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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Expression and distribution of β amyloid precursor protein immunomarkers in the detection of diffuse axonal injury

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SUMMARY

Introduction/Objective The diffuse axonal injury has a very important place in clinical and forensic aspects of neurotraumatology. A special challenge is proving it in situations of short survival (less than two hours) after a craniocerebral injury.

The aim of this study was to determine the efficacy of beta-amyloid precursor protein (βAPP) immunohistochemical staining in postmortem diagnosis of axonal injuries in head injury survival shorter than two hours, its expression, and distribution through the brain tissue of the deceased.

Methods 36 adult fatalities, both sexes, injured by acceleration-deceleration mechanisms were divided into two groups: died up to two hours and died more than two hours after the injury. Immunostaining of brain tissue samples (frontal parasagittal white mass, genu and splenium of the corpus callosum and rostral pons) was used to register βAPP positivity. Data were processed by methods of descriptive and inferential nonparametric statistics, and $p < 0.05$ was considered statistically significant.

Results The βAPP immunopositivity was shown in 88.9% of cases (82.3% of ≤ two hours group *vs*. 94.7% of > two hours group). βAPP expression was enhanced towards the posterior structures of the brain. The shortest survival period with detected βAPP immunopositivity was 20–25 minutes, in three cases. There was an association of βAPP expression in the brainstem and interhemispheric/perimesencephalic subarachnoid hemorrhage ($p = 0.035$).

Conclusion βAPP immunohistochemical staining is effective in proving diffuse axonal injury in casualties that survived less than half an hour. Interhemispheric/perimesencephalic subarachnoid hemorrhage may indicate a more severe form of axonal injury.

Keywords: craniocerebral trauma; diffuse axonal injury; fatal outcome; amyloid beta-peptides

INTRODUCTION

Diffuse axonal injury (DAI) is one of the most common forms of diffuse brain injury. It is often present in traffic traumatism and can also be seen after falls from height, and blows to the head. Regardless of the mechanism of injury, the basic biomechanical principle of DAI is the acceleration-deceleration mechanism with elements of head rotation [1, 2]. The injury begins with the direct action of mechanical forces that cause the primary axonal injury followed by the pathophysiological cascading process of secondary axonal injuries that develops over time (for hours and days). The primary axonal injury occurs less frequently and to a lesser extent, while the secondary axonal injury is the dominant pathophysiological event [1, 3]. Clinically DAI is characterized by a prolonged comatose state (over six hours) in the absence of increased intracranial pressure or ischemic processes. Modern magnetic resonance imaging (MRI) techniques are sensitive enough to register the presence of an axonal injury in patients who have survived craniocerebral trauma long enough. Unfortunately, MRI is not a routinely applicable diagnostic method in the acute phase of diagnosis, immediately after injury. The reason is the incompatibility of the technical and procedural properties of MRI and the unstable condition of the traumatized patient who requires different types of support for vital functions in the initial phase of care. In this phase, computed tomography (CT) is still an indisputable radiological diagnostic method, but unfortunately it is insufficiently powerful in the detection of axonal injuries. Macroscopic pathomorphological manifestation of the axonal injury is often absent and not visible during autopsy. Pathomorphologically DAI is manifested by irregular axonal thickening, swelling (so-called varicosities) of partially damaged axons, and axonal bulbs at the ends of complete axonal tears [4]. These lesions are disseminated and scattered mainly along with the central brain structures: the parasagittal white matter of the frontal lobes, corpus callosum, internal capsule, thalamus, brainstem, and sometimes in the white matter of the cerebellum. Besides βAPP there are also other biomarkers in DAI diagnostic: N-acetylaspartate, glial fibrillary acidic protein, S100 calcium-binding

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protein B, ubiquitin C-terminal hydrolase, neurofilament-light etc. [5, 6, 7].

DAI is not exclusively of traumatic origin, but can also occur as a part of the development of brain edema, cerebral hypoxia, intoxication, etc. [8, 9]. Therefore, the term DAI is often changed to the more specific name of a traumatic axonal injury, which emphasizes its traumatic etiology. In literature, the classification of axonal injuries according to Adams is generally accepted [10]:

- grade 1: microscopically verified axoplasmic damage in the white matter of the cerebrum, corpus callosum, or brainstem;
- grade 2: grade 1 + focal lesions (bleeding) in the corpus callosum;
- grade 3: grade 1 + grade 2 + focal lesions in the rostral part of the brainstem and the upper cerebellar peduncles.

Special forensic significance is the fact that DAI very often ends in death, sometimes in a short period of several hours. In these situations, the primary axonal injury and the rapid decay of axons in certain, vital regions of the brain play an important role. This is especially true in cases where the axonal injury is the only intracranial pathological change. Its presence proves the viability of the injury (vital reaction), determines the exact cause of death and the possible mechanism of the injury, which are all crucial forensic issues.

The aim of this study was to test the efficacy of βAPP immunohistochemical staining in casualties that survived less than two hours after the injury, to examine the prevalence, distribution, and characteristics of the axonal injury of the deceased.

METHODS

Brain tissue was collected during regular forensic autopsies at the Institute for Forensic Medicine of the Republic of Srpska in Banja Luka, in the period from June 2017 to the end of 2019. The study included fatalities in acceleration-deceleration mechanisms (traffic accidents, fall from height, blows to the head), whose autopsies were performed up to 24 hours after death. A total of 36 brains of the deceased, both sexes, aged 18–81 years were collected. Brain tissue samples from the parasagittal white matter of the frontal lobe, anterior (genu) and posterior (splenium) parts of the corpus callosum, the rostral part of the pons with the upper cerebellar peduncles were taken for the study. The sex, age distribution, and the mode of injury (drivers/passengers in the vehicle, pedestrians, cyclists, motorcyclists, falls from height and blows to the head), brain mass, the Glasgow Coma Scale (GCS), fractures of cranial bones, focal lesions, and the presence of microbleeds in the brain tissue were observed. Exclusion criteria were: deceased under 16 years of age, and cases with confirmed neurodegenerative, inflammatory, or global ischemic-hypoxic changes of the central nervous system. The control group (10 subjects) consisted of brain tissue of deceased who died from causes not related to neurotrauma, without verified neurodegenerative, hypoxic-ischemic, hypoglycemic, and inflammatory changes of the central nervous system. The police reports and medical documentation determined the exact time and manner of the injury, the time of death, and the period of survival. During autopsy, all macroscopic craniocerebral injuries were carefully registered. The brain was examined on 1 cm thick cross-sections while brainstem and cerebellum were analyzed on 0.5 cm thick horizontal sections. Then, tissue blocks were taken from: the parasagittal white matter of the frontal lobe, the genu and splenium of the corpus callosum, and the rostral part of the pons. After fixation in a buffered solution of 10% formalin, standard molding and preparation of histological specimens was performed. Hematoxylin-eosin staining excluded pathological changes in brain tissue such as diffuse edema, brain atrophy, degenerative, inflammatory, general hypoxic-ischemic changes, and verified possible microbleeds.

For immunohistochemical staining we have used: monoclonal antibodies to βAPP (βAPP - Monoclonal Mouse Anti-Human β-Amyloid, clone 6F/3D, dilution 1:50 DAKO, Glostrup, Denmark), labeling of the antigen-antibody complex with streptavidin-biotin method (DAKO, K 0690), and visualization of tissue sections by dropping diamino-benzidine (DAKO, code 3466). Immunohistochemical staining registered ßAPP positivity on the analyzed sections through the presence of axonal bulbs and/or varicosities, and it was semiquantitatively assessed by the Gentleman`s scale [11]:

- "0" no staining;
- "+" weak positivity (isolated, sporadic scattered axonal varicosities);
- "++" typical positivity (grouped axon bulbs and varicosities);
- "+++" strong positivity (diffusely spread, involving larger parts of the visual field or bundles of nerve fibers).

DAI grading was performed according to the Adams classification [10]. To check the efficacy of the selected βAPP immunohistochemical technique in cases of short survival, two groups were formed, adjusted by sex, age, and mechanism of injury: group I – 17 brains of deceased who died within two hours after the injury, and group II – 19 deceased who survived over two hours. The consent of the Ethics Committee of the University Clinical Center of the Republic of Srpska in Banja Luka was obtained for this research. Statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Sex, age distribution, and mode of injury were presented numerically, as a percentage and medians. Nonparametric χ^2 and Fisher's exact test were used to compare categorical variables and to check the interconnection of individual variables. The Kruskal–Wallis and Mann–Whitney U tests were used to test the differences in the distribution of independent features with the ordinal scale of measurement. The Spearman's rank correlation coefficient was used to analyze the correlation of the two features with the ordinal scale of measurement. The statistical significance threshold was $p < 0.05$.

Table 1. Demographic characteristics, mechanism of casualties and associated craniocerebral injuries in observed groups and total

Variables	Total (36 cases)	≤ two hours (17 cases)	> two hours (19 cases)	p
Age (years): median (range)	51 (18–81)	45 (19–81)	54 (18–81)	0.302
Sex:				
male	30 (83.3)	15 (88.2)	15 (78.9)	0.472
female	6 (16.7)	2 (11.8)	4 (21.1)	
Mechanism of injury:				
driver/passenger	12 (33.3)	7 (41.2)	5 (26.3)	
pedestrian	10 (27.8)	4 (23.5)	6 (31.6)	0.372
motorcyclist/cyclist	8 (22.2)	4 (23.5)	4 (21.1)	
fall from height	4 (11.1)	2 (11.8)	2 (10.5)	
blow to the head	2 (5.6)	0 (0.0)	2 (10.5)	
Glasgow coma score:				
≤ 8	30 (83.3)	14 (82.4)	16 (84.2)	0.475
9–12	6 (16.7)	3 (17.6)	3 (15.8)	
AssCranioInjuries:				
Cranial fractures	28 (77.8)	13 (76.5)	15 (78.9)	0.323
HED	2 (5.6)	0 (0)	2 (10.5)	0.169
HSD	30 (83.3)	14 (82.4)	16 (84.2)	0.881
SAH	28 (77.8)	14 (82.4)	14 (73.7)	0.532
IVH	23 (63.9)	12 (70.6)	11 (57.9)	0.429
CCH	11 (30.6)	6 (35.3)	5 (26.3)	0.559
BSH	4 (11.1)	3 (17.6)	1 (5.3)	0.238
Contusions	18 (50.0)	4 (23.5)	14 (73.7)	**0.003 (0.000*)**
Pet. hemorrhage	11 (30.6)	5 (29.4)	6 (31.6)	0.888

Values are presented as numbers (%), median and range for years; AssCranioInjuries – associated craniocerebral injuries; HED – epidural hemorrhage; HSD – subdural hemorrhage; SAH – subarachnoid hemorrhage; IVH – intraventricular hemorrhage; CCH – corpus callosum hemorrhages; BSH – brainstem hemorrhages; Pet. hemorrhages – petechial hemorrhages; p value – χ^2 and Kruskal–Wallis* test; boldface type indicates statistical significance

Table 2. Distribution of βAPP immunoexpression in the observed brain regions (total)

βAPP	Front. (N)	CCG (N)	CCS (N)	Pons (N)
+	15	8	8	7
++	10	16	17	9
+++	2	2	3	12
0	9	10	8	8

βAPP – beta amyloid precursor protein; Front. – parasagittal white matter of the frontal lobe; CCG – genu of corpus callosum; CCS – splenium of corpus callosum; + – weak positivity; ++ – typical positivity; +++ – strong positivity; 0 – negative βAPP immunoexpression

RESULTS

Table 1. shows basic demographic data, mechanism of injury and associated craniocerebral injuries by group and in total. In our sample, male gender and traffic accidents as a mechanism of injury were dominant. The most common associated craniocerebral injuries were subdural hemorrhage (HSD), subarachnoid hemorrhage (SAH) and skull bone fracture. Statistical comparison of these variables between groups revealed a significant difference only in the presence of brain tissue contusions, which were significantly more often present (14 contusions) in the group that survived longer (χ^2 p = 0.003; Kruskal–Wallis p = 0.000, Mann–Whitney U p = 0.000).

Axonal injury

On a total sample of 36 brains, βAPP immunopositivity was demonstrated in 32 cases or 88.9%. According to the observed brain regions, in the parasagittal white matter, the axonal injury was confirmed in 75% of cases, in the genu of the corpus callosum 72.2%, in the splenium of the corpus callosum 77.8% and in the pons 77.8%. According to the Adams classification of the severity of the axonal injury, grade 1 of DAI was found in 20 (55.6%) cases, in nine cases grade 2, and grade 3 in three cases. βAPP immunostaining revealed a fairly even distribution of the axonal injury through the observed regions of brain tissue, with a noticeable shift in the expression intensity to the posterior structures of brain tissue and the highest frequency of strong immunopositivity in the pons region (Table 2, Figure 1). Spearman's correlation test confirmed a weak relationship between the presence and intensity of axonal injuries in the parasagittal white matter of the frontal lobe and the anterior parts of the corpus callosum (r = 0.395), and a moderately strong association between the posterior parts of the corpus callosum and the rostral region of the brainstem (r = 0.627).

In the group of deceased that survived up to two hours, βAPP immunostaining showed the presence of a DAI in 14 brains (82.3%), while in the group where the deceased survived more than two hours, βAPP immunopositivity was present in 18 of 19 cases (94.7%). The shortest survival period in which βAPP immunostaining confirmed an axonal injury was about 20–25 minutes, in three cases. The first case was a 30-year-old man, a cyclist, with a weak expression in the splenium of the corpus callosum (+) and a typical immunopositivity in the pons (++). Second case was a 35-year-old male, pedestrian, with weak immunopositivity in the white matter of the frontal lobe (+), typical through the corpus callosum (++) and very strong in the pons (+++). The third case was a 34-year-old man, a car driver, with a verified axonal injury in the frontal white matter (++), splenium of the corpus callosum (+), and pons (+++) (Figure 2). In the first

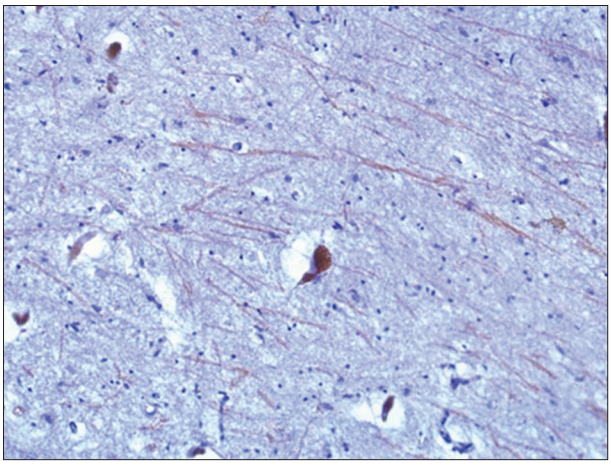

Figure 1. βAPP immunopositivity in the pons of a deceased who survived trauma for 50 hours; numerous, almost parallel-oriented, varicose thickened axons; a 54-year-old male, cyclist; βAPP immunostaining, 40 × magnification

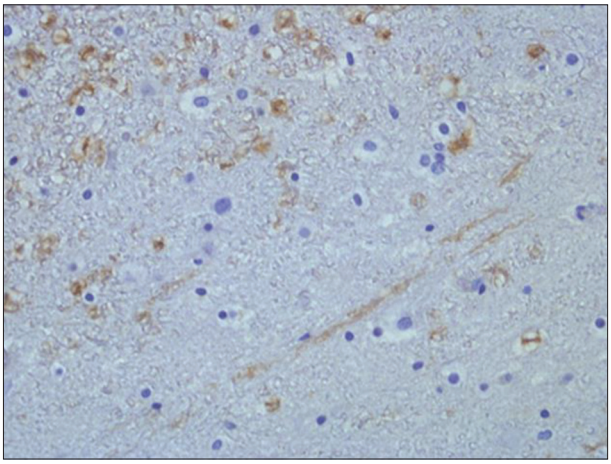

Figure 2. βAPP immunopositivity in the pons of deceased who survived trauma for 20–25 minutes; there are pronounced axonal bulbs and more discreet axonal varicosities that extend through the pons; a 34-year-old male, car driver; βAPP immunostaining, 40 × magnification

two cases, there were similar associated craniocerebral injuries: fracture of the roof and base of the skull, HSD, SAH, intraventricular hemorrhage and brainstem hemorrhage, with petechial hemorrhage in the white matter and corpus callosum in the first case. In the third case, in addition to the axonal injury, less pronounced HSD and SAH were found. When comparing the efficacy of βAPP immunostaining in the both groups, there was no statistically significant difference between observed regions (Table 3). The χ^2 test showed statistical significance between the presence of petechial hemorrhages in the parasagittal white matter of the frontal lobes and the axonal injuries in the anterior part (genu) of the corpus callosum ($p = 0.023$) but the association of petechial hemorrhages with the axonal injury in the white matter of the frontal lobes has not been confirmed ($p = 0.064$). The association of βAPP immunopositivity expression in the brainstem with diffusely disseminated and interhemispheric/perimesencephalic localized SAH was found ($p = 0.035$).

Table 3. Comparison of βAPP immunopositivity in the observed brain regions by groups

βAPP	Front. (N)		CCG (N)		CCS (N)		Pons (N)	
	≤ 2 h	> 2 h	≤ 2 h	> 2 h	≤ 2 h	> 2 h	≤ 2 h	> 2 h
+	8	7	2	6	2	6	2	5
++	5	5	7	9	11	6	5	4
+++	0	2	1	1	0	3	7	5
0	4	5	7	3	4	4	3	5
p	0.557 0.706*		0.290 0.051*		0.095 0.283*		0.547 0.394*	

βAPP – beta amyloid precursor protein; Front. – parasagittal white matter of the frontal lobe; CCG – genu of corpus callosum; CCS – splenium of corpus callosum; + – weak positivity; ++ – typical positivity; +++ – strong positivity; 0 – negative β APP immunostaining; p – χ^2 test, *Kruskal–Wallis test

In the control group (five males and five females, aged 21–77 years), six died of natural causes, three due to asphyxia and one of the consequences of stab wounds to the chest, without verified mechanical head injuries. Brain tissue immunostaining for βAPP was negative in all observed brain regions in all ten cases.

DISCUSSION

Over two-thirds of the victims with DAI are active workers. Men in traffic accidents are the most common casualties. This is in line with the results of other studies on DAI and craniocerebral trauma [12, 13]. The measured GCS values in the vast majority of casualties in this research indicated severe craniocerebral injury. The remaining, smaller number of cases with more favorable GCS (9–12) showed milder forms of DAI. Although not statistically significant, such distribution of GCS values is quite consistent with the fatal injury outcome and views that GCS is one of the indicators of severity of craniocerebral injury and recovery outcome predictor [14, 15]. In severe craniocerebral injuries with a fatal outcome, we most often saw a polymorphic pathomorphological image: skull fractures, various types of intracranial hemorrhage, and brain contusions with associated diffuse injuries of brain tissue, including axonal injuries. The available literature describes links between intraventricular hemorrhage and the presence of an axonal injury in the corpus callosum and pons [16]. In our study, such results were not confirmed and there was a significant association between DAI and most of the observed associated craniocerebral injuries. The connection between the expression of the axonal injury in the pons with SAH (interhemispheric and perimesencephalic), as well as diffusely spread SAH is distinguished. Similar results are described in recent studies [17, 18] which emphasizes the need for further research on whether this type of SAH can serve as a marker of severe DAI already in the initial phase of diagnosis (CT) and treatment of these patients. In forensic terms, this could indicate a very similar mechanism of injury by shearing forces of different layers of brain tissue and accompanying blood vessels. The association of petechial hemorrhage in the white matter of the frontal lobes and the axonal injury in the anterior segment of the corpus callosum, in this study, was not followed by the same relationship between petechial hemorrhage and axonal injury in the same localization (white matter of the parasagittal frontal region), although the significance level ($p = 0.064$) is at the very border of significance. These results do not contradict current views on the connection between microbleeds and axonal injuries [19, 20], but also do not confirm them with certainty. Reasons for this may be found in inconsistent research methodologies, the method of measuring the severity of certain types of injuries, etc. From the forensic point of view, cases where the axonal injury is a solitary craniocerebral injury are especially interesting. There were only two such cases in our study; the first is a 19-year-old young man, a car driver who survived for about 50 minutes, with a very strong immunopositivity in the brainstem and weak expression in the frontal white matter. The other was a 50-year-old man, a driver who survived six and a half hours, with typically pronounced immunopositivity in the corpus callosum and brainstem while a weak βAPP immune reaction was found in the frontal white matter. However, in both cases, there were associated injuries of other organ systems (contusions of lung tissue, fractures

of long bones, lacerations of the liver, etc.) which were not necessarily fatal but certainly contributed to the fatal outcome. We believe that the strong expression of the axonal injury in the brainstem in the first case, as well as the typical expression of the axonal injury through the brainstem and corpus callosum in the second case, can be considered as the immediate cause of death within the experienced polytrauma. No significant differences were found between the observed groups in terms of the prevalence of associated craniocerebral injuries, except in the case of brain contusions. Brain contusions were significantly more common in the group that survived longer. The probable explanation is that brain tissue contusions develop as a function of time and often progress in the posttraumatic period. In that way, the minor contusion injury, seen at the beginning, spreads over time and becomes easier to see visually. This expansion of the contusion focus was registered during the first 12 hours, sometimes the first 3–4 days after the injury, in which microvasculature lesions in the contused region and the release of transcription factor 1 and nuclear factor kappa B play an important role [21, 22, 23]. Expression of βAPP immunopositivity on a total sample close to 90% confirms the view of Gentleman et al. [11] that DAI is almost a regular finding in the case of a fatal blunt force head injury. In casualties that survived up to two hours, the immunopositivity is only slightly lower, but still over 80%, so we did not establish a significant difference between the observed groups. This study confirmed that βAPP immunostaining showed high sensitivity and efficacy in detecting axonal injuries in persons who died in a period much shorter than two hours. The shortest survival period with a positive βAPP immune reaction was about 20–25 minutes, confirmed in three cases. All three cases had been associated with various focal craniocerebral injuries. A similar short survival period in which βAPP immunostaining was successfully obtained is reported by Hortobágyi et al. [24].

The distribution of βAPP immunopositivity is fairly even throughout the observed regions of the brain, which corresponds to the notion of axonal injury as a diffuse cerebral injury. However, it is evident that the expression of βAPP immunopositivity increases from the anterior to the posterior structures of the brain (weak positivity is most common in the frontal white matter, typical through the corpus callosum and strong in the rostral parts of the brainstem). This finding is expected given the vital importance of brain regions such as the brainstem and corpus callosum. In the literature, the classification of the axonal injury severity according to Adams is generally accepted, which connects the most severe, grade 3 of axonal injury with the worst outcome [10], but this was not proven to be optimal in our research. There were only three cases (8.3%) of DAI grade 3, two cases of survival for half an hour and one case of survival for 17 hours. This is unusual in a sample of 36 severe craniocerebral injuries with a fatal outcome and deserves more careful analysis. Are macroscopically noticeable focal hemorrhages in the corpus callosum and brainstem really a relevant parameter or perhaps the presence of smaller, microscopically noticeable hemorrhages should be considered as valid criteria in this classification?

The limitations in this study were the relatively small total sample and the suboptimal number of different localizations from which brain tissue samples were taken for specific immunostaining. Data on the presence of axonal injuries in other predilection regions of the brain such as the thalamus, internal capsule, parahippocampal region, cerebellum, and lower parts of the brainstem would certainly be welcome and provide valuable information on the overall prevalence of this injury and possible association with other types of cerebral trauma. Finally, it was not possible to completely rule out the impact of serious injuries to other organ systems on the fatal outcome. In future research, it would be desirable to provide a larger sample without associated injuries to other body systems, in which multiple predilection cerebral regions for axonal injury development would be observed. Of particular forensic significance would be the study of an axonal injury as a solitary craniocerebral injury.

CONCLUSION

In postmortem proofing of DAI, βAPP immunohistochemical staining is a very powerful forensic diagnostic tool and shows efficacy in cases of survival less than half an hour. The observed link between interhemispheric/perimesencephalic SAH and axonal injury in the brainstem directs the focus of further research towards the interrelationship of these two types of craniocerebral injuries, which could, besides forensic, have an undeniable clinical significance in facilitating the diagnosis of more severe forms of axonal injuries in initial stages of treatment of these patients.

Conflict of interest: None declared.

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Експресивност и дистрибуција бета амилоидног прекурсорног протеинског имуномаркера у доказивању дифузне аксонске лезије

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САЖЕТАК

Увод/Циљ Дифузна аксонска лезија има важну улогу у савременој неуротрауми, у клиничком као и форензичком аспекту. Посебан изазов је њено доказивање у ситуацијама кратког надживљавања краниоцеребралне повреде, испод два сата.

Циљ овог рада је утврдити ефикасност имунохистохемијског бојења бета амилоидног прекурсорног протеина (*βAPP*) у постморталној дијагностици аксонске лезије код надживљавања повреде главе краћег од два сата, његову експресију и дистрибуцију кроз мождано ткиво смртно страдалих.

Методе Узорак од 36 смртно страдалих, одрасле доби, оба пола, у акцелерацијско-децелерацијским механизмима, подељен је у две групе: умрли до два сата и умрли после више од два сата од повређивања. Имунохистохемијским бојењем узорака мозданог ткива (фронтална парасагитална бела маса, гену и спленијум корпус клалозума, ростралнi део понса) регистрована је *βAPP* позитивност анализираних исечака. Добијени подаци обрађени су методама дескриптивне и инференцијалне непараметријске статистике, са нивоом статистичке значајности $p < 0,05$.

Резултати *βAPP* имунопозитивност потврђена је код 88,9% случајева (82,3% умрлих до два сата и 94,7% умрлих после више од два сата). *βAPP* имунопозитивност се појачава ка задњим структурама мозга. Најкраћи период надживљавања са детектованом *βAPP* имунопозитивношћу је 20–25 минута, у три случаја. Уочена је повезаност *βAPP* експресије у мождaном стаблу са интерхемисферично/перимезенцефаличном субарахноидалном хеморагијом ($p = 0,035$).

Закључак *βAPP* имунохистохемијско бојење показује ефикасност у доказивању дифузне аксонске лезије код надживљавања краћег од пола сата. Интерхемисферично/перимезенцефалично локализована субарахноидална хеморагија може указивати на теже форме дифузне аксонске лезије.

Кључне речи: краниоцеребрална траума; дифузна аксонска лезија; смртни исход; бета амилоидни пептид