Detection of diffuse axonal injury in forensic pathology

Sorin Hostiuc1,*, Daniel Pirici2, Ionut Negoț3, Daniela Adriana Ion4, Mihai Ceausu5

Abstract: Even if βAPP remains the gold standard in identifying diffuse axonal injuries, other histological, ultrastructure, histochemical, or immunohistochemical markers may aid the diagnosis and may better characterize this traumatic brain pathology. Moreover, associated changes, including neuronal body changes, the presence of microglial clusters, and so on, when identified in a traumatic context, should suggest at least the need of using specific markers for identification of DAI. The purpose of this article is to present the main microscopy techniques used in forensic practice to detect diffuse axonal injury.

Key Words: diffuse axonal injury, beta-APP, ubiquitin, forensic pathology.

Diffuse cerebral injuries (DCIs) are one of the most important types of closed brain trauma and one of the most common causes of vegetative state and post-traumatic neurologic disability [1]. There are four main types of DCIs: ischemic brain injury, caused by an insufficient blood supply of the brain, vascular injury – usually caused by the generalization of a hemorrhage secondary to a focal vascular lesion, brain edema, and diffuse axonal injury (DAI). Axonal injuries in trauma may occur either in the area of a bleeding (focal axonal injury) or at remote sites (diffuse axonal injury, DAI) [2], and are caused by angular, rotational or translational acceleration/deceleration of the head, causing axons various magnitudes of tensile stress [2-6]. The initial axonal stretch causes a temporary ondulation and cytoskeletal misalignment associated with a mechanical injury of the sodium ion channels, causing a massive Na influx toward the axoplasm mainly through tetrodotoxon sensitive Na+ voltage dependent channels [7]. This influx causes a depolarization of the axonal openings for conotoxin-sensitive Ca2+ voltage dependent channels, causing a massive influx of Calcium ions from the extracellular space [8] that in turn causes the activation of various metabolic pathways and cytoskeletal changes [7-9], including the activation of calpain, a protease known to degrade microtubule-associated proteins and monomeric tubulin [10]. Recently was suggested an additional mechanism for the development of the
diffuse axonal injury, mitigated by neuronal integrins. Neuronal integrins are involved in the modulation of the synaptic strength [11], remodeling of the cytoskeleton [12], stabilization of long-term memory potentiation [13], etc. Integrin stimulation causes the activation of the Rho-ROCK pathway [11], that determined cytoskeleton remodeling by regulating actin filament reorganization [14], polymerization of neurofilament proteins [11], or polymerization of actin [15], and subsequently the appearance of focal neuronal swelling, reminiscent of DAI in vitro [11].

These lesions were first described by Chisolm, a US military surgeon in 1861[16]. His description of cerebral contusion focused however, mostly on the clinical aspects of this pathology, and did not differentiate axonal from other types of diffuse closed head injuries. Jakob, in 1913, published an experiment in which he concussed monkeys and rabbits by letting a hammer of known weight fall on their heads from a well-established height. The procedure was repeated until the experiment animals showed various neurological signs, including nervousness, loss of appetite, or clumsiness. During the histological examination of the brain were found degenerative lesions in the cerebral hemispheres, brainstem, cerebellum, and around the margins of the pons and medulla [17]. Cajal, in 1928 proved that an axon, subjected to a certain amount of tensile stress would become swollen, beaded, and varicose upon release. If the axon fiber was to rupture, at the end of the proximal stub would be found a mass of axoplasm called retraction balls (clubs) [18]. Rand and Courville described neurofibrillar changes associated with commotio cerebri, by using reduced silver stains, on cadaveric material: “varicosities and fusiform enlargements were observed, apparently as preliminary changes leading to segmentation and coarse and fine granulation of these structures” [19]. The interest in diffuse degenerative lesions of the white matter in brain trauma was sparked by an article written by S. Stritch in 1956, describing nerve fiber degeneration, not due to cortical cell loss, nor to infarction or laceration of brain substance, that caused extreme dementia [20]. Adams coined the term diffuse axonal injury, and described them as primary traumatic events: “diffuse damage to white matter may occur as a primary event at the moment of the impact damage to the brain” [21]. More recently however, this hypothesis was disputed by various authors who proved that axonal disconnection is a slow progressive process [22-25], primary axotomy being a relatively rare event [26].

Macroscopically, DAI may be undetectable unless they are associated with micro vascular ruptures determining the appearance of petechial hemorrhages [27][Fig.1].

Classically the histological identification of diffuse axonal injury relied heavily on macroscopically evidence of contusive areas in predefined locations and the use of silver stains to detect microscopic changes. Recently however, a set of new diagnostic markers, mostly immunohistochemical, have been used in order to detect DAI with various degrees of success. The purpose of this article is to present the main microscopy techniques used in forensic practice to detect diffuse axonal injury.

Localization and grading of DAI
According to Gennarelli, DAI can be classified into three stages:

1. Axonal abnormalities restricted mainly to the parasagittal white matter of the cerebral hemispheres (axonal retraction balls and, in cases with a longer survival – small clusters of microglia in the white matter). Experimentally, this grade was associated with mild or moderate post-traumatic coma (16-19 minutes and 2 to 6 hours respectively).

2. Focal lesions of the corpus callosum in addition to the axonal abnormalities identified in the first grade. Experimentally this grade was associated with moderate posttraumatic coma, severe disability and lateral injury.

3. Focal lesion in the region of the superior cerebellar peduncle in addition to the abnormalities identified in the second grade. Experimentally this grade was associated with severe posttraumatic coma (lasting more than 6 hours), and a poor outcome (severe disability or prolonged coma until death) [28]. Adams considered that a severe DAI can be identified through the presence of three main signs: (1) a focal lesion in the corpus callosum, often with involvement of the interventricular septum and sometimes with associated intraventricular hemorrhage, (2) focal lesions at the level of the dorsolateral quadrant(s) of the rostral brainstem and (3) microscopic proofs of diffuse damage to axons [29]. Blumberg however, showed that these features of distribution are uncharacteristic – from an analysis of 34 cases he only found 15 (44%) complying with this definition. All cases had however, lesions in both cerebrum and the brainstem in different locations; moreover, the most frequent locations of DAI were: corpus callosum (94%), pons (88%), cerebral white matter (79%), fornices (76%), midbrain (78%), cerebellum (72%), and medulla (36%) [30]. Oehmichen et al. found that simultaneous proof of DAI in the pons and corpus callosum is specific for physical trauma [31], and that pons is the most frequently involved area, being affected in 74.5% cases of traumatic brain injuries [31].

Histology of the DAI
Axonal changes are identifiable in the white matter or in large fiber bundles (corpus callosum, internal capsule, or corticospinal fibers of the brainstem) [32]. There are two distinct types of morphological lesions suggestive for DAI: axonal varicosities and retraction balls (Figs 2-4). Axonal varicosities are caused by axonal elongation without rupture and are characterized by a periodic arrangement of enlargements separated by shrunken
areas [33]. Retraction balls are identifiable as round or elliptical eosinophilic masses, with varying sizes from 5 to 40 microns, and are seen as early as 2 hours after the initial injury [34]. Classically, DAIs were identified using either HE or silver stains. DAIs are difficult to identify using HE, and are also significantly underestimated. Silver stains are more sensitive. However, they positively label all axons, making sometimes difficult to interpret the presence or axonal diameter irregularities [35]. In silver stains, at two hours post-injury are identifiable sinusoidally enlarged neurons (Fig. 3); at 6-10 hours these sinusoidal changes are more preeminent; after 16 hours are apparent early retraction balls, that are significantly larger at 72 hours (Fig. 4) [36]. For survival periods of more than 2-3 months, becomes identifiable a secondary, Wallerian degeneration, usually bilateral, but not necessarily symmetrical [1, 27].

Visualization of the peroxidase reaction products with tetramethylbenzidine. Peroxidase reaction was used by Erb and Povlishock to identify early DAIs using an experimental model with cats; they found specific patterns of expression depending on the time since injury, as seen in Table 1.

Immunostaining with anti-ubiquitin antibody. Anti-ubiquitin antibody is able to identify DAIs starting from 6h after injury. However, reactive axonal changes

![Figure 1. Multiple hemorrhagic foci of various shapes and sizes disseminated in the nervous tissue of the frontal lobe, VG, 100x, car accident; these lesions may accompany axonal injuries, but are not mandatory (personal collection).](image1)

![Figure 2. Diffuse axonal injury. Left – normal axonal appearance. Middle – axonal varicosities. Right – retraction bulbs.](image2)

![Figure 3. Axonal disruptions (arrows) in corpus callosum and “wavy fibers” in occipital and parietal lobes after a gunshot wound (close contact range), (male, 40 y.o., survival less than 24 h), Bielschowsky silver stain, 400x (personal collection).](image3)
(retraction balls, axonal varicosities) are found significantly more frequent 24h after injury in the corticospinal tract near the decussation, and the diencephalon. Retraction balls have a stronger immunopositivity at the periphery and a weaker one at the center [38]. Differentiation should be sought here between DAIs and dystrophic neurites, especially if the subject is an older patient or was suffering from a neurodegenerative disorder.

Immunostaining with neurofilament (NF) antibody. The 68kDa NF is considered the most useful NF antibody for detecting DAIs, as it has minimal concomitant immunoreactivity with cell nuclei, somata, and dendritic processes, and is best for detecting reactive changes [39]. Also, studies using 68kDa NF found that the frequency of axonal changes was highest in the areas

**Table 1. Identification of DAIs using peroxidase reaction products with tetramethylbenzidine**

<table>
<thead>
<tr>
<th>Time</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td>Focal alterations in the peroxidase profile, typically located within the caudal diencephalon throughout the thalamus, cerebral peduncles, and pons; the most common focal alteration – a local, lobular pooling of intra-axonal peroxidase, of 10 microns in diameter</td>
<td>[37]</td>
</tr>
<tr>
<td>2h</td>
<td>Peroxidase foci increased in diameter, and had a greater complexity (some being multilobular); moreover some axons presented a discontinuity between the proximal, peroxidase-laden axonal segment and the distal segment</td>
<td>[37]</td>
</tr>
<tr>
<td>4h</td>
<td>Further increase in diameter (now up to 16 microns) and complexity of the peroxidase-laden lobules, often with thinning or disappearance of interconnecting peroxidase bands, increased distance between lobules, and the presence of uni or multilobular peroxidase pools, without distal axonal continuity</td>
<td>[37]</td>
</tr>
<tr>
<td>6h</td>
<td>Further increase in diameter of the peroxidase-laden lobules (now being up to 18 microns); the majority presented no continuity with the distal peroxidase containing segments</td>
<td>[37]</td>
</tr>
</tbody>
</table>

**Figure 4.** Axonal injuries in corpus callosum after a car accident with 3 days survival: retraction balls and axonal varicosities, Bielschowsky silver stain, 400x (personal collection).

**Figure 5.** Strong IHC reaction for NFT in neuronal elongations of the brainstem, in the context of diffuse axonal injuries, 200x (personal collection).
where axons changed their anatomical course (nearby penetrating vessels, about to enter target nuclei, or decussated within brain parenchyma)[39]. Staining with NF antibodies can be used to detect the age of the DAIs (See Table 2, Fig. 5).

**Immunostaining for beta amyloid precursor protein (βAPP) antibody.** βAPP is a transmembrane glycoprotein, transported by fast anterograde axoplasmic flow[35, 41-44], having amongst other, a plethora of neurotrophic functions, essential for neuronal survival after axonal damage (promotion of axonal sprouting, synaptogenesis, neurite outgrowth) [44]. It is currently the gold standard in detecting DAIs, as it is very sensitive, and a positive reaction is identifiable less than 2 hours after traumatic brain injury [45]. A positive βAPP reaction in the neurites is a sign of vitality, as the reaction is negative in the absence of blood [31]. According to Gentleman, the grading of the βAPP immunopositivity in DAIs is as follows: 0 – normal, 1 – any staining of axons, however, light, 2 – scattered paths of axonal damage, and 3 – extensive damage through large areas of the white matter [46]. The time since injury can be obtained using βAPP by using the descriptors presented in Table 3, and Fig 6.

Electron microscopy studies showed a specific pattern of events occurring after traumatic brain injury at the axonal level[37] (see Table 4). However, its usefulness in forensic practice is limited.

**Table 2**

<table>
<thead>
<tr>
<th>Time</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min-1h</td>
<td>Some neurons are showing waving of the axons, and a small number of axons are enlarged, especially in the deep white matter and the brain stem</td>
<td>[40]</td>
</tr>
<tr>
<td>1-2h</td>
<td>Increased number of enlarged axons, especially in the deep white matter and the brain stem</td>
<td>[40]</td>
</tr>
<tr>
<td>4-6h</td>
<td>Large number of waved and enlarged neurons (coarse varicosities), and retraction balls, not identifiable by using Bodian stain</td>
<td>[40]</td>
</tr>
<tr>
<td>6h – 12h</td>
<td>Focal intra-axonal accumulation of reactive neurofilament subunits</td>
<td>[39]</td>
</tr>
<tr>
<td>12-24h</td>
<td>Continued expansion of immunoreactive neurofilament mass, correlated with evidence of disconnection (complete axotomy). Axonotomized neurons are significantly larger (swollen) compared to non-axotomized neurons</td>
<td>[39]</td>
</tr>
<tr>
<td>&gt;24h</td>
<td>Reactive swellings had a non-immunoreactive cap of axoplasm encompassing an expanded immunoreactive neurofilament core, and sometimes lobulation of the axon.</td>
<td>[39]</td>
</tr>
</tbody>
</table>

**Figure 6.** Diffuse axonal injuries in the brainstem in the context of cerebral trauma due to a car accident, strong IHC reaction for APP of the damaged axons (it is physiological in the perikarya), showing fusiform swellings, thickened filaments and globules, 200x (personal collection).

**Figure 7.** Central chromatolysis associated with DAIs. Left – normal appearance, with an increased number of tigroid bodies. Middle – moderate loss, mostly central, of tigroid bodies, at 12 hours. Right – significant loss of tigroid bodies, 24h after injury.

**Figure 8.** Chromatin changes associated with DAIs. Upper left – arrangement of chromatin in chain-like strands; upper right – map-like pattern nearby nuclear membrane; Lower left – ring agglutination, Lower right – mass-fibril agglutination.
ASSOCIATED PATHOLOGICAL FINDINGS

Changes in the neuronal body

Central chromatolysis. At about two hours after injury is identifiable a loss of tigroid material, mostly in the perinuclear area. At 12 hours the loss is fairly noticeable, and after 24 hours the loss is widespread. After a few weeks of survival this granulation is still underdeveloped, suggesting a slow recuperative process [19] (see Fig. 7).

Nuclear changes. Within two hours after injury the nuclei are often found in an eccentric position, having an irregular contour, with folds and thickness irregularities in the nuclear membrane. In longer survival periods both the nucleus and the nucleolus assume an eccentric position [19].

Chromatin changes. At 1-2 hours after injury the chromatin becomes arranges in chain-like strands; after 24 hours it forms map-like patterns or agglutinations adherent to the nuclear membrane [19]. After two weeks the chromatin arranges itself in bizarre configurations (rings, multiple masses, mass-fibril combination patterns). See Fig. 8 for some configurations.

Immunostaining for neuronal specific enolase (NSE). Normally NSE labels both normal and neoplastic cells of neuronal and neuroendocrine origin. Post-injury, at 1-2 hours the positive staining starts to decrease, and at 4-6 hours are identifiable neuronal bodies with weak or no staining [40]. This decrease in immunostaining is associated with an increased release of NSE, whose levels in venous blood was found to peak one hour after injury [49].

Immunostaining for heat shock protein 70 (hsp70). Hsp70 are strongly upregulated by heat and chemical stresses (especially heavy elements like cadmium, arsenic or mercury), but a positive immunostaining was also proven in diffuse axonal injury with secondary insults [50]. In the latter case hsp70 proteins were undetectable until 30 minutes after injury, whereas positive staining was positive at 3 hours, reached a peak at 24h, and decreased after 72h. Staining was positive on the cell membrane and cytoplasm, mostly on the pyramidal cortical neurons [50].

Immunostaining for oligomerized stromal interaction molecule 1 (STIM 1), a sensor for Ca\textsuperscript{2+} concentration in the ER. STIM 1 is normally expressed in the neuronal bodies in the cerebral cortex near the sagittal sinus, and in the cytoplasm and envelopes at the beginning of axons. The expression is higher in neurons from the layers II, III, V. In association with DAI, the expression of STIM-1 is increased almost twofold at 6 hours postinjury, reaches a peak at 12 hours (about 2 and a half the normal expression), and starts to decrease after 2 days [51].

Non-neuronal cells

Adams found microglial stars (clusters of hypertrophied microglia) in patients with a survival of at least 14 days. In cases with a long survival, the microglial stars, even if still identifiable, were largely obscured by the presence of reactive changes associated with the Wallerian-type degeneration of the white matter [21]. Immunohistochemically, Geddes et al., by studying the
presence of the CD68 immunopositivity, found no changes compared to controls at 24 hours. After 36 hours however, they noticed an increased size and number of microglia in areas with damaged axons, and to a lesser extent in negative βAPP areas. After 6 days they found aggregates of CD68 positive microglial cells around axonal bulbs. After 10-11 days were identifiable microglial clusters, that were significantly more frequent at 14 days. In cases with a longer survival (more than 5 months), was identifiable an association between foamy fibers and microglial proliferations [48].

**Immunostaining for glial fibrillary acidic protein (GFAP).** GFAP is an intermediate filament protein expressed in various CNS (astrocytes, ependymal cells), and non-CNS cells. Associated with diffuse axonal injuries, Vanezis et al. found at 2 hours an increased positivity in the end-feet processes of astrocytes around blood vessels. After 6-10h was identifiable an immunopositivity of the se end-feet processes of astrocytes in the white matter, but no reactive astrocytes. At 16h were found positive reactive astrocytes in the white matter and at 72h intensely positive reactive astrocytes in the white matter [36]. Li et al. found intensely positive astrocytes, with large bodies at 4 hours postinjury [40]. Geddes however, found no significant changes compared to controls within 5 days from the injury. After 8 days they found marked reactive astrogliosis, but no aggregation around the damaged axons. After 3 months, diffuse astrocytic gliosis was identifiable in the degenerating corpus callosum, cerebral white matter, internal capsule and long tracts [48].

**Immunostaining for Galectin-3/Mac-2.** Galectin-3/Mac-2 stains a subset of microglia involved in myelin phagocytosis [52]. In traumatic brain injury associated with diffuse axonal injury the density of galectin-3/Mac-2 positive cells is maximum in cells beneath the site of impact, and decreased both posteriorly and anteriorly from that site. The maximum increase was noted at 24h postinjury [52].

**Immunostaining for βAPP can identify macrophages incorporating βAPP material at 4-10 days after injury [31].**

**Immunostaining for ionized calcium-binding adapter molecule (IBA-1).** IBA-1 (a widely used microglia marker)immunoreactivity after traumatic brain injury increases significantly compared to normal controls in days 1, 8, 14 after injury [52].

**CONCLUSION**

Even if βAPP remains the gold standard in identifying diffuse axonal injuries, other histological, ultrastructure, histochemical, or immunohistochemical markers may aid the diagnosis and may better characterize this traumatic brain pathology. Moreover, associated changes, including neuronal body changes, the presence of microglial clusters, and so on, when identified in a traumatic context, should suggest at least the need of using specific markers for identification of DAIs.

**Acknowledgment.** This work was possible with the financial support of the Sectoral Operational Programme for Human Resources Development 2007-2013, co-financed by the European Social Fund, under the project number POSDRU/159/1.5/S/137390 for SH.

---

**References**


18. y Cajal SR. Degeneration & regeneration of the nervous system: Oxford University Press, Humphrey Milford; 1928.


