A Histopathological and Immunohistochemical Study of Traumatic Diffuse Axonal Injury in Albino Rats

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Abstract:
Diffuse axonal injury (DAI) is a frequent result of traumatic deceleration injuries and a frequent cause of persistent vegetative state in patients. DAI is the most significant cause of morbidity in patients with traumatic brain injuries, which most commonly are the result of high-speed motor vehicle accidents. Axonal injury (AI) has been described as a phenomenon which can be induced as a diffuse change, i.e. diffuse axonal injury (DAI) caused by traumatic brain injury. It is only when obvious lesions are absent or minimal; the occult effect of DAI can be invoked. The aim of this study is to evaluate the vitality of the brain and approximately the survival time of rats subjected to sublethal head trauma, using some ordinary and immunohistochemical stains for the injured brains.

Sixty adult male albino rats were used in this study, 8 of them were kept as a control group and the others were subjected to sublethal head trauma under light ether anaesthesia. Then they were grouped after exclusion of cases with skull fracture or intracranial hemorrhage, according to their survival time into 4 groups: group I (0—<30 minutes), group II (0.5 hour—<2 hours), group III (2 hours—<4 hours) and group IV (4 hours—< 6 hours). Sections from different brain areas were prepared with different stains and examined.

Gross examination revealed petechial hemorrhages scattered in all brains, especially brain stem compared to the control group. Microscopic examination pointed to the possibility of detecting the neuronal injury as early as 0.5-2 hours using immunoreactivity against neurone specific enolase (NSE). Also B-amyloid precursor Protein (B-APP) immunoreaction could detect the axonal injury as early as 2-4 hours. Silver stain and H & E stains could reveal these neuronal traumatic changes at later times (4-6 hours). So, immunohistochemical stains are useful to detect neuronal changes for short survivors while ordinary stains are suitable to detect neuronal changes for victims surviving longer times after trauma. It is to be noted that the diffuse nature of these neuronal injuries is necessary for the diagnosis of traumatic head injury, since ischemic hypoxic insults can lead to localized neuronal changes. So, detection of DAI is helpful in the diagnosis of sudden obscure deaths, especially when occult head trauma is suspected.

Introduction:
Head trauma of varying severity may induce diffuse axonal injury. Half of the patients with severe head injury have diffuse axonal injury, but this type of injury also occurs in patients with moderate and mild head injury. The clinical presentation and prognosis will therefore vary.1 In many cases of TBI widespread disruption of the axons occurs through a process known as diffuse axonal injury (DAI) or traumatic axonal injury (TAI).2

The term DAT is a misnomer. It is not a diffuse injury to the whole brain, rather it is predominant in discrete regions of the brain following high-speed, long-duration deceleration injuries. DAI is a consistent feature of TBI from transportation-related injuries, as well as some sports injuries. The pathology of DAI in humans is characterized histologically by widespread damage to the axons of the brainstem, parasagittal white matter of the cerebral cortex, corpus callosum, and the gray-white matter junctions of the cerebral cortex. Computed tomography and magnetic resonance imaging scans taken initially after injury are often normal. The deformation of the brain due to plastic flow of the neural structures associated with DAI explains the micropathologic findings, radiologic findings, and medical and neuro-psychologcial complications from this type of injury mechanism. There is evidence that the types of cellular injury in TBI (DAI, anoxic, contusion, hemorrhagic, perfusion-reperfusion) should be differentiated, as all may involve different receptors and biochemical

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pathways that impact recovery. These differing mechanisms of cellular injury involving specific biochemical pathways and locations of injury may, in part, explain the lack of success in drug trials to ameliorate TBI. The pathophysiology of DAJ, was first described by Holbourn in 1943, using 2-dimensional gelatin molds. His work led to the understanding that shear injury is not induced by linear or translational forces but rather by rotational forces. Sudden acceleration-deceleration impact can produce rotational forces that affect the brain.

Histological studies revealed increased spacing, tearing of axons, impaired axoplasm transport and occurrence of hemorrhage to be strain and displacement rate dependent. Linear relationships existed between the increasing strain and the occurrence rate of axonal injury as evidenced by multiple indicators (IAT, hemorrhage, torn fibers or primary axotomy) at both rates. It has been indicated that the severity of both functional and structural injury increased with increases in strain and displacement rate. A characteristic feature of severe diffuse axonal injury in man is radiological evidence of the "shearing injury triad" represented by lesions, sometimes haemorrhagic, in the corpus callosum, deep white matter and the rostral brain stem. The lesions were most easily seen at 24 h when they appeared as foci of tissue rarefaction, in which there were a few polymorphonuclear leucocytes. At the margins of these lesions, large amounts of accumulated amyloid precursor protein (APP) were found in axonal swellings and bulbs. By 1 week post-injury, there was macrophage infiltration with marked astrocytosis and early scar formation. This lesion is considered to be due to severe deformation of white matter and this is the first time that it has been identified reproducibly in a rodent model of head injury under controlled conditions.

Immunohistochemistry using beta-amyloid precursor protein (APP) N-terminus antibodies is routinely used to detect traumatic axonal injury (TAI). In an immunohistochemical study, monoclonal antibody to 70 kilodalton neurofilament subunit was used in a standard Avidin-Biotin Complex Kit (DAKO). Microscopic findings revealed subarachnoid hemorrhage, lateral 3rd ventricular hemorrhage, and rarefaction and petechial hemorrhage of the local contusional lesion. In the medium level injury, there was a marked petechial hemorrhage in the corpus callosum and subependymal area. In the high level injury, there was marked edema in the white matter of the ipsi- and contralateral cerebral hemisphere, and multiple petechial hemorrhage in the brain stem and cerebellum. Microscopic findings in the corpus callosum, subependyma and brain stem in the vicinity of petechial hemorrhage revealed a large number of axonal swellings, but in these specimens only a few typical axonal retention balls were seen with Bodian and immunohistochemical stains.

The aim of this study is to detect the early neuronal injury, using ordinary and immunohistochemical stains, for evaluating the vitality of brain injury and estimating the survival time, which might help in the diagnosis of some obscure deaths.

Materials and methods

Animals:
Sixty adult male albino rats (150-200 gm each) were used in this study. They were housed under the standard laboratory environmental conditions as regard light, temperature and feeding for 5 days prior to the experiment.

Experimental design:
All animals were exposed to light ether anaesthesia. 8 of them were kept as a control group. Others were subjected to sublethal head injury; each rat was caught from the hind limbs, then its head was stroked once against the wall (knight, 1996 and Cotran et al., 1999). The tested animals were observed for apnea, seizures and recovery from anaesthesia then classified into 4 equal groups (13 rats each).

Group I: It included rats which died or sacrificed from 0-30 minutes after head injury.

Group II: It included the rats which died or sacrificed after 30 minutes till 2 hours from head injury.

Group III: It included the rats which died or sacrificed between 2-4 hours from head injury.

Group IV: It included the rats which died or sacrificed between 4-6 hours from head injury.

Methods:
After recovery from anaesthesia, the control animals were sacrificed. The skulls of all animals were exposed and observed for any fracture. The brain was extracted and observed for hemorrhage. Cases with skull fracture or
intracranial hemorrhage were excluded from the study. So, four rats were excluded from group I, three rats from group II, two rats from group III and three rats from group IV (table I).

The selected brains from different cases were fixed by formaline (10%) immersion for 2 weeks prior to microscopic examination. Blocks of brain tissue were cut from at least 3 regions; (cerebrum, cerebellum and brain stem) and embedded in paraffin. The paraffin sections were then subjected to the following stains.

1- Hematoxylin and eosin (H & E), according to Bencroft and Stevens (1977).
2- Silver stain, according to Drury and Wallington (1980).
3- Immunohistochemical stains:
   b) Anti-B-APP antibody: antibodies against beta amyloid precursor protein, according to the method of Sheriff et al. (1994).

**Results**

**Clinical observations:**

Most rats developed apnea for few seconds, mild generalized convulsions for several seconds and/or signs of decortication (bilateral flexion deformity of the fingers and wrist joints in the forelimbs) after trauma. Recovery from anaesthesia was delayed for several minutes compared with control animals.

**Gross pathologic findings:**

- Peticheal hemorrhages were found to be scattered throughout the brain, especially brain stem.
- Various degrees of congestion and cerebral swelling were seen in all brains of test groups.
- In control animals: no changes were seen in the brain.

**Microscopic pathologic changes** (fig. 1,2,3,4, 5, and table 1). The brain tissue stained with H & E stain in group I and II showed intact neurons compared with the control group (Fig. I). While that of group III and IV showed central chromatolysis (Fig. 2).

The brain tissue stained with silver stain showed intact axons in group I and II, whereas in group III and IV there were retraction balls in the form of oval shaped structures (Fig. 3).

Immune-reaction for NSE in group I (++++) was normal like the control group (Fig. 4) and it started to fade in group II (+++), group III (+) and completely disappeared in group IV.

- B-APP immuno-reactivity could not be visualized in both control and group I as it stains only the injured axons and needed 3 hours after injury to be detected. Immuno-reactivity for B-APP started to appear in group III and became more evident in group IV (fig. 5).

**Table (I): The reaction of different groups to different stains.**

<table>
<thead>
<tr>
<th>Group</th>
<th>A-NSE Reaction</th>
<th>B-APP Reaction</th>
<th>H&amp;E Stain</th>
<th>Silver Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>normal reaction (++++)</td>
<td>No reaction</td>
<td>Intact neurons</td>
<td>Intact axoni</td>
</tr>
<tr>
<td>Group I (n=9)</td>
<td>Normal (++++)</td>
<td>No reaction</td>
<td>Intact neurons</td>
<td>Intact axo</td>
</tr>
<tr>
<td>Group II (n=11)</td>
<td>Moderate (+++)</td>
<td>No reaction</td>
<td>Intact neurons</td>
<td>Intact axo</td>
</tr>
<tr>
<td>Group III (n=10)</td>
<td>Mild (+)</td>
<td>Moderate (+++)</td>
<td>Chromatolysis</td>
<td>Axonal balls &amp; injured axons</td>
</tr>
<tr>
<td>Group IV (n=10)</td>
<td>No reaction</td>
<td>Severe (++++)</td>
<td>Chromatolysis</td>
<td>Axonal balls &amp; injured axons</td>
</tr>
</tbody>
</table>
Fig. (I): Photomicrograph of brain tissue in group I, II showing intact neurons. (H. & E. x 200).

Fig. (2): Photomicrograph of brain tissue in group III, IV showing central chromatolysis. (H. & E. x 250).

Fig. (3): Photomicrograph of brain tissue in group III, IV showing retraction balls in the form of oval shaped structures (silver stain x 200).
Discussion:
Traumatic Brain Injury (TBI) is the consequence of the spatiotemporal pressure variations occurring inside the brain during head traumas. The spatial distribution of the pressure gradient (PG) is responsible for the cerebral lesions’ localisation and the consequent neurological signs. Beside skull's deformation caused by the contact loading and determining skull vibrations and/or fractures, current biomechanical theories concern two inertial phenomena: the linear acceleration and the rotational head movements. So, in this study the design of experiment was based on that observation, i.e. by striking the head of the rat against a wall.

In traumatic brain injury (TBI) rapid deformation of brain tissue leads to axonal
injury and cell death. In many significant head injuries, even if there is no visible gross damage to meninges or cerebral tissue, microscopic lesions may be demonstrated. It seems probable that even in relatively slight injury, causing only transient concussion, this occult damage also occurs (Knight, 1996). The lesions are due to shearing stresses within the soft brain substance, so that adjacent layers move relative to each other. The axons of neuron become broken and within a few hours spherical retraction balls form. The neuron may recover, but many will degenerate, which can be seen later as cellular or axonal reaction (chromatolysis).

The present study depended on the detection of the axonal reactions by different staining methods. In this study, we excluded cases with skull fracture or intracranial hemorrhage because many studies showed that these cases had no or little DAI. Also, the cause of death in these cases is obvious.

Our results showed that the different brain areas examined (Cerebrum, cerebellum, brain stem) were affected even away from the impact site. This indicates that the brain injury was diffuse in nature. Evidence for diffuse traumatic axonal injury (TAI) in clinical cases and animal models of traumatic brain injury (TBI) indicate that pathophysiological mechanisms extend to regions remote from the injury epicenter. The potential for indirect cerebellar trauma contributing to TBI pathophysiology is of significance, since impairment of motor function and coordination is a common consequence of TBI but is also a domain associated with cerebellar function. Axonal accumulation of amyloid precursor protein, indicative of traumatic axonal injury TAI, was observed in the corpus callosum and lateral aspects of the white matter above the site of impact, and in the thalamus. From a mechanical point of view, the interhemispheric formations and the rostral portion of the brainstem act as fixating structures for the cerebral hemispheres during rotational acceleration of the head. It is known that the motion of the cerebral hemispheres is delayed at the points of fixation, where greater stress would be produced, particularly on the side subjected to greater displacement. The frequent involvement by DAI of deep, centromedial brain structures, usually to one side of the midline, supports the mechanism proposed above.

Neuron-specific enolase (NSE) is a glycolytic enzyme that is localized primarily to the neuronal cytoplasm. Neuron-specific enolase (NSE) is a sensitive marker of brain injury after stroke, global ischemia, and coma. This enzyme is released into the CSF when neural tissue is injured. A serum NSE level of 21.2 ng/dL was 86% sensitive and 74% specific in predicting poor outcome. It appears that the serum NSE level can be used as a predictor of global short-term physical disability in children following closed TBI. The neuronal immuno-reactivity of NSE in this study started to be lost 30 minutes after injury, (group II). At 4–6 hours, (group IV), almost all injured neurons had lost their NSE immuno-reactivity.

Both, patients with and without invisible intracerebral pathology in CT scans exhibited elevated concentrations of NSE after TBI and a significant decrease in the follow-up blood samples. Release patterns of NSE differed in patients with primary cortical contusions, diffuse axonal injury (DAI), and signs of cerebral edema (ICP) without focal mass lesions. All serum concentrations of NSE were significantly correlated with the volume of contusions. The data of the present study indicate that the early release patterns of NSE may mirror different pathophysiological consequences of traumatic brain injury. In recent years, the immunohistochemical stain P-amyloid precursor protein (P-APP) has been used to assess the extent of axonal injury in a variety of pathological processes but in forensic practice is of greatest utility in the assessment of traumatic brain injury. Beta-amyloid precursor protein (beta-APP) is a marker of specific features of traumatic axonal injury. B-amyloid percursor protein (b-APP) is a low molecular protein which the normal values of are not to be found in axons detected by standard immunohistochemistry. It has been noticed that an increased frequency of appearance of this protein substance in axons changed by injury, while a reactive positivity to anti-body b-APP was to be found only rarely at the brain hypoxo without mechanical injury CNS. In our study, its immuno-reactivity started to appear 2–4 hours after head injury, (group III), and became marked in group IV. Diffuse traumatic axonal injury (TAI) in humans has been demonstrated by
beta-APP immunoreactivity in patients surviving at least 2 h after head injury. This could be explained by the slow accumulation of B-APP at damaged ends of axons, since normal axons are not immuno-reactive for B-APP.

The beginning of a new era in brain research came with the recognition that distinct silver-impregnated morphologic changes occurring in damaged axons could be used for tracing axon pathways in experimental animals with specifically placed lesions. Improvements in staining methods used to selectively impregnate the disintegrating axons but to leave normal axons unstained were achieved by Nauta and Gygax (early workers with these procedures) and spawned a host of method variations known as the “Nauta” methods. Of these, the Fink-Heimer and de Olmos cupric-silver methods were able to unambiguously demonstrate disintegrating synaptic terminals, thereby allowing complete tracing of axon pathways.

Suppressive A silver methods evolved from empirical observations about 50 years ago that argyrophilia of normal nerve fibers can be suppressed by a short period of oxidation of tissue sections, whereas degenerating nerve fibers in the same preparations were still clearly visible. It has been suggested that suppressive silver methods allow visualization of different processes of neuronal degeneration, and therefore may be a useful adjunct for identifying axotomy-induced neuronal degeneration. In this study axonal balls and injured axons could be detected 2-4 hours after injury, (group III), with silver stains as black structures and more manifest in group IV, i.e. 4-6 hours after injury. However, using Garvey's silver axon stain eosinophilic neurons were noted in many cases surviving < 1 hour after injury and increased in frequency and severity with time. On the other hand, longer time (12-18 hours) after injury with silver impregnation was recorded.

Moreover, the literature shows that the posttraumatic interval can be determined by other methods for demonstration of AI, such as by ubiquitin immunostaining (360 min), silver staining (15-18 h), hematoxylin and eosin staining (about 24 h), or by demonstration of a microglial reaction (about 4 to 10 days) or of a few remaining isolated bulbs, without accompanying fibers, which can be detected after a survival time of up to 17 months.

Radiologically, axons seldom rupture at the moment of injury, but it is more common that it takes hours or a few days until the axons are detached.

Chromatolysis could be detected only in group III and IV because it was a secondary reaction to axonal damage. These results were concomitant with that of Foda and Marmarou (1994) who observed massive diffuse axonal swellings as early as 6 hours after head injury reaching a maximum after 24 hours. However, shorter time (< 1 hour) was recorded, (Anderson and Opskin, 1998) and longer time (15 hours), was also reported. Also, Milovanovic and Dimiaio (1994), 27 stated that the individual must survive 12-24 hours before retraction balls can be seen on II and E stained slides of the brain. However, immunohistochemistry and Western blot analyses of the brains were performed using antibodies specific for amyloid precursor protein (APP), beta peptides, beta-site APP-cleaving enzyme (BACE), presenilin-1 (PS-1), caspase-3, and caspase-mediated cleavage of APP (CCA). Substantial co-accumulation for all of these factors was found in swollen axons at all time points up to 6 months following injury.

From this study, it can be concluded that the early detection of neuronal changes after head injury is not only important for the diagnosis of some obscure deaths, but also for estimating the survival time. It is to be noted that immuno-stains, although more sensitive than ordinary stains, are not specific for detection of traumatic neuronal injury, since they give also positive results with ischemic hypoxic brain injury. Fortunately, the simultaneous detection of this neuronal injury in different brain regions (Cerebrum, cerebellum and brain stem) must be regarded as evidence of DAI, a phenomenon that until now was thought to be traumatically induced.

So, it is recommended to use the immuno-stains for the detection of brain injury in short survivors (less than 3 hours), while ordinary stains are useful after that. Also, the pathologist must stain sections from different brain regions to verify the nature of the cause of the brain injury, whether traumatic or not especially when there is no obvious brain lesion.
References:


