maze performance was observed. This observation is consistent with the return to baseline performance in Morris water maze by 10–14 days post-injury reported by DeRoss and colleagues (2002) in adult rats.

These earlier studies utilized rodent animal models to study repetitive brain injury. Rodent models have a limited utility in providing the relevant insight to improve understanding of white matter injury in the pediatric human population. An immature animal with salient features of the pediatric human brain such as overall shape, gyral pattern, distribution of grey and white matter, degree of myelination, regional cerebral metabolism, and blood flow would offer a more appropriate model to study injury mechanisms and diagnosis and treatment strategies for children. Based on detailed information from previous investigations, strong parallels have been established between brain development in the pig and the human (Armstead and Kurth, 1994; Hoehner et al., 1994). The neonatal (3- to 5-day-old) piglet's brain development and response to injury has been reported to most closely model the human infant (Duhaime et al., 2000).

We have published our acute (6 h survival) non-impact neonatal (3- to 5-day-old) piglet model of closed head injury, with its marked increase in distribution of injured axons following two consecutive mild axial accelerations of 130–150 rad/s spaced 15 min apart compared to single accelera-

tions at 6 h survival (Raghupathi et al., 2004). Furthermore, we developed a survival piglet model of non-impact head injury with quantitative functional neurobehavioral assessments (Friess et al., 2007). We observed no significant axonal injury or neurobehavioral deficits at 12 day survival for piglets experiencing two mild axial head rotations less than 15 min apart. Animals experiencing single moderate (≈ 190 rad/s) axial head rotations demonstrated transient and persistent neurobehavioral deficits that correlated with neuropathology. In this communication, we present new data at this higher level of velocity: single and double (1 day or 1 week inter-injury interval) moderate velocity (≈ 190 rad/s) axial head rotations in a survival piglet model. In addition we have expanded our functional assessment battery and developed a cognitive composite dysfunction score for piglets. Finally, neurobehavioral functional data and histopathology from piglets experiencing single rotations, double rotations one week apart, and double rotations one day apart were compared to instrumented shams over a 12 day survival period.

Material and Methods

All protocols were approved by the Institute of Animal Care and Use Committee of the University of Pennsylvania. Neonatal (3- to 5-day-old) farm piglets were studied in seven littermate groups of five pigs (three or four females, one male). Each littermate group was housed together throughout the 2 week period. Animals in each littermate group were randomly assigned to single injury (SINGLE), instrumented sham (SHAM), double injury one day apart (24HR), or double injury one week apart (WEEK) ($n=7$ for each group). The male sibling was utilized only for assessing socialization and was housed with others in the group. Two days prior to the start of the study (day -3), piglets were numbered using color-coded nail polish on their backs and front hooves to facilitate identification. While piglets were placed together in the empty test space (4' \times 8') with a bowl of milk replacer (Litter-milk, Land O Lakes, Arden Hills, MN), they were allowed to explore the space to become acclimated to their environment and the research staff. One day prior to the start of the study (day -2), the previous day's protocol was repeated with the addition of training individual piglets to ambulate a 9 inch wide balance beam to a bowl containing 1 mL of milk replacer. The balance beam was repeated until proficiency at the task was displayed.

Brain injury and physiologic measurements

On study days -1, 0, and 7, all female piglets were anesthetized with 4% isoflurane via a snout mask. When a pinch reflex was absent, the piglets were orally intubated with a 3.0 mm endotracheal tube. End tidal CO₂ (VetCap model 2050081; SDI, Waukesha, WI) was utilized to confirm endotracheal tube placement and was continually monitored until extubation. Core body temperature (rectal probe) and arterial oxygen saturation (oxymeter probe) were monitored continuously (MDE Escort II; MDE, Arieta, CA). Animals were ventilated (1–2% isoflurane, Hallowell EMC, Pittsfield, MA) until return of spontaneous respirations. Uninjured sham piglets and injured animals not scheduled for injury on that study day were anesthetized, intubated, and placed on the bite plate. These animals were then taken off isoflurane and removed from the bite plate to simulate the timing of the

injury protocol. Injured animals experienced rapid axial head rotations with HYGE pneumatic actuator (angle rotation 110° over 10–12 ms), as described previously (Raghupathi and Margulies, 2002; Raghupathi et al., 2004; Friess et al., 2007). All injured groups had similar velocity and head accelerations (Table 1).

After injury or sham anesthesia, animals were placed on heating blankets to maintain core body temperature between 36°C and 38°C. Oxygen saturation was maintained between 95% and 100% at all times with supplemental oxygen and ventilatory support as needed. Return of pinch reflex was recorded in all piglets. Upon return of respiratory effort and airway protective reflexes, the animals were extubated. Animals were returned to the animal care unit after the following criteria were met: vocalization (without squealing), steady ambulation, no aggression or avoidance behavior, no piloerection, and presence of proper feeding/drinking.

Behavioral and functional tests

All procedures involved operant conditioning techniques with milk replacer as a positive reward, and no aversive conditioning was performed. A broad range of neurobehavioral functions including open field testing (executive function), glass barrier task (visual-based problem solving), food cover task (olfactory-based problem solving), and balance beam (motor), as previously described (Friess et al., 2007), were evaluated in all groups on study days 1, 4, 6, 8, and 11. All handlers and assessment scorers were blinded to the animals' group identification.

T-maze

Additionally on study days 1 and 8, animals' learning and anterograde memory were tested with a T-maze system adapted from Bolhuis and colleagues (2004). Food bowls in each arm of the T-maze were only visible after the animal had fully committed to the arm of the maze (Fig. 1). Behind each arm of the T-maze was a bowl of milk replacer (never visible to the animal) to eliminate smell as a confounder (C, Fig. 1). The T-maze was divided into 10 imaginary zones (Fig. 1, dashed lines). Animals were first shown the arm of the maze with no food reward and then the arm with food. During the training trials, each piglet was then given as many as 20 opportunities to successfully find the food reward. A trial was considered a success if the pig found the food reward within 15 s without entering the other T-maze arm. Pigs were required to complete at least five correct trials to proceed further in T-maze testing. During training trials, if the piglet chose the wrong arm of the T-maze, the piglet was given 60 s to find the correct arm. At the end of 60 s, if the animal still had not found the correct food bowl, they were gently directed to the food re-

ward. After successful training, the intra-maze change trial began, where a moveable pie plate was placed in the arm of the T-maze with the food reward (Fig. 1D). There was sufficient room in the arm of the T-maze for each piglet to easily pass by the pie plate without interacting with it. Intra-maze change trials measurements obtained included latency to food reward, number of errors, and length of time in physical contact with pie plate. An error was defined as each time the test subject entered a zone (Fig. 1, dashed lines) that placed the piglet farther from the food reward. Each animal was given a maximum of 5 min to reach the food reward. The pie plate was removed, and pigs were then run through five more trials. Finally the food reward was switched to the other arm of the T-maze and six additional trials were performed. Measurements obtained included latency to food reward, number of errors, and number of visits to the original food site. Each animal was given a maximum of 5 min to reach the food reward.

Histology and immunohistochemistry

The animals were euthanized on study day 12 to evaluate regional patterns of traumatic axonal injury. Animals were anesthetized with 4% isoflurane. Once pinch reflex was absent, animals were given a lethal dose of intravenous sodium pentobarbital (150 mg/kg). Animals were transcardially perfused through a midline sternotomy with 1 L heparinized saline (5000 U/L), and brains were fixed *in situ* by perfusion with a buffered solution of 10% formalin (3.5 L; Sigma Chemical, St. Louis, MO). Brains were removed from the cranial vault and post-fixed overnight at 4°C. Formalin-fixed brains were examined for neuropathology by a neuropathologist blinded to animals' group assignments. Macroscopic examination included documentation of focal pathology including presence of subdural and subarachnoid hemorrhages and surface contusions. Brains were sectioned into sixteen 3 mm thick coronal blocks through the cerebrum, brain stem, and high cervical spinal cord; sections were grossly examined for tissue tears, intracerebral hemorrhage, and subarachnoid hemorrhage. Following routine processing, tissue was embedded in paraffin wax, and two 6 μ m thick sections were cut from every block for microscopic evaluation. Sections were stained with hematoxylin and eosin (H&E) or with the immunohistochemical markers for axonal injury beta-amyloid precursor protein (β -APP) (dilution of 1:5000; Chemicon 22C11; Millipore, Billerica, MA) and counterstained with Meyer's hematoxylin. Slides were examined by a blinded reviewer at scanning power (5–10 \times magnification). Specific fields were examined at 20–40 \times magnification. Locations of axonal injury, subarachnoid and parenchymal hemorrhage, and cell death were noted on digital photographs of the coronal sections.

TABLE 1. AVERAGE AXIAL ANGULAR VELOCITY AND ACCELERATIONS^a

| Group | Velocity 1 (rad/s) | Acceleration 1 (rad/s ²) | Velocity 2 (rad/s) | Acceleration 2 (rad/s ²) |
|--------|--------------------|--------------------------------------|--------------------|--------------------------------------|
| Single | 193.7 (182–219) | 58,511 (53,696–69,798) | | |
| 24HR | 196.7 (186–212) | 55,172 (44,999–80,630) | 195.9 (178–232) | 54,346 (39,964–66,674) |
| Week | 190.3 (169–210) | 57,316 (47,015–75,718) | 187.6 (165–201) | 56,135 (40,920–75,000) |

^aGroup range provided in parentheses. There were no significant differences in loading conditions between groups or between first and second injury days.

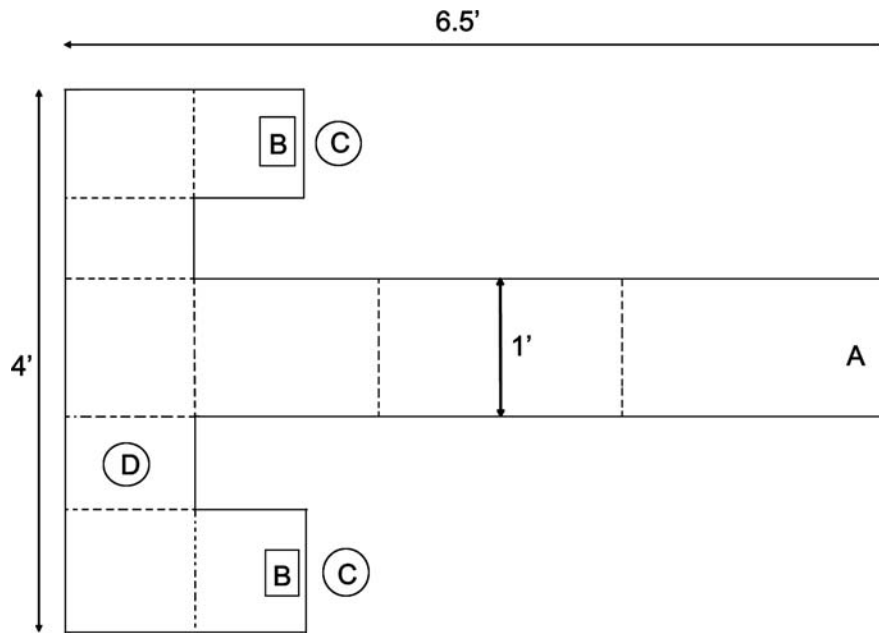


FIG. 1. T-maze test. (A) Piglet starting position. (B) Food reward troughs (only one had food). (C) Filled food dishes not visible to the piglet. (D) Intra-maze change novel object.

Total brain area was measured by tracing the brain area in scaled digital images of each of the 16 slices (ImageJ; NIH, Bethesda, MD) and summing the results from each slice. Regions of β -APP reactivity were noted by the neuropathologist on these images, and the locations of white matter damage were traced in each slice using the same procedure to determine total area. Total and injured areas were multiplied by section thickness to determine total and injured brain volumes. White matter lesion volume (%) was calculated by dividing the total volume of injured white matter by the total brain volume. Brain stems were evaluated separately for white matter injury based on β -APP reactivity and scored on a 3-point scale by the neuropathologist (0 = no injury, 1 = mild, 2 = moderate, and 3 = severe brain stem injury). Ischemic brain damage was assessed as mild, moderate, or severe using a grading system described by Graham et al. (1989). The designation of severe ischemia was assigned to those cases in which the lesions were diffuse, multi-focal, and large within arterial territories; moderate when the lesions were limited to the arterial boundary zones, singly or in combination with subtotal infarction in the distribution of the cerebral arteries, or if there were six to ten subcortical lesions; and mild if there were five or fewer subcortical lesions in the piglet brain.

Statistical analysis

The percent of epochs each behavior was observed in the open field testing was initially analyzed by two-way analysis of variance (ANOVA) to evaluate the higher level interaction of group and day on every behavioral measure from every assessment. The number of zones entered and lines crossed in the open field was evaluated in the same manner. T-maze functional outcomes (latency to food reward, errors, and number of visits to old food) were evaluated in a similar manner. Glass barrier, food cover, and balance beam were initially

analyzed by three-way ANOVA to evaluate higher level interaction of group, day, and trial on latency and errors. Normality was assessed by the Kolmogorov-Smirnov test. If the data was not normally distributed, it was log-transformed prior to analysis. If on three-way ANOVA a variable was not found to exert significance on outcome or significantly interact with the other variables, data were consolidated across that variable for a complete post hoc analysis. Consolidated data were examined using a Tukey-Kramer with significance defined at $p < 0.05$. A two-way ANOVA to evaluate higher level interaction of group and day on number of failures/day was also performed. Again Tukey-Kramer tests were utilized with significance defined at $p < 0.05$.

Results

Loss of consciousness

On study day 0, 24HR had a significantly longer unconscious time (pinch latency) after its second injury compared to that of anesthetized SHAM on study day 0 (10.1 ± 3.4 vs. 2.8 ± 0.7 min) (Fig. 2). Also on day 7, WEEK also had a significantly longer unconscious time compared to that of SHAMs after their first injury (5.1 ± 0.7 vs. 2.8 ± 0.7 min). Interestingly, on study day 7 the 24HR had a significantly longer unconscious time after anesthesia only (AO) compared to that of SHAM (8.2 ± 2.6 vs. 3 ± 0.7 min). All other comparisons were not significant. Groups had no difference in weight gain over the study period, with an average weight gain of 1.7 kg over the study period (days -2 to 12).

Mortality

The 24HR group had a significantly higher mortality ($n = 3$, 43%) compared to that of SINGLE ($n = 1$, 13%), WEEK (0%), or SHAM (0%) groups ($p < 0.05$). The SINGLE mortality was euthanized on study day 1 with inability to feed and ambu-

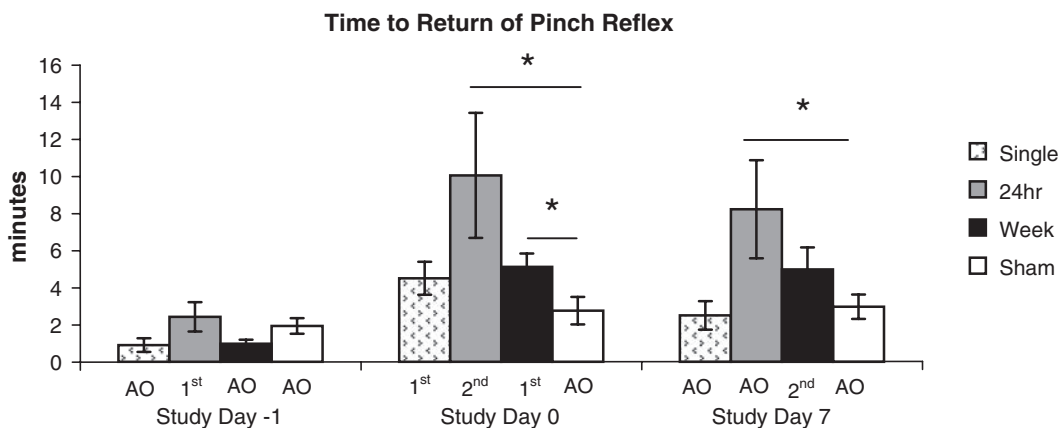


FIG. 2. Return of pinch reflex on each study day after first injury (1st), second injury (2nd), or anesthesia only (AO). *Statistical differences ($p < 0.05$).

late, but had received a much higher velocity rotation (Table 2). Two of the 24HR animals were euthanized on study day 0 due to loss of respiratory drive and poor neurologic outcome. The third animal died on study day 4 and pathologic analysis was not performed. Two animals from the SINGLE group and one animal from the WEEK group were sacrificed on the same day as rapid head rotation because they sustained non-neurologic injuries (palate fractures) incompatible with returning to general husbandry care.

Pathology

Macroscopic and microscopic findings were compared across groups. One animal from the 24HR was unavailable for examination due to unexpected mortality. We note that SINGLE and 24HR were sacrificed 12 days after injury, whereas WEEK was sacrificed 5 days after injury. Thus for a comparative group we included five additional animals that were euthanized 5 days after receiving a single similar axial head rotation (velocity 192 ± 1 rad/s and acceleration $52,552 \pm 1743$ rad/s², comparable to loads in Table 1) to evaluate for temporal progression of white matter injury determined by β -APP immunoreactivity (SINGLE-5). These animals did not participate in any training or behavior studies.

All brains were evaluated for ischemic injury using the classification system described earlier. Absent was used when there was no evidence of ischemic lesions. Only three brains were classified as having severe ischemia (two from 24HR group and one SINGLE injury), and all three had not survived the complete study period due to poor neurologic outcome

(loss of respiratory drive, inability to ambulate to food, or feed). Moderate ischemia was observed in two brains (one SHAM and one SINGLE), and both survived the entire study period. Mild ischemia was found in five animals, three of which were sacrificed early due to palate fractures.

Brain stem injury (either ischemic injury or β -APP immunoreactivity) was a less common occurrence, found only in six animals (three 24HR, two SINGLE, and one WEEK). Mortality was 50% for animals with brain stem injury.

A total of 23 brains from animals that had survived the 12 day study period were available for analysis (24HR, $n = 4$; SINGLE, $n = 6$; WEEK, $n = 6$; SHAM, $n = 7$). The majority of the axonal injury, demonstrated with β -APP immunoreactivity (Fig. 3), was observed in the frontal lobes of the injured animals and generally limited to white matter bundles and at grey-white matter interfaces (Fig. 4). None of the SHAM animals had any β -APP staining ($0 \pm 0\%$). White matter β -APP injury volume, expressed as percent of total brain volume, in all three of the injury groups was significantly greater than SHAM (24HR, $0.36 \pm 0.18\%$; SINGLE, 0.07 ± 0.03 ; WEEK, $0.37 \pm 0.13\%$; $p < 0.01$) (Fig. 5). An additional five animals experienced a single head rotation and were examined 5 days after injury (SINGLE-5) to assess the temporal relationship of β -APP immunoreactivity. SINGLE piglets surviving 12 days had significantly less β -APP staining compared to that of SINGLE-5 ($0.07 \pm 0.03\%$ vs. $0.25 \pm 0.07\%$; $p < 0.03$), revealing that, all things being equal, less white matter injury is detected for longer post-injury survival periods. While WEEK had significantly more white matter damage than SINGLE, when survival time was comparable (SINGLE vs. 24HR, SINGLE-5

TABLE 2. LOADING CONDITIONS OF ANIMALS THAT DID NOT COMPLETE 12-DAY SURVIVAL

| Group | Velocity 1 (rad/s) | Velocity 2 (rad/s) | Study day of death or euthanasia | Reason |
|--------|--------------------|--------------------|----------------------------------|-------------------------|
| Single | 169 | | Day 0 | Palate fracture |
| Single | 196 | | Day 0 | Palate fracture |
| Single | 219 | | Day 1 | Poor neurologic outcome |
| 24HR | 212 | 232 | Day 0 | Apnea |
| 24HR | 200 | 199 | Day 0 | Poor neurologic outcome |
| 24HR | 197 | 201 | Day 4 | Found dead in cage |
| Week | 169 | 165 | Day 7 | Palate fracture |

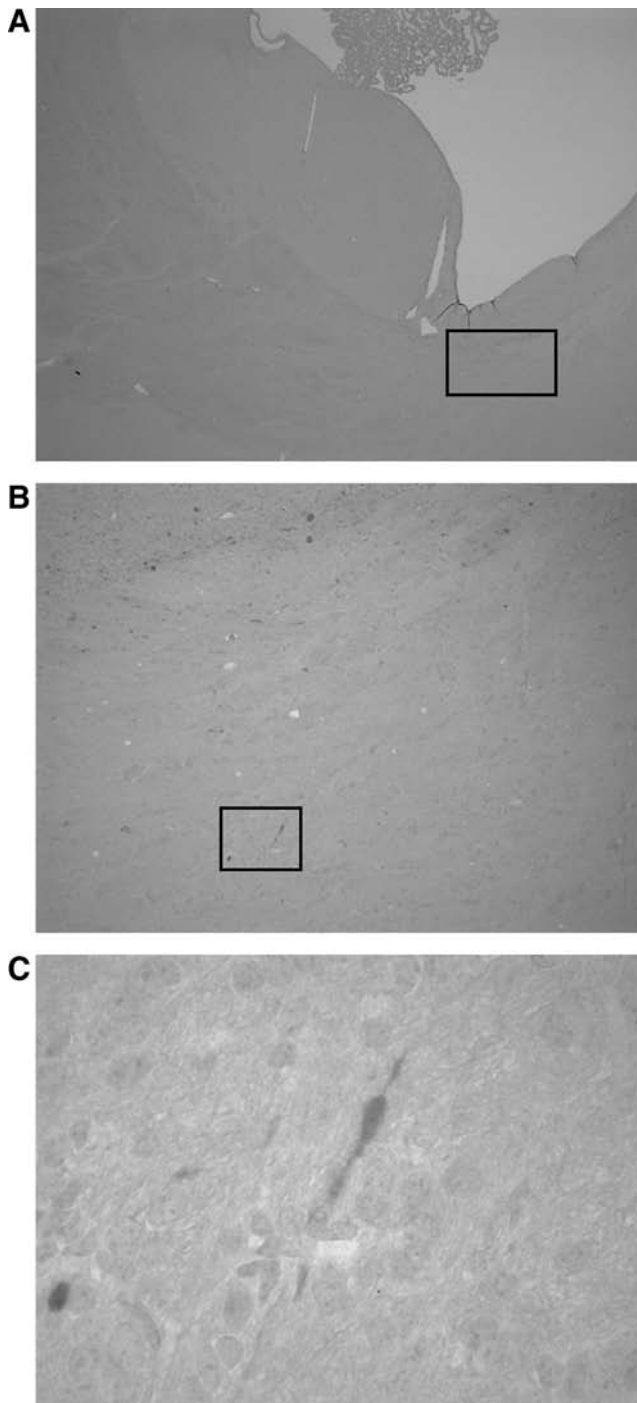


FIG. 3. Representative slice from parietal-temporal lobe near the lateral ventricle at magnifications of 2× (**A**), 10× (**B**), and 60× (**C**), demonstrating areas of β -APP immunohistochemistry reactivity.

vs. WEEK), white matter injury volume did not reach significance between single and repeated rotations.

Neurobehavioral assessments

All neurobehavioral assessments were performed on study days 1, 4, 6, 8, and 11, except for the T-maze, which was only performed on study days 1 and 8. All groups demonstrated a

significant improvement in performance (latency to food reward, errors, and failures) over successive days for balance beam and food cover; however there were no significant differences between the groups. For example, all groups had difficulty with the food cover test on study day 1, averaging 5.2 failures/day (out of 6 attempts/day). There was a significant improvement with study day effect, and by study day 11 animals were averaging 1.1 failures/day.

Open field testing

Throughout the survival period (study days 1–11), no significant differences between the groups was observed for a range of behaviors including sniffing floors, sniffing toys, moving toys running, standing, or escaping. In addition during the 10 min interval of social interaction with Pig X, the uninjured con-stimulus, there were no significant differences on a range of behaviors (sniffing, nudging, and mounting) between the groups. There was no significant difference in proximity to Pig X across groups. On subset analysis of open field activity on day 8, 24HR and WEEK had decreased sniffing activity (26% and 27%, respectively) compared to SHAM (40%) but this did not reach statistical significance.

T-maze testing

T-maze testing was performed on study days 1 and 8. No significant difference between groups was observed on day 1 on any outcome measures. All groups demonstrated a strong learning effect, with decreased latencies to food reward over trials and days. There was no significant difference in this learning effect across groups utilizing a two-way ANOVA. By study day 8, all animals were successfully trained on the T-maze, and differences between groups emerged. During intramaze change, 24HR and WEEK spent significantly more time with the novel object compared to SHAM (Table 3). In addition, 24HR and WEEK had longer latencies than SHAM to the food reward on normal and reversal trials but this finding did not reach statistical significance (Table 3). Interestingly, although the double injured groups had longer latencies to food reward on reversal trials, this did not coincide with increasing visits to the “old food,” but rather a prolonged period spent in the incorrect arm of the T-maze. One could interpret this as difficulties with adaptability and problem solving.

Glass barrier

All groups demonstrated a learning effect over days and trials. There was no difference between the injured groups on latency to the food reward or errors on study day 1, 4, 6, 8, or 11. However failures reached significance across groups on day 1. Specifically, 24HR had a significantly higher failure rate than SHAM (Fig. 6), while animals that had received only a single injury by day 1 (SINGLE and WEEK) were observed to have comparable failures rates (0.8 failures/ five trials) to our previous published results (Friess et al., 2007).

Cognitive composite dysfunction score

To improve the description of the overall neurobehavioral performance of piglets and correlate it with severity of neuropathology, we developed a composite cognitive dysfunction score. The behavior performance on study day 8 was used in the composite score, as this was the first day that reflected

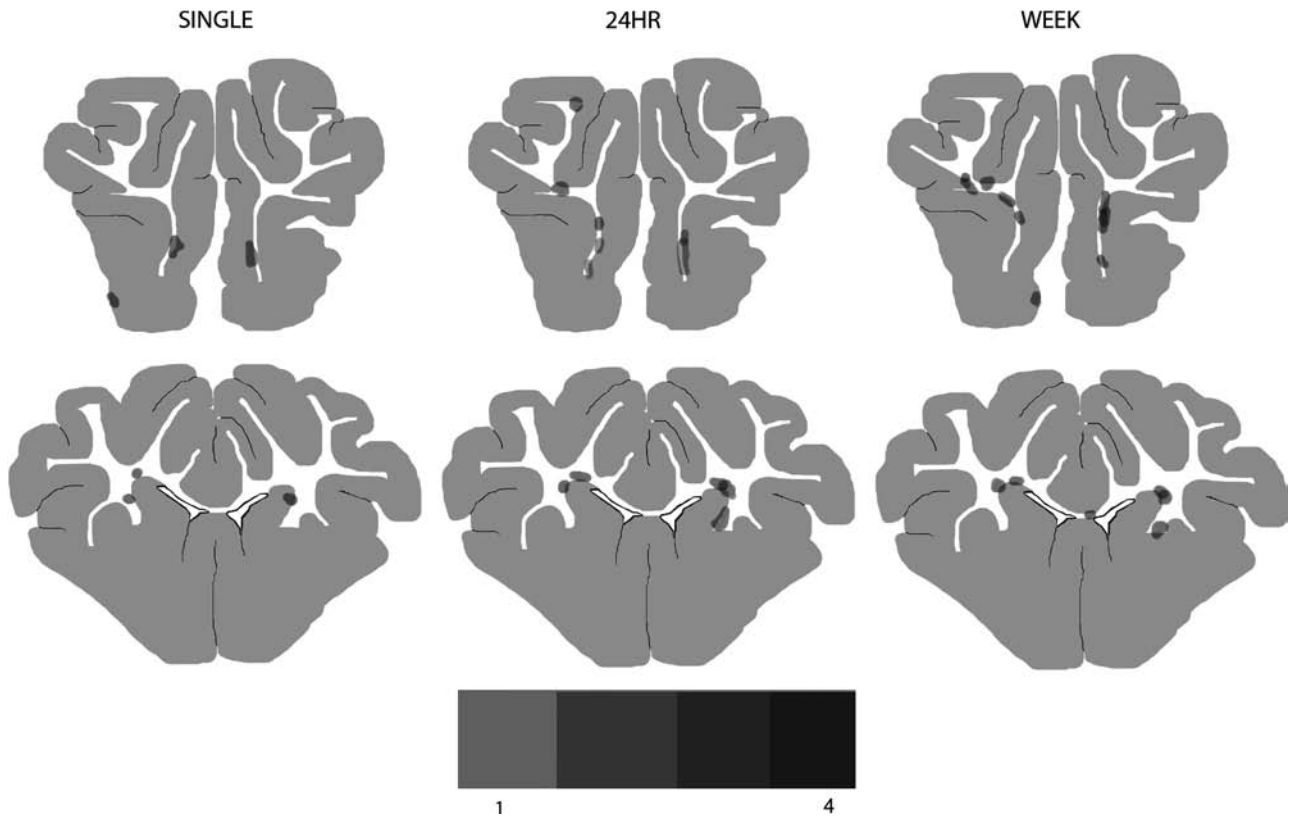


FIG. 4. Schematic depicting composite of the regional distribution of axonal injury of each injury group. Shading represents number of animals with axonal injury at the specific location.

the changes in neuropathology across groups. Neurobehavioral outcome measures exhibited a wide range of variability within each animal group (coefficient of variance, 15–220%). The basis for the composite score was a set of neurobehavioral tests with the most consistent responses among the SHAM

group on study day 8 (coefficient of variance, $\leq 40\%$). Five neurobehavioral measures from day 8 were included: T-maze training failure rate, T-maze intra-maze change time in contact with novel object, latency to food reward for T-maze normal trials and T-maze reversal trials, and sniffing the walls

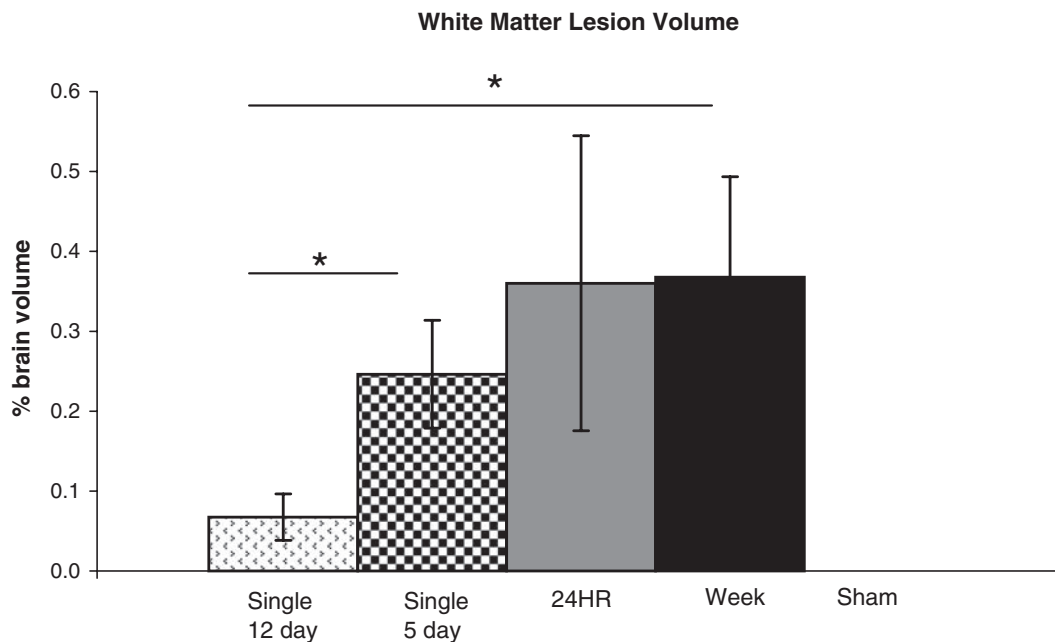


FIG. 5. Percent white matter injured for each group. *Statistical differences ($p < 0.05$).

TABLE 3. T-MAZE RESULTS, DAY 8

| | Single | Sham | 24HR | Week |
|-------------------------------|------------|------------|--------------------------|-------------------------|
| Intra-maze time in contact, s | 9.5 ± 4.6 | 4.0 ± 1.6 | 21.5 ± 13.3 ^a | 22.3 ± 8.9 ^a |
| Normal trials, s | 3.5 ± 0.3 | 3.3 ± 0.2 | 12.5 ± 4.9 | 8.8 ± 2.4 |
| Reverse trials, s | 11.4 ± 0.4 | 17.2 ± 2.4 | 25.8 ± 4.9 | 24.5 ± 6.7 |
| Visits to "old food"/trial | 1.1 ± 0.2 | 0.9 ± 0.2 | 0.9 ± 0.2 | 0.8 ± 0.2 |

^aDenotes significant difference ($p < 0.05$) compared to sham.

from open field testing. Together, these outcome measures assess executive function, memory, learning, reverse learning, and problem solving.

First, for each of the five measurements, mean and standard deviation for SHAM are calculated for each behavior. Next, each injured animals' performance on the selected behaviors is compared to the SHAM mean and standard deviation. The injured animal's z-score is calculated for each behavior by taking the differences between the individual animal's performance and the SHAM mean, and divided by the standard deviation of the SHAM group. Negative scores are used for individual performances that fall below the SHAM mean for T-maze intra-maze change, T-maze normal trials, and T-maze reversal trials, and for individual results that are above the SHAM mean for T-maze pass rate and sniffing the walls in open field testing. Scores for each of the five behavior measures for an individual subject are summed to calculate the composite score for that animal. Cognitive composite dysfunction scores correlated well with percent white matter injured as assessed by β -APP immunoreactivity, with a correlation coefficient of 0.83 (Fig. 7). 24HR and WEEK were observed to have significantly higher cognitive composite dysfunction scores compared to SINGLE.

Discussion

We have developed a cognitive composite dysfunction score that correlates white matter injury severity in neonatal piglets with neurobehavioral assessments of executive function, memory, learning, and problem solving. Neonatal piglets undergoing repeated moderate axial head rotation (190 rad/s) were observed to have neurobehavioral deficits and significant amounts of axonal injury detected by β -APP immunoreactivity. Worsening performance on cognitive

composite score correlated well with increasing severity of white matter axonal injury.

Animals experiencing two rotations within 24 h demonstrated a much higher mortality (43%) than SINGLE (13%) or WEEK (0%). Only one of the three 24HR animals that died had a markedly higher velocity of rotation compared to that of the rest of the group (222 rad/s compared to group average of 196 rad/s) that might contribute to increased risk of mortality. All three premature deaths in the 24HR group had impaired respiratory drive (apnea) and temperature regulation after injury, which correlates with the findings of brain stem injury on pathology.

Previously, we reported that animals experiencing two mild rapid axial head rotation (≈ 140 rad/s) less than 15 min apart had more axonal injury (β -APP reactivity) at 6 h following injury than did animals experiencing single loads (Raghupathi et al., 2004). However we had observed no β -APP reactivity when assessing mild double injuries 12 days after injury (Friess et al., 2007). Taken together with our morbidity in the 24HR group, WEEK group, and our previous group of double injuries 15 min apart, we conclude that there is an increase in susceptibility to catastrophic injury for repeated rapid rotations within 24 h at moderate axial loads. These studies are limited in the amount of physiologic data (intracranial pressure, arterial blood gas measurements, invasive arterial pressure) that can be obtained without compromising the neurobehavioral function outcome measures. Alterations in cerebral blood flow, oxygenation, metabolism, and neurochemistry, following repetitive brain injury may be partially responsible for these observations and acute non-survival studies investigating these variables are currently underway.

Latency for return of pinch reflex after sham anesthesia on day 8 was markedly delayed in the surviving members of

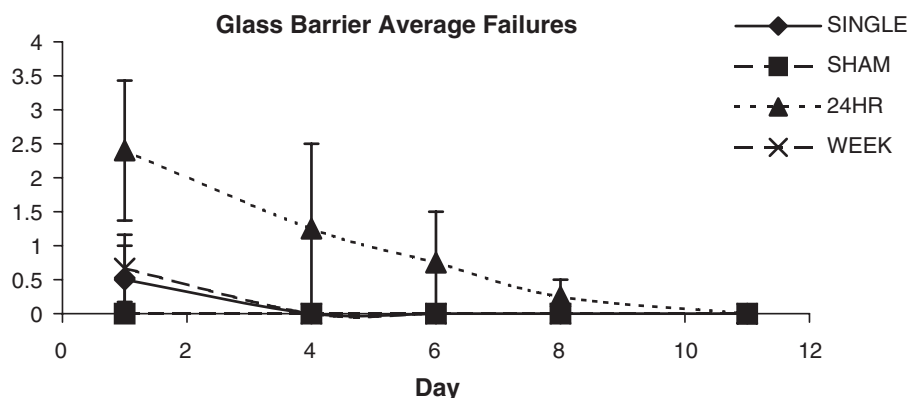


FIG. 6. Glass barrier failures. 24HR was significantly higher than SHAM on study day 1.

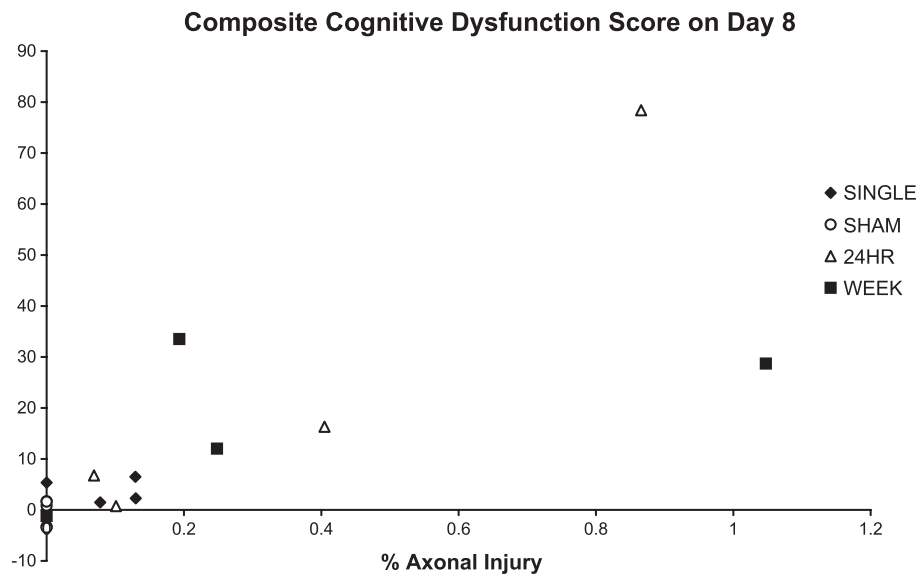


FIG. 7. Cognitive composite dysfunction score on day 8 correlated well (0.83) with white matter injury. Animals failing T-maze training were excluded.

the 24HR group compared to that of SINGLE and SHAM. It is unknown what effect previous TBI in adult or pediatric patients has on minimum alveolar concentration (MAC) of inhalation anesthetics. However, pediatric patients with a history of cerebral palsy and severe mental retardation have been reported to have significantly lower MAC compared to that of matched controls (Frei et al., 1997).

We observed that animals receiving two head rotations had more axonal injury based on β -APP immunohistochemistry compared to that of SINGLE, although only the WEEK group reached statistical significance, due to the relatively small number of animals in the 24HR group that survived the entire study period. In humans, post-mortem brain examination correlation showed increasing axonal swelling with increasing post-injury interval up to 84 h post-injury, after which time axonal swelling appears to plateau or decrease (Wilkinson et al., 1999). We previously reported β -APP reactivity following double injuries at 6 h after survival but no evidence of axonal injury at 12 day survival (Raghupathi et al., 2004; Friess et al., 2007). In the present communication, we included a comparative group of single rotation pigs at 5 days post-injury to assess progression/resolution of β -APP reactivity. We observed significantly more white matter injury at 5 days post-injury (SINGLE-5) compared to that at 12 days post-injury (SINGLE), suggesting a resolution or repair of axonal injury. Thus, if the interval between two rotations had no effect, one would expect that the WEEK group's brains examined at 5 days post-injury should show more severe pathology than 24HR, which we examined at 12 days post-injury, but the relationship did not reach significance. Thus, we postulate that a 24 h injury interval may retard recovery or injury resolution. In addition, when comparing single to double rotations with matched survival time of the second injury (WEEK vs. SINGLE-5, and 24HR vs. SINGLE), we conclude that the effect of the repeated rotations did not reach statistical significance at survival times of 5 or 12 days, although we previously noted significance at 6 h (Raghupathi et al., 2004).

Functional deficits in the sniffing behavior in open field in the injury groups were consistent with our previously reported results (Friess et al., 2007), with statistically significant decreases in activity seen in both double injured groups (24HR and WEEK) compared to those with SHAM. In addition, the 24HR animals demonstrated significantly higher failure rates compared to those with SHAM on the glass barrier problem solving test. Interestingly, WEEK did not demonstrate a rise in failure rates on post-injury day 8 (i.e., one day after the second injury). Possible explanations for this observation include a learning effect overwhelmed the injury effect, or that the week-long interval between injuries had less of an effect on neurological deficits than a 24 h interval. The majority of outcome measures in the T-maze test did not demonstrate statistically significant differences between the groups. This may be attributable to the high mortality rate in the 24HR group, which limited the statistical power of the tests, and future study designs should consider re-randomization to overcome the increased mortality of this repeat injury group.

We sought to develop neurobehavioral functional outcome metrics that sampled a broad range of higher cortical functions, and which correlated with graded neuropathology. Previously, our neurobehavioral outcomes measures assessed executive function, visual- and olfactory-based problem solving, and motor coordination. With the addition of the T-maze test, we now evaluate memory and reversal learning, as well as improve our assessment of executive function and learning (Bolhuis et al., 2004). In the T-maze, double injured pigs were observed to spend more time with the novel object during the intra-maze change task, which could possibly be attributed to deficits in attention and short memory observed in adults and children following mild TBI (Power et al., 2007; Malojcic et al., 2008). With our novel cognitive composite dysfunction score, we now correlate neurobehavioral outcomes with the extent of white matter injury. Utilizing a variety of outcome measures from T-maze—training success rates (learning), intra-maze change (short term memory and attention), normal trials (learning and memory), and reversal

trials (reversal learning and behavioral flexibility)—we produced a composite score that demonstrated good correlation (0.83) with white matter damage (β -APP staining) across all injury groups. Prospective validation of this cognitive composite dysfunction score is currently underway in our laboratory. This battery of tests could have broad applications in evaluating longer term neurofunctional capacity in other large animal models with diffuse brain injury (e.g., hypoxic ischemia, cardiopulmonary bypass, cardiopulmonary arrest).

In our immature large animal model of TBI, two head rotations produced poorer outcome, as assessed by neuropathology and neurobehavioral functional outcomes, compared to that with single rotations. More importantly, we have observed an increase in injury severity and mortality when the head rotations occur 24 h apart compared to 7 days apart. These observations have important clinical translation to the management of head injury, infants subjected to repeated inflicted head trauma, and return to play guidelines in the pediatric population. Further investigations should focus on better understanding of the time dependence of the mechanisms involved in repeated brain injury in the pediatric population, as well as investigations with juvenile pigs (1–2 month old), to better model head injuries during athletic activities in school age children.

Acknowledgments

These studies were supported by an endowed chair in critical care medicine from The Children's Hospital of Philadelphia (M.A.H.) and an NIH grant (no. R01-NS39679 [S.S.M.]). The authors are grateful to Ahn Truong and Kristin Owens for their assistance in behavior testing and data analysis.

Author Disclosure Statement

No competing financial interests exist.

References

- Armstead, W.M., and Kurth, C.D. (1994). Different cerebral hemodynamic responses following fluid percussion brain injury in the newborn and juvenile pig. *J. Neurotrauma* 11:487–497.
- Barrow, I.M., Hough, M., Rastatter, M.P., Walker, M., Holbert, D., and Rotondo, M.F. (2006). The effects of mild traumatic brain injury on confrontation naming in adults. *Brain Inj.* 20:845–855.
- Biasca, N., and Maxwell, W.L. (2007). Minor traumatic brain injury in sports: a review in order to prevent neurological sequelae. *Prog. Brain Res.* 161:263–291.
- Bolhuis, J.E., Schouten, W.G., de Leeuw, J.A., Schrama, J.W., and Wiegant, V.M. (2004). Individual coping characteristics, rearing conditions and behavioural flexibility in pigs. *Behav. Brain Res.* 152:351–360.
- De Monte, V.E., Geffen, G.M., May, C.R., McFarland, K., Heath, P., and Neralic, M. (2005). The acute effects of mild traumatic brain injury on finger tapping with and without word repetition. *J. Clin. Exp. Neuropsychol.* 27:224–239.
- DeRoss, A.L., Adams, J.E., Vane, D.W., Russell, S.J., Terella, A.M., and Wald, S.L. (2002). Multiple head injuries in rats: effects on behavior. *J. Trauma* 52:708–714.
- Duhaime, A.C., Christian, C.W., Rorke, L.B., and Zimmerman, R.A. (1998). Nonaccidental head injury in infants—the “shaken-baby syndrome.” *N. Engl. J. Med.* 338:1822–1829.
- Duhaime, A.C., Margulies, S.S., Durham, S.R., O'Rourke, M.M., Golden, J.A., Marwaha, S., and Raghupathi, R. (2000). Maturation-dependent response of the piglet brain to scaled cortical impact. *J. Neurosurg.* 93:455–462.
- Ewing-Cobbs, L., Prasad, M., Kramer, L., Louis, P.T., Baumgartner, J., Fletcher, J.M., and Alpert, B. (2000). Acute neuroradiologic findings in young children with inflicted or noninflicted traumatic brain injury. *Childs Nerv. Syst.* 16:25–33; discussion, 34.
- Frei, F.J., Haemmerle, M.H., Brunner, R., and Kern, C. (1997). Minimum alveolar concentration for halothane in children with cerebral palsy and severe mental retardation. *Anaesthesia* 52:1056–1060.
- Friess, S.H., Ichord, R.N., Owens, K., Ralston, J., Rizol, R., Overall, K.L., Smith, C., Helfaer, M.A., and Margulies, S.S. (2007). Neurobehavioral functional deficits following closed head injury in the neonatal pig. *Exp. Neurol.* 204:234–243.
- Giza, C.C., and Hovda, D.A. (2001). The neurometabolic cascade of concussion. *J. Athl. Train.* 36:228–235.
- Graham, D.I., Ford, I., Adams, J.H., Doyle, D., Teasdale, G.M., Lawrence, A.E., and McLellan, D.R. (1989). Ischaemic brain damage is still common in fatal non-missile head injury. *J. Neurol. Neurosurg. Psychiatry* 52:346–350.
- Guskiewicz, K.M., McCrea, M., Marshall, S.W., Cantu, R.C., Randolph, C., Barr, W., Onate, J.A., and Kelly, J.P. (2003). Cumulative effects associated with recurrent concussion in collegiate football players: the NCAA Concussion Study. *JAMA* 290:2549–2555.
- Guskiewicz, K.M., Marshall, S.W., Bailes, J., McCrea, M., Cantu, R.C., Randolph, C., and Jordan, B.D. (2005). Association between recurrent concussion and late-life cognitive impairment in retired professional football players. *Neurosurgery* 57:719–726; discussion, 719–726.
- Hoehner, P.J., Kirsch, J.R., Helfaer, M.A., Ganunis, T.F., Murphy, M.T., and Traystman, R.J. (1994). Dihydropyridine ligand binding decreases earlier in adolescent than in infant swine after global cerebral ischemia. *Stroke* 25:2060–2065; discussion, 2066.
- Hoyert, D.L., Heron, M.P., Murphy, S.L., and Kung, H.C. (2006). Deaths: final data for 2003. *Natl. Vital Stat. Rep.* 54:1–120.
- Huh, J.W., Widing, A.G., and Raghupathi, R. (2007). Basic science, repetitive mild non-contusive brain trauma in immature rats exacerbates traumatic axonal injury and axonal calpain activation: a preliminary report. *J. Neurotrauma* 24:15–27.
- Iverson, G.L., Brooks, B.L., Collins, M.W., and Lovell, M.R. (2006). Tracking neuropsychological recovery following concussion in sport. *Brain Inj.* 20:245–252.
- Kirkwood, M.W., Yeates, K.O., and Wilson, P.E. (2006). Pediatric sport-related concussion: a review of the clinical management of an oft-neglected population. *Pediatrics* 117:1359–1371.
- Laurer, H.L., Bareyre, F.M., Lee, V.M., Trojanowski, J.Q., Longhi, L., Hoover, R., Saatman, K.E., Raghupathi, R., Hoshino, S., Grady, M.S., and McIntosh, T.K. (2001). Mild head injury increasing the brain's vulnerability to a second concussive impact. *J. Neurosurg.* 95:859–870.
- Longhi, L., Saatman, K.E., Fujimoto, S., Raghupathi, R., Meaney, D.F., Davis, J., McMillan, B.S.A., Conte, V., Laurer, H.L., Stein, S., Stocchetti, N., and McIntosh, T.K. (2005). Temporal window of vulnerability to repetitive experimental concussive brain injury. *Neurosurgery* 56:364–374; discussion, 364–374.
- Lovell, M.R., Collins, M.W., Iverson, G.L., Field, M., Maroon, J.C., Cantu, R., Podell, K., Powell, J.W., Belza, M., and Fu, F.H. (2003). Recovery from mild concussion in high school athletes. *J. Neurosurg.* 98:296–301.

- Lovell, M.R., Collins, M.W., Iverson, G.L., Johnston, K.M., and Bradley, J.P. (2004). Grade 1 or "ding" concussions in high school athletes. *Am. J. Sports Med.* 32:47–54.
- Malojic, B., Mubrin, Z., Coric, B., Susnic, M., and Spilich, G.J. (2008). Consequences of mild traumatic brain injury on information processing assessed with attention and short-term memory tasks. *J. Neurotrauma* 25:30–37.
- McCrea, M., Guskiewicz, K.M., Marshall, S.W., Barr, W., Randolph, C., Cantu, R.C., Onate, J.A., Yang, J., and Kelly, J.P. (2003). Acute effects and recovery time following concussion in collegiate football players: the NCAA Concussion Study. *JAMA* 290:2556–2563.
- McCrory, P., Johnston, K., Meeuwisse, W., Aubry, M., Cantu, R., Dvorak, J., Graf-Baumann, T., Kelly, J., Lovell, M., and Schamasch, P. (2005). Summary and agreement statement of the Second International Conference on Concussion in Sport, Prague 2004. *Br. J. Sports Med.* 39:196–204.
- Power, T., Catroppa, C., Coleman, L., Ditchfield, M., and Anderson, V. (2007). Do lesion site and severity predict deficits in attentional control after preschool traumatic brain injury (TBI)? *Brain Inj.* 21:279–292.
- Raghupathi, R., and Margulies, S.S. (2002). Traumatic axonal injury after closed head injury in the neonatal pig. *J. Neurotrauma* 19:843–853.
- Raghupathi, R., Mehr, M.F., Helfaer, M.A., and Margulies, S.S. (2004). Traumatic axonal injury is exacerbated following repetitive closed head injury in the neonatal pig. *J. Neurotrauma* 21:307–316.
- Schnadower, D., Vazquez, H., Lee, J., Dayan, P., and Roskind, C.G. (2007). Controversies in the evaluation and management of minor blunt head trauma in children. *Curr. Opin. Pediatr.* 19:258–264.
- Standaert, C.J., Herring, S.A., and Cantu, R.C. (2007). Expert opinion and controversies in sports and musculoskeletal medicine: concussion in the young athlete. *Arch. Phys. Med. Rehabil.* 88:1077–1079.
- Wall, S.E., Williams, W.H., Cartwright-Hatton, S., Kelly, T.P., Murray, J., Murray, M., Owen, A., and Turner, M. (2006). Neuropsychological dysfunction following repeat concussions in jockeys. *J. Neurol. Neurosurg. Psychiatry* 77: 518–520.
- Wilkinson, A.E., Bridges, L.R., and Sivaloganathan, S. (1999). Correlation of survival time with size of axonal swellings in diffuse axonal injury. *Acta Neuropathol.* 98:197–202.

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