

Infant Rat Model of the Shaken Baby Syndrome: Preliminary Characterization and Evidence for the Role of Free Radicals in Cortical Hemorrhaging and Progressive Neuronal Degeneration

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ABSTRACT

Infants subjected to repeated episodes of violent shaking develop brain damage characterized by intracranial hemorrhage and progressive cortical atrophy. We have developed an animal model that mimics this pathological state and investigated its etiology and treatment. Anesthetized male rats, 6 days of age, were subjected to one episode of shaking per day for 3 consecutive days. Separate groups of rats were sacrificed 1 h postinjury on the third day of shaking for HPLC quantification of cortical •OH and vitamin E levels, and histological assessment of cortical hemorrhaging. Additional groups were sacrificed 7 or 14 days postinjury to demonstrate progressive neuronal degeneration via cortical wet weight comparisons. In comparison to noninjured shams, the results indicated that cortical vitamin E and •OH levels rose 53.7% ($p < 0.005$) and 457.1% ($p < 0.001$), respectively, in shaken infant rats. Brain histologies revealed a moderate-to-severe degree of cortical hemorrhaging in these animals 1 h postinjury. By 7 and 14 days postinjury, there was a 13.3% and 28.7% ($p < 0.0001$ vs. sham) loss of cortical tissue in shaken infants, respectively, indicating progressive neuronal degeneration. Treatment with 10 mg/kg (ip) of the 21-aminosteroid antioxidant, tirilazad mesylate, 10 min before and 2 h after each episode of shaking, resulted in a 53.1% attenuation of cortical •OH levels and a 34.9% decrease in brain hemorrhaging ($p < 0.05$ vs. vehicle). Tirilazad treatment did not, however, significantly effect cortical vitamin E concentrations at 1 h postinjury or the extent of progressive neuronal degeneration at either 7 or 14 days postinjury. The present animal model mimics the brain pathology seen in abused children. Our observation that tirilazad mesylate, an antioxidant-lipid peroxidation inhibitor, significantly reduces cortical •OH levels and brain hemorrhaging in shaken infant rats supports a role for oxygen radicals in the pathophysiology of this type of CNS injury. The failure of tirilazad to block progressive cortical degeneration suggests that mechanisms other than free radicals may be of prime importance in the mediation of this aspect of the pathology.

Key words: cortical hemorrhaging; free radicals; shaken baby syndrome; tirilazad mesylate

INTRODUCTION

INFANTS SUBJECTED TO REPEATED EPISODES OF VIOLENT SHAKING develop brain damage characterized by subarachnoid, subdural, and intrahemispheric bleeding followed by progressive cortical atrophy which may continue up to 1 year postinjury (Alexander et al., 1989; Spaide et al., 1990; Duhaime et al., 1996). Mortality from the shaken baby syndrome has been estimated to be as high as 33% and significant morbidity is seen in over half of the victims (Duhaime et al., 1987; Spaide et al., 1990). As with other types of head trauma, treatment is primarily supportive and consists of maintaining proper ventilation and adequate cerebral perfusion pressure, in addition to decreasing intracranial pressure and brain edema when necessary. At this time, there are no neuroprotective drugs on the market that target this particular type of CNS injury. In order to develop an appropriate drug therapy, it is necessary to understand both the mechanics and the pathophysiology of this unique type of head trauma. The purpose of the present study was to lay the foundation for future drug development by providing such insights.

Shaking is a mechanical, acceleration/deceleration type of injury in which the brain shifts violently, back and forth in the skull, causing the superficial pial vessels to burst. Whether enough force is created during the act of shaking to tear the bridging veins, crossing from the brain to the sagittal sinus, is subject to debate. Using dolls, constructed with varying head sizes and neck-muscle strengths, Duhaime et al. (1987) concluded that the g forces associated with violent shaking were not great enough to rupture the major blood vessels of the brain. And indeed pilot work, during the development of the present animal model, demonstrated that shaking alone does not produce significant brain hemorrhaging in infant rats. Rather, the added insult of hypoxia had to be combined with shaking in order to mimic the subdural hemorrhaging and progressive cortical degeneration seen in abused children.

Hypoxia may be a very real secondary insult in the shaken baby syndrome, which serves to exacerbate brain damage due to the primary mechanical injury of shaking. Apnea, respiratory distress, lethargy, and coma are all common symptoms seen in abused children upon admission to the ER (Duhaime et al., 1987, 1988; Whyte et al., 1989). In addition, autopsy reports have revealed evidence of high spinal cord injury (Hadley et al., 1989) and brain stem damage (Bostrom et al., 1992) in these children. Cumulative damage to the brain stem respiratory center could conceivably cause the child to experience longer and longer periods of apnea with each successive episode of shaking. Hence, the secondary insult of hy-

poxia would become superimposed on the primary mechanical act of shaking.

Postinjury periods of hypoxia and hypotension are associated with an increase in mortality and morbidity in severely head-injured patients (Price and Murray, 1972; Gentleman, 1992; Chestnut et al., 1993). Experimentally, posttraumatic hypoxia has also been shown to exacerbate head injury, both biochemically and histologically (Ishige et al., 1987a,b; Tanno et al., 1992; Cherian et al., 1996). For example, rats subjected to fluid percussion head injury followed by 30 min of hypoxia reportedly have more extensive cortical injury, including blood-brain barrier damage and edema, and greater postinjury reductions in high-energy phosphates and cerebral blood flow (CBF), than nonhypoxic injured controls (Ishige et al., 1987a,b). When hypotension is combined with fluid percussion injury or impact injury, a similar potentiation occurs. High-energy phosphates (Ishige et al., 1988) and CBF are greatly depressed, cerebral oxygen delivery is reduced, and EEG levels are suppressed (DeWitt et al., 1992). In neonatal rats, hypoxia triggers progressive cortical degeneration when combined with unilateral carotid occlusion (Andine et al., 1990). The sensitivity of the neonate to hypoxia-ischemia is most pronounced around postnatal day 7 (Grafe, 1994) and correlates with similar peaks in the postnatal brain growth spurt (Dobbing and Sands, 1979) and sensitivity to NMDA toxicity (Hagberg et al., 1994; Ikonomidou et al., 1989).

In this paper, we describe an infant rat model of traumatic subarachnoid hemorrhage that mimics the histopathology of the shaken baby syndrome. Rat pups were subjected to one episode of shaking + hypoxia per day for 3 consecutive days and then sacrificed anywhere from 1 h to 14 days later to quantify cortical •OH and vitamin E levels, brain hemorrhaging, and progressive cortical degeneration. Free radicals and, in particular, •OH are considered important triggers of lipid peroxidative injury and have been convincingly implicated in the pathophysiology of both head injury and subarachnoid hemorrhage (SAH) (Hall et al., 1993; Smith et al., 1994; Hall, 1996). For example, following concussive head injury in the rat (Smith et al., 1994) and mouse (Hall et al., 1993), cortical •OH concentrations increase markedly within 5 min postinjury and remain elevated for as long as 60 min. This is followed by a parallel, although slightly delayed, opening of the blood-brain barrier (BBB). Tirlazad mesylate is an antioxidant, lipid peroxidation inhibitor that remains localized within the vascular endothelium (Hall and McCall, 1993). Its antioxidant activity is conferred by multiple mechanisms, including membrane stabilization, decreased formation and/or scavenging of peroxy and hydroxyl radicals, and protection of endogenous vitamin E levels (Audus et al.,

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1991; Hinzmann et al., 1992; Hall, 1997). Data from our own and other laboratories indicate that tirilazad decreases cortical •OH concentrations and protects the BBB following head injury (Hall et al., 1992, 1993; Smith et al., 1994) and SAH (Zuccarello and Anderson, 1989; Smith et al., 1996) in small rodents. The drug also significantly attenuates cortical degeneration in neonatal rats subjected to hypoxia + unilateral carotid occlusion (Bagenholm et al., 1996). In addition, tirilazad has been shown to enhance the preservation of vitamin E levels following blunt spinal cord injury in the cat and focal ischemia in the gerbil (Hall, 1997), presumably by competing for the same reaction as vitamin E during the inhibition of lipid peroxidation. Hence, we chose to test the neuroprotective effects of tirilazad in the present infant rat model of the shaken baby syndrome.

MATERIALS AND METHODS

All experiments received prior approval by the Institutional Animal Care and Use Committee of Pharmacia & Upjohn, Inc. to ensure that they were performed in strict compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Animals

Male Sprague Dawley rats, 6 days of age, were used in this experiment. They were housed in litters of eight in hard-bottom cages with wood chip bedding. The mothers were given *ad libitum* access to Purina Rodent Diet and tap water throughout the study. A 12-h light-dark cycle was maintained in the colony room, with lights on 6:00 a.m. to 6:00 p.m.

Apparatus

The shaker (Fig. 1) consisted of four polypropylene tubes, each 2 inches in diameter, mounted to a piston driven by compressed air (D. Gleason, Research Laboratory Instrument Support, Pharmacia & Upjohn). The piston moved horizontally, back and forth, at a rate of 20 times per 6 sec. This movement created enough force to snap the head of an infant rat back and forth in a manner similar to that of a child being shaken (g forces calculated as mass × length × time × 10⁻² = 9.05). Hypoxic air (8% O₂, bal N₂) was piped in from the bottom of each tube, and the entire chamber housing the shaker was warmed with a heat lamp. Individual tube temperatures were monitored using an Anritherm Digital Handheld Thermometer (Model HL-500, ANRIYSU Meter Co., LTD; Physitemp Type IT-18 probe; both from Cole-Pharmer Instrument Co., Niles, IL). Since rat pups 6–8

days of age are poikilothermic (Schmidt et al., 1987; Gordon, 1990), a tube temperature of 39–40°C was needed to maintain their body temperature at 36–37°C (Intraperitoneal temperature monitor, Model BAT-12, with 26 g probe; IITC Life Science USA, Woodland Hills, CA). Body temperatures were recorded only during the pilot stage of model development.

Procedure

Injury (shaking + hypoxia). Rats were anesthetized with 14 mg/kg Brevital (i.p.) 3–5 min before being placed into the shaker. They were wrapped in light-weight foam rubber vests, hung upside down in a tube, and allowed to equilibrate to the hypoxic conditions for 5 min. Following this equilibration period, all pups were subjected to a total of 12 min of intermittent shaking (one episode). They were shaken for 6 sec, then rested for 6 sec. This cycle was repeated 60 times. Upon removal from the chamber, pups were placed in an incubator maintained at 36–37°C for 2–3 h and then returned to the nest. In the present study, pups were subjected to one episode of shaking per day for 3 consecutive days and then sacrificed anywhere from 1 h to 14 days later.

Rats were dosed with 10 mg/kg tirilazad (i.p.), or an equal volume of citric acid vehicle (2 ml/kg), 10 min before and 2 hr after each episode of shaking. Separate groups of rats were sacrificed 1 h postinjury on the third day of shaking for HPLC quantification of cortical •OH and vitamin E levels, and histological assessment of cortical hemorrhaging. Additional groups were sacrificed 7 or 14 days postinjury to demonstrate progressive neuronal degeneration via cortical wet weight comparisons.

Measurement of cortical hydroxyl radical and vitamin E levels. Hydroxyl radical and vitamin E levels were quantified in the right cortices of noninjured (sham) and injured infant rats treated with either citric acid vehicle or tirilazad. The method for determining cortical •OH levels, via the salicylate trapping method, has been described previously (Althaus et al., 1993). Briefly, rats were given an i.p. injection of 300 mg/kg salicylic acid 10 min before sacrifice (sodium salt, Aldrich Chem Co., Milwaukee, WI). A 3 × 4 mm block of tissue was then cut out of the fresh, nonperfused frontal cortex and immediately frozen in liquid nitrogen. On the day of assay, tissue samples were weighed and immediately placed in 1.5-ml microcentrifuge tubes containing 100 µL of ethanol (1% ascorbate). The sample were homogenized in a vortex mixer for 2 min and then centrifuged, at 14,000 RPMs (10,950 g's), for an additional 2 min. A 30-µL aliquot of supernatant was injected onto a BAS ODS 5-µm column via a Perkin Elmer ISS-100 autosampler. The mobile phase contained 15% acetonitrile, 10% tetrahydrofuran, and 14.15 g chloroacetic acid per

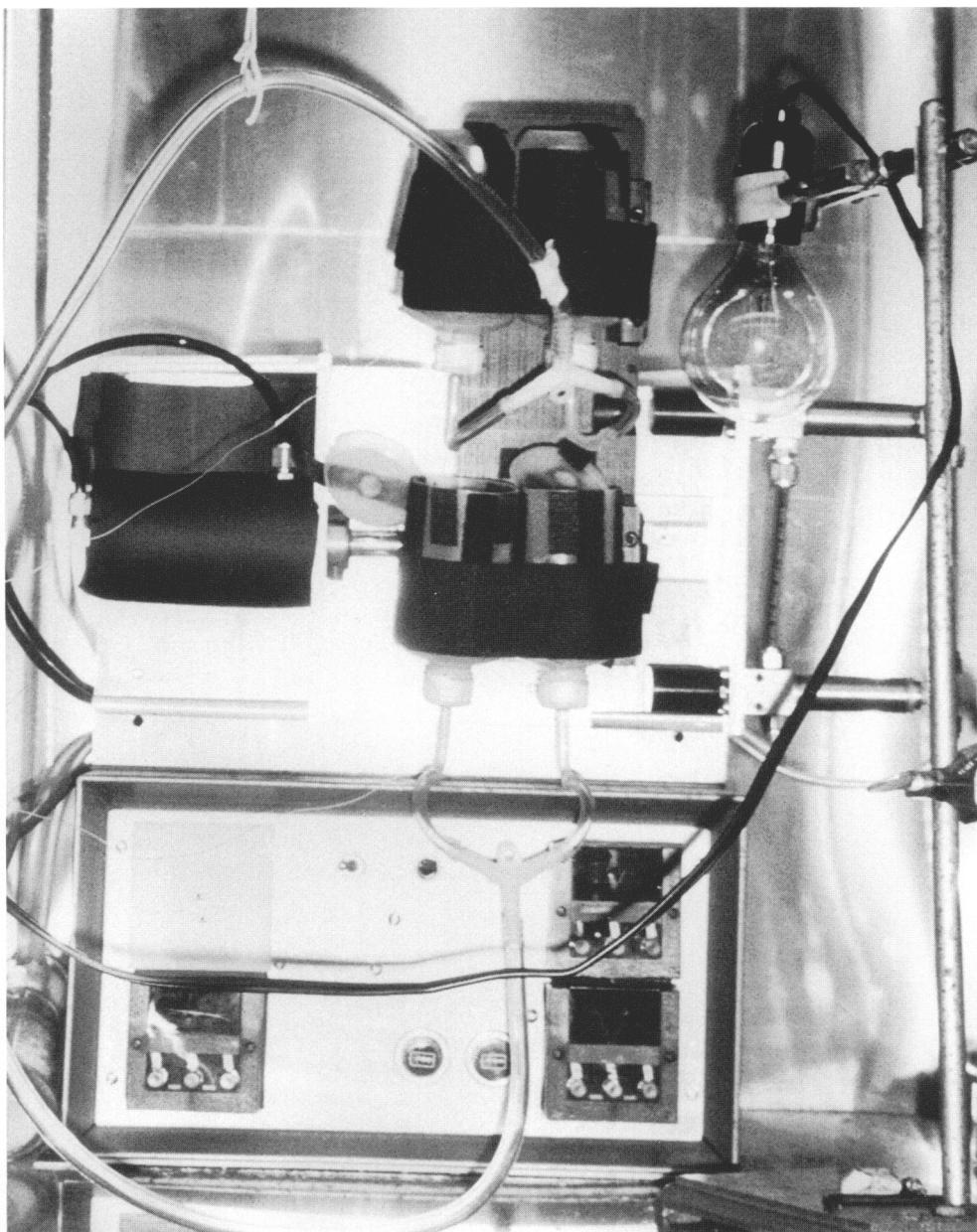


FIG. 1. The shaker consisted of four polypropylene tubes, each 2 inches in diameter, mounted to a piston driven by compressed air. The piston moved horizontally, back and forth, at a rate of 20 times per 6 sec. Hypoxic air (8% O₂, bal N₂) was piped in from the bottom of each tube.

liter. The column effluent was directed first through a BAS LC-4C electrochemical detector (650 mv) for quantification of 2,3- and 2,5-dihydroxybenzoic acid (DHBA), and then through a Waters 486 UV detector (295 nm) for the spectrophotometric identification of salicylate. Sample contents were read against external standards of 2,3-DHBA, 2,5-DHBA and salicylate. Peaks were integrated using Waters Maxima 820 software, and the final •OH concentrations were expressed as a ratio of

DHBA to salicylate. This normalized the absolute level of DHBA with brain concentrations of salicylate, which tended to vary from animal to animal. Moreover, use of the ratio lessens the variability observed with measurement of 2,3-DHBA and 2,5-DHBA alone.

The method for vitamin E quantification has also been previously described (Zhang et al., 1993). In the present study, 20–50 mg of cortical tissue was homogenized in 80–200 µL of ethyl acetate containing 100 µg/ml of the

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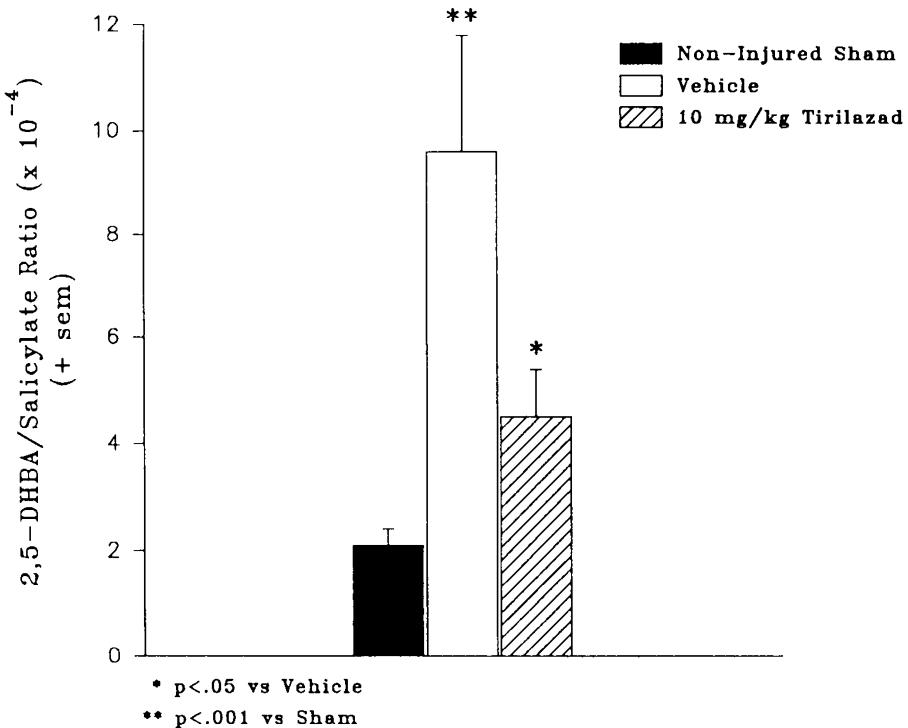


FIG. 2. The effects of tirilazad treatment on cortical •OH levels in shaken infant rats sacrificed 1 h after the third episode of shaking + hypoxia. Values are expressed as the ratio of 2,5-DHBA to salicylate \pm sem.

internal standard (a normal C₂₂ alcohol esterified to Trolox, synthesized at Pharmacia & Upjohn, Inc.). The tissues were sonicated (VirTis sonicator with a microprobe tip, speed setting of 4) for 15–20 sec and then cen-

trifuged at 15,000 rpm (4°C) for 10 min. A 5-µL aliquot of supernatant was analyzed by HPLC using an Inertsil C-8 column (3.0 × 250 mm, 5-µm particle size) equipped with an Inertsil C-8 guard column. An isocratic

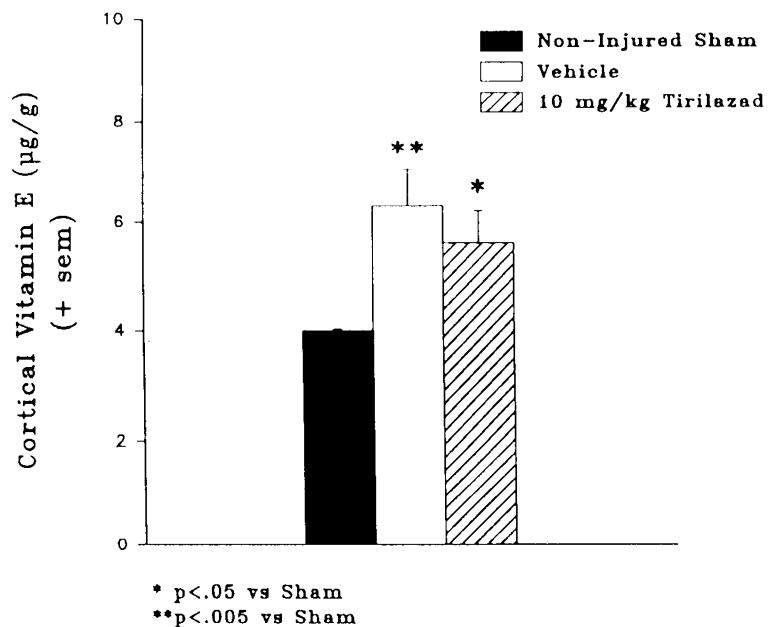
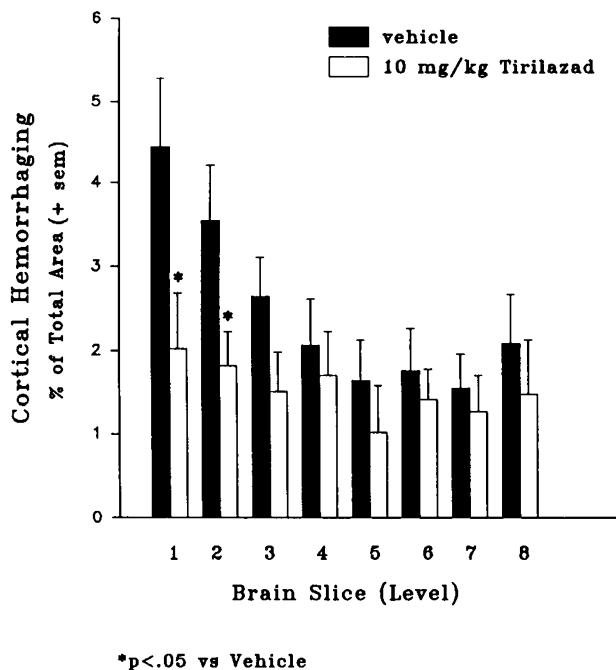


FIG. 3. Cortical vitamin E levels ($\mu\text{g/g} \pm \text{sem}$) in sham, and vehicle- and tirilazad-treated rats sacrificed 1 h after the third episode of shaking + hypoxia.



* $p < .05$ vs Vehicle

FIG. 4. Cortical hemorrhaging at each of eight levels cut sequentially through the brains of vehicle- and tirilazad-treated rats. All rats were subjected to three episodes of shaking + hypoxia and sacrificed 1 h after the third episode. Values are expressed in terms of percent of total area \pm sem.

mobile phase, consisting of methanol/water (95:5), 0.1% pyridine and 0.3% sodium perchlorate, with a flow rate of 0.7 mL/min was used. Vitamin E and the internal standard eluted at approximately 8.2 and 13.6 minutes, respectively, and were monitored by a Waters 470 fluorescence detector (EX = 290 nm; EM = 340 nm). The data were collected using the Waters Millennium Chromatography System, 2.1. Ratios were calculated by dividing the peak height of the vitamin E by the peak height of the internal standard. Concentrations of vitamin E were reported in units of $\mu\text{g/g}$.

Quantification of cortical hemorrhaging. Following perfusion with 10% formalin, 5- μm coronal sections were cut at 750- μm intervals through the brain (total of eight levels) and stained with H&E. The area of each cortical hemorrhage was determined using an image analysis system (Inquiry[®] Quantitative Autoradiography for Windows[™], Loats Assoc., Inc., Westminster, MD, 1996). The sum total of all hemorrhaged areas within each level was then computed and expressed as a percent of the entire area of each brain slice. The data were also compiled across all eight levels to give an index of the total amount of cortical hemorrhaging in each brain.

Quantification of cortical degeneration. Cortical wet weights were used to quantify the extent of neuronal degeneration at 7 or 14 days postinjury. At the time of sac-

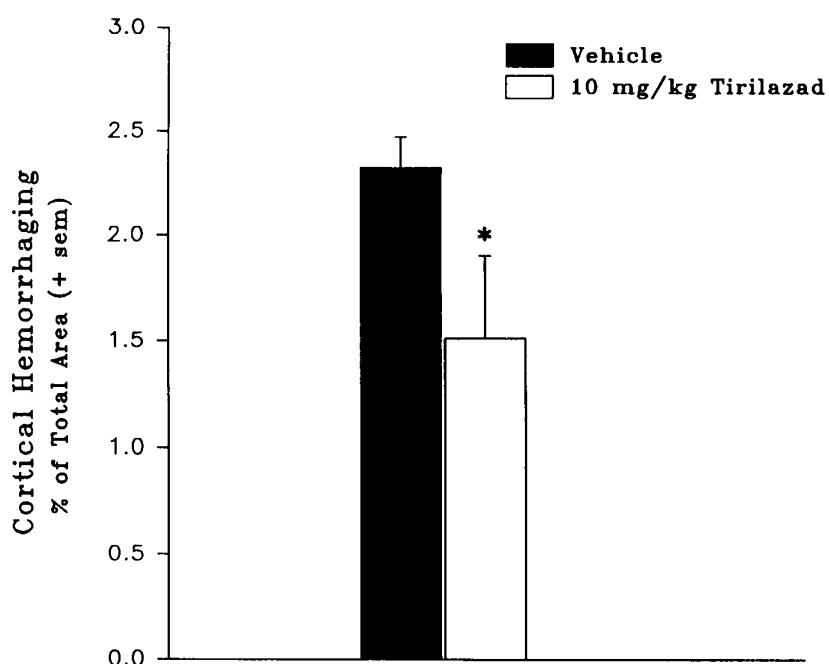
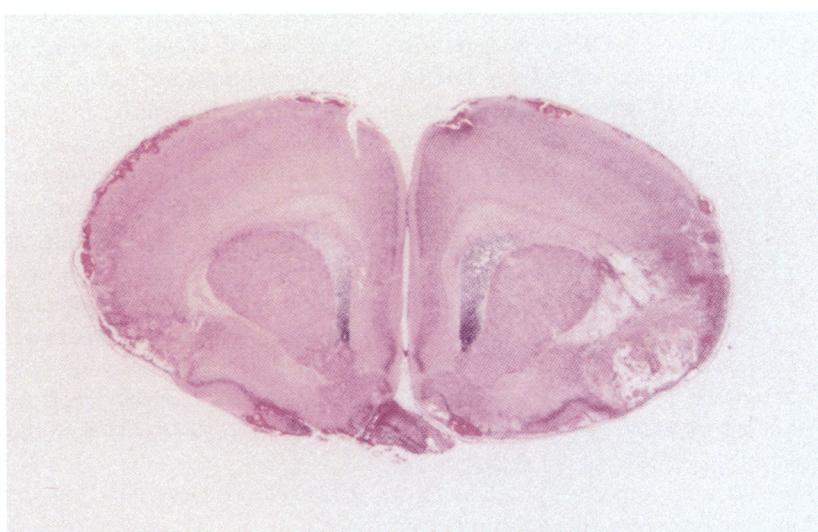


FIG. 5. Cortical hemorrhaging in vehicle- and tirilazad-treated rat pups sacrificed 1 h after the third episode of shaking + hypoxia. Values are expressed in terms of percent of total area \pm sem.

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A



B

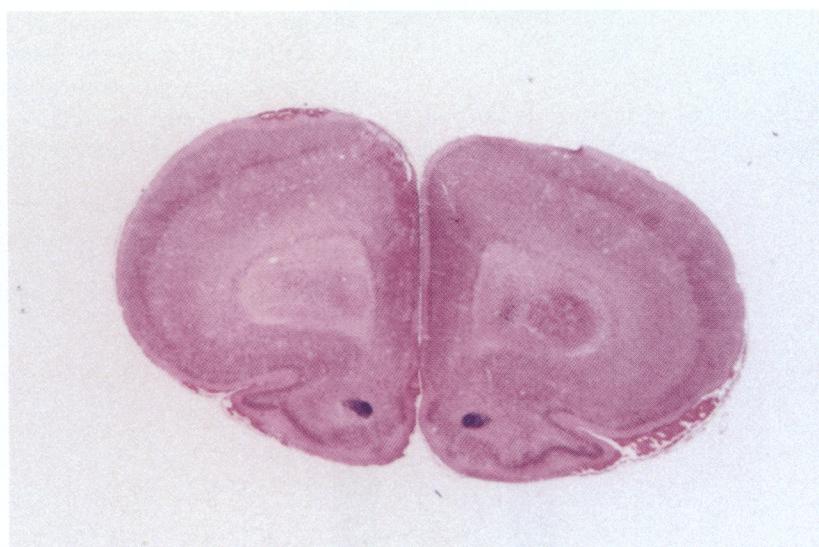


FIG. 6. H&E-stained brain histologies from shaken infant rats that were treated with vehicle (A) or tirilazad (B), and sacrificed 1 h after the third episode of shaking + hypoxia.

rifice, the left and right nonperfused cortices of drug- and vehicle-treated rats were weighed and expressed as a percent of noninjured age-matched controls. The cortices were then dried overnight (100°C) and reweighed to determine the presence/absence of edema. Since the weights of the left and right cortices did not differ significantly, the two were combined and the total cortical weight was used to assess treatment effects.

In the assessment of progressive neuronal degeneration, both the raw cortical weights and the ratio of cortical weight to body weight were also compared. Both methods of data analysis yielded similar results to that obtained when the cortical wet weights were

expressed as a percent of noninjured control weights. In order to make discussion of the data more understandable, we chose to present the data in terms of the latter method, as a percent of noninjured control values.

RESULTS

Cortical Hydroxyl Radical and Vitamin E Levels

In Fig. 2, cortical •OH levels are presented as the ratio of 2,5-DHBA to salicylate for rats shaken once a day for 3 consecutive days and then sacrificed 1 h

postinjury on the last day of shaking ($n = 8$ per group). Although the ratio of 2,3-DHBA to salicylate was approximately one-third that of the 2,5-DHBA, similar results were obtained when it was used to quantify cortical •OH levels. In comparison to noninjured shams, cortical •OH levels increased 457.1% in shaken infant rats ($p < 0.001$). Treatment with 10 mg/kg tirilazad, 10 min before and 2 h after each episode of shaking, resulted in a significant, 53.1%, attenuation in •OH formation relative to vehicle-treated rats ($p < 0.05$ vs. vehicle).

Cortical vitamin E levels are presented for sham, and vehicle- and tirilazad-treated rats in Fig. 3 ($n = 8$ per group). Vitamin E concentrations rose 53.7% ($p < 0.005$) and 36.6% ($p < 0.05$), above that of noninjured shams, in vehicle-and tirilazad-treated rats, respectively. Although a trend toward lower vitamin E concentrations was observed in tirilazad-treated animals, the difference between drug and vehicle groups was not statistically significant.

Cortical Hemorrhaging

Figure 4 compares cortical hemorrhaging at each of eight levels cut sequentially through the brains of vehicle-

and tirilazad-treated rats ($n = 6$ per group). Although there was an overall decrease in the amount of cortical hemorrhaging observed in tirilazad-treated rats at all levels, statistically significant effects were observed in only the two most rostral levels ($p < 0.05$ vs. vehicle). When these data were compiled across all eight levels to give an index of the total amount of hemorrhaging present in each brain (Fig. 5), a significant 34.9% attenuation of cortical hemorrhaging was demonstrated with tirilazad treatment ($p < 0.05$ vs. vehicle). In Fig. 6, brain histologies from vehicle- and tirilazad-treated rats are compared. Cortical hemorrhages were typically observed in at least two to three successive levels, and very often in as many as four to five levels in both drug and vehicle groups.

Progressive Cortical Degeneration

The cortical wet weights of shaken infant rats are expressed as a percent of noninjured age-matched controls and presented as a function of drug treatment and time of sacrifice in Fig. 7 ($n = 6-8$ per group). At 7 and 14 days postinjury, the cortical wet weights of vehicle-treated rats were 86.7 ± 5.2 and $71.3 \pm 6.9\%$ ($p < 0.0001$) of noninjured controls, respectively, thus demon-

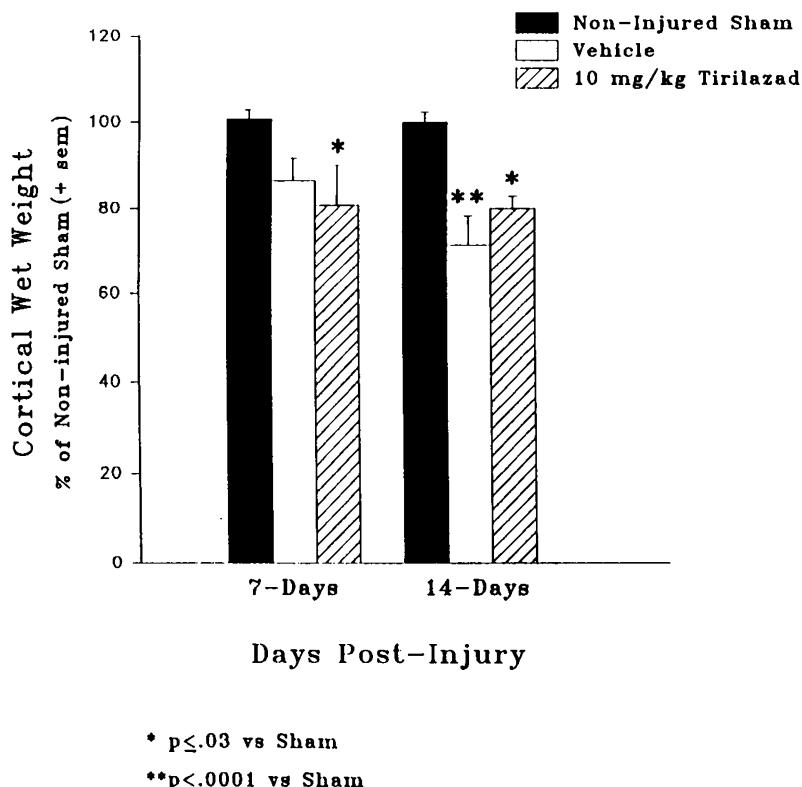


FIG. 7. Cortical wet weights of vehicle- and tirilazad-treated rats that were subjected to three episodes of shaking + hypoxia and sacrificed 7 or 14 days postinjury. Wet weights were expressed as a percent of noninjured, age-matched controls (\pm sem) in order to quantify the extent of progressive cortical degeneration.

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strating the presence of progressive cortical degeneration. Tirilazad treatment did not significantly effect the degree of degeneration at either 7 ($80.7 \pm 9.5\%$) or 14 days ($79.9 \pm 3.0\%$) postinjury.

In comparison to noninjured controls, analysis of the cortical dry weights from vehicle- and tirilazad-treated rats, sacrificed 7 or 14 days postinjury, indicated that there was no edema present in either group ($n = 6-8$ per group). The mean percent water content in the combined left and right cortices of vehicle- and tirilazad-treated rats were 84.2 ± 0.4 and $84.3 \pm 0.8\%$ at 7 days postinjury (vs. noninjured controls, $83.7 \pm 0.3\%$) and 81.9 ± 0.7 and $82.0 \pm 0.2\%$ at 14 days postinjury (vs. noninjured controls, $81.9 \pm 0.2\%$), respectively. The failure to observe brain edema may be due to a number of factors: mild dehydration, loss of cortical tissue, or the timing of tissue sampling.

At the outset of the experiment (e.g., 6 days of age), all rats weighed 11–15 g and the mean weights between groups did not differ significantly. The mean body weights of vehicle- and tirilazad-treated, and noninjured age-matched controls, sacrificed at 7 and 14 days postinjury, are shown in Table 1. The results indicated that there were no significant differences between groups sacrificed at 7 days postinjury. However, at 14 days postinjury, vehicle-treated rats weighed slightly less than noninjured controls (45.7 ± 3.6 vs. 51.8 ± 0.8 g; $p < 0.05$).

DISCUSSION

The present animal model of the shaken baby syndrome mimics the histopathology seen in severely abused children. Rat pups subjected to violent episodes of shaking develop moderate-to-severe cortical hemorrhaging and progressive neuronal degeneration, which continues up to 14 days postinjury. The current study demonstrates the appropriateness of using the rat in modeling infant head injury. The neonatal rat brain undergoes rapid postnatal development, with peak growth velocities occurring between postnatal days 5 and 10. Whereas the rat brain

is 12% of its adult weight at birth, by postnatal day 7 it is over 50% of its adult weight (Dobbing and Sands, 1979). It is at this age, postnatal day 7, that the neonatal rat is also most sensitive to hypoxia-ischemia- and glutamate-induced neurotoxicity (Grafe, 1994; Hagberg et al., 1994). In comparison, peak growth velocities occur between birth and 3 months in humans. The human brain is 27% of its adult weight at birth and nearly 60% at 3 months of age (Dobbing and Sands, 1979). Rapid postnatal brain growth is thought to increase the vulnerability of the CNS to certain types of injury. Hence, the rat is a very appropriate species to use in modeling the pathophysiology of the shaken baby syndrome.

Results from the present study demonstrate that oxygen radicals play an important role in the pathophysiology of secondary cortical hemorrhaging in shaken infant rats. This is supported by our observation that cortical hemorrhaging was apparent, histologically, at a time when $\bullet\text{OH}$ levels were dramatically increased in the injured tissue. In addition, both cortical hemorrhaging and $\bullet\text{OH}$ concentrations were significantly reduced, 34.9% and 53.1%, respectively, following tirilazad treatment. Tirilazad is a 21-aminosteroid with potent antioxidant, iron-induced lipid peroxidation inhibiting properties (Hall and McCall, 1993; Hall et al., 1994). It does not readily cross the blood-brain barrier (BBB), but rather, remains largely within the vascular endothelium, limiting the propagation of free radical reactions within the lipid bilayer (Hinzmann et al., 1992). This is one mechanism by which the drug protects the BBB following CNS injury. Free radicals and, in particular, $\bullet\text{OH}$ have been shown to play an important role in the mediation of BBB damage due to head injury and SAH (Hall et al., 1994; Smith et al., 1994, 1996). Reports indicate that cortical $\bullet\text{OH}$ concentrations peak 1–5 min following head injury in the rat (Smith et al., 1994) and mouse (Hall et al., 1992, 1993) and remain elevated for as long as 60 min postinjury. This is followed by a slightly delayed opening of the BBB. Treatment with tirilazad has been shown to attenuate the rise in $\bullet\text{OH}$ concentration and to protect the BBB in both species. Similarly, tirilazad has been shown to reduce BBB damage and vasogenic edema following SAH in the rat (Zuccarello and Anderson, 1989; Smith et al., 1996), and to significantly improve microvascular hypoperfusion following SAH in the cat (Hall and Travis, 1988). Reports indicate that tirilazad also effectively reduces the degree of post-SAH angiographic vasospasm seen in a variety of species (Hall, 1996). Evidence supports the conclusion that these pathophysiological events are mediated, in part, by an oxygen radical-, iron-induced increase in lipid peroxidation that begins in the SAH clot (Kanamaru et al., 1991; Hall and McCall, 1993) and then spreads to the microvascular smooth muscle and en-

TABLE 1. MEAN BODY WEIGHTS (g) OF VEHICLE- AND TIRILAZAD-TREATED, AND NONINJURED AGE-MATCHED CONTROLS

Time of sacrifice	Control	Vehicle	Tirilazad
7-day postinjury	41.0 ± 0.9	38.7 ± 0.9	38.2 ± 1.4
14-day postinjury	51.8 ± 0.8	$45.7 \pm 3.6^*$	50.0 ± 1.4

Injured rats were subjected to one episode of shaking per day for 3 consecutive days and then sacrificed 7 or 14 days later.

* $p < 0.05$ vs. 14-day control.

Values = mean \pm sem.

dothelium (Sasaki et al., 1985, 1986). The efficacy of tirilazad in SAH has been further demonstrated in phase III clinical trials showing a significant decrease in mortality and morbidity in both men and women, particularly in the most severe neurogrades.

Tissue vitamin E levels have been shown to decline significantly following blunt spinal cord injury in the cat and focal ischemia in the gerbil (Hall, 1997). Treatment with tirilazad, in both cases, enhances the preservation of vitamin E in the injured tissue, presumably by competing for the same reaction as vitamin E during the inhibition of lipid peroxidation. However in the present study, cortical vitamin E levels increased, rather than decreased, following injury due to shaking + hypoxia in vehicle-treated rats. Vitamin E's rate of entry into the uninjured brain is normally slow (Machlin and Gabriel, 1982), due to its association with lipoproteins and retention in brain endothelium. However as the BBB breaks following each episode of shaking, brain penetration of vitamin E may increase. Thus, the elevated brain levels of vitamin E observed in infant rats following shaking + hypoxia may reflect post-traumatic changes in BBB permeability. The fact that tirilazad treatment tended to lessen the increase in brain vitamin E (although not significantly), is consistent with the drug's demonstrated ability to protect the BBB in animal models of head injury (Hall et al., 1992; Smith et al., 1994) and SAH (Zuccarello and Anderson, 1989; Smith et al., 1996).

Although the use of cortical wet weights appears to be a rather simplistic way to assess cortical degeneration, it is nonetheless an appropriate, accurate, and well-documented measure. Typically, this approach has been used to quantify cortical damage in neonatal rats 14 days after unilateral carotid occlusion plus 2 h of hypoxia (e.g., neonatal hypoxia-ischemia model). Using this model, researchers have demonstrated a high degree of correlation between the loss of cortical weight in the injured hemisphere and the loss of brain tissue evaluated histologically (Andine et al., 1990; Hagberg et al., 1994; Silverstein et al., 1986). Good correlations have also been demonstrated between the reduction in cortical weight, infarct volume, morphological score and various biochemical markers of cell death following both hypoxia-ischemia and NMDA-induced neurotoxicity (Gillard et al., 1994; MacDonald et al., 1986). The sensitivity of cortical wet weight measures is further underscored by data indicating that the percent of neurons injured (as assessed histologically or via cortical weights) increases as a function of the duration and severity (e.g., concentration of oxygen) of the hypoxic insult (Grafe, 1994). Histological assessment of the injured hemisphere in these rat pups revealed that the cortical damage consisted primarily of

focal neuronal damage with select areas of cortical infarction and necrosis (Grafe, 1994; Hagberg et al., 1994). In the current study, progressive cortical degeneration was clearly demonstrated in vehicle-treated rats subjected to shaking + hypoxia. However, treatment with 10 mg/kg tirilazad, 10 min before and 2 h after each of three episodes of shaking, did not attenuate the neuronal degeneration at either 7 or 14 days postinjury. In an additional study (data not included), twice a day dosing with tirilazad (10 mg/kg, i.p.) was extended for a full 10 days after injury. At 14 days postinjury, there was still no evidence of neuroprotection in the drug-treated group. This is in contrast with data from our own laboratory demonstrating that 7.5 mg/kg tirilazad (i.p.) significantly decreases cortical damage in neonatal rats subjected to unilateral carotid occlusion + 2 h of hypoxia treatment. Although hypoxic-ischemic events contribute to the pathophysiology of both types of injury, the balance or relative contribution of these and other mechanistic factors may differ. The fact that tirilazad failed to attenuate progressive cortical degeneration in shaken infant rats suggests that free radicals may not be critical in the mediation of this aspect of the pathology of the shaken baby syndrome. Rather other factors, such as glutamate toxicity, must be considered. Glutamate neurotoxicity has been implicated in the pathophysiology of both head injury and cerebral ischemia (Park et al., 1988; Faden et al., 1989; McIntosh et al., 1990; Povlishock, 1993). In the neonate, NMDA antagonists have been shown to significantly attenuate brain damage and edema in each case (Hagberg et al., 1994; Ikonomidou and Turski, 1996). This is particularly relevant to the present model since reports indicate that the sensitivity to NMDA-induced neurotoxicity peaks around postnatal day 7 in neonatal rats (Hagberg et al., 1994; McDonald and Johnston, 1990).

The present rat model of the shaken baby syndrome mimics the brain pathology and clinical symptoms seen in abused children in a number of ways. Infants subjected to violent shaking typically display retinal hemorrhages, in addition to subdural, subarachnoid, and intrahemispheric bleeding (Duhaime et al., 1987, 1992; Alexander et al., 1989). Common clinical symptoms include lethargy, difficulty breathing, and poor feeding (Duhaime et al., 1987). In our rat model of the shaken baby syndrome, the body weights of shaken infant rats were significantly less than those of noninjured controls at 14 days postinjury, indicating a "failure to thrive." A pilot histological study also revealed evidence of retinal hemorrhaging 1 h after the third episode of shaking (data not shown). This is not surprising considering the fact that the rats were hung upside down in the shaker. The in-

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verted position should cause an increase in intraocular pressure, which, when combined with violent shaking and hypoxia, would tend to cause the retinal vessels to burst. Additional studies are needed to verify this.

Extensive subdural/subarachnoid hemorrhaging was also observed in shaken infant rats 1 h after the third episode of shaking, in addition to intrahemispheric bleeding in the most severe cases. Shaking is an acceleration/deceleration type of injury similar to whiplash, but of longer duration. Both accidental and experimentally induced whiplash injury have been shown to produce diffuse axonal injury, with varying degrees of severity (Gennarelli, 1983; Adams et al., 1989; Liau et al., 1996). Diffuse axonal injury (DAI) is best demonstrated histologically through the visualization of axonal swellings and retraction bulbs, but can also be inferred from MRIs, which identify hemorrhagic lesions and focal areas of necrosis in cerebral white matter, the corpus callosum, and dorsal brain stem. As with any type of injury, DAI is more than likely triggered by the primary injury and then exacerbated by secondary biochemical events. Repeated MRI/CT scans lend support to this notion by demonstrating diffuse axonal injury and progressive cortical atrophy in severely abused children subjected to shaking alone or shaking plus impact injury (Alexander et al., 1989; Spaide et al., 1990; Francel et al., 1996). In the present model, shaking + hypoxia triggered progressive cortical degeneration which continued up to 14 days after the initial injury. This was demonstrated by a significant reduction in cortical wet weights over time, a measure that has been shown to correlate with the percent of injured neurons (Grafe, 1994) and size of the cortical infarct (Hagberg et al., 1994) produced by hypoxic-ischemic insult in the neonatal rat. Although we did not directly show, through histological examination, that shaking + hypoxia causes DAI in the present model, it is certainly a likely possibility. It should be pointed out that the early signs of DAI (e.g., axonal swellings, retraction bulbs) may be difficult to demonstrate in the neonatal rat because the brain is undergoing rapid development in terms of myelination and the growth of nerve fibers, particularly in respect to diameter. Nonetheless, this type of study is needed to completely characterize the model.

In summary, we have developed an infant rat model of the shaken baby syndrome characterized by significant brain hemorrhaging and progressive cortical degeneration similar to that seen in severely abused children. The present results suggest that free radicals play a critical role in the pathophysiology of secondary brain hemorrhaging, but may not be of prime importance in the mediation of the subsequent cortical neurodegeneration.

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