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Histological and immunohistochemical study of brain damage in traumatic brain injuries in children, depending on the survival period

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Abstract

Numerous studies showed that, at present, traumatic brain injury (TBI) is one of the main causes of death in young adults, but also a main cause of disabilities at all ages. For these reasons, TBI are continuously investigated. In our study, we evaluated the histopathological (HP) and immunohistochemical (IHC) changes that occurred in the brain in underage patients after a severe TBI depending on the survival period. We histopathologically and immunohistochemically analyzed a number of 22 cases of children, deceased in Dolj County, Romania, following some severe TBI, undergoing autopsy within the Institute of Forensic Medicine in Craiova between 2015–2020. Patients were divided into three groups depending on the survival period, namely: (*i*) patients who died during the first 24 hours of the accident; (*ii*) patients who died after seven days of survival; (*iii*) patients who died after 15 days of survival. Microscopic examinations of the brain fragments, collected during the necropsy examination, showed that the traumatic agent caused primary injuries in all brain structures (cerebral parenchyma, meninges, blood vessels). However, HP injuries ranged in size and intensity from one area to another of the brain. In patients with a longer survival period, there was observed the presence of smaller primary injuries and larger secondary injuries. There was also observed a growth in the number of meningo-cerebral microscopic injuries, depending on the increase of the survival period.

Keywords: traumatic brain injury, immunohistochemistry, pediatrics, neuroinflammation, head trauma.

⊟ Introduction

Traumatic brain injury (TBI), defined as disturbance of the brain function and/or damage of its structure, caused by an external physical force, represents, at present, a medical and socio-economic problem. The annual TBI incidence is estimated at about 50 million cases worldwide, figures suggesting that about half of the global population could have an episode of TBI during their lives. Statistical data show that, annually, TBI costs the global economy about $ 400 billion, representing 0.5% of the world gross product [1, 2]. Other studies consider that the TBI number is much higher, most brain traumas not being recorded due to their less severity and, as such, they do not address a hospital service. Epidemiological studies in recent years showed that only in the US, TBI are responsible for over 50 000 deaths every year [2, 3]; also, they are responsible for about 30.5% of all deaths caused by trauma [4]. In Europe, in 2012, 1 375 974 TBI patients were admitted and 33 415 people died because of this [5].

TBI are a massive burden for worldwide health systems, not only because of the organic or mental injuries they cause, but also because of the economic costs that include medical expenses, as well as indirect expenses, such as productivity losses [6–8].

Experimental studies and observations in clinical trials showed that there are two distinct phases of TBI injuries: (*i*) the acute primary event in which brain tissue injury is closely related to the energy that the brain receives at the time of trauma; (*ii*) the secondary stage, which includes a cascade of injuries like glutamatergic excitotoxicity, calcium (Ca) overload and vascular dysfunction; these

secondary injuries usually last for several months or even years, resulting in a model of chronic post-traumatic brain disease [9, 10].

Several clinical studies showed that after TBI there are long-term behavioral dysfunctions, including cognitive decline or the onset of mental disorders, for a longer period of time after TBI [10]. Even if patients have a good physical recovery, 30% of them reported a change in the cognitive function, such as memory and concentration deficiency, up to three months after a mild TBI [11]. Also, TBI occurring in the first years of life could lead to a higher risk of mortality, independent of its severity [12]. Repeated TBI, especially in sports (boxing) are known as a risk factor for dementia [13].

Aim

The objective of our study was the histopathological (HP) and immunohistochemical (IHC) evaluation of meningo-cerebral injuries, depending on the post-traumatic survival period in underage patients who died after a severe TBI and were considered cases of forensic medicine.

⊟ Materials and Methods

The group of patients in this study included 22 underage patients, who died following a TBI and underwent autopsy within the Institute of Forensic Medicine in Craiova, Dolj County, Romania, between 2015–2020. Of these, 12 patients died during the first 24 hours after the accident, six died after seven days of survival, and four died after a period of 15 days of survival.

Every case underwent necropsy according to the present legal requirements, to establish the causes of death. After the macroscopic examination of the brain, there were harvested brain fragments of about 2/2/2 cm from the injured area, but also from a distance from the TBI, for the HP and IHC study, to complete the death diagnosis.

The harvested biological material was fixed in a 10% neutral buffered formalin, for three days, and included in histological paraffin according to the classical HP protocol. After the paraffin inclusion, there were performed 4 μm-thick sections in the microtome, stained with Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) green light trichrome.

For the IHC study, we used special histological blades covered with poly-L-lysine debris, to avoid detaching the biological material from the histological blade, during the use of the immunohistochemistry technique.

In our study, for highlighting neuronal and astrocyte changes, we used the following antibodies: anti-neuronal nuclei (NeuN) (monoclonal rabbit anti-human, clone 13E6, 1:150 dilution, Dako); anti-neurofilament (NF) (monoclonal mouse anti-human NF protein, clone 2F11, 1:100 dilution, Dako); anti-neuron-specific enolase (NSE) (monoclonal mouse anti-human NSE, clone BBS/NC/VI-H14, 1:100 dilution, Dako); anti-glial fibrillary acidic protein (GFAP) (polyclonal rabbit anti-human GFAP, 1:1000 dilution, Dako); anti-ionized Ca-binding adaptor molecule 1 (IBA1) [mono-clonal mouse immunoglobulin (Ig)G2b κ, Iba-1, ab15690, 1:200 dilution, Abcam].

The study was performed after the prior approval of the management of the Institute of Forensic Medicine in Craiova, complying with the measures for confidentiality and protection of personal data, according to the present legislation.

⊟ Results

In our study, in the deceased patients within the first 24 hours after the accident, we identified a multitude of HP changes in all parts of the brain (cerebral parenchyma, blood vessels, leptomeninges), which were primarily caused by the impact between the vulnerate agent and the skull and less by the secondary pathophysiological processes, respectively the vascular dysfunction, glutamatergic excitotoxicity or Ca overload of neurons and glial cells.

The macroscopic and microscopic aspects of the brain injuries had maximum intensity on the impact area of the cranial box with the vulnerate agent, but similar injuries were identified in other areas of the brain, including the opposite pole to the traumatic agent action.

In patients exposed to severe TBI and who died immediately after the accident, we identified areas of brain laceration characterized by fragmentation of the cerebral parenchyma and rupture of the leptomeninges, associated with subdural hemorrhage and cerebral edema (Figure 1A). The most common and most obvious microscopic lesions observed in deceased people within 24 hours after TBI were represented either by the intraparenchymal blood accumulation, in the form of intracerebral hematomas (more or less voluminous), or by diffuse hemorrhages, either in the liquid spaces of the brain as leptomeningeal or ventricular hemorrhages (Figure 1, B and C) or subdural hematomas. In some patients, perivascular hemorrhage was identified in the Virchow–Robin space, the microscopic aspects denoting the rupture of the blood-brain barrier (BBB) (Figure 1D), while in other patients there were identified areas with perivascular edema, showing the permeability of the BBB (Figure 1E).

Regarding changes in the nervous tissue, even if the time between TBI and the death of individuals was reduced, there were identified areas where neurons and glial cells presented pericellular edema, increased neuroplasm acid ("red neurons", specific to brain ischemia), condensation of nuclei and nuclei fragmentation of neuronal extensions with the presence of a heterogeneous neuropil (Figure 1F).

The IHC examinations performed on the brain fragments, harvested from children who died in less than 24 hours after the traumatic impact, showed significant changes in neurons or glial cells in the areas of TBI and lower intensity in other brain areas. Thus, the IHC marking of the NeuN protein showed an important decrease of the reaction (until disappearance) in neurons around the TBI area (Figure 2A), but the reaction to NeuN was unchanged in the brain areas located remote from the TBI area (Figure 2B).

The assessment of the reaction of NF in the structure of neurons allowed us to observe that the IHC reaction in small neurons was reduced but was maintained at a higher level in larger neurons and in thick neural extensions (Figure 2C), showing the disintegration of NFs in the structure of the cytoskeleton of small neurons, especially those in the TBI area.

The reaction of NSE in the healthy neurons from the brain was intense, even in the areas near the TBI, in patients who died within the first 24 hours after the accident, which shows an increase of its synthesis in neurons, immediately after the TBI (Figure 2D). The astrocyte reaction was quite low in the subjects of this group, being observed only a moderate reaction around the cerebral dilaceration areas (Figure 2E), while the microglia reaction was absent (Figure 2F).
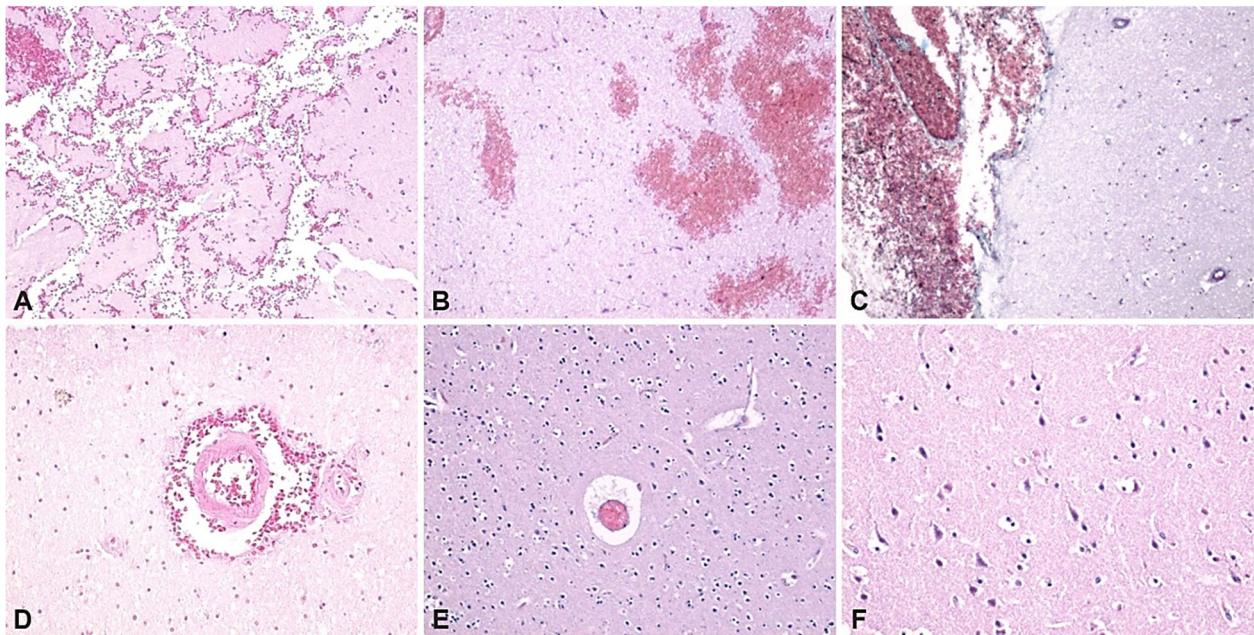
Figure 1 – *Histopathological changes of the brain in deceased patients within the first 24 hours after TBI: (A) Dilacerated cerebral parenchyma area, associated with diffuse brain hemorrhage, in a 12-year-old boy, victim of a traffic accident; (B) Microscopic aspect of extended interparenchymal hemorrhage, present in the same case as in the previous image; (C) Image of leptomeningeal hemorrhage in a 6-year-old boy, victim of a traffic accident; (D) Microscopic image of perivascular hemorrhage through the Virchow–Robin space, in a 6-year-old boy, victim of a traffic accident; (E) Brain area with post-traumatic perivascular edema, in a 6-year-old boy, victim of animal aggression; (F) Encephalic nervous tissue with moderate pericellular edema, nuclear condensation, neuroplasm acidification and incipient fragmentary of neuropil in a 6-year-old boy, victim of animal aggression. HE staining: (A, B and E) ×100; (D and F) ×200. GS trichrome staining: (C) ×100. GS: Goldner–Szekely; HE: Hematoxylin–Eosin; TBI: Traumatic brain injury.*
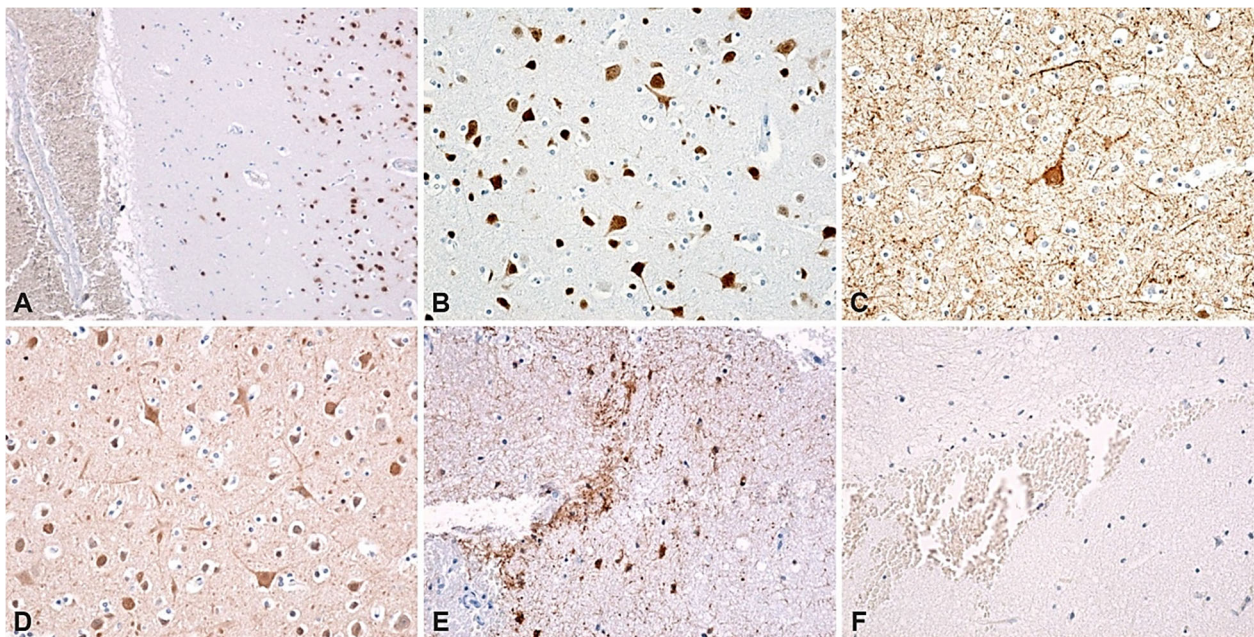

Figure 2 – *IHC changes of cerebral parenchyma in deceased patients within the first 24 hours after TBI: (A) IHC image from a severe cranial trauma with leptomeningeal hemorrhage, in a 6-year-old boy who died four hours after the traumatic impact, in which the reduction or lack of IHC reaction is observed in neurons in the superficial layers of the brain; (B) Normal IHC image of NeuN protein in the neurons of contralateral hemisphere in the same case; (C) Image of cerebral parenchyma in the immediate vicinity of the TBI area, where there is observed a low reaction of the IHC reaction for NF protein in small neurons, but with its high level in larger neurons and in thick neuronal extensions; (D) Brain area near the TBI, with moderate perineurial edema, but with intense IHC reaction of NSE; (E) Moderate reaction of astrocytes around a cerebral dilaceration area; (F) Absent reaction of microglia 24 hours after the TBI. Immunomarking with anti-NeuN antibody: (A) ×100; (B) ×200. Immunomarking with anti-NF antibody: (C) ×200. Immunomarking with anti-NSE antibody: (D) ×200. Immunomarking with anti-GFAP antibody: (E) ×100. Immunomarking with anti-IBA1 antibody: (F) ×100. GFAP: Glial fibrillary acidic protein; IBA1: Ionized calcium-binding adaptor molecule 1; IHC: Immunohistochemical; NeuN: Neuronal nuclei; NF: Neurofilament; NSE: Neuron-specific enolase; TBI: Traumatic brain injury.*

In individuals who died seven days after TBI, the HP examination highlighted the same types of lesions, but of higher intensity. Intracerebral or subdural hematomas or hemorrhages had a heterogeneous aspect due to the partial hemolysis of red cells and hemoglobin release (Figure 3, A and B). Around the hemorrhagic lesions, the inflammatory reaction was minimal or absent. The small-sized vessels, arterioles, venules, and capillaries were almost entirely with collapsed walls and with marked perivascular edema (Figure 3C).

The cerebral parenchyma presented areas of brain necrosis, through the necrosis and apoptosis of neurons and neural extensions, with the formation of spongy aspect areas (Figure 3D). In areas near the TBI, neurons presented nuclear and cytoplasmic condensation, neuronal debris in cavities that mimic neuronal forms (neuronal ghosts), neuropil with granular aspect resulting from fragmentation and partial lysis of neuronal extensions (Figure 3E). In areas more distant from the TBI, there were identified neurons with preserved aspect, but with a heterogeneous, fine granular, sometimes acidophilic neuroplasma, with an aspect of "red" or ischemic neurons. The neuropil presented a disorganized aspect due to the damaging of some neuronal extensions (Figure 3F).

IHC examinations in patients who died after seven days from TBI highlighted the increased reduction and even absence of the NeuN reaction, which is equivalent to the onset of cellular death processes (Figure 4A). NFs presented a reduced number both in neurons and neuronal extensions, being visible only in the larger neurons and in thick neuronal extensions (Figure 4B). NSE presented a low IHC reaction, being highlighted in some neurons, thus showing the intensity of neural suffering with the reduction of enolase synthesis and its release in the perineurial space as the neuronal suffering is prolonged (Figure 4C). In contrast, in the subjects of this group, there was observed an increase in the number and activity of astrocytes, both in the TBI areas and around them (Figure 4, D and E). The IHC reaction of microglia was reduced, being identified rare positive cells to the anti-IBA1 antibody (Figure 4F).

The microscopic study of the brain fragments, harvested from people who died 15 days after TBI, showed the presence of complex brain injury. At the level of the leptomeninges, there was highlighted the presence of congested, sometimes thrombosed vessels (Figure 5A), associated with a moderate inflammatory reaction and perivascular fibrosis (Figure 5B). Perivascular hemorrhages through Virchow–Robin spaces were transformed into thrombotic structures (Figure 5C), while in the areas of brain dilaceration the necrosis of cerebral parenchyma was in advanced stages and the glial cells or neurons could not be identified (Figure 5D). In the areas around the TBI, there were identified areas with neurons with acidophilic, condensed cytoplasm, with pericellular and perivascular edema and with fragmented neuropil (Figure 5E), but also areas of cerebral parenchyma with a spongy aspect because of neuronal death (Figure 5F).

IHC examinations of the brain in people who died 15 days after TBI showed the complete absence of reaction to NeuN (Figure 6A) and of NFs (Figure 6B); instead, NSE was observed to be moderately positive in areas of cerebral parenchyma remote from the TBI area (Figure 6C). Astrocytes presented intensely positive, hypertrophied, with irregular, heterogeneous margins, with few extensions (Figure 6D). Microglia presented in high number, intensely positive, size-increased, with heterogeneous cytoplasm both in the TBI areas (Figure 6E) and remote from them (Figure 6F).
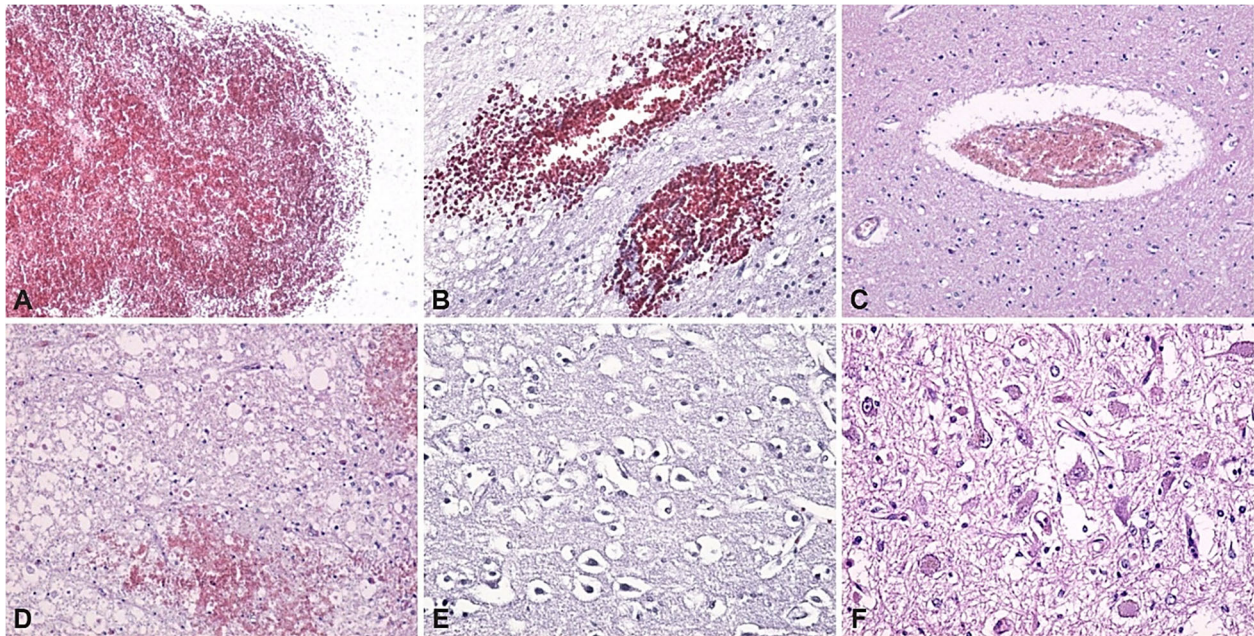

Figure 3 – *Histopathological changes of the brain in patients deceased seven days after TBI: (A) Image of an old cerebral hematoma, directly limited by the cerebral parenchyma, with partially hemolyzed hematoma, in a 9-year-old boy, a victim of a railway accident; (B) Intraparenchymal microhemorrhages, associated with perineural edema and incipient cell necrosis in a 9-year-old boy, victim of a railway accident; (C) Microscopic image of a cerebral vein, with disorganized wall and intense perivascular edema; (D) Diffuse parenchymal microhemorrhages, associated with important cell necrosis, in an 8-year-old boy, victim of a traffic accident; (E) Brain area with intense perineuronal edema and with a granular aspect of the neuropil, in a 9-year-old boy, victim of a railway accident; (F) Area of cerebral parenchyma located remote from the TBI area; there is observed the presence of neurons with fine granular, heterogeneous, acidophilic neuroplasma and disorganization of neuropil. HE staining: (A, C and D) ×100; (F) ×200. GS trichrome staining: (B and E) ×200. GS: Goldner–Szekely; HE: Hematoxylin–Eosin; TBI: Traumatic brain injury.*
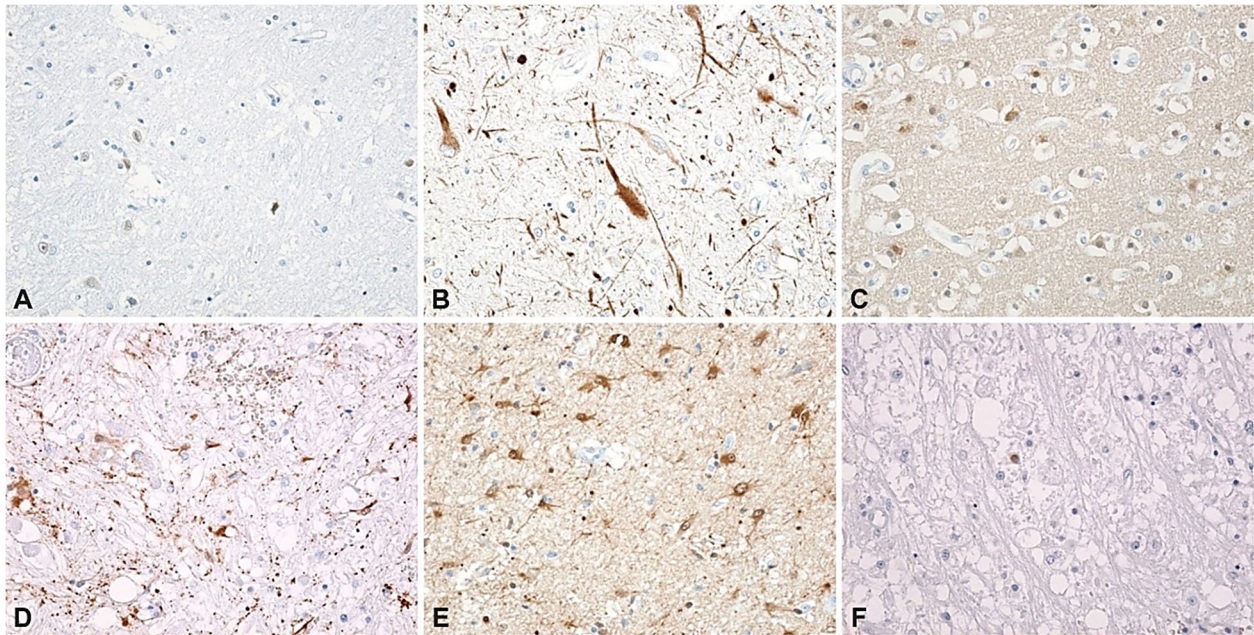
Figure 4 – IHC changes in cerebral parenchyma in patients deceased seven days after TBI: (A) Area of cerebral parenchyma with low or even absent neuronal reaction to NeuN; (B) Low IHC reaction of NFs in both neuronal bodies and the neuropil (neuronal extensions); (C) Brain area reduced reaction of NSE; (D) Area of cerebral parenchyma from the TBI area with multiple areas of cell necrosis and intense reaction of astrocytes; (E) Cerebral parenchyma in the vicinity of the TBI area, with an increased number of astrocytes and intense IHC reaction (reactive gliosis aspect); (F) Low reaction of microglia in the cerebral parenchyma in the immediate vicinity of the TBI area. Immunomarking with anti-NeuN antibody: (A) ×200. Immunomarking with anti-NF antibody: (B) ×200. Immunomarking with anti-NSE antibody: (C) ×200. Immunomarking with anti-GFAP antibody: (D) ×200. Immunomarking with anti-GFAP antibody: (E) ×200. Immuno-marking with anti-IBA1 antibody: (F) ×200. GFAP: Glial fibrillary acidic protein; IBA1: Ionized calcium-binding adaptor molecule 1; IHC: Immunohistochemical; NeuN: Neuronal nuclei; NF: Neurofilament; NSE: Neuron-specific enolase; TBI: Traumatic brain injury.
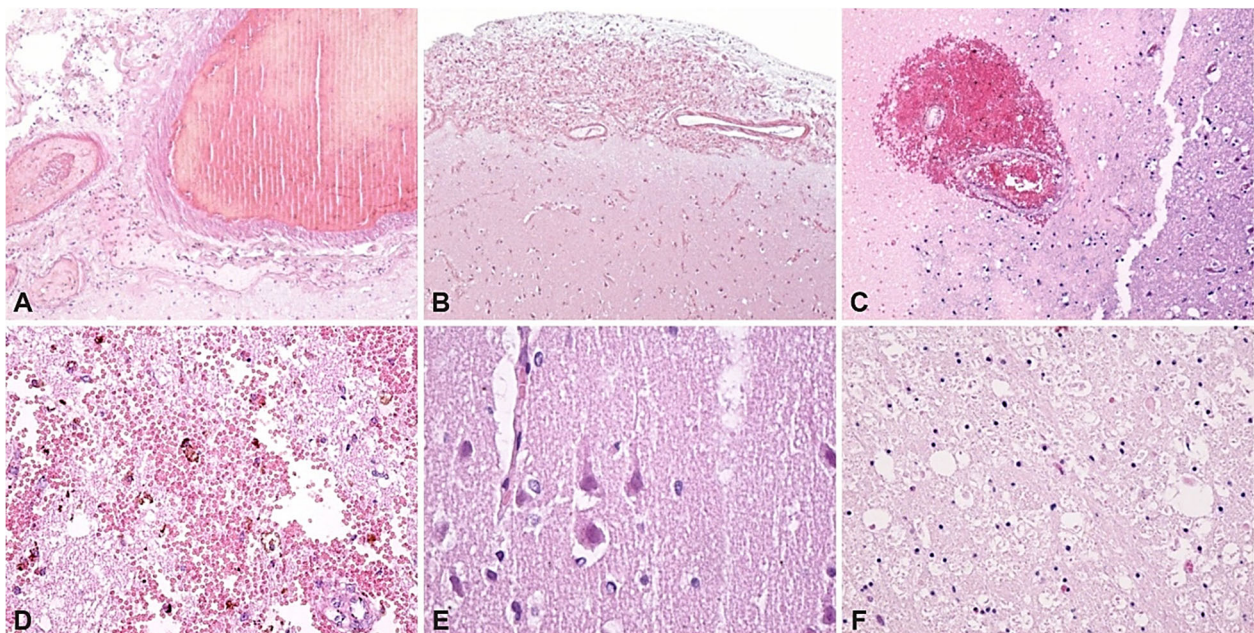

Figure 5 – Microscopic brain changes highlighted in children with TBI who died 15 days after the accident: (A) Congested and thrombosed vessels in the leptomeninges; (B) Blood vessels in the leptomeninges, with perivascular fibrosis and moderate inflammatory reaction; (C) Microscopic image of an old perivascular hemorrhage through the Virchow–Robin space, transformed into a perivascular thrombus; (D) Cerebral dilaceration area with necrotic cerebral parenchyma and intraparenchymal hemorrhage; (E) Cerebral parenchyma in the vicinity of the TBI area with red (ischemic) neurons, with perineuronal and perivascular edema, with collapsed blood capillaries and fragmented neuropil; (F) Area of brain parenchyma with spongy aspect resulting from neuronal death. HE staining: (A, B, C and F) ×100; (D and E) ×200. HE: Hematoxylin–Eosin; TBI: Traumatic brain injury.
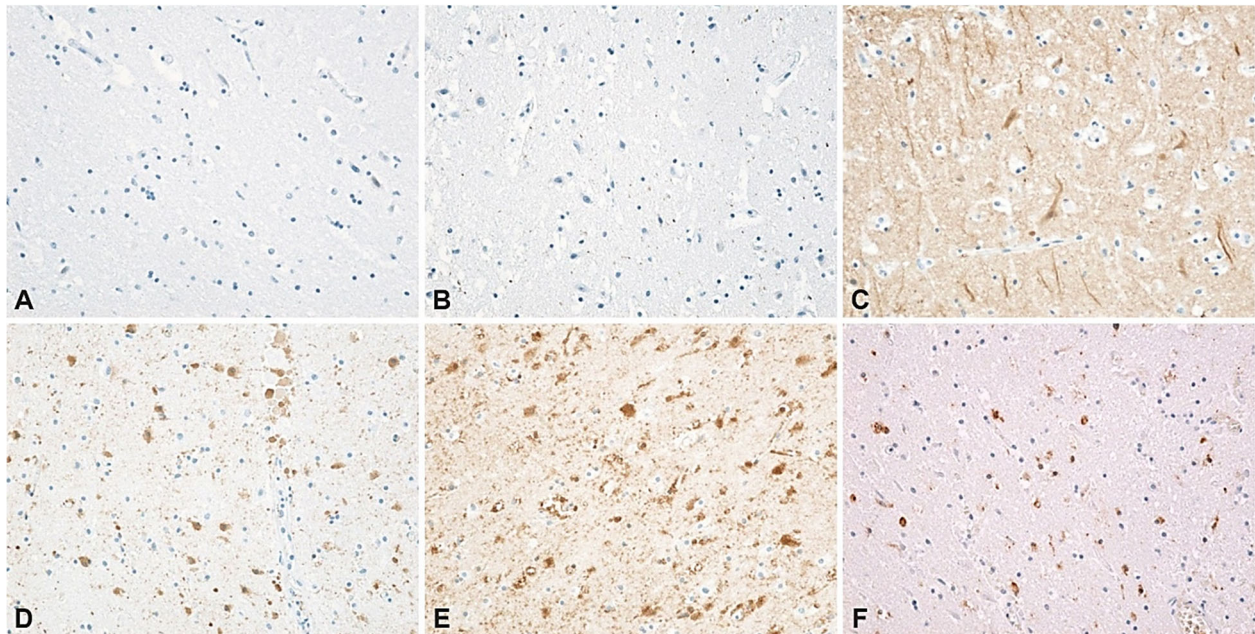
Figure 6 – *IHC changes in cerebral parenchyma in patients deceased 15 days after TBI: (A) Neuronal negative reaction to the anti-NeuN antibody 15 days after TBI; (B) Area of cerebral parenchyma with negative reaction of neurons to the anti-NF antibody, 15 days after TBI; (C) Cerebral parenchyma area with moderately positive reaction for NSE, 15 days after TBI; (D) Image of cerebral parenchyma with intense reaction of astrocytes; (E) Image of cerebral parenchyma from the TBI area with intense microglia reaction; (F) Cerebral parenchyma remote from the TBI area with an increased number of microglia, intensely positive to the anti-IBA1 antibody. Immunomarking with anti-NeuN antibody: (A) ×100. Immunomarking with anti-NF antibody: (B) ×100. Immunomarking with anti-NSE antibody: (C) ×200. Immunomarking with anti-GFAP antibody: (D) ×200. Immunomarking with anti-IBA1 antibody: (E) ×200. Immunomarking with anti-IBA1 antibody: (F) ×200. GFAP: Glial fibrillary acidic protein; IBA1: Ionized calcium-binding adaptor molecule 1; IHC: Immunohistochemical; NeuN: Neuronal nuclei; NF: Neurofilament; NSE: Neuron-specific enolase; TBI: Traumatic brain injury.*

⊟ Discussions

TBI is a major neurological problem reaching epidemic worldwide proportions nowadays [14, 15]. Clinically, TBI occurs in different forms, from mild changes in the state of consciousness, to a deep coma state and death [16], depending on the violence of the individual contact with the traumatic agent. In our study, the traumatic agents were road vehicles in 17 cases, three animal aggressions, height falling in one case and one railway accident.

All the patients in our group underwent severe aggression, but not all died immediately after aggression, which leads us to believe that the intensity of aggression varied from one case to another.

TBI pathogenicity is a complex process, caused by primary lesions (produced by the impact of the skull with a mechanical agent) and secondary lesions (a cascade of chemical, molecular and inflammatory changes induced by the TBI). These brain changes lead to temporary, permanent neurological damage or death [16].

In subjects deceased within the first 24 hours after the injury, death occurred because of quite severe primary lesions; in subjects who died seven and 15 days, respectively, after the TBI, primary lesions of cerebral parenchyma were amplified by secondary lesions.

In general, the primary damage of the skull may result in injury of the brain tissue, change in the brain blood flow, and changes in the metabolism of neurons and glial cells. These processes eventually lead to ischemia, generalized cerebral edema and neuronal death [17, 18].

It is established that the immediate impact of moderate or severe mechanical TBI can cause focal and diffuse brain damage [19, 20], whose intensity correlates with the intensity of the TBI.

As we presented here, a TBI with a high energy can directly damage brain tissue, causing brain contusions, with nervous tissue dilacerations, axonal shearing, but also damage of blood vessels in the brain, causing local hemorrhages, of various sizes [21–23].

Intracranial hematomas are an additional substance that, in the closed and hard space of the cranial box, leads to compression of the nervous tissue and brain blood vessels causing the reduction of brain blood flow, hypoxia, ischemia and exacerbation of the initial brain injury [24, 25].

In our study, cerebral vascular lesions, characterized by more or less large hematic extravasations, were present in all groups of patients. If in the group of patients deceased within the first 24 hours after the TBI, we identified leptomeningeal hemorrhages, intracranial hematomas and diffuse microhemorrhages, without hemolysis, in the groups of patients who died after seven and 15 days, respectively, there were identified vascular and perivascular thrombosis (in the Virchow–Robin spaces), heterogeneous hematomas with partially hemolyzed red cells and perivascular edema. In all the studied groups of patients, there was observed the collapse of the small (capillary) intraparenchymal blood vessels, which shows an increase of brain hypoxia, due to the presence of intracranial hematic overflows and cerebral edema.

If in the group of children who died in the first day, the

microscopic lesions of neurons and cells were more reduced, in the groups with patients deceased after seven and 15 days, respectively, the microscopic changes of neurons, glial cells and neuronal extensions were more significant. Thus, neurons presented nuclear and cytoplasmic condensation, nuclear pyknosis, karyorrhexis, vacuolar or fine granular cytoplasm, acidophilic cytoplasm, cell fragmentation, cell lysis with neuronal debris, cell necrosis, etc. Neuronal extensions presented as fragmented, with a heterogeneous structure. We consider that all these microscopic changes are due to the presence of secondary lesions, which are extremely complex, being induced by neuronal excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, neuro-inflammation and degeneration of axons.

It is known that excitotoxicity is a cascade of intra-neuronal events initiated by the excessive stimulation of neurons by neurotransmitters, which causes an excessive intracellular Ca accumulation. The presence in neuroplasm of Ca^{2+} ions in a large quantity activates a series of proteins and enzymes (calpain, caspase) that induce the apoptotic death of neurons [26]. Of the neuromediators, it is believed that glutamate is the main molecule that determines the excessive accumulation of intracellular Ca. Several studies on laboratory animals, as well as on humans, showed a substantial increase in extracellular glutamate and cerebrospinal fluid (CSF), proportional to the severity of the TBI [27, 28]. The presence of excessive glutamate seems to be due to several factors: alteration of the BBB by the traumatic agent, with the onset of hypoxia or brain ischemia; alteration of the neuronal membrane; neuronal death directly caused by TBI with the release of excitatory amino acids; reduction of glutamate capture and metabolism by astrocytes, etc. [29–31].

The excess of Ca^{2+} ions in the cytosol of the neuronal cell, the disturbance of the BBB function of the neural membrane penetrating large quantities of sodium (Na^+) intracellular ions, are responsible for disturbing the function of the mitochondria, resulting in the direct consequence of reducing or inhibiting adenosine triphosphate (ATP) synthesis and the nervous cell death [32, 33].

Other factors that play essential roles in TBI pathogenesis, are reactive oxygen species (ROS). They are formed either in the neurons, because of the intracellular growth of Ca^{2+} ions, or they are produced by activated leukocytes, astrocytes, microglia, because of altering the integrity of the BBB. ROS reacts with some proteins, enzymes, and cellular deoxyribonucleic acid (DNA), causing morphological and functional changes. They also react with polyunsaturated fatty acids from membrane phospholipids, changing the activity of intracellular organelles, followed by cellular death [34–36]. Increasing the permeability of the mitochondrial membrane and the oxidation of membrane proteins leads to a change in ionic transport. As such, the persistent release of very reactive oxygen free radicals and the associated increase in the level of lipid peroxidation mediated by ROS in TBI, reduces neuronal plasticity, but also the cerebral blood flow. All these biochemical changes amplify the neuronal death mechanisms [37].

TBI do not only alter the neuronal bodies, but also their extensions, and, in particular, the axons. According to some studies, TBI can produce by direct mechanism the destruction of axons "primary axotomy" or indirectly

"secondary axotomy", by proteolysis, by ROS, accumulation of Ca^{2+} or by disorganization of the axonal cytoskeletal network [38–40].

We consider that, at present, it is almost impossible to accurately establish the involvement of secondary factors in TBI pathogenesis.

In our study, IHC examinations brought additional data on brain lesions after TBI. Thus, the assessment of NeuN protein showed that the IHC reaction was normal in patients who died within the first 24 hours, except for the traumatized area, where it was reduced. The intensity of the reaction was greatly diminished with the increase of the survival time of patients, so that in the group of deceased after seven days, the reaction was poorly positive or absent in the neurons, and 15 days after the TBI the IHC reaction was absent in all neurons. These IHC aspects allowed us to conclude that the reaction to NeuN correlates with the viability of neurons.

The reaction to the anti-NeuN antibody is frequently used to assess the functional status of neurons under normal and pathological conditions. Several studies showed that the nervous system lesions can affect the expression of the NeuN protein. Thus, the axonal lesion of the motor neurons of the facial nerve nucleus determines the almost complete reduction of the NeuN immunoreactivity in the respective neurons, although they survived axotomy [41]. Other studies showed that the IHC reaction to NeuN diminished or completely disappeared in the neurons affected by ischemia or hypoxia [42]. All studies performed so far confirmed that the loss of NeuN immunoreactivity indicates their deterioration, but it cannot be a definitive proof of neuronal death [43].

As the HP study highlighted changes in the neuronal morphology, we proposed the evaluation of the neuronal cytoskeleton by studying the changes of NFs. The IHC study showed that NFs disintegrate, diminish, and disappear from neurons with the increase of the survival period, during which the secondary lesions induced by the TBI intensify.

NFs are part of intermediate filaments, being specific microscopic structures, which belong to the cytoskeleton of mature neurons [43–46]. Although, initially, it was believed that NFs only play a mechanical role of neuronal support, especially of axons, being a structure of their protection to mechanical stress [47, 48], at present it was established that NFs have multiple roles: allow the adaptation of neurons to cellular signaling or in case of lesions, facilitate the growth of axons both in the central nervous system (CNS) and peripheral nervous system, they facilitate the management of depolarization wave in the large, myelinated axons, facilitate the distribution of intracellular organelles (mitochondria, the endoplasmic reticulum, microtubules, synaptic vesicles), while regulating the activity of some synapses, etc. [49–52].

The changes in NFs in TBI were less studied. It is believed that in the TBI of the axons there is a wrapping of the NFs through the intracellular growth of Ca^{2+} or due to the activation of some intraneuronal enzymes [53, 54]. Other studies claim that a few minutes after TBI there occur axonal degeneration processes that consist of the disorganization of the axonal cytoskeletal network due to the destruction of NFs [40]. Together with processes of proteolysis mediated by Ca^{2+} ions, acute axonal damage can progress and develop as delayed and secondary axotomy,

days and months after the initial traumatic event, a process characterized by the degradation of myelin sheath, damage of axonal transport and the accumulation of axonal transport proteins in various areas of the axons. Axonal lesions may induce apoptotic death of neurons [55]. We consider that the reduction of the IHC reaction of NFs following severe TBI is due to their destructuring. Some studies showed that proteins in damaged axonal NFs pass into the CSF and blood, being used as biomarkers for axonal lesions and neuronal death [56].

The assessment of the NSE after severe TBI in our patients, depending on the survival period, showed that the IHC reaction is reduced until disappearance with the survival period increase, both in the TBI and remotely. This IHC aspect shows a profound damaging of the neuronal metabolism and even their death. It is known that NSE is an enzyme specific to neurons and the neuroendocrine tissue, having a molecular weight of 75 kDa [57, 58]. Several studies showed that NSE is the only marker that directly indicates the functional damage of neurons, its value being increased in CSF and blood after TBI, because of secondary TBI [59]. As some studies previously showed, the serum level in the CSF of NSE increases in the first hours after the TBI and, therefore, the measurement of NSE may be useful in identifying a TBI or monitoring their progression. The serum levels of NSE are considered to reflect the extent of primary brain injury, but also the progression of neuronal lesions [60–62]. Moreover, in the last decade, NSE was considered a peripheral biomarker of the permeability of the BBB [63–65].

Regarding the reaction of astrocytes, in our study we observed a minimum reaction in deceased persons within the first 24 hours and an increase in their number and dimensions as the survival time increases. However, in patients who died after 15 days we reported an amputation of astrocyte extensions, a reaction considered a sign of astrocyte impairment.

Astrocytes are the most numerous glial cells in the CNS, providing both structural and functional support to neurons. They may be identified and characterized relatively easy due the presence of GFAP, a unique structural protein, present in the intermediate filaments of the cellular skeleton [66]. Under physiological conditions, astrocytes play essential roles in maintaining the homeostasis of ions, synapses, transmitters, water, and blood flow, etc., for the normal functioning of neuronal circuits [67]. In TBI, more studies highlighted an increase in the immunostaining with GFAP, indicating the activation and proliferation of astrocytes [68, 69]. Also, there was observed that astrocytes not only proliferate in response to TBI, but also increase their size, a process known as astrogliosis [70].

In our study, a reaction similar to astrocytes was found in microglia. The number, size and reactivity of microglia intensified with the increase of the survival period. Microglia represents the major cellular component of the innate immune system of the brain and constitutes up to 10% of the total adult CNS cells [71]. Until recently, microglia were considered macrophages of the CNS, however, a series of recent discoveries established that microglia have a distinct molecular line and signature compared to circulating monocytes but have almost the same pathophysiological functions as the cells [72]. The increase in the number

and activity of the cells of the macrophagic system denotes the existence of cellular and tissue debris that require phagocytosis.

Our study shows that brain changes in the case of severe TBI are complex, which requires special therapeutic measures to save the patient's life and recovery after neurological sequelae. Most of the studies in recent years showed the severity and complexity of TBI [73–76] and the need to continue investigations to identify the biological markers that can offer information on the severity of neurological lesions and the efficiency of therapeutic measures.

⊟ Conclusions

TBI causes complex lesions that train both the nervous tissue (neurons, neuronal extensions, glial cells) and blood vessels and meningeal structures. Primary lesions directly caused by trauma are amplified by secondary injuries as the survival period increases. Neuronal death is intensified as the survival time extends, acquiring complex aspects of necrosis, apoptosis, and even neuronal autophagy. IHC examinations confirmed that neuronal impairment in the TBI is amplified with the extension of the patient's surviving period, neurons losing NeuN protein, NF proteins and NSE. The increase in the number and size of astrocytes, as well as the intensification of the IHC reaction to GFAP represents a normal reaction, of tissue repair, of astrocytes. The increase in the number of microglia and monocyte macrophage cells, with the extension of the survival period, indicates the presence of high quantities of cellular and tissue debris that needed to undergo a phagocytizing process.

Conflict of interests
The authors declare that they have no conflict of interests.

Authors' contribution
Răzvan Ştefan Ţolescu and Marian Valentin Zorilă have contributed equally to this paper.

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