A new model of diffuse brain injury in rats

Part II: Morphological characterization

MONTASSER A. ABD-ELFAATTI FODA, M.S., M.D., AND ANTHONY MARMAROU, PH.D.

Richard Roland Reynolds Neurosurgical Research Laboratories, Division of Neurosurgery, Medical College of Virginia, Richmond, Virginia

A new model producing diffuse brain injury, without focal brain lesions, has been developed in rats. This has been achieved by allowing a weight of 450 gm to fall onto a metallic disc fixed to the intact skull of the animal which is supported by a foam bed. Two levels of injury were examined by adjusting the height of the falling weight to either 1 m or 2 m. Two groups of animals were studied. Group 1 animals were separated into three subgroups: 10 received a 1-m weight drop, 58 received a 2-m weight drop, and 13 served as controls; all were allowed to breathe spontaneously. Group 2 animals were separated into the same subgroups: four received a 1-m weight drop, six received a 2-m weight drop, and four served as controls; all of these were mechanically ventilated during the procedure. In Group 1, morphological studies using light and electron microscopy were performed at 1, 6, 24, or 72 hours, or 10 days after insult; all Group 2 rats were studied at 24 hours after injury.

Results from Group 1 animals showed that no mortality occurred with the 1-m level injury, while 59% mortality was seen with the 2-m level injury. On the other hand, no mortality occurred in Group 2 animals regardless of the level of trauma induced. However, the morphological changes observed in both groups were similar. Gross pathological examination did not reveal any supratentorial focal brain lesion regardless of the severity of the trauma. Petechial hemorrhages were noticed in the brain stem at the 2-m level injury. Microscopically, the model produced a graded widespread injury of the neurons, axons, and microvasculature. Neuronal injury was mainly observed bilaterally in the cerebral cortex. Brain edema, in the form of pericapillary astrocytic swelling, was also noted in these areas of the cerebral cortex and in the brain stem. Most importantly, the trauma resulted in a massive diffuse axonal injury that primarily involved the corpus callosum, internal capsule, optic tracts, cerebral and cerebellar peduncles, and the long tracts in the brain stem. It is concluded that this model would be suitable for studying neuronal, axonal, and vascular changes associated with diffuse brain injury.

KEY WORDS • head injury • experimental model • neuronal injury • traumatic brain edema • diffuse axonal injury • immunocytochemistry • rat

SEVERELY head-injured patients may suffer from widespread brain damage secondary to trauma in the absence of focal mass lesion, which is not a consequence of herniation or perfusion failure. This type of diffuse brain injury has long been emphasized as the most common cause of a persistent vegetative state and severe disability after closed head injury.12,24 Adams, et al.,1,16 observed this type of brain injury in 13% to 28% of fatal head injuries and they introduced the term “diffuse axonal injury” to express its pathological nature. Gennarelli, et al.,29 in a multicenter study, indicated that 43% of severely head-injured patients suffered a diffuse brain injury. More recently, Marshall, et al.,30 found that 55% of patients with severe head injury in the Traumatic Coma Data Bank suffered from diffuse head injury, of whom 12.6% had an entirely normal computerized tomography (CT) scan. In patients with diffuse head injury, CT shows either diffuse brain swelling, occasionally associated with petechial hemorrhage, or an entirely normal brain.18,43,50,51

Unfortunately, studying this type of diffuse brain injury in animals has been difficult as the currently available models of severe head trauma essentially produce a focal brain contusion more than a diffuse injury.3,13,14,17,27,31,32,41,42 In addition, laboratory studies of angular acceleration, which have been shown to produce diffuse axonal injury, have been curtailed.21,23,33 The goal of our research was to produce a new model of closed brain injury in rodents with combined impact and acceleration.26 This report documents the morphological characterization of the rodent brain exposed to this form of brain injury.
Materials and Methods

Injury Device

A simple device was designed to induce blunt trauma to the protected skull of the rat. A cylindrical column of brass weighing 450 gm was allowed to fall through a clear Plexiglas tube onto a small rounded stainless-steel disc fixed to the central portion of the skull vault of the rat. The severity of trauma was adjusted by changing the height of the falling weight. Figure 1 demonstrates the trauma device, the site of the metallic disc on the skull of the rat, and the relation of the rat to the trauma device just before and after the impact. The biomechanics and pathophysiological changes of this model have been discussed in a companion report.29

Induction of Head Trauma

Ninety-five Sprague-Dawley rats, each weighing 350 to 375 gm, were studied. All rats underwent general anesthesia using a mixture of isoflurane (1% to 2%), nitrous oxide (66%), and oxygen (33%). A midline scalp incision was performed followed by perioveal elevation to expose the central area of the skull vault between the coronal and lambdoid sutures. A stainless-steel disc 1 cm in diameter was firmly fixed by dental acrylic to this central portion of the skull vault. When the trauma device was ready, the rat was placed in the prone position on a foam bed with the disc centered immediately under the lower end of the Plexiglas tube of the trauma device. Two belts were fastened around the trunk of the rat to prevent it from falling off the foam bed after trauma induction. The weight was allowed to drop freely from the designated height through the Plexiglas tube onto the disc; the foam bed together with the rat were moved away from underneath the tube immediately after impact to ensure a single hit. The rat was then transferred back to the operating table and observed for a couple of minutes. The skull vault was inspected for the presence of any fracture. The scalp was sutured and the rat was allowed to recover from anesthesia. Rats that died on impact and those with skull fractures were excluded from the morphological study, although they were subjected to immediate postmortem gross pathological examination.

Animals in the control groups were surgically prepared for impact in the same way as above, but were not subjected to the head trauma.

Animal Groups

Two groups of animals were studied. Group 1 animals were allowed to breathe spontaneously during the procedure and Group 2 animals received mechanical ventilation.

Spontaneously Breathing Animals (Group 1). To investigate the effect of diffuse head trauma on animals without respiratory assistance, the 81 rats in Group 1 were anesthetized via a mask and were allowed to breathe spontaneously during and after the impact. After the metallic disc was fixed to the vault of the skull, the rat was disconnected from the anesthesia mask and within 30 seconds was placed on the foam bed and injured as described above. The rat was reconnected to anesthesia within 5 seconds after trauma and observed

Fig. 1. Photographs demonstrating the steps for inducing diffuse brain injury using our impact acceleration model. A: The trauma device is a simple one consisting of brass weighing 450 gm (straight arrows) falling by gravity through a Plexiglas tube onto the protected skull vault of the rat that lies prone on a foam bed (curved arrow). B: The rat received general anesthesia via a mask. A small metallic helmet was then fixed to the central part of the skull vault using dental acrylic. C: When the trauma device was ready, the rat was disconnected from the anesthesia mask and rapidly positioned on the foam bed with the helmet centered underneath the lower end of the Plexiglas tube. From the designated height, the weight was allowed to fall freely on the helmet. D: Immediately after the first hit, the foam bed with the rat on it was rapidly moved away from underneath the tube to prevent the weight from bouncing on top of the metallic disc. Note that the rebound of the weight was so high that it did not appear in the picture.
Diffuse brain injury model morphology

for the return of spontaneous respiration. Survivors were allowed to recover from anesthesia and followed during the designated period of survival.

Mechanically Ventilated Animals (Group 2). In a companion report, it was observed that mortality in severely injured animals decreased from approximately 50% to zero when animals were intubated and mechanically ventilated during the impact. A second group of 14 animals was intubated and mechanically ventilated during and after the impact to investigate the effects of diffuse head trauma on respiratory-assisted animals and the possible causes of the high mortality rate observed in spontaneously breathing animals. The rats in this group were allowed to recover within 10 to 15 minutes following impact.

Brain Fixation

Survivors from Group 1 were separated into three subgroups: 10 rats received a 1-m injury (Group 1A); 24 rats received a 2-m injury (Group 1B); and 13 rats received a sham injury (Group 1C). The rats in each subgroup were sacrificed and studied after 1, 6, 24, or 72 hours, or 10 days after insult. Similarly, survivors from Group 2 were also separated into three subgroups: four rats received a 1-m injury (Group 2A); six rats received a 2-m injury (Group 2B); and four received a sham injury (Group 2C). All animals in Group 2 were studied 24 hours after insult.

At the designated period of survival in both sets, the rat was deeply anesthetized in a controlled chamber using a mixture of isoflurane (5% to 10%), nitrous oxide (66%), and oxygen (33%). The chest was rapidly opened, a perfusion catheter was introduced into the ascending aorta, and the right atrium was incised. Perfusion pressure was monitored throughout the procedure and maintained at a pressure of 80 to 90 mm Hg. At first, 200 ml normal saline was perfused during 2 minutes, followed by 400 to 500 ml fixative perfused over a period of 20 minutes. The fixative used was either 10% formaldehyde in 0.1 M sodium phosphate buffer (for light microscopy (LM)) or 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (for electron microscopy (EM) and immunocytochemistry). The brain was carefully removed and placed in the same fixative for 4 hours, after which it was examined for gross morphological changes.

Light Microscopic Procedure

For LM studies, brains were sectioned either in the coronal or sagittal planes, before being embedded in paraffin. Sections 7 μm thick were cut with a rotary microtome and stained with hematoxylin and eosin (H & E). To confirm the early neuronal changes, additional sections were stained with 0.1% cresyl violet and then with 1% acid fuchsin fortified with several drops of glacial acetic acid. Another set of sections were prepared with Bielschowsky stains to verify the axonal changes.

Electron Microscopic Procedure

For EM, sections 100 μm thick were cut in the coronal plane using a Vibrotome, postfixed in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol and propylene oxide, then embedded in Epon 812. Samples were taken from areas of interest that included: the parasagittal supratentorial cortical areas posterior to the level of the foramen Monro, the brain stem, and the cerebral and cerebellar peduncles. Semithin sections 1 μm thick were cut from these samples with a glass knife and were stained with toluidine blue for LM studies. Next, 50-nm thin sections were cut with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 10 electron microscope.

Immunocytochemical Procedure for Neurofilament Antibody

Recently, antibodies to the 68-kD neurofilament subunit have been used for the detection of axonal change following traumatic brain injury. We employed 68-kD immunoreactivity to confirm and map axonal injury in our model, in both respiratory-assisted and spontaneously breathing animal groups.

For this approach, brains were cut into 50-μm sections in the sagittal plane. Sections were first rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.2) three times for 10 minutes each. Blocking of nonspecific binding was achieved with 10% normal horse serum (NHS) which had been heated, treated, and ultrafiltered, then dissolved in PBS for 60 minutes. The blocking solution contained 0.1% Triton X-100 for EM sections and 0.3% Triton X-100 for LM sections. The sections were incubated with agitation in the 68-kD neurofilament monoclonal antibody, diluted 1:2000 with 1% NHS in PBS (1% NHS/PBS), for 16 to 18 hours at 4°C. Subsequently, after three 10-minute rinses with 1% NHS/PBS, the sections were reincubated for 60 minutes in biotinylated antimouse immunoglobulin G, diluted 1:200 in 1% NHS/PBS. After a brief rinse with PBS, the sections were immersed in avidin-biotin peroxidase complex at room temperature for 60 minutes. The sections were then rinsed three times with PBS for 10 minutes each, followed by two rinses of 10 minutes each in 0.1 M phosphate buffer (PB). Immunolabeled peroxidase was visualized by immersing the section in freshly prepared 0.05% 3,3-diaminobenzene tetrahydrochloride (DAB) and 0.1 M PB for 10 minutes, followed by 3% hydrogen peroxide, diluted 3:1000 in 0.05% DAB, for 10 to 20 minutes according to the progress of the reaction as seen in LM. Lastly, sections were rinsed three times for 10 minutes each in 0.1 M PB.

Sections prepared for LM were then mounted on gelatin-coated slides, dehydrated in ascending concentrations of ethanol followed by xylene, and coverslipped. Sections for EM were osmicated, dehydrated, em-
bedded on plastic slides in Medcast resin, cut in semithin and ultrathin sections, stained, and examined following exactly the same procedure as we have described above.

Results

Clinical Observations

Spontaneously Breathing Animals (Group 1). All 10 animals receiving a 1-m injury (Group 1A) survived the trauma and had no skull fracture, so animals injured from this height were considered as the "mild brain injury group." Immediately after the head trauma, occasional rats in this group developed apnea for a few seconds, mild generalized convulsions that lasted for several seconds, and/or signs of decortication (manifested as bilateral flexion deformity of the fingers and wrist joint in the forelimbs). In addition, recovery from anesthesia was delayed for several minutes compared with control animals. However, the signs of decortication improved clinically within 1 or 2 days after injury.

On the other hand, 34 (58.6%) of the 58 rats with a 2-m injury (Group 1B) died either rapidly within a few minutes after trauma (91%) or after they had survived prolonged unconsciousness that lasted for several hours (9%). Because of this high mortality rate, rats injured from the 2-m height were considered as the "severe brain injury group." Immediately after trauma, all rats in this group had a period of apnea for 10 to 20 seconds that was associated in the majority of animals with severe generalized convulsions that lasted for 15 to 30 seconds. These convulsions occurred only once immediately after trauma. In addition, all rats in this group developed a decortication flexion deformity in their forelimbs. In the 24 survivors, these manifestations improved over a period of 3 to 5 days. In addition, we observed that recovery from anesthesia in this group was more protracted (30 minutes to 2 hours) than that noted in either the control or the mild brain injury groups.

Mechanically Ventilated Animals (Group 2). In Group 2, all animals injured from either 1 or 2 m survived the trauma. The convulsions observed in these animals were less severe than those observed in the spontaneously breathing group. However, the course and severity of other posttraumatic clinical manifestations, including delayed recovery from anesthesia and the decortication flexion deformity observed in this group, were similar to those noted in Group 1.

Pathological Changes

Although the gross and microscopic pathological changes observed in both Groups 1 and 2 were related to the level of trauma induced, these observations did not differ with the method of ventilation employed. Hence, the following pathological findings represent both groups of animals and they will be presented with relation to the severity of trauma.

Gross Pathological Observations. In animals injured from the 1-m level, apart from a mild subarachnoid hemorrhage (SAH) in the basal cisterns, the brains looked normal without contusion or focal lesions. In animals injured from the 2-m level, SAH was massive and extensive, occupying the basal cisterns and cisterna magna and extending to the subarachnoid spaces over the cerebral hemispheres. Intraventricular hemorrhage was also noted in the majority of animals in this group. Severely injured brains showed no focal lesion other than petechial hemorrhages, which were frequently observed in the dorsal portions of the lower brain stem and occasionally in the midbrain (Fig. 2). Congestion, in the form of diffuse pinkish coloration and engorged vessels, was also seen in the cortical areas of the severely injured brains examined within 72 hours of injury.

Gross pathological examination of brains from the 29 animals that died from the impact, without skull fracture, showed the presence of subarachnoid, intraventricular, and brain-stem petechial hemorrhages similar to those observed in the survivors of the 2-m injury. No additional focal brain contusions or hemorrhages could be found in this group of animals. The
other five animals that died on impact suffered from a transverse fracture perpendicular to and crossing the sagittal suture. The subarachnoid, intraventricular, and brain-stem petechial hemorrhages in these five animals were also similar to those observed in the survivors of the 2-m brain injury. In addition, these animals suffered from epidural hemorrhage due to injury of the superior sagittal sinus and cortical brain contusion located directly underneath the fracture site. Death in these animals would be related to injury of the superior sagittal sinus.

Microscopic Changes. In spite of these minimal gross pathological findings, microscopic changes of the neurons, axons, astrocytes, and small blood vessels were striking both in the mild and severe brain injury groups. However, these changes were more severe and much more extensive in the severe brain injury group animals.

Neuronal Changes. Neuronal changes were quite common in the supraventricular areas of the cerebral cortex of both mild and severe brain injury groups. However, these changes were directly related to the severity of the trauma induced. In other words, the neuronal changes in severely injured animals were more severe than those observed in the mildly injured ones (Fig. 3).

The central portion of the area involved was located under the site of the metallic disc. In sections prepared with H & E or acid fuchsin and cresyl violet stain, "pink shrunken neurons" associated with perineuronal vacuolation could be observed in these areas as early as 1 hour after trauma. In addition, dark contracted neurons with corkscrew-like processes were observed in the same sections stained with either H & E or acid fuchsin and cresyl violet. It was observed that the density of both pink and dark neurons gradually reduced as one approached the normal cortical areas at the periphery (Fig. 4). Neither pink nor dark neurons could be seen in the section from the control animals or from injured animals examined 10 days after trauma.

Under EM, a group of neurons in these cortical areas were observed with coarse clumping of hyperchromatic nuclear chromatin and with granular or amorphous gray cytoplasm. In these neurons, the nuclear membrane and later the plasma membrane could not be identified (Fig. 5A). Another set of contracted hyperchromatic neurons with pronounced electron density could also be observed in these areas. However, the nuclear and cytoplasmic membranes and the cytoplasmic organelles of such neurons were found to be intact (Fig. 5B).

Parenchymal Microvascular Changes. Parenchymal microvascular changes following this form of impact acceleration of head trauma consisted of brain edema and vascular congestion. These changes were evident after either the mild or the severe level of trauma. However, they were also directly related to the severity of the trauma in that the brain edema and vascular congestion observed in the severely head-injured rats were much more extensive than those found in mildly injured rats.

Two forms of brain edema were observed. In all head-injured rats, pericapillary brain edema was noted at the LM level in the supraventricular cortical areas under-
FIG. 4. Photomicrographs of the cerebral cortex under the site of impact of a severely brain-injured animal (450-gm weight dropped 2 m) 24 hours after trauma. H & E, × 670. A: In the central part of the injured area, neurons were pink, shrunken, and surrounded by perineuronal vacuolation (open arrows), while capillaries were congested with red blood cells (solid arrows) and associated with pericapillary astrocytic swelling (arrowheads). B: At the periphery of this injured area, the density of injured neurons and capillaries decreased and normal neurons could be observed as well. C: Neurons and capillaries looked normal in cortical areas distant from the injured area.

FIG. 5. Electron micrographs of injured neurons in the cerebral cortex of severely brain-injured rats. × 7410. A: A group of these injured neurons developed the electron micrographic characteristics of cell death including the amorphous appearance of the cytoplasm, the presence of flocculent densities seen in the mitochondria (open arrowheads), and the disappearance of the nuclear membrane with the escape of coarse clumps of nuclear chromatin (closed arrowheads) in the cytoplasm. A macrophage (M) was also seen in the same field. B: Another set of dark neurons was observed to have intact nuclear membranes (arrows) and cytoplasmic organelles; however, they were surrounded with perineuronal edema (asterisk) and they had swollen mitochondria (arrowheads).
Diffuse brain injury model morphology

Fig. 6. Electron micrographs of the capillary changes in the injured animals. A: Brain edema in the form of pericapillary astrocytic swelling (asterisks) was observed in the cerebral cortex. × 6800. B: In the white matter (corpus callosum) widening of the extracellular space by the edema fluid (open asterisks) could also be seen. × 2500. C: At higher magnification, tight junctions and basement membranes of the capillary endothelium (asterisk) were ultrastructurally intact. × 66,000. D and E: The lumina of many capillaries in the cerebral cortex were disfigured and narrowed by vasoconstriction or by the surrounding edema. × 4000.

neath the metallic disc, in association with injured neurons (Fig. 4). In addition, this perivascular edema was frequently observed in the thalamus and brain stem of severely injured animals only. At the EM level, this perivascular edema was found to be a marked swelling of the pericapillary foot processes of the astrocytes (Fig. 6A). However, the endothelial cells, basement membranes, and intercellular tight junctions of these capillaries were morphologically normal (Fig. 6C).

The second type of brain edema was widening of the extracellular space, frequently observed in the corpus callosum of severely injured animals (Fig. 6B). Both forms of brain edema could be seen as early as 6 hours after trauma, and appeared to reach a maximum after 24 hours. Thereafter, the edema resolved in a few days. However, brain edema lasted for a longer period in the severely injured rats compared to those with mild injury.

In addition to the neuronal injury and brain edema observed in the supraventricular cerebral cortex, several capillaries were noted on LM to be congested, with sludging red blood cells (Fig. 4A and B). It was observed on EM that the lumen of many capillaries
in this area of cerebral cortex was narrowed by vasoconstriction (Fig. 6D) and/or by perivascular edema (Fig. 6E).

**Axonal Changes.** Massive diffuse axonal swelling was observed in this model as early as 6 hours after injury, reaching a maximum after 24 hours. The severity of diffuse axonal injury was also directly related to the level of head trauma induced (Fig. 7). In sections stained with the conventional LM stains (H & E and Bielschowsky stains), diffuse axonal injury in the form of retraction balls could be seen in the long tracts of the brain stem (especially in the pyramidal, rubrospinal, gracilis, and cuneate tracts). These retraction balls continued to be visible, although smaller in size and number, until the 10th day after trauma.

Employing antibodies to the 68-kD neurofilament subunit was very useful not only in the anatomical localization of diffuse axonal injury, but also in the demonstration of its initial pathogenesis. Based on the 68-kD immunoreactivity, axonal injury was observed in the optic tracts (Fig. 8A), cerebral peduncles (Fig. 8B), superior cerebellar peduncles (Fig. 8C), rubrospinal tract (Fig. 8D), corticospinal tracts in the pons and medulla oblongata (Fig. 8E and F), and in the pyramidal decussation in the medulla oblongata (Fig. 8G-I). Injured axons could also be observed, although to a lesser extent, in the corpus callosum, internal capsule, and the spinothalamic, gracilis, and cuneate tracts.

Different stages of diffuse axonal injury could be recognized 24 hours after trauma in the same animal. Thus, some injured axons were found at the stage when they were swollen due to focal impairment in the anterograde transport before disconnection (Fig. 8E and F), others had already been disconnected (Fig. 8G), still others showed accumulation of the organelles in the peripheral parts of the axons with internalization of the 68-kD immunoreactive neurofilaments to the core of the axon (Fig. 8H), and in others rupture was imminent (Fig. 8I). Diffuse axonal injury was also observed in the mildly head-injured rats (Fig. 8J). This sequence of morphological changes in the injured axons (axonal constriction and swelling, axonal disconnection, internalization of neurofilaments with the aggregation of the other axonal organelles toward the periphery, disruption of axonal balls, and lastly, the phagocytosis of dead axons) was more easily appreciated at the EM level (Fig. 9).

**Discussion**

**A Diffuse Head Injury Model**

The modeling of diffuse brain injury in experimental research has been challenging. As Adams, et al.,\(^1\)^ have stated, it is unfortunate that so much emphasis has to be placed on focal lesions in defining the severity of a diffuse process. This criticism is applicable to the majority of currently available head trauma models. In many of these models, trauma is subjected to the intact skull, producing depressed skull fracture with a focal brain contusion and/or laceration.\(^{32,41}\) In other such models, including the widely used fluid-percussion injury model, the high-velocity impacts to the exposed dura produce ipsilateral focal cortical contusions.\(^{15-17,19,27,31,42,49}\) Thus, due to the focal nature of lesions produced, results derived from these models have been criticized because of their inability to faithfully replicate the range of diffuse axonal injuries seen in man.\(^1\) Experimental diffuse brain injury identical to that occurring in man has been produced by subjecting the head of the monkey to angular acceleration,\(^{21,23,33}\) but this model has not been widely used.

In our model, an impact acceleration was designed to be directed to the protected skull of the rat, thus avoiding focal injuries of the cerebral cortex that could result from either a depressed skull fracture or directing the trauma to the dura mater. The foam bed, which is key to the model, allows freedom of movement and acceleration.

In severely head-injured animals, although the mortality rate dramatically decreased from 59% in spontaneously breathing rats to zero in mechanically ventilated ones, the severity of posttraumatic clinical observations and pathological changes in both respiratory groups was similar. In a companion study,\(^29\) fol-
Diffuse brain injury model morphology

A wide-spread diffuse axonal injury was observed in the severely brain-injured rats 24 hours after injury. Examples of the tracts involved are shown in the optic tract (A); cerebral peduncles (B); superior cerebral peduncles (C); rubrospinal tract (D); corticospinal tract in the pons (basis pontis) (E and F); and pyramidal decussation (G, H, and I). At severe head-injury levels, different stages of diffuse axonal injury could be seen in the same animal where some axons were swollen and showed marked constriction but were still intact (white arrows), others had already disconnected (black arrow), others formed retraction balls (white arrowheads), others showed internalization of the immunoreactive neurofilaments with migration of organelles toward the periphery, forming a nonimmunoreactive cap (black arrowhead), and in some others rupture was imminent (open black arrow). Diffuse axonal injury was also observed in the mild brain-injured rats (J), but was not as pronounced as that in the severely head-injured group.

Fig. 8. Photomicrographs with 68-kD immunoreactivity. × 340.
lowing trauma in all spontaneously breathing severely injured animals, a progressive increase in pCO₂ levels was observed that was much more dramatic and rapidly reached lethal levels in those that died. These changes in blood gas levels could be easily avoided by mechanical ventilation. In addition, brain-stem auditory evoked potentials of these severely head-injured animals were intact with only a transient inconsistency in wave IV. All of these observations relate the high mortality rate in spontaneously breathing animals (Group 1B) to a transient central respiratory dysfunction that was readily reversible by respiratory support.

The finding of brain-stem petechial hemorrhage in severely injured rats in our study is not new and has been reported in both human and animal studies. However, our observations at the microscopic level support the notion that these brain-stem petechial hemorrhages do not occur in isolation and are part of a wide spectrum of neuronal, axonal, and microvascular abnormalities. In addition, although the mildly brain-injured animals in our study did not suffer brain-stem petechial hemorrhage, they did develop neuronal, axonal, and microvascular abnormalities similar to, but less extensive than, those observed in the severely injured rats. The development of diffuse axonal injury caused by a falling weight in our study and that reported in humans as a result of a fall, indicates that impact acceleration would represent another mechanism of diffuse brain injury besides the previously suggested angular acceleration.

**Neuronal Injury in the Cerebral Cortex**

While the presence of pink (acidophilic) neurons has been considered a sign of neuronal death, the interpretation of dark neurons seen in the cerebral cortex is controversial. Some investigators have considered the dark and contracted appearance of neurons to be the hallmark of an artifactual change related only to poor fixation. On the other hand, several other reports stated that such neurons represent a reversible neuronal injury after hypoglycemia.

In our study, the presence of dark and pink neurons was limited to the cortical areas located underneath the site of impact and was associated with perivascular brain edema and vascular congestion. At the EM level, the finding of two groups of neurons with increased electron density in these cortical areas (one with intact nuclear and cytoplasmic membranes and the other with large defects in their membranes) matches the observations of others who considered these neurons to represent reversibly injured and dead disintegrating neurons, respectively. Furthermore, the presence of macrophages in association with these injured neurons, and the absence of dark and pink neurons in the supratentorial cortical areas 10 days after injury would indicate the removal of dead neurons by phagocytosis and the recovery of the reversibly injured neurons by that time, a finding observed in other head-injury models. These observations further suggest that the neuronal response to diffuse trauma would vary, where some neurons (pink neurons) would be irreversibly injured, others (dark neurons) would be injured but able to recover, and the remaining would be morphologically intact.

We have found that, when morphological studies were conducted on animals injured by our model 6 weeks after impact, there is significant reduction in neuronal density (number of neurons/sq mm) and reactive astrocytosis in the supratentorial cerebral cortex in a severity that was directly related to the degree of head injury (unpublished data). These late observations represent the sequelae of the neuronal injury observed in the supratentorial cortex in the first few days after impact.

**Brain Edema Formation**

In addition to the overt forms of traumatic vascular injury, posttraumatic brain edema formation in the absence of parenchymal hemorrhage has been reported and is considered as a subtle form of injury to the blood-brain barrier. Two forms of brain edema, astrocytic swelling in cerebral cortex and extracellular edema in the white matter, could be produced by other models of head trauma.

In our study, pericapillary astrocytic swelling was observed primarily in areas of the cerebral cortex located underneath the metallic disc in association with injured neurons, while capillaries, astrocytes, and neurons in the rest of the cerebral cortex were morphologically intact. The severity and extension of this astrocytic swelling were directly related to the degree of head trauma. In addition, astrocytic swelling could not be observed in the injured animals 10 days after trauma or in control animals. These observations indicate that astrocytic swelling in our material developed as a result of posttraumatic brain edema. Furthermore, the occurrence of neuronal injury and brain edema in the cortical areas directly underneath the site of impact would represent a coup injury that is only evident at the cellular level.

Our observation that brain edema was evident as early as 6 hours after injury, reaching a maximum after 24 hours, supports the findings reported by van den Brink, et al., who determined the extent of brain edema in the same model by measuring the brain water content. However, the absence of obvious morphological abnormalities in the blood-brain barrier in our study together with the development of this edema in the absence of a blood-pressure surge would suggest either a physiological dysfunction of the blood-brain barrier in the first few hours after injury or cytotoxic edema. Further investigation is necessary to clarify the mechanisms responsible for the posttraumatic brain edema in this model.

A recent report demonstrated the development of ischemia in the first 12 hours in severely head-injured patients. They related this ischemia to vasospasm of the conducting arteries, which had been demonstrated in other angiographic studies and confirmed by transient Doppler ultrasound studies. Bouma, et al., treated their severely head-injured patients with induced hypertension; this therapy was beneficial in one group of patients but not effective in another group.

M. A. A. Foda and A. Marmarou

J. Neurosurg. / Volume 80 / February, 1994
FIG. 9. Electron micrographs demonstrating different stages of diffuse axonal injury at severe injury levels 24 hours after trauma.  A: Sagittal section of a markedly swollen axon with an early indentation of its myelin sheath (arrowheads). Note the uptake of the 68-kD antibodies by the electron-dense neurofilaments. × 8000.  B: Sagittal section of a swollen axon showing marked indentation of its myelin sheath (open arrows). The axon had disconnected and the organelles had been accumulated in the swollen proximal segment (black arrow). × 2500.  C: Plain electron micrograph of an axial section of a swollen axon showing the internalization of neurofilament (asterisk) with the accumulation of the organelles in the periphery (curved arrow). × 6800.  D: Sagittal section of a markedly swollen axon showing disruption of its myelin sheath (small arrows). Macrophages (M) are seen inside the myelin sheath. × 2000.  E: Macrophages (M) have migrated within the myelin sheath of an injured axon engulfing its dead debris. × 5000.
In our study, the vasospasm of arterioles and capillaries frequently observed in severely head-injured rats was not the sole etiological factor for brain ischemia. In addition, the lumen of many capillaries was compromised and disfigured by the surrounding edema, and trapping of red blood cells in the narrowed capillaries was observed in the supraventricular cortical areas in association with injured neurons. Diffuse axonal injury has been described by various authors.31,32,33

**Diffuse Axonal Injury**

To the best of our knowledge, this is the first trauma model that induces a widespread axonal injury in rats. Recent reports have shown that, in the rat, the use of fluid-percussion and cortical impact models of traumatic brain injury have yielded relatively few damaged axons.36,37

Adams, et al., classified diffuse axonal injury into three grades: Grade 1 was characterized by histological evidence of axonal injury in the white matter of the cerebral hemisphere, corpus callosum, brain stem, and (less commonly) the cerebellum; Grade 2 was associated with focal lesion in the corpus callosum; and in Grade 3 there was an additional focal lesion in the dorsolateral quadrants of the rostral brain stem.3 In our model, mildly injured rats are consistent with Grade 1 diffuse axonal injury as, in these animals, axonal injury involved the cerebral hemisphere and brain stem, and no focal lesions were observed in the corpus callosum or the brain stem. Further, our severely injured rats can be categorized as having a Grade 3 diffuse axonal injury, where axonal abnormalities were more global and involved the cerebellum, and petechial hemorrhages were observed in the brain stem. However, the absence of petechial hemorrhage in the corpus callosum in these severely injured rats might be related to the mechanics of trauma used in our model (that is, oriented in the sagittal plane), where it has been shown that focal lesions in the corpus callosum could be produced only by a head acceleration oriented in the coronal plane and not in the sagittal plane.21

From our findings, we share Povlishock’s observation27 that axons decussating in their intra-axial course, changing anatomical direction to target nuclei, or taking oblique turns around penetrating vessels appear most prone to damage. However, we have to emphasize that the diffuse axonal injury in our model was not limited to the large-caliber axons, as was noticed by others,14,35,36 but involved axons of different calibers. Finally, our observations in the mildly head-injured animals also support findings by others that minor head trauma could produce diffuse axonal injury and hence it could be associated with subtle neurological dysfunction.23,34,38

**Conclusions**

A new model of graded severity for diffuse brain trauma has been developed in rats. The model is economical and technically simple, and produces diffuse axonal injury similar to that described in man. This type of brain trauma produces graded widespread subtle abnormalities of the neurons, axons, and blood-brain barrier that are more easily appreciated at the ultrastructural level. Further investigations are required to complete the scenario of diffuse brain injury.

**Acknowledgments**

We acknowledge the advice of William Rosenblum, M.D., John Povlishock, Ph.D., and Larry Jenkins, Ph.D., during this research, and also the assistance of Mrs. Susan Walker and Mrs. Virginia Godsey in preparation of the study materials.

**References**

Diffuse brain injury model morphology


Manuscript received July 21, 1992. Accepted in final form July 26, 1993.
This work was supported by National Institutes of Health Grants NS-19235 and NS-12587 and by Grant H133BB0029 from the National Institute on Disability and Rehabilitation Research. Additional facilities and support were provided by the Richard Roland Reynolds Neurosurgical Research Laboratories and Margaret Harrison Reynolds.
Address reprint requests to: Anthony Marmarou, Ph.D., Division of Neurosurgery, Medical College of Virginia, Box 508, MCV Station, Richmond, Virginia 23298.