Pathological Spectrum and β-APP Immunoreactivity as a Diagnostic Tool of Diffuse Axonal Injury following Traumatic Brain Injury: A Novel Classification

Meenakshi Sharma[1] Arulselvi Subramaniam[2] Kangana Sengar[2] Vaishali Suri[3] Deepak Agrawal[4] Nabarun Chakraborty[1] Ravindra Mohan Pandey[5] Rajesh Malhotra[6] Sanjeev Lalwani[1]

[1] Division of Forensic Pathology and Molecular DNA Laboratory, Jai Prakash Narayan Apex Trauma Centre, All India Institute of Medical Sciences, New Delhi, India
[2] Department of Laboratory Medicine, Jai Prakash Narayan Apex Trauma Centre, All India Institute of Medical Sciences, New Delhi, India
[3] Neuropathology Laboratory, Center for Neurosciences, All India Institute of Medical Sciences, New Delhi, India
[4] Department of Neurosurgery, All India Institute of Medical Sciences, New Delhi, India
[5] Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, India
[6] Department of Orthopaedics, Jai Prakash Narayan Apex Trauma Centre, All India Institute of Medical Sciences, New Delhi, India

Address for correspondence Sanjeev Lalwani, MD, Division of Forensic Pathology and Molecular DNA Laboratory, Jai Prakash Narayan Apex Trauma Centre, All India Institute of Medical Sciences, New Delhi 110029, India (e-mail: drsanjeevlalwani@gmail.com).

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Abstract

Aim Different deposition patterns and grading systems used to define and identify DAI remain discordant and to date these are a challenge in clinical practice. Our main objective was to study the post-mortem axonal changes and develop a grading system to identify DAI on the basis of histopathological and immunoreactive β-amyloid precursor protein (β-APP) observations in severe TBI cases.

Methods Prospective study with 35 decedents with sTBI (GCS score $\leq$ 8) was conducted and samples were collected from three different sites–corpus callosum, thalamus and brain stem. Serial sections from each site were stained with hematoxylin and eosin (H&E), and immunohistochemistry (IHC) of β-APP.

Results We developed a grading system based on histopathological characteristics to assess the overall damage of axonal injury. We found maximum histopathological changes in cases with prolonged stay. Corpus callosum showed maximum changes in both gradings. Curiously, we also detected axonal swellings with H&E staining. Usually neglected, the thalamus also showed significant histopathological and immunoreactive changes for sTBI.

Conclusion Our study based on histopathological and β-APP scoring system to define and identify DAI thus facilitates accurate diagnosis of DAI post mortem, which has forensic implications, and may further contribute toward survival and improvement of quality of life of sTBI patients.

Keywords
- traumatic brain injury
- diffuse axonal injury
- β-app
- H&E stain
- forensic pathology

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Highlights

1. Methods used for identification of DAI are incoherent.
2. Developed histopathological characteristics-based grading to assess axonal injury.
3. Detection of axonal swellings with H&E staining.

Introduction

Traumatic brain injury (TBI), a silent epidemic of modern times, is one of the leading causes of mortality and life-changing morbidities worldwide, with enormous socio-economic consequences.[1] Sixty-nine million (95% CI 64–74 million) individuals worldwide are estimated to sustain a TBI each year, with the Southeast Asian and Western Pacific regions experiencing the greatest overall burden of the disease. Head injury following road traffic collision is more common among all trauma cases in lower middle-income countries (LMICs).[2] For instance, in India alone, ~1 million people are injured each year with around 200,000 deaths.[3,4] Its high prevalence coupled with poor prognosis, necessitates more scientific research in the field of TBI.[5]

TBI, defined as a sudden blow or jolt to the head that produces permanent and/or temporary damage in neurological function, is known to affect individuals across all spectrums of gender, ethnicity, age and socioeconomic status.[6] Road traffic collisions, fall, and violence are a significant source of TBI,[7] that is classified using the Glasgow coma scale into mild,[8–10] moderate,[11–14] and severe[3–7,15] varieties according to the severity of injury.[15]

Severe TBI shows characteristic pathologic features [axonic karyorrhexis, hemorrhage, gliosis, axonal swellings, gemistocytic astrocytes, lipid-laden macrophages, neovascularization] in multiple anatomical regions, in the brain. Widespread evidence of primary and secondary axonal injury is clinically defined as diffuse axonal injury (DAI), which is mainly caused by high-impact accelerating/decelerating forces that damage white matter tracts, resulting in severe neurological dysfunction along with impaired cognitive and psychiatric abilities.[11–13] DAI occurs preferentially in the corpus callosum,[14] Interestingly, underlying skull fractures have little to no association with DAI.

Although computed tomography (CT) scan is the useful first-level radiological examination to detect and identify DAI, it has a rather low yield when compared with magnetic resonance imaging (MRI) and diffuse tensor imaging (DTI).[8] MRI is more sensitive than CT scans, but MRI may also miss DAI, as it identifies the injury using signs of edema, which may not be present. Moreover, there are limitations to the application of MRI immediately post-injury in critical survivors. Because most TBI subjects suffer from cognitive impairments, it can be difficult to determine whether imaging findings are attributable to differences in cognitive abilities, structural changes, or true functional differences.[9] This is true for postmortem settings as well and histopathological scoring is the only resort.

Microscopic devastation of brain tissue by focal/diffuse, following brain trauma is defined as posttraumatic encephalopathy (PTE). Transfer of acceleration–deceleration forces to the brain following episodic, or repetitive blunt, mild to severe impacts to the head and leads to progressive neurodegenerative syndrome termed as chronic traumatic encephalopathy (CTE). PTE and CTE are both distinct pathologies in the posttraumatic spectrum of brain diseases, although a definite study of the manifestations of the entire spectrum of brain injury warrants pathological examinations of brain tissues after postmortem. The spectrum of PTE changes comprises persistent sequelae of primary and secondary brain trauma, examined in the present study. The brain of a CTE sufferer may appear grossly unremarkable without any focal or lobar necrosis, infarct, acute and chronic hemorrhage, or significant cortical atrophy. That refers, routine hematoxylin and eosin (H&E) staining of CTE affected brain sections may appear typical.[10,16] For PTE, however, conventional staining methods (H&E) have been employed for the microscopic detection of axonal damage. These methods, however, may miss any signs of injury in patients who survive for less than 12 hours[17,18] and underrate the degree of axonal damage due to relatively poor visibility of injured neurons. β-APP immunostaining is the current gold standard for detecting axonal changes at the earliest to objectify diffuse axonal damage following TBI.[19]

Different deposition patterns and grading systems have been used by various studies, but not all studies have specified the methods used for grading the amount of APP.[20–25] At present, there is a large body of literature dealing with incidence, specificity, and biomechanical significance for determining vitality and survival time of DAI patients after sTBI; however, identifying DAI still remains a challenge in clinical and forensic practice. Moreover, as these studies show inconsistent results, there is a lack of worldwide consensus on the definition and classification of DAI.

Our main objective was to study the postmortem axonal changes in the corpus callosum, thalamus, and brain stem region, associated with diffuse axonal injury (DAI) post sTBI. Further, to develop a grading system to identify DAI on the basis of both histopathological and immunoreactive β-APP findings (in terms of amount and deposition pattern) in severe TBI cases compared with control cases. Our study can contribute to the discussion on the role of such important histopathological findings as routine investigative tools in forensic settings, which may aid in the reconstruction of the traumatic event as well as assess the severity of the injury with relation to the cause of death and provide with an estimate of the survival time. In the near future, studies such as these could also lay the foundation stone of sTBI treatment regimens as these pave the way to better comprehend the biomechanical events taking place post sTBI.

Materials and Methods

A prospective study was conducted at Jai Prakash Narayan Apex Trauma Centre, AIIMS, New Delhi. A total of 35

autopsy cases of patients who died due to sTBI during the period of December 2017 to December 2018 were included in the study. All demographical and clinical details were retrieved, including Glasgow coma score (GCS) and CT findings.Cases with GCS score $\leq$ 8 (severe TBI cases) at the time of admission and age above 18 years with positive CT findings were included in the study. Patients who have had post-trauma craniotomy or any surgical intervention related to the brain, neuropsychological illness, penetrating injury to the head, post resuscitation GCS score $>$ 8, and autopsy performed more than 24 hours after death were excluded from the study. Clinical records of all patients in both study and control groups were also studied to analyze demography, clinical status at presentation, mechanisms of injury, surgeries performed, other injuries, the presence of fractures, sepsis or any other diseases and survival time. CT scans were also analyzed for the detection of any hematomas, contusions, fractures, mass effect, mid-line shift (MLS) or other structural lesions and evidence of DAI. Picture archiving and communication system (PACS) was used to generate clinical DAI CT score.[19]

Controls

Ten post mortem brain tissues were also taken from same sites as study samples from age and gender matched control individuals having no past record of any TBI, and/or neuropsychological illness. We tried to avoid inclusion of patients with probable hypoxic or asphyxial changes. None of the control patient had skull fracture or any positive brain CT findings.

After death, the body of the patient was stored in a refrigerated body cabinet at a circumjacent temperature of −5°C. Autopsy in each case was conducted according to standard protocols. During autopsy, the brain was drawn out as a whole and cut in the mid sagittal plane superiorly at the corpus callosum and anteriorly at the anterior commissure. Samples were collected from three different sites—corpus callosum, thalamus, and brain stem, after informed consent from the legally authorized representative (LAR) of the patient. Two LARs (Legally Authorised Relatives) of each patient were asked to sign participant informed consent form (PICF). Participant information sheets (PIS) that described the project and its utility in brief were also handed out to the LARs.

After sample collection, all tissues were fixed in 10% formaldehyde for at least 4 to 6 weeks. Formalin was changed after every 2 weeks to ensure the fixation of the brain tissue in cases of study samples of sTBI. After ensuring proper fixation of the tissue, gross examination was performed. Transverse sections measuring 1 to 2 cm from the region of interest from every site in each case were taken.

After tissue processing, 4 µm (using Microm HM 355 S) thick paraffin-embedded serial sections from each site were stained with H&E, and immunohistochemistry (IHC) of β-APP. H&E helped in assessing the general brain morphology and examining other post traumatic pathological changes in the corpus callosum, thalamus, and brainstem. Sections were immunostained for β-APP on Ventana Benchmark XT (Roche tissue diagnostics) using XT ultraview DAB V3 detection kit according to manufacturer's instructions. Briefly, 4 µm thick serial paraffin sections from different sites were obtained on poly-L-lysine-coated slides. Sections were immunostained for β-APP (β-amyloid precursor protein) with anti-β-APP antibody diluted 1:100 (rabbit polyclonal to amyloid precursor protein, ab-15272, Abcam) The sections were incubated at 37°C for 32 minutes for both anti-β-APP and NFP antibodies. To visualize the reaction products, sections were reacted with 0.05% 3,3-diaminobenzidine-tetrahydrochlroride (DAB), and H_2O_2. The histological sections were counterstained using Meyer's hematoxylin. For positive controls, histological sections of normal brains were used and for negative controls, the phosphate buffer solution or normal rabbit serum were used instead of primary antibody. The sections were examined using high magnification to assess the distribution and pattern of β-APP/NFP immunoreactivity at the same time. All observations were conducted blind to the demographic and clinical information by two independent observers.

Two different grading systems were used to categorize sTBI cases. First grading was based on routine H&E staining, where varied pathological features assessed in sTBI cases were graded to give a definite score. Second grading system, in accordance with Jenson et al[26] was based on immunoreactivity of β-APP where microscopic features, a hallmark of axonal injury, were identified through immunohistochemical examination of β-APP.

Grading used for Hematoxylin and Eosin staining

We had developed a grading system (►**Table 1**) based on histopathological characteristics to assess the overall damage after axonal injury followed by sTBI in all sites collected for both study and control samples (►**Fig. 1**).

Each slide was examined to observe and grade hypoxic changes including neuronal changes, axonal bulbs, cellularity (gliosis) characterized by an increase in microglial cells, oligodendrocytes, and other glial cells; infarction; neovascularization; lipid-laden macrophages; gemistocytic astrocytes (glial scars); congestion/edema, hemorrhage, blood vessel features such as vasculitis/hyalinized vessel walls; and transection changes.[27]

Each feature was studied from each site such as corpus callosum, thalamus, and brain stem. Scores for each of the pathological changes pertaining to each region were added and divided by the maximum possible core to give a percentage.

The maximum score was 16 = [3 (degenerative changes) + 1 (cellularity) +3 (axonal swellings) + 1 (infarction) + 1 (neovascularization) + 1 (lipid laden macrophages) + 1 (gemistocytic astrocytes) + 1 (congestion/edema) + 1 (hemorrhage) + 1 (vasculitis) + 1 (hyalinized blood vessels) + 1 (transaction changes)] for the corpus callosum, thalamus, midbrain, pons, and medulla oblongata].

Grading the Amount of β-APP Staining

The distribution (amount and pattern) of β-APP stain was recorded for each slide (►**Fig. 2**) and scored as per the grading system developed by Jensen et al[26] given below:

Amount per field at 200x magnification		Score
Granular Changes	**1–5**	**1**
	6–20	2
	>20	3
Swellings	1–5	1
	6–20	2
	>20	3
Retraction Bulbs	Present	1
Bands	Granular	1
	Non-granular	2

All gradings, whether histopathological or β APP immunoreactive, were performed by an individual masked to patient history.

Data Management and Statistical Analysis

Data were recorded in a pre-designed performa and managed on an excel spread sheet. All the entries were checked for any possible keyboard error. Categorical variables were summarized by frequency (%) and χ2/Fisher's exact test, as appropriate, were used to compare the frequencies between sTBI and control subjects. Quantitative variables were assessed for approximate normality of quantitative variables. Variables following normal distribution were summarized by mean ± SD and student's *t*-test was used to compare mean between sTBI and controls. Effect size (95% confidence interval) was also computed. Variables following nonnormal distribution were summarized by median and range/interquartile range. Wilcoxson's sum rank test was used to compare the distribution of nonnormal variables between sTBI and controls. STATA 14.0 statistical software was used for data analysis. In this study, p-value ≤ 0.05 were considered statistically significant. An analysis of the area under curve (AUC) of receiver operating characteristic (ROC) graph was performed using an online program developed at the John Hopkins University.[28]

All forms and pamphlets were printed in both English and Hindi. The described work has been performed in accordance with the 'Declaration of Helsinki'.[29]

Results

Demographics

The average age of all patients included in the study was 40 years with GCS score of 3 (3–8). In the study, 83% were males (29 males and 6 females). The mode of injury was RTA in 74% cases and fall (26%) in rest of the cases. In 43% of cases, skull fracture was present ($n = 15$) (►**Table 2** and ►**Supplementary Table S1**, available online only).

Controls

The average age of control cases was 33 years with a GCS score of 3 (3–15). Among control cases, 90% were males, the mode of injury in 50% of cases was trauma without head injury, and 50% were dead due to other causes (hanging and

Table 1 Grading used for hematoxylin and eosin staining

S. No.	Microscopy		Corpus callosum, thalamus, midbrain, pons, and medulla
1	Hypoxia Changes	Degenerative changes (anoxic neurons/red neurons/karyorrhexis)	Absent-0 Mild degenerative changes-1 Moderate degenerative changes-2 Severe degenerative changes-3
		Cellularity (gliosis)	Absent-0 Present-1
2	Focal infarct changes	Axonal swellings	Absent-0,present 1–5 = 1 6–20 = 2 >20 = 3
		Transection changes	Absent-0, Present-1
		Infarction	Absent-0, Present-1
		Neuvascularization	Absent-0, Present-1
		Lipid-laden macrophages	Absent-0, Present-1
		Gemistocytic astrocytes (glioscars)	Absent-0, Present-1
4	Congestion/ edema		Absent-0, Present-1
5	Hemorrhage		Absent-0, Present-1
6	Others	Vasculitis	Absent-0, Present-1
		Hyalinized blood vessels	Absent-0, Present-1

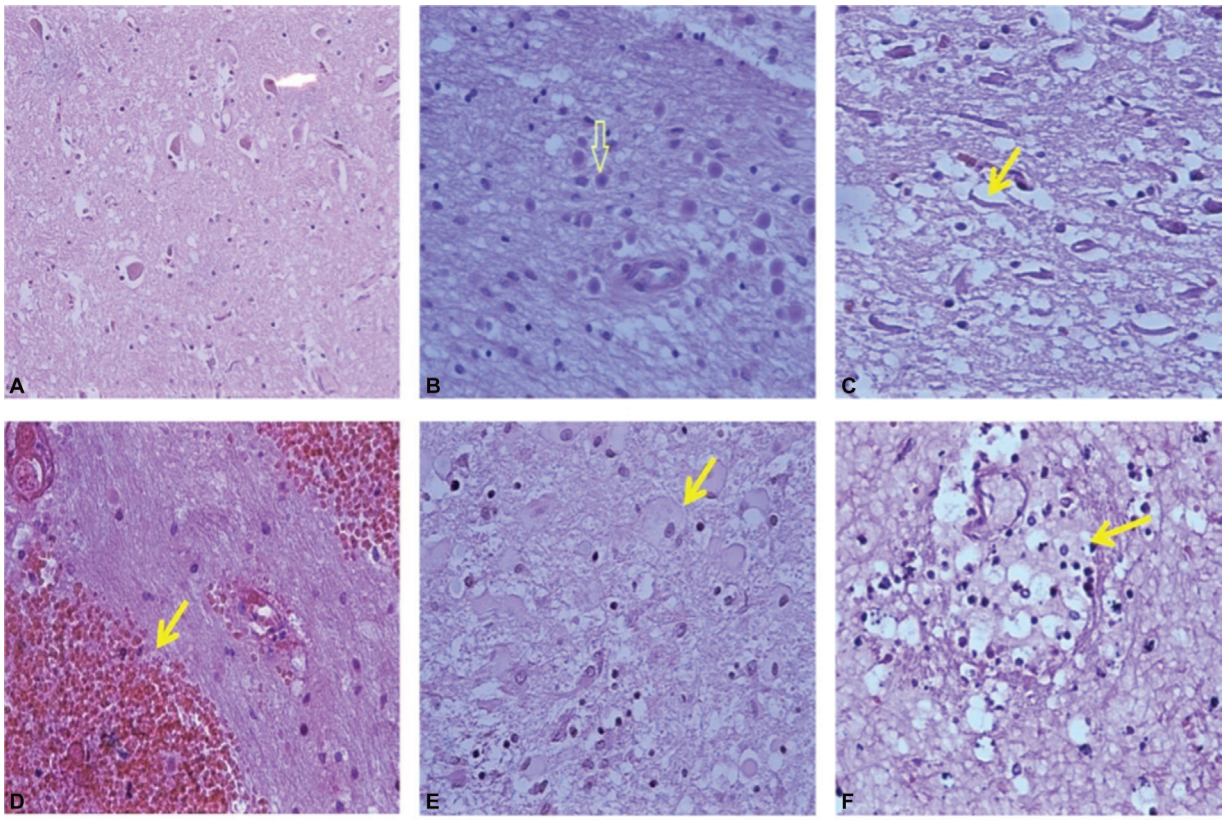

Fig. 1 Histopathological characteristics post-severe TBI: (**A**) Section from thalamus, arrow showing neuron with shrunken nucleus and dense eosinophilic cytoplasm(200x). (**B**) Sections from the corpus callosum: showing many axonal bulbs (20–50/HPE)(400X). (**C**) Section from pons showing severe degeneration of neurons(400X). (**D**) Section from corpus callosum showing areas of hemorrhage (200X). (**E**) Section from corpus callosum showing collection of gemistocytic astrocytes (400X). (**F**) Section from mid brain showing collection of foamy macrophages (400X).

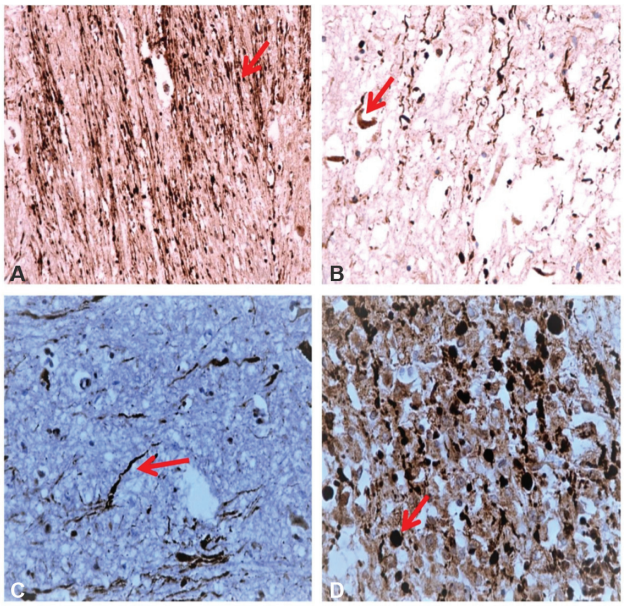

Fig. 2 Site-wise β-APP distribution post severe TBI: (**A**) Section from the pons showing axonal swellings. (**B**) Section from corpus callosum showing non-granular bands. (**C**) Section from the midbrain: arrow showing the granular band. (**D**) Section from the corpus callosum: arrow showing retraction bulb.

drowning). Histopathological and β-APP score were less than 50% in all control cases (►**Supplementary Table S2**, available online only).

CT Findings

In CT brain findings, 16 patients had SDH (46%), 3 had EDH (8%), 12 had SAH (34%), 6 had IVH (17%), 12 had basal cisterns (34%) open, 4 cases had white cerebellar sign (11%) positive and mass effect. Midline shift was found to be evident in 12 cases, 22% had less than 5 mm shift, while 11% had more than 5 mm shift. Among our study patients, 51% solely had head injury and 25% had polytrauma (►**Supplementary Table S3**, available online only).

Correlation of Histopathological Score and β-App Score with Length of Stay

Histopathological changes were maximum in cases with prolonged length of stay (> 1 month) followed by patients who had survived up to 2 weeks. Histopathological grading indicated least changes in cases with ≤ 1 day survival. β-APP scores were found to be maximum in cases with 5 days survival (►**Fig. 3** and ►**Supplementary Table S4**, available in the online version).

Site-wise Histopathological Changes in post TBI Autopsy Brain Tissue

Corpus callosum showed the maximum cellular changes such as the presence of infarction, gliosis [18(51.4)], presence of axonal bulbs (>20 nos. in 1 HPF) [(7^{20}] and hemorrhage [(19 (54.3)] as compared with the thalamus and brain stem.

Similarly, in β-APP scoring, the corpus callosum showed the maximum changes such as granular changes (>20 nos in

Table 2 Demographic profile of severe traumatic brain injury (sTBI) patients as compared with non-TBI controls

S. No.	Variables	TBI $n=35$ f (%)	non TBI $n=10$ f (%)	*p*-Value
1	Age (mean ± S.D)	39.9 ± 12.3	33 ± 10.1	0.109
2	GCS score [(median) (min-max)]	3 (3–9)	3 (3–15)	0.83
3	Sex			
	Male	29 (82.9)	9 (90)	0.999
	Female	6 (17.1)	1 (10)	
4	Mode of injury			
	RTA	26 (74.3)	2 (20.0)	<0.001*
	Fall	9 (25.7)	2 (20.0)	
	Gunshot	0 (0.0)	1 (10)	
	Hanging	0 (0.0)	4 (40)	
	Drowning	0 (0.0)	1 (10)	
5	Skull#			
	Absent (0)	20 (57.1)	10 (100)	0.01*
	Present[1]	15 (42.9)	0 (0.0)	

Abbreviations: f, frequency; GCS, Glasgow coma score; RTA, road traffic accident; SD, standard deviation; TBI, traumatic brain injury.
#fracture; % percentage; *Significant *p*-value

1 HPF); [16 (45.7)], axonal swellings (>20 in 1 HPF) [$n=15$ (42.9)], retraction bulbs [$n=28$ (80)], presence of bands (granular bands [$n=29$ (82.9)], and nongranular bands [$n=25$ (71.4)].

Histopathological Changes in Post TBI Autopsy Brain Tissue in Comparison to Controls

Infarction were significant in the corpus callosum [infarction ($p=0.003$)]. Gliosis was seen in the corpus callosum ($p=0.02$)] and thalamus ($p=0.04$)] in TBI cases. Thalamus and brain stem showed more degenerative neuronal changes as compared with controls (►**Supplementary Tables S5** and **S6**, available online only).

Results of Grading for Amount of β-APP Staining

The distribution of β-APP staining was recorded for each slide and scored according to the grading scheme developed by Jenson et al.[26] We saw statistically significant changes such as granular changes, axonal swellings, retraction bulbs, granular and nongranular bands in all three sites—corpus callosum,

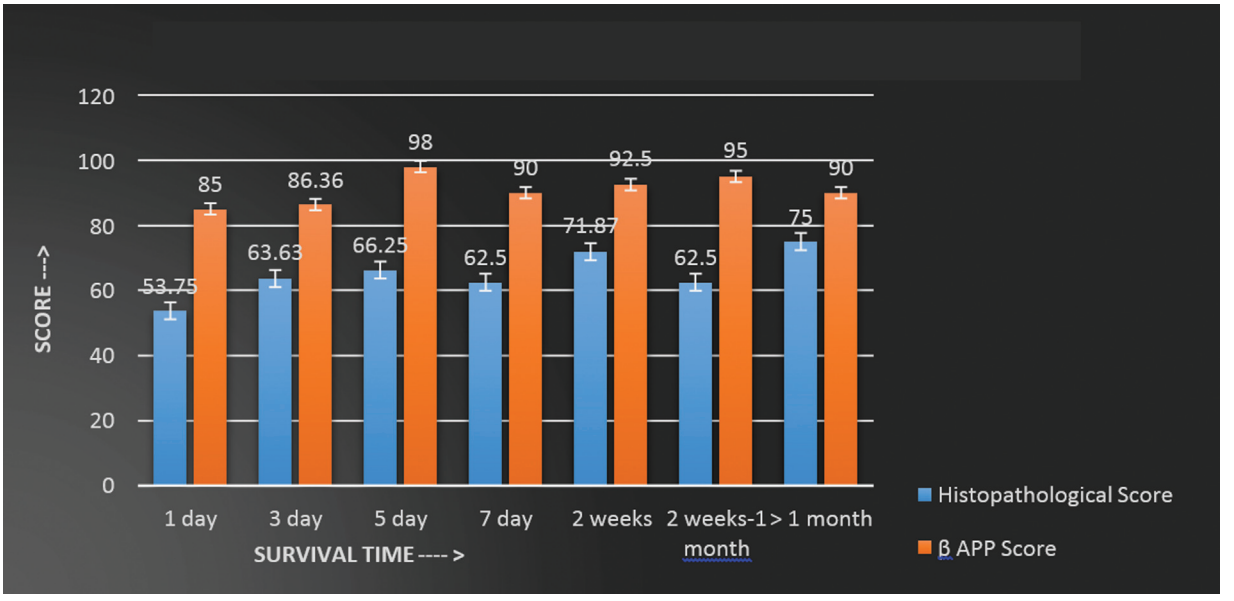

Fig. 3 Correlation between histopathological score and β-APP score with the length of stay.

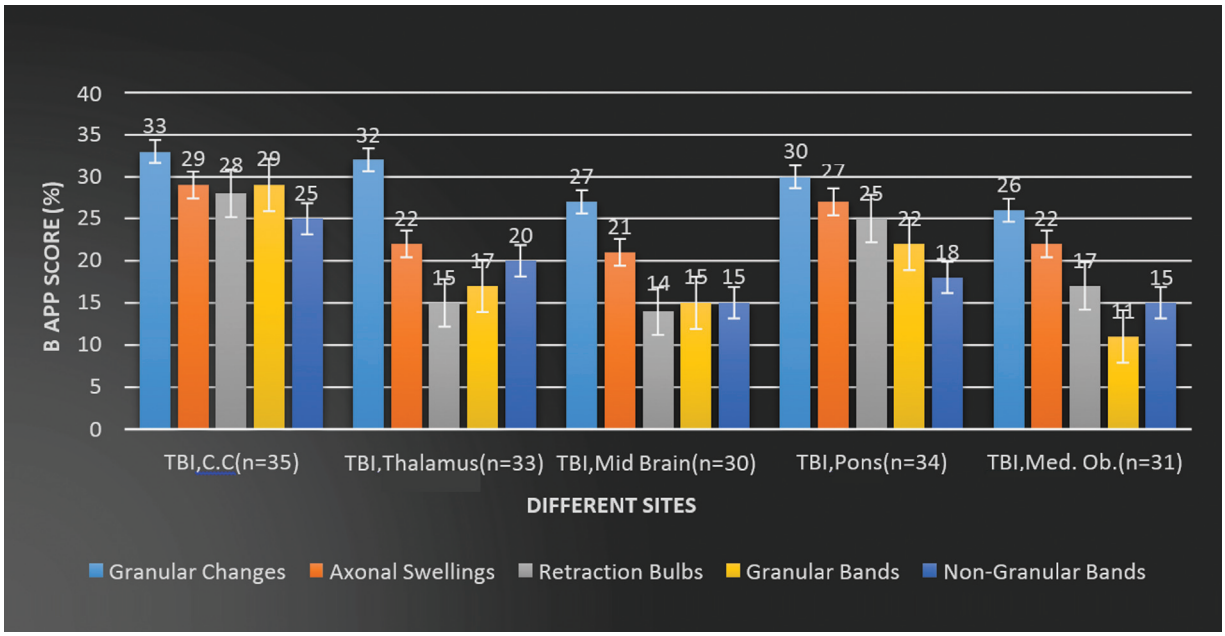

Fig. 4 Site-wise β-APP distribution: sTBI versus nonTBI.

thalamus, and brain stem) (►**Fig. 4**) (►**Supplementary Tables S7** and **S8**, available online only).

Histopathological Score and β-APP score in TBI Study Patients

Histopathological characteristics were studied and graded according to ►**Table 1** in different areas of the brain such as the corpus callosum, thalamus, and brain stem. The average histopathological score (with the maximum score of 16) of all TBI cases were 9.97 (62.3) ± 1.85 (11.5). The average score (with the maximum score of 10) of β-APP in the studied cases is 8.91 (89.1) ± 1.03 (10.4). The $p < 0.001$, in both the scoring systems which is statistically significant (►**Table 3**).

Correlation of Clinical DAI score with H&E scoring and β-APP scoring

The degree of correlation between histopathological scoring with clinical DAI scoring was found to be statistically significant ($p = 0.001$). Concurrent with previous studies, βAPP grading also tallied well with clinical DAI score with a p of 0.001.

AUC Analysis of the Novel Classification

AUC analysis of the novel grading/classification of DAI based on histopathological scoring gave us generally favorable results. The area under the curve of the ROC plot came out to be 0.995, with 93.3% accuracy, 91.2% sensitivity, and the algorithm missed 3 positive cases (►**Supplementary Fig. S1**, available online only).

Discussion

Diffuse axonal injury occurs when the brain rapidly moves back-n-forth inside the skull in response to accelerating and decelerating forces, causing axonal swellings and progression to secondary axon disconnections and Wallerian degeneration.[30] The presence of DAI after TBI is rather unfavorable with regard to functional outcome.[31] The pathological mechanism of DAI is complicated and there is no uniform standard for its clinical diagnosis.

We had 35 severe TBI patients and 10 control patients, with different histopathological features, such as degenerative changes, cellularity/gliosis, infarction, neovascularization, lipid-laden macrophages, gemistocytic astrocytes, congestion/edema, hemorrhage/vacuities, hyalinized blood vessels, and transection changes. Each feature from each site such as corpus callosum, thalamus, and brain stem (midbrain, pons, and medulla oblongata) was studied. The average histopathological score (with the maximum score of 16) of all TBI cases was

Table 3 Histopathological score and beta-APP score in TBI study patients compared with nonTBI

Samples	Histopathological ($n \pm$ SD)		*p*-Value	β APP ($n \pm$ SD)		*p*-Value
	Score (max.16)	%		Score (Max.10)	%	
Case ($n = 35$)	9.97 ± 1.85	62.32 ± 11.59	<0.001*	8.91 ± 1.03	89.14 ± 10.39	<0.001*
Control ($n = 10$)	4.3 ± 1.05	26.87 ± 6.62		2.3 ± 1.05	23 ± 10.59	

Abbreviations: Max, maximum; β APP, beta-amyloid precursor protein.
% Percentage, *Significant *p*-value.

9.97 (62.3) ± 1.85 (11.5) with $p < 0.001$, which is statistically significant.

Maximum post TBI histopathological cellular changes were evident in corpus callosum followed by thalamus and brain stem. Compared with nonTBI controls, the maximum significant changes of infarction, gliosis, axonal swellings, and congestion were evident in the corpus callosum and thalamus, whereas neuronal degenerative changes were more prominent in the thalamus and brain stem. Compared with histopathological grading, β-APP grading showed significant changes in all sites; hence, β-APP serves as a better marker for identifying more cellular changes (►**Supplementary Tables S7** and **S8**, available online only).

The maximum damage occurred preferentially in the corpus callosum and brainstem, usually on one side of the midline. Likewise in our study, corpus callosum showed maximum cellular changes such as the presence of infarction, gliosis [18 (51.4%)], presence of axonal bulbs (>20 no. in 1 HPF) [(7 (20%)] and hemorrhage [(19 (54.3%)] as compared with the thalamus and brain stem. Infarctions and gliosis were significant in the corpus callosum with 0.003 and 0.02, *p*-values, respectively. Gliosis was also observed in the thalamus ($p = 0.04$)] in TBI cases. As Bisht et al had shown that thalamic injury was evident in 87.5% of patients with severe TBI using NF and myelin stain.[32] Similarly, in our study, the thalamus and brain stem (midbrain, pons, and medulla oblongata) showed more degenerative neuronal changes ($p = 0.01$ and 0.006, 0.01, 0.05, respectively) as compared with controls and cannot be ignored while identifying DAI-associated sTBI. (►**Supplementary Tables S5** and **S6**, available online only).

β-APP staining can detect axonal damage within 35 minutes after severe head injury($n = 7$), but for Group 2 (severe head injury [= 4] with a recorded survival time of less than 30 minutes and Group 3 cases ($n = 4$), where death was not primarily ascribed to head injury but survival was between 45 and 109 minutes, all sections were negative for β-APP staining.[33] In our study, histopathological changes were the maximum in cases with prolonged length of stay (> 1 month) followed by patients who had survived up to 2 weeks. Histopathological grading indicated less changes in cases with ≤ 1 day survival. β-APP score was 80 to 100% (in cases 9, 14, 24, 29, and 32) and was found to be the maximum in cases with 5 days survival.

We used the Jensen et al grading system[26] to identify the DAI in each β-APP stained section from five different anatomical regions (corpus callosum, thalamus, midbrain, pons, and medulla oblongata). All these characteristics were found to be statistically significant in all sites (that is corpus callosum, thalamus, and brain stem). The average score (with the maximum score of 10) of β-APP in the studied cases was 8.91 (89.1) ± 1.03 (10.4). We have found positivity of β-APP staining in 100% of the sTBI cases and least degree of β-APP accumulation in cases of drowning, hanging, and blunt trauma abdomen.

Furthermore, we observed a statistically significant correlation of the histopathological grading system developed by us with clinical DAI scoring ($p = 0.001$). This prompts us to propose that in cases of dubious history of unknown bodies, our study may contribute toward correct determination of cause of death via histopathological testing postmortem, especially in cases where radiological investigations may not be entirely plausible.

Consistent with other studies, we also observed that the β-APP grading related well with clinical DAI grading, reiterating the fact that the former can be used as the gold standard to diagnose DAI. Thus, we can say that with suitable sampling from the corpus callosum, thalamus, midbrain, pons, and medulla oblongata, examination of sufficient number of blocks and detailed examination of sections using both histopathological and β-APP score will facilitate reliable and more precise diagnosis of DAI, resulting from PTE, especially in victims with unknown histories.[18] For an sTBI survivor with unremarkable gross appearance and normal brain CT scan, but poor cognitive outcome, a brain biopsy may be of immense utility in determining the extent of axonal damage, progression of primary to secondary injury, presence of DAI etc., eventually assisting doctors/surgeons in preparing further action plan.

Limitations

This study was conducted at JPNATC, AIIMS, owing to which it was rather difficult to include people who died of natural causes. Control patients were those who died by hanging, which could lead to hypoxic changes. Repeated hypoxia leads to an accumulation of βAPP as hypoxia increases Aβ generation by altering β- and gamma-cleavage of APP,[34] which is why we observed βAPP deposition in control samples, although significantly lesser than that in the sTBI group.

Conclusion

Our study, based on histopathological and β-APP scoring system to identify DAI, will facilitate the accurate diagnosis for DAI in forensic settings aiding in the criminal justice system. In the near future, studies such as these could also pave the way toward novel sTBI treatment regimens through enabling us to better elucidate the biomechanical events taking place post sTBI. Further research in this area may also enable in deciphering reliable information regarding the intensity of kinetic forces, thus helping in correlation with the data which may not be known to the forensic expert.

Author's Contributions

M.S., A.S., K.S., V.S. helped in conceptualization, design, literature review, data acquisition, manuscript layout, analysis, writing and editing, and gaining ethical approval. N.C. helped in analysis, writing, manuscript layout and editing. D.A. and R.M.P. helped in data acquisition and statistical analysis. R.M. helped in review. S.L. helped in concept, screening of intellectual content, and review. The manuscript has been reviewed and approved by all authors.

Data Availability Statement
The datasets used and/or analyzed during the current study can be made available by the corresponding author on request.

Ethical Approval
The study was approved by the Institute Ethics Committee (IEC), AIIMS, New Delhi (Ref. No.: IEC-69/2017).

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Conflict of Interest
None declared.

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