

Traumatic Axonal Injury is Exacerbated following Repetitive Closed Head Injury in the Neonatal Pig

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ABSTRACT

Inflicted brain injury is associated with widespread traumatic axonal injury (TAI) and subdural hematoma and is the leading cause of death in infants and children. Anesthetized 3–5-day-old piglets were subjected to either a single ($n = 5$) or double ($n = 6$, 15 min apart) rapid (<15 msec), non-impact, axial rotations of the head. Peak rotational velocities (averaging 172 rad/sec for single and 138 rad/sec for double loads) were lower than those utilized to induce severe injuries (240–260 rad/sec; Raghupathi and Margulies, 2002). At 6 h post-injury, brains were evaluated for the presence TAI using immunohistochemistry for the 200-kDa neurofilament protein (NF200). Accumulation of NF200 was observed in both contiguous (swellings) and in disconnected axons (axon bulbs) predominantly in central deep and peripheral subcortical white matter regions in the frontal, temporal, and parietal lobes of all injured piglets. Although the density of injured axons did not significantly increase after two rotational loads, the distribution of injured axons shifted from a few foci (2.2 ± 2.3 per animal) with 1–2 swellings/bulbs following a single rotation to significantly more foci (14.7 ± 11.9), and additional foci (2.5 ± 1.9) containing 3 or more axon swellings/bulbs following two rotational loads. The density and distribution of injured axons following a single mild rotation were significantly reduced compared with those obtained previously following a single more severe rotational load. Collectively, these data are indicative of the graded response of the immature brain to rotational load magnitude, and importantly, the vulnerability to repeated, mild, non-impact load-injury conditions.

Key words: children; mild head injury; neurofilament; white matter

INTRODUCTION

INFLECTED HEAD INJURY or “shaken impact syndrome” occurs predominantly in infants 3–6 months of age and results in a high mortality (Duhaime et al., 1998). In ad-

dition, more than 50% of the survivors develop chronic neurological problems, including epilepsy and cognitive deficits (Luerssen et al., 1991; Duhaime et al., 1998; Marin-Padilla et al., 2002). The intracranial injuries commonly associated with the shaken impact syndrome in-

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clude subdural hemorrhage, brain swelling, white matter tears, and diffuse axonal injury (Shannon et al., 1998; Geddes et al., 2001a,b). As indicated by the terminology, the hallmark of inflicted head injury appears to be the repetitive nature of the loading conditions—or “shaking.” Considerable debate surrounds the degree of severity of the repeated injuries (Hymel et al., 1998; Macciocchi et al., 1998). The mortality and morbidity associated with repeated concussions has been established in both adult and young athletes, and clinical evidence suggests that repeated mild head injuries can result in accumulated brain damage (Salcido and Costich, 1992; Rabadi and Jordan, 2001).

Contusional and/or diffuse brain injury in immature animals have utilized single impact injuries in 7-day-old to 17-day-old rats (Ikonomidou et al., 1996; Adelson et al., 1996, 2001; Prins et al., 1996), in 21-day-old mice (Tong et al., 2002), and in 1–6-week-old piglets (Armstead and Kurth, 1994; Durham et al., 2000; Brodhun et al., 2001). Diffuse brain injury in the rat resulted predominantly in axonal injury and hemorrhage in the brainstem, mild ventriculomegaly, and little to no neuronal loss (Prins et al., 1996; Adelson et al., 2001). When neuronal injury was present (i.e., in contusional injuries), the subcortical white matter in both hemispheres contained degenerating axons (Tong et al., 2002), as identified by silver and Fluoro-Jade labeling. Models of repeated mild brain injuries have been developed in adult rodents (Kanayama et al., 1996; Allen et al., 2000; Uryu et al., 2002; DeRoss et al., 2002; DeFord et al., 2002). These studies have demonstrated that multiple (two to seven) concussive brain injuries, spaced anywhere from 1 to 3 days apart, result in transient impairment of cognitive function (DeRoss et al., 2002; DeFord et al., 2002), and accumulation of microtubule-associated proteins and neurofilament proteins in cortical neurons (Kanayama et al., 1996). A model of “shaken baby syndrome” was developed using either neonatal rats (Smith et al., 1998) or neonatal mice (Bonnier et al., 2002), employing either manual techniques or a horizontal shaker, respectively. Injured animals exhibited hemorrhagic lesions, white matter tears, periventricular cysts, and cell death in the white matter (Bonnier et al., 2002). However, the extent to which these models reflect the clinical neuropathology of shaken impact syndrome is unclear.

We have developed a model of pediatric head injury by subjecting the head of a 3–5-day-old piglet to a rapid, non-impact rotational velocities of 240–260 rad/sec (Raghupathi and Margulies, 2002). At 6 h following head rotation, traumatic axonal injury (TAI) was observed predominantly in the deep and subcortical white matter regions in the frontal and temporal lobes, along with evidence of subarachnoid hemorrhage. Quantification of

the extent of TAI and comparison to similar data available from adult pigs that experienced rotational velocities of 214–286 rad/sec (Smith et al., 2000) revealed that piglets were subjected to severe loading conditions. Our objective in the current study was to evaluate the effect of reducing the loading conditions on the extent of regional TAI, and, additionally, to develop a model of repeated mild brain trauma. Brains were evaluated at 6 h post-injury for microscopic contusional tissue tears and hemorrhages (using Cresyl violet), and TAI using an antibody to the 200-kDa subunit of the neurofilament protein and histopathology of a single mild rotation was compared with that of two consecutive mild rotational accelerations.

MATERIALS AND METHODS

Brain Injury and Physiologic Measurements

Neonatal (3–5 day, $n = 11$) farm piglets were anesthetized with 3% isoflourane delivered via a snout mask. When pinch reflex was absent, tracheostomy was performed and a 3.5-mm endotracheal tube was placed. A rectal thermometer (Cole Parmer model 70002H) was inserted in order to measure core body temperature. Arterial oxygen saturation was measured using an oxymeter probe on the hind limb (Vet/OX™ model 4402, San Diego Instruments, CA), and mean arterial pressure was monitored using a pressure cuff on the hind leg (DINAMAP™ model 8300, Critikon, Tampa, FL). All measurements were periodically evaluated (≤ 30 -min intervals) prior to injury and following injury. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Studies were performed with head rotational acceleration in the axial plane using the HYGE pneumatic actuator as previously described (Raghupathi and Margulies, 2002). Animals were taken off anesthesia and 1–3 min later received either a single, rapid (< 15 msec), non-impact rotational load ($n = 5$) or two rotational loads spaced 10–15 min apart ($n = 6$); the head was rotated in the same direction for both events. As control, three piglets were anesthetized, but were not surgically prepared and did not receive injury. Immediately following the rotational load, the snout of the animal was removed from the bite plate, and the animal was placed on heating blankets to maintain core body temperatures between 36°C and 38°C. Upon return of the pinch reflex, anesthesia was re-administered (3% isoflourane for induction and 1.5% for maintenance). When the diastolic BP fell below 25 mm Hg, animals received an intravenous fluid bolus of normal saline (10 mL/kg). Oxygen saturation was maintained at 95–99% at all times.

Histology and Immunohistochemistry

The animals were euthanized 6 h after injury in order to evaluate and compare the regional patterns of traumatic axonal injury to previously published patterns of axonal damage at 6–8 h following axial rotation of the adult and pediatric porcine head. Piglets were taken off isoflurane anesthesia and reanesthetized with sodium pentobarbital (200 mg/kg). Animals were transcardially perfused with 1 L of heparinized saline (5000 units/L), and brains were fixed *in situ* by perfusion with a buffered solution of 10% formalin (3.5 L, Sigma Chemical Co., St. Louis, MO). Brains were removed from the cranial cavity and post-fixed overnight at 4°C and blocked into 1-cm coronal sections for gross examination and photography. Blocks were cryoprotected in sucrose, and 40- μ m-thick sections from the cryoprotected blocks were stained with Cresyl violet, and evaluated using a light microscope for tissue tears in the white matter, and presence of intracerebral hemorrhage in white and gray matter. Microscopic evidence of axonal injury was investigated in various white matter regions using neurofilament (NF) immunohistochemistry. Sections were incubated with anti-NF200 antibody (1:400, clone N52, Sigma Chemical Co.) overnight at 4°C, followed by biotinylated donkey anti-mouse IgG (1:2000, Jackson ImmunoResearch Labs, West Grove, PA). Antibody complexes were detected using avidin-biotin-peroxidase (ABC) histochemistry (Vector Labs, Burlingame, CA) and 3,3'-diaminobenzidine as chromogen. Omission of primary antibody on selected sections of uninjured and injured pig tissue provided a negative control. Reactive axonal changes, indicative of both primary and secondary axotomy, were determined by identifying the presence of swollen axons and terminal axonal bulbs containing NF-200 protein (Fig. 1). White matter regions in the frontal, parietal, and temporal lobes were evaluated throughout each of four coronal sections (Fig. 2) between plates 1 and 12 (Yoshikawa, 1968) and identified as central (deep white matter, below the sulci) and peripheral (subcortical, within the gyri) white matter regions, external capsule, midbrain, hippocampus, and basal ganglia. The entire area of each white matter region was evaluated in both hemispheres using a Leica microscope at 20 \times magnification (area of viewing field = 0.71 mm²), and counts and location of injured axons were mapped onto schematics of the four coronal sections. Data from a viewing field were included if at least one swollen axon/axonal bulb was observed. In order to compare the data in the present study to those in Raghupathi and Margulies (2002), the number of regions exhibiting three or more injured axons were tabulated separately. The numbers of injured axons in each region were summed in each animal, and

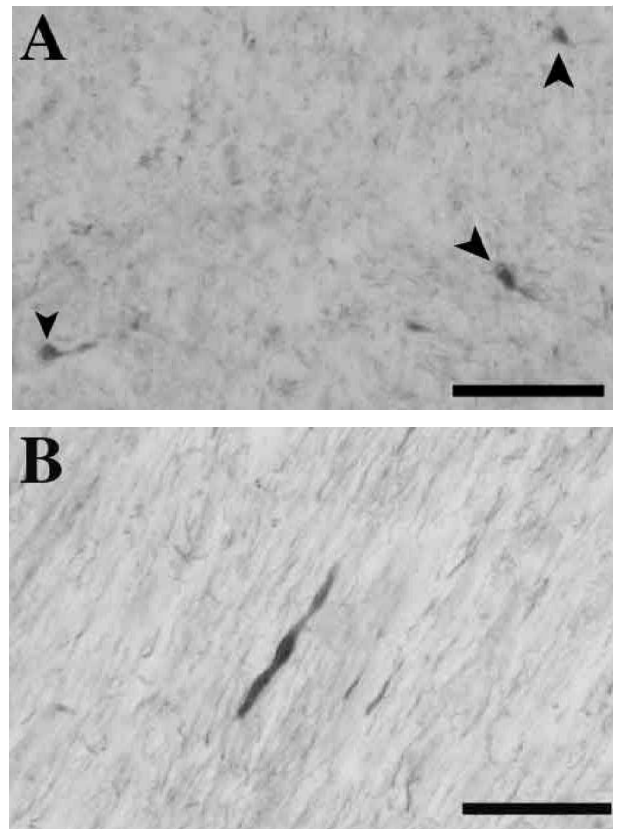


FIG. 1. Representative examples of traumatically injured axons in white matter regions of the 3–5-day-old piglet following a rotational load. **(A)** Representative photomicrograph depicting accumulation of NF200 in injured axons (axon bulbs, arrowheads) following a single load. **(B)** Representative photomicrograph depicting NF200 accumulation in a contiguous axon (swelling) following two consecutive rotational loads. Note NF200-positive axons exhibiting normal caliber surrounding the injured axon. Bars = 50 μ m.

then averaged across animals. In addition, the number of microscopic viewing fields in each region containing injured axons were summed. The density of injured axons (injured axons/mm²) was calculated by dividing the total number of axons in both hemispheres by the total field area, where total field area = number of fields included, times the viewing field area of 0.71 mm².

Data Analysis and Statistics

Physiological data in injured animals were compared to baseline values using a repeated measures one-way ANOVA, followed by a Student-Neuman Keuls post-hoc *t*-test. Because uninjured animals did not contain any injured axons, counts of injured axons and density were compared between animals that were subjected to a sin-

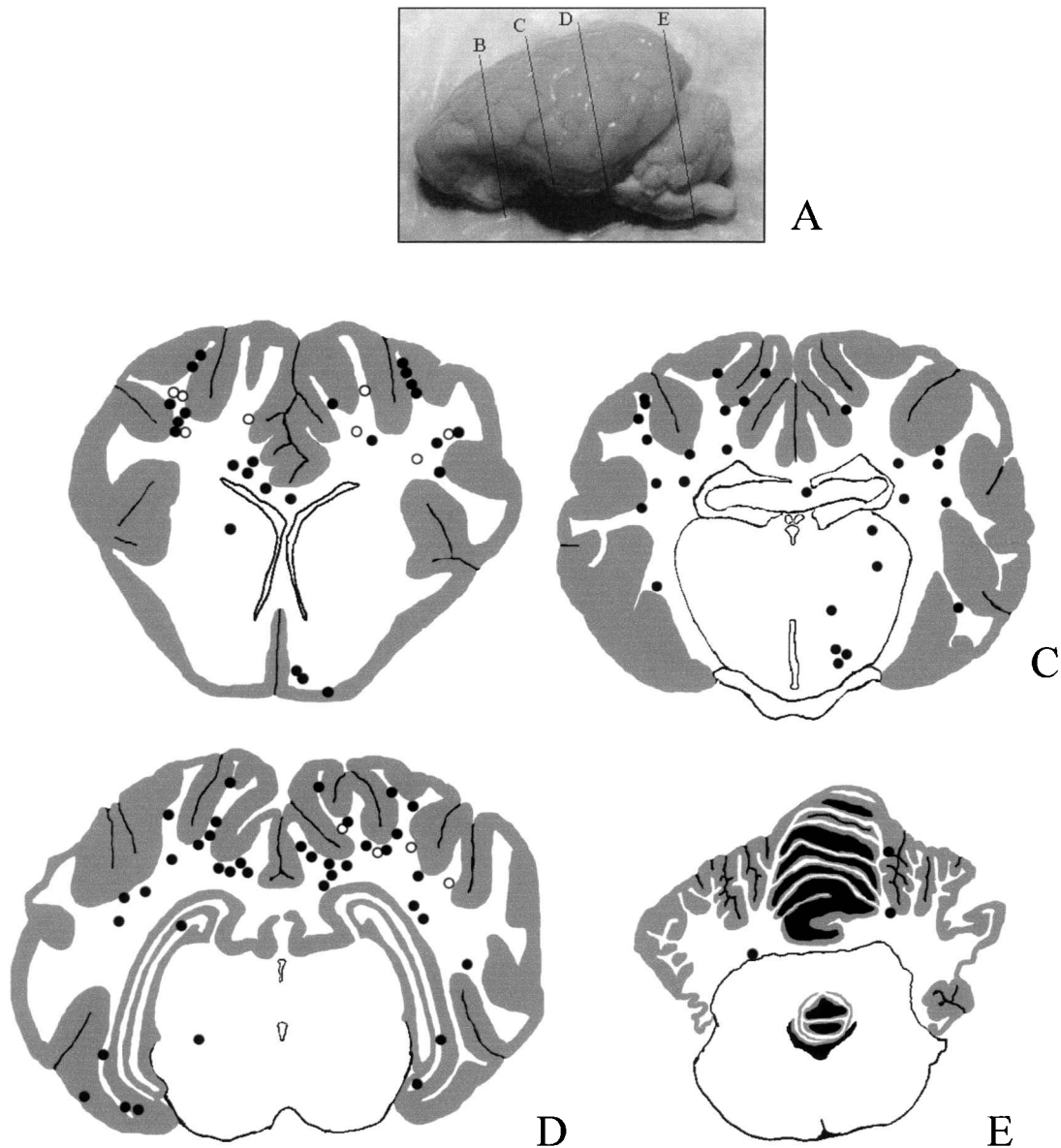


FIG. 2. Schematic depicting the regional distribution of injured axons in white matter regions following a single (open circles) or two (filled circles) rotational loads. All fields shown contained at least one axon swelling and/or axon bulb. White matter regions were examined at 20 \times magnification encompassing a field of view of 0.71 mm², which is represented in the schematic as an open or filled circle. (A) Sections examined. (B–E) Plates P3, P6, P10 and P13 redrawn from the *Atlas of Brains of Domestic Animals* (Yoshikawa, 1968).

gle load to those that experienced two consecutive loads using a two-tailed *t*-test. A *p* value of less than 0.05 was considered significant.

RESULTS

Physiology

The average peak angular velocity for the rotation of piglet heads in the axial plane was 172 ± 17 rads/sec

(*n* = 5) for the animals that received a single rotational load, while the peak angular velocities for the first and second rotations in the animals that received two rotations (*n* = 6) were 136 ± 8 and 140 ± 6 rads/sec, respectively (Table 1). Although the values for peak angular velocity and maximal deceleration were not different between the first and second loads for the piglets receiving two injuries, these values for were significantly lower than those for piglets receiving a single injury (*p* < 0.01 for peak angular velocity, and *p* < 0.02 for maxi-

AXONAL INJURY FOLLOWING REPETITIVE HEAD TRAUMA

TABLE 1. BRAIN INJURY PARAMETERS IN 3–5-DAY-OLD PIGLETS

| Animal no. | Body weight, kg | Brain weight, g | First load | | | Second load | | |
|------------|-----------------|-----------------|-------------------------|-------------------------|-----------------------------|-------------------------|-------------------------|-----------------------------|
| | | | Peak ang. velocity, r/s | Total Δt , msec | Max decel, r/s ² | Peak ang. velocity, r/s | Total Δt , msec | Max decel, r/s ² |
| 1 | 2.1 | 38 | 188 | 15.0 | 54,948 | — | — | — |
| 2 | 2.4 | 34 | 160 | 17.8 | 41,351 | — | — | — |
| 3 | 1.8 | 39 | 161 | 19.0 | 50,857 | — | — | — |
| 4 | 1.8 | 34 | 193 | 19.4 | 54,629 | — | — | — |
| 5 | 1.8 | 35 | 159 | 19.4 | 52,391 | — | — | — |
| Average | 2.0 | 36 | 172 | 18.1 | 50,835 | | | |
| SD | 0.3 | 2 | 17 | 1.9 | 5,561 | | | |
| 6 | 2.5 | 38 | 144 | 20.2 | 46,502 | 148 | 20 | 44,844 |
| 7 | 1.9 | 33.5 | 130 | 18.0 | 33,575 | 132 | 19 | 35,102 |
| 8 | 2.2 | 36 | n.d. | n.d. | n.d. | 141 | 19 | 30,391 |
| 9 | 1.7 | 39.5 | n.d. | n.d. | n.d. | 141 | 21 | 26,775 |
| 10 | 2.1 | 33 | 129 | 19.0 | 32,223 | 141 | 20.2 | 43,158 |
| 11 | n.d. | 30 | 141 | 20.2 | 25,199 | 134 | 20.2 | 35,597 |
| Average | 2.1 | 35 | 136* | 19.4 | 34,375* | 140* | 19.9 | 35,978* |
| SD | 0.3 | 4 | 8 | 1.1 | 8,879 | 6 | 0.8 | 7,028 |

For each load, peak angular velocity (peak ang vel) is denoted in radians/sec (r/s), maximum deceleration (max decel) is denoted in radians/sec² (r/s²), and, duration of the load (total Δt) is denoted in milliseconds (msec). n.d., not determind. * $p < 0.05$, compared to the average value in piglets receiving a single load.

mum deceleration, Table 1). All piglets resumed spontaneous breathing immediately following either one injury or two consecutive injuries. The mean latency for the return of the pinch reflex was approximately 19 min following one injury and 5 min following the second rotational injury. All brain-injured piglets exhibited a non-significant decrease in core body temperature in the first 60 min following injury, and recovered to 37–38°C

over the remainder of the evaluation period (Table 2). Although there was no significant change in heart rate over the 6-h survival period (Table 2), piglets that received two mild rotational accelerations exhibited a significant decrease in mean arterial pressure from 1 to 5 h post-injury. At 3 h post-injury, the diastolic pressure in 3/5 piglets receiving a single rotation and 6/6 piglets receiving two rotations fell below 25 mm Hg; these piglets re-

TABLE 2. PHYSIOLOGIC VARIABLES FOLLOWING A SINGLE MILD ROTATIONAL LOAD ($n = 5$) OR TWO CONSECUTIVE MILD ROTATIONAL LOADS ($n = 6$) IN THE INFANT PIGLET

| Time (min) | Single load | | | Two loads | | |
|------------|-------------|----------------|------------|------------|----------------|------------|
| | MAP (mm Hg) | HR (beats/min) | Temp (°C) | MAP(mm Hg) | HR (beats/min) | Temp (°C) |
| Baseline | 45 ± 9 | 163 ± 17 | 36.4 ± 0.9 | 45 ± 9 | 156 ± 17 | 36.1 ± 0.5 |
| 0–5 | 50 ± 13 | 164 ± 34 | 35.6 ± 1.4 | 43 ± 7 | 157 ± 18 | 35.8 ± 0.8 |
| 5–30 | 46 ± 8 | 157 ± 15 | 35.5 ± 0.8 | 41 ± 11 | 154 ± 19 | 35.8 ± 1.0 |
| 30–60 | 39 ± 9 | 138 ± 23 | 35.8 ± 1.0 | 38 ± 7 | 151 ± 18 | 36.2 ± 0.6 |
| 60–120 | 39 ± 3 | 152 ± 22 | 36.9 ± 1.0 | 31 ± 4* | 156 ± 19 | 36.9 ± 0.7 |
| 120–180 | 34 ± 7 | 163 ± 35 | 37.3 ± 0.7 | 32 ± 4* | 152 ± 13 | 37.3 ± 0.9 |
| 180–240 | 41 ± 3 | 161 ± 32 | 37.7 ± 0.7 | 32 ± 2* | 152 ± 15 | 37.5 ± 1.2 |
| 240–300 | 43 ± 7 | 156 ± 30 | 37.8 ± 1.0 | 32 ± 5* | 152 ± 15 | 37.6 ± 1.4 |
| 300–360 | 41 ± 7 | 163 ± 31 | 37.7 ± 1.3 | 36 ± 6 | 152 ± 16 | 37.5 ± 1.3 |

The mean arterial pressure (MAP), heart rate (HR), and core body temperature (Temp) were measured using non-invasive techniques in anesthetized piglets for 60 min prior to (baseline) and up to 6 h following application of the load(s). Values are presented as means ± standard deviation. * $p < 0.05$ compared to baseline values using a post-hoc Dunnett's t -test.

ceived a single intravenous bolus of normal saline (10 mL/kg), after which the diastolic pressure returned to pre-injury baseline levels.

Histology

All macroscopic and microscopic findings noted were in comparison to the three uninjured controls. While gross examination revealed no blood on the surface of the uninjured brain, blood was present to a limited extent over the frontal lobe and brainstem following either a single rotation (3/5 animals) or double rotation (6/6 animals) (data not shown). Cerebral tissue tears were not seen on gross inspection of all coronal brain blocks. Microscopic evaluation of the coronal sections stained with Cresyl violet showed no subarachnoid hemorrhage or focal hemorrhages in any brain-injured animal.

Immunohistochemistry for Axonal Injury

The white matter regions in the brains of the three uninjured piglets exhibited normal patterns of immunoreactivity for the 200-kDa neurofilament protein, indicated by the absence of axonal swellings and terminal bulbs. Evidence of traumatic axonal injury (axonal swellings and/or terminal bulbs) was noted following either a single mild rotation (Fig. 1A) or two consecutive mild injuries (Fig. 1B). Injured axons were defined as having diameters greater than two times the largest axons in the same regions in the control animals. Injured axons were

interspersed between normal-appearing axons in all white matter regions evaluated, and occurred in the absence of intraparenchymal hemorrhages. At 6 h following a single mild rotational load, evidence of traumatic axonal injury was present in four of five piglets (Table 3). In three of five piglets, axonal injury was restricted to the peripheral subcortical and central deep white matter regions in the frontal and parietal lobes (Fig. 2A,D). Five of six piglets subjected to two consecutive mild rotational accelerations exhibited traumatic axonal injury (Table 3). As illustrated in Figure 2, injured axons were present in peripheral subcortical and central deep white matter regions in the frontal, parietal, and temporal lobes, and, in the midbrain, hippocampus, fornix, corpus callosum, and cerebellum. One piglet receiving two loads contained injured axons exclusively in the form of axonal swellings. In the group that received two consecutive rotations, five of the six piglets had axonal bulbs and contiguous axonal swellings, and the remaining had axonal swellings exclusively. As observed for the group receiving a single mild rotational load, axonal injury in the piglets subjected to two consecutive mild loads predominated in the peripheral subcortical and central deep white matter regions (Fig. 2).

Quantitative analysis revealed that the number of NF200-positive injured axons summed across all white matter regions was significantly greater in the piglets that received two rotations ($p < 0.02$, 29 ± 19) compared to those that received a single rotation (3 ± 3 , Table 3).

TABLE 3. REGIONAL QUANTIFICATION OF INJURED AXONS IN BRAINS OF INFANT PIGLETS FOLLOWING EITHER A SINGLE, MILD ROTATIONAL LOAD (PIGLETS 1–5) OR TWO, CONSECUTIVE MILD ROTATIONAL LOADS (PIGLETS 6–11)

| Piglet | WM-c | WM-p | MB | HC | CC | FF | CG | Total |
|---------------|---------|---------|-------|-------|-------|-------|-------|--------------|
| 1 | 0 | 4 (2) | 0 | 0 | 0 | 0 | 0 | 4 (2) |
| 2 | 1 (1) | 2 (1) | 0 | 0 | 0 | 0 | 0 | 3 (2) |
| 3 | 1 (1) | 1 (1) | 0 | 0 | 0 | 0 | 0 | 2 (2) |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 3 (3) | 4 (3) | 0 | 0 | 0 | 0 | 0 | 7 (6) |
| Mean \pm SD | | | | | | | | 3 \pm 3 |
| 6 | 24 (14) | 23 (17) | 5 (4) | 2 (2) | 0 | 0 | 0 | 54 (37) |
| 7 | 17 (12) | 23 (9) | 3 (3) | 3 (3) | 1 (1) | 1 (1) | 0 | 48 (29) |
| 8 | 17 (8) | 7 (4) | 0 | 0 | 0 | 0 | 3 (2) | 27 (14) |
| 9 | 6 (3) | 15 (6) | 0 | 3 (3) | 0 | 0 | 2 (1) | 26 (13) |
| 10 | 10 (4) | 6 (5) | 0 | 0 | 0 | 0 | 0 | 16 (9) |
| 11 | 0 | 1 (1) | 0 | 0 | 1 (1) | 0 | 0 | 2 (2) |
| Mean \pm SD | | | | | | | | 29 \pm 19* |

Injured axons were identified in four coronal planes (illustrated in Fig. 2) using immunohistochemistry for the 200-kDa neurofilament (NF200) protein and defined based on the appearance of accumulated NF-200 protein in contiguous axons (swellings) or in disconnected axons (axonal bulbs). Numbers in parentheses denote the total number of microscopic fields (0.7 mm²) in each region containing the injured axons. CB, cerebellum; CC, corpus callosum; FF, fimbria-fornix; HC, hippocampus; MB, mid-brain; WM-c sub-gyral white matter regions in deep central locations of the cerebrum; WM-p, peripheral or subcortical white matter regions within the gyri. * $p < 0.02$ compared to corresponding values in piglets receiving a single load.

Compared to brains of piglets receiving a single mild rotation, brains of piglets receiving two consecutive mild rotational loads contained significantly greater number of foci containing either one or two injured axons/foci ($p < 0.05$, $F = 5.22$, Fig. 3A), or greater than three injured axons ($p < 0.05$, $F = 7.09$, Fig. 3A). Specifically, following a single rotation the axonal injury pattern can be characterized as a few regions with one to two injured axons, while following two rotational loads we found significantly more regions exhibiting one to two injured axons and the appearance of many regions with three or more injured axons. The density of injured axons in foci containing one to two injured axons in brains of piglets re-

ceiving one mild load was 1.3 ± 0.8 injured axons/mm² (mean \pm SD), and was statistically not different from that in piglets receiving two consecutive injuries (1.8 ± 0.4 SD axons/mm², $F = 2.07$, Fig. 3B). However, when the foci that contained three or more injured axons were compared, piglets that received two consecutive rotational loads exhibited a significantly greater density of injured axons (5.9 ± 3.4 injured axons/mm²) compared to those that received a single rotation (0.8 ± 1.9 axons/mm², $p < 0.05$, $F = 7.47$, Fig. 3B).

DISCUSSION

Traumatic axonal injury was observed following inertial rotation of the head of a 3–5-day-old piglet in the axial plane under mild (160–190 rad/sec) loading conditions. The distribution of injured axons following a single, mild rotational acceleration was restricted to the peripheral subcortical and central deep white matter regions in the frontal lobes only. When piglets were subjected to two consecutive (spaced 20 min apart) loads of mild severity (120–150 rad/sec each), the number of foci exhibiting injured axons in the frontal lobes significantly increased. In addition, injured axons were also noted in white matter regions in the parietal and temporal lobes, in the corpus callosum, hippocampus and basal ganglia. Although the overall density of injured axons in each of these foci did not significantly increase after two consecutive rotational loads, the phenotype of injured axons appeared to shift from a few regions with one to two injured axons, to significantly more regions exhibiting one to two injured axons and the appearance of many regions with three or more injured axons. Piglets subjected to two consecutive loads also exhibited an approximately 30% decrease in mean arterial pressure from 60 min post-injury. Despite the observation that the rotational velocity in the single-load group was significantly greater than either the first or the second rotational velocities in the double-load group, the physiologic and immunohistochemical data are indicative of the vulnerability to repeated, mild, non-impact loading conditions.

Comparison of the present observations to those obtained following a single rotational accelerations under peak loading conditions of 240–260 rad/sec (Raghupathi and Margulies, 2002) revealed that a single rotation at significantly lower (160–190 rad/sec) peak loads resulted in both a markedly lower density of injured axons ($p < 0.05$ compared to the data in Table 3 from Raghupathi and Margulies, 2002), and, a demonstrably reduced distribution (compare Fig. 2 in this study to Fig. 4 from Raghupathi and Margulies, 2002). Injured axons in the brains of mild-injured piglets were predominantly char-

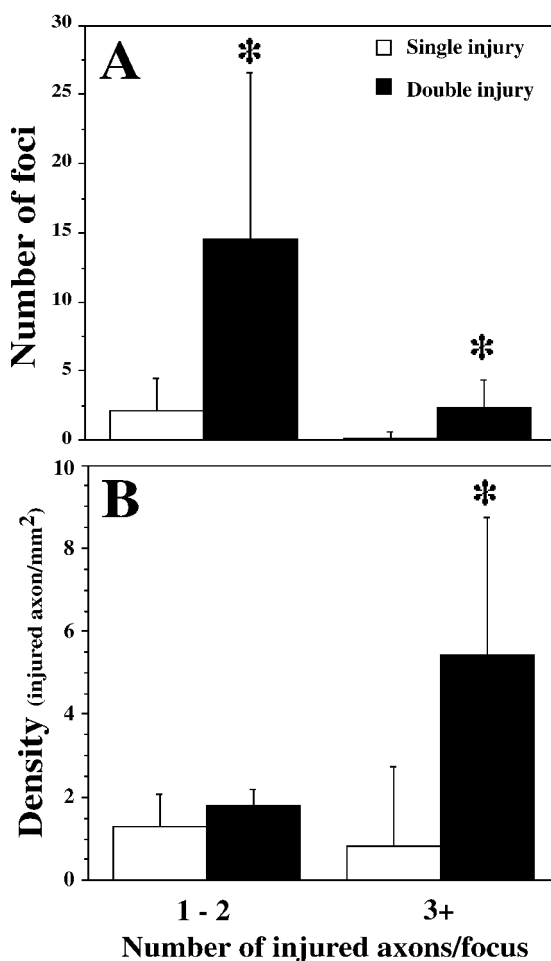


FIG. 3. Quantitative analyses of number of regions (foci) containing traumatically injured axons and the density of injured axons in piglet brains. (A) Foci (each focus depicts a field of view of 0.71 mm²) containing injured axons were separated into either those that contained one to two swelling and/or axon bulbs or those that contained three or greater injured axons. (B) The density was calculated as described in text. * $p < 0.05$, compared to brains receiving a single load.

acterized by intra-axonal swellings, while severe loads gave rise to both terminal bulbs and swellings. In addition, while single severe loads resulted in subarachnoid and subdural bleeding over the frontal lobes and brain stem of the immature piglets, single or double mild loads resulted in only a limited accumulation of subarachnoid blood. Petechial hemorrhage in brain parenchyma was not observed following rotation under mild conditions, but was prevalent after severe loads. Interestingly, two consecutive rotations under loading conditions of 130–180 rad/sec per load resulted in a density of injured axons (5.9 ± 3.4 injured axons/mm²) similar to that following a severe load (6.6 ± 1.5 , Table 3 in Raghupathi and Margulies, 2002). However, the higher single loading conditions did result in a significantly ($p < 0.05$) greater number of foci containing injured axons (17.5 ± 8 , calculated from data in Table 3 from Raghupathi and Margulies, 2002) compared to two loads of lower intensity (2.5 ± 1.9 , Fig. 3). Moreover, when considering foci with three or more injured axons, only higher single loading conditions result in injured foci in the midbrain and hippocampus (data not shown).

Inflicted head injury in infants and children may be characterized by repeated episodes of mild brain trauma (Duhaime et al., 1998; Geddes et al., 2001a; Marin-Padilla et al., 2002). Although multiple models of repetitive brain injury have been developed in adult rodents, models of repetitive head injury in the immature animal are limited to recent studies in which unrestrained 8-day-old rats or mice were subjected to shaking in a horizontally rotating shaker at 200 or 900 cycles per minute, respectively (Smith et al., 1998; Bonnier et al., 2002). Smith et al. (1998) reported that one episode of shaking (12 min per episode) each for 3 days under hypoxic conditions resulted in an immediate increase in tissue free radicals, cortical hemorrhage and a 15–30% loss of cortical tissue by 2 weeks post-injury. Recently, using a similar apparatus, Bonnier et al. (2002) demonstrated that a single episode of shaking under normoxic conditions in 8-day-old mice resulted in cystic lesions and cell death in periventricular white matter, corpus callosum, and brainstem by 3 weeks post-injury. Interestingly, we did not observe any evidence of tissue tearing or intracerebral hemorrhage, suggesting that the piglets may have sustained mild brain injury.

Injured (swollen) axons positive for APP and neurofilament have been observed in white matter regions of post-mortem brains from cases of shaken baby syndrome (Shannon et al., 1998; Vowles et al., 1987; Gleckman et al., 1999). More recently, Geddes et al. (2001) have suggested that the axonal injury observed in the cerebral hemispheres of infants who died from the shaken-baby

syndrome was of a vascular origin and may occur as a result of hypoxia, while the axonal injury that is present in the craniocervical region and the lower brainstem may be of traumatic origin. These data are supported by observations of increased immunoreactivity for the amyloid precursor protein (APP) in the white matter regions in the basal ganglia, brainstem and neocortex following a single episode of shaking of the 8-day-old mouse (Bonnier et al., 2002). Although we did not observe evidence of systemic hypoxia, the data from our study suggest that traumatic axonal injury in cerebral white matter regions appeared not to be of vascular origin, and may be an important component of the histopathological alterations following either a single episode or two consecutive episodes of mild head injury in the immature animal.

Models of repetitive head injuries in adult rodents using impact-based apparatus have demonstrated that two to four concussions resulted in impaired cognitive function, cytoskeletal derangements, and axonal injury (Kanayama et al., 1996; Laurer et al., 2001; DeRoss et al., 2002; DeFord et al., 2002). Similar to the observations in the present study, two mild impact injuries in the adult mouse spaced 24 h apart resulted in a greater degree of axonal swelling in the midbrain compared to a single mild injury (Laurer et al., 2001). Similarly, cognitive impairment was greater in mice that received four separate concussions compared to mice that received a single injury (DeFord et al., 2002). In contrast, it was observed that a subacute level of closed head injury in adult rats conditioned the brain such that the brain appeared resistant to subsequent impact(s) (Allen et al., 2000; DeRoss et al., 2002). Analysis of the density of injured axons in the foci that were present in similar regions of the single- and double-injured piglet brains in the present study did not reveal a propensity for either sensitization (increased density) or tolerance (decreased density). However, qualitative observations suggested that the preponderance of injured axons following a single rotational load exhibited a swollen, contiguous phenotype, while in the doubly injured piglet brains the profile of injured axons included terminal swellings. These observations may suggest that two consecutive injuries may accelerate the progression of secondary axotomy, or alternatively, may increase the incidence of primary axotomy.

We have demonstrated that non-impact rotation of the piglet brain under low loading conditions resulted in significant traumatic axonal injury in the cerebral white matter regions, the density and distribution of which was significantly greater after two consecutive rotational injuries. Future efforts focusing on the effect of a temporal separation between the inflicted loads and the effect of superimposed hypoxia are warranted.

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