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Time-related morphometric studies of neurofilaments in brain contusions

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Abstract

In forensic pathology age determination of injuries is of key importance. The purpose of the study was to analyze morphometrically changes in neurofilaments following the brain contusion and relate them to the length of the time of survival. To do this, the authors analyzed specimens of brains collected during medicolegal autopsies. According to the available literature, no such study involving material from deceased humans was conducted. The researched material was divided into nine subgroups (10 cases each) according to the time of death of persons: immediately at the crime site, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days after head trauma. Neurofilaments were immunohistochemically stained and evaluated quantitatively using the Met-Ilo computer application. The initial results were then analyzed statistically with the one way analysis of variance (ANOVA) and the least significant difference (LSD) tests. It was calculated that there are significant differences in numbers and area fractions of neurofilaments within 7 days after head trauma. It must be concluded that morphometric analysis of neurofilaments is a promising method but further studies are required.

Key words: *brain contusion, neurofilaments, morphometry, survival time.*

Introduction

In medicolegal traumatology determination of the age of injuries is extremely important, as it allows to establish the time of trauma. Basing on this knowledge, in the course of legal investigation the public prosecutor can substantiate his allegations that the offense being inquired was committed at a given date.

The issue of age determination of brain contusions has been within the scope of interest of forensic pathologists and neuropathologists since the beginning of the twentieth century. The usual study method consists in analysis of morphological changes within the site of contusion which follow from complicated processes of resorption and organization of the contused nerve tissue.

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In everyday practice it is posttraumatic changes in the nerve tissue with concomitant cell-mediated reactions that are analyzed. Apart from classic histologic stainings immunohistochemical procedures are utilized as well. They allow for univocal identification of cells which have migrated to the site of contusion [8,30].

The literature offers tabular comparisons of morphological changes with regard to the age of brain contusion, i.e. the time that has lapsed since the trauma [2,13,17]. However, laboratory practice has shown that these methods are not accurate enough and therefore pose a danger of diagnostic errors.

Study of several different morphologic elements within the site of brain contusion increases the accuracy of analysis and, as a consequence, decreases the range of uncertainty of evaluations. Nowadays, progress in immunohistochemistry makes it possible to study structural elements of cells, including nerve tissue cells.

The authors have attempted to determine the applicability of histopathologic examination of posttraumatic changes of morphologic structures of nerve cells in victims of fatal intracranial injuries.

Because of postmortem changes, mainly autolysis, which usually obscure pathological findings, alterations in neurofilaments (NF) – structural proteins of nerve cells, which are more resistant to autolysis than other cell proteins, e.g. enzymes – have been chosen for study [11,23].

What is worth emphasizing, the available literature lacks any data on temporal changes in the neuronal cytoskeleton after brain trauma which could be used for forensic wound age estimation. This observation has been made by neuropathologists as well [8].

Material and methods

The researched material comprised specimens of sites of contusions in cortical-subcortical regions of cerebral hemispheres of 90 deceased persons whose corpses have undergone medicolegal autopsies in the Department of Forensic Medicine of the Medical University of Gdańsk in accordance with proper rulings of local public prosecutors. The comparative material consisted of specimens collected during medicolegal autopsies of 10 deceased persons who died immediately after the head trauma. This time specimens were collected from brain regions that showed neither macroscopic nor microscopic posttraumatic changes. The presence of diffuse axonal injury (DAI) was excluded by an experienced neuropathologist.

There was no need to obtain any prior approval for human subjects' research from a local bioethics committee due to the judgment of the Supreme Court which ruled that both paraffin blocks and microscopic slides comprise health records (case no. V CSK 256/10, judgment of 9 February 2011).

Circumstances of head traumas were as follows: car accidents (pedestrians, drivers and passengers), falls from height, uncontrollable falls from erect position, and assaults.

The researched material was divided into nine subgroups (10 cases each) according to the time of death of a person: immediately at the site, 12 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days after head trauma. All people who did not die immediately at the site and were treated in hospital got a similar pattern of treatment, typical for craniocerebral injuries (antiedematous drug – mannitol; steroid – dexamethasone; general anesthetic – thiopental; antitetanic anatoxin; antibiotics). One of the above-mentioned drugs – dexamethasone has a potential protective influence on neurofilament degradation what is discussed below.

The specimens were being fixed in 4% neutral buffered formaldehyde solution for 48 to 72 hours and the basic histologic tissue preparation method was applied with the use of the Shandon-Citadel 2000 autotechnicon tissue processor. Afterwards, the specimens were embedded in paraffin blocks (paraffin melting point being 56°C) and sliced in the Leica microtome into 4-micron-thick sections which were routinely stained with hematoxylin and eosin. Immunohistochemical reaction was performed using the Dako FLEX Monoclonal Mouse Anti-Human Neurofilament Protein Clone 2F11 without labelling of subunits. This antibody is specific for high-weight neurofilaments, which are considered to be the most resistant to degradation of all neurofilament types because of their highest degree of phosphorylation and ability to bind calmodulin [11,23]. The specimens were then mounted on glass slides and treated in the Dako FLEX Target Retrieval Solution High pH for demascation of the antigen. Deparaffinization and epitope revealing procedures were carried out in the PT-LINK machine for 20 minutes at 97°C. Staining was performed in the Dako Autostainer

Link 48 automaton. The specimens were dehydrated through a series of alcohol solutions of ascending concentration, passed through xylene and enclosed in Canada balsam.

In screening studies, highly fragmented and chaotically arranged NF and diversely stained background were observed which prevented from quantitative (morphometric) evaluation of changes. To circumvent this problem, in the study morphometric analysis was performed using "Met-Ilo", the image analysis application written in the Institute of Materials Science of the Silesian University of Technology [27]. Full color photomicrographs, taken at 400-fold magnification with the Axio Cam Erc 5s camera attached to the Carl Zeiss Scope A1 light microscope, were analyzed. To facilitate the analysis of biostructural image of NF additional modules were introduced.

In every histologic specimen of the nerve tissue, apart from NF there are other structures usually accompanied by information noise which is due to image artifacts produced in the course of tissue preparation and image digitalization. Therefore, before morphometric analysis is carried out it is necessary to select only such pixels that represent the analyzed structures. The end result of data processing is a binary image in which pixels of the analyzed structures are assigned a value of 1 and pixels of the background (not to be analyzed) are assigned a value of 0. The binary image is displayed as an overlay of the input image in such a way that pixels with value 0 are transparent and pixels with value 1 have a color which is determined by a user beforehand (Fig. 1).

The procedure of binarization can be carried out either manually, by a researcher, or automatically, basing on the data contained in the gray level histogram of the analyzed image. It is only in few cases that binarization immediately yields a correct binary image of analyzed objects. Therefore, the detection process has certain stages:

- modification of the input image,
- binarization,
- modification of the binary image.

Neurofilaments, which can be seen in images, are morphologically diverse as to their brightness,

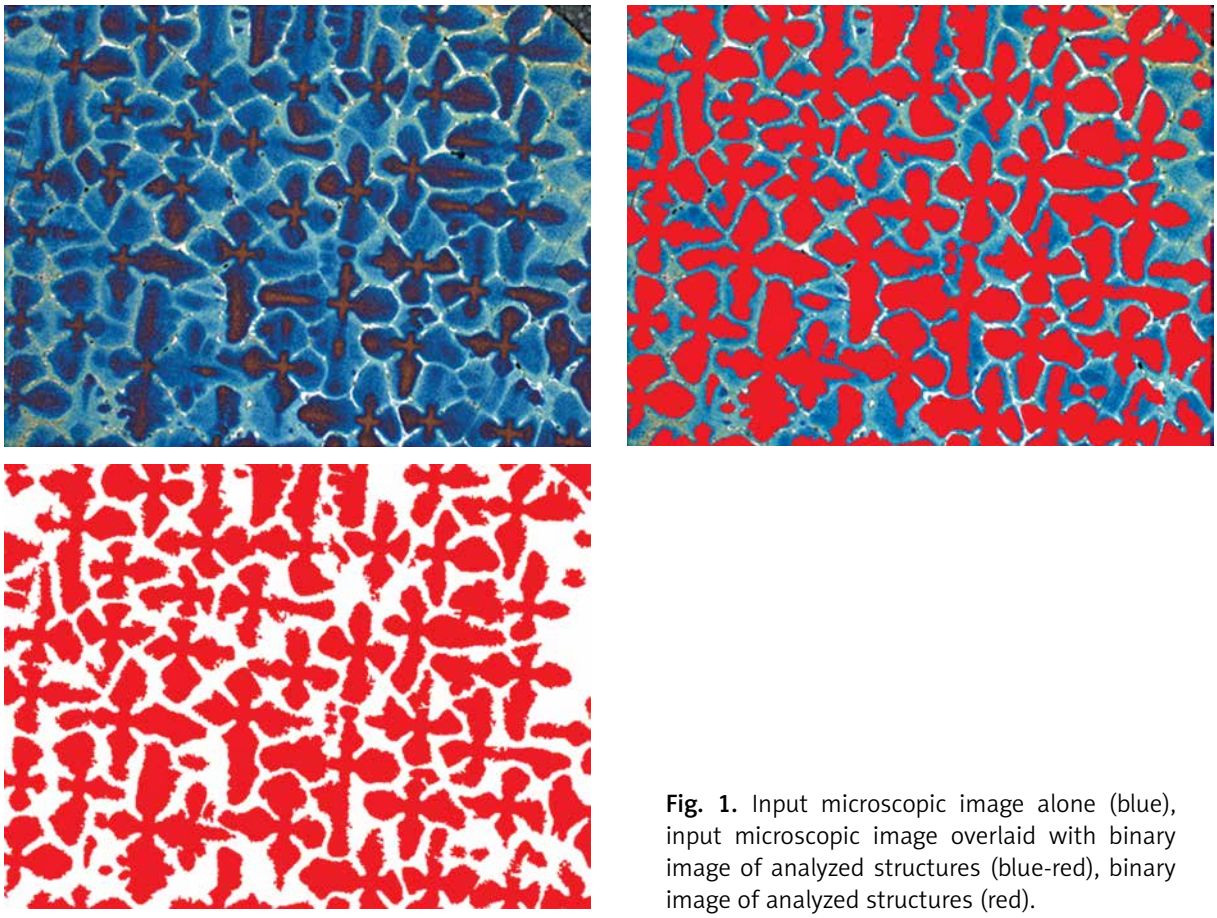

Fig. 1. Input microscopic image alone (blue), input microscopic image overlaid with binary image of analyzed structures (blue-red), binary image of analyzed structures (red).

color and area fraction (i.e. the proportion of the surface area of the detected neurofilaments to the surface area of all images). To minimize the impact of diverse brightness on results of quantitative analysis of the NF structure, the gray-scale histograms of the channels R, G and B were normalized in input images. The visual effect (Fig. 2) of this procedure is not breathtaking, but the procedure itself is critically important for correct binarization and morphometry.

Analyzed structures variably bind labelled NF antibodies. To objectify evaluation, it has been decided that only those structures would be assessed quantitatively which are intensely stained. Initial analyses have suggested that binarization of NF is most effective if performed in two stages.

In the first stage, an initial binary image, the so-called mask, is produced using the Ridler and Calvard *k*-means method [21]. In this method a histogram is divided into two areas. One area is made of pixels of the gray level lower than the threshold value *k*, the other comprises pixels of the gray level higher than the threshold value *k*. Output value of this threshold equals the arithmetic mean of minimal and maximal gray levels of the analyzed image. Each of these two areas is assigned a mean gray level (g_1 and g_2, respectively) and a new threshold value *k* is calculated from the formula $k = (g_1 + g_2)/2$. This value is used to divide the histogram into two separate areas. The procedure repeats with thresholds *k* computed in subsequent measuring loops till the threshold value *k* becomes constant.

In the second stage the *k*-means method is applied again. At this point the gray level histogram is constructed on the basis of those pixels whose value in the mask equals 1 (Fig. 3C). This kind of binarization is called geodesic binarization.

Neurofilaments and their fragments which had become visible in the course of binarization were selected with respect to their size. Structures which counted less than 10 pixels were removed from binary images, because they were most likely artifacts.

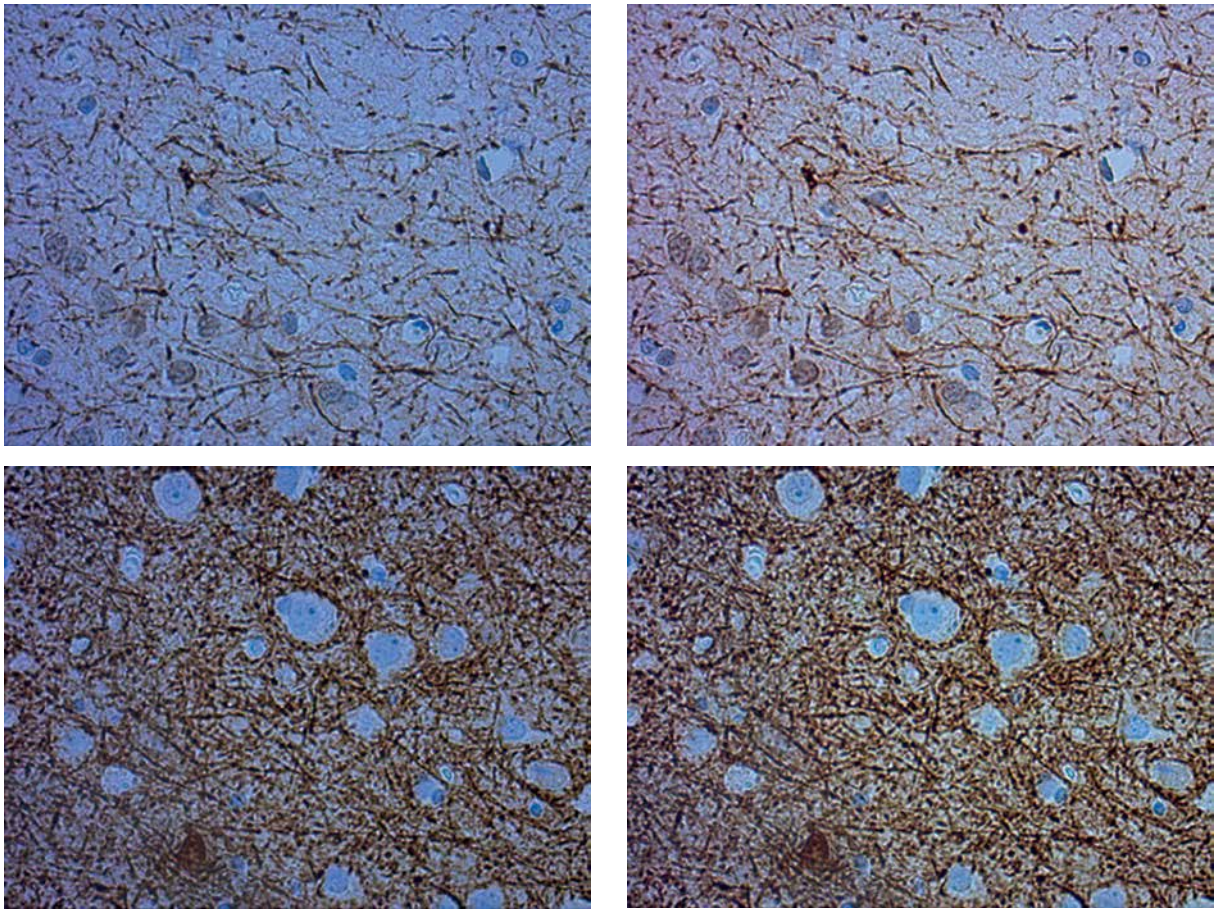

Fig. 2. Input images (left) and input images after R, G and B channel histogram normalization (right).

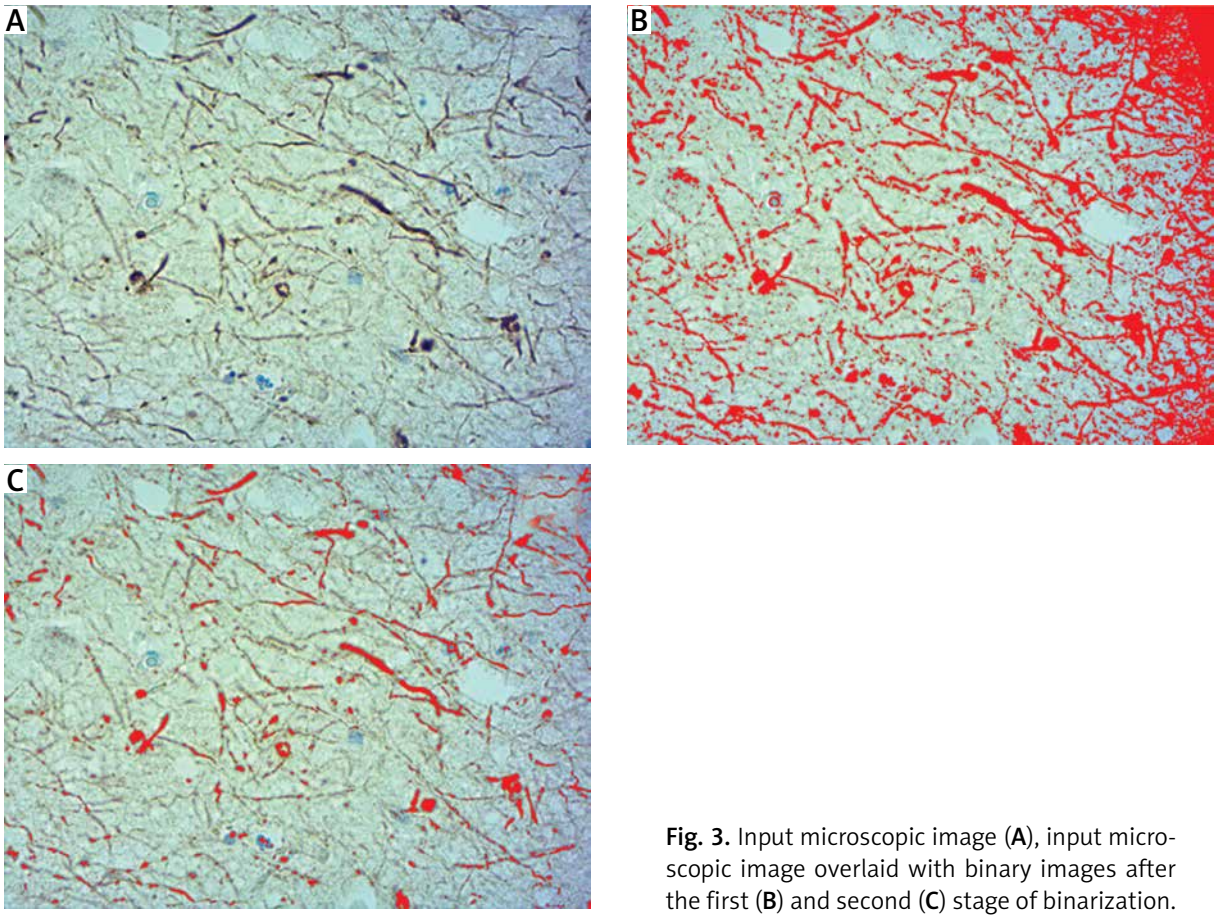

Fig. 3. Input microscopic image (**A**), input microscopic image overlaid with binary images after the first (**B**) and second (**C**) stage of binarization.

All of the above procedures were fully automated and, once initialized, operated without any participation of researchers.

Measurement was made using the Met-Ilo surface method, all available magnitudes were calculated.

Quantitative characteristics of NF comprised two features: the area fraction and the number in an analyzed field. In order to verify hypotheses that both features are normally distributed, the Shapiro-Wilk test was conducted. As the results were positive, one way analysis of variance was applied. The results are presented in Figures 4 and 5. As in both cases there were significant differences of average values ($p < 0.05$), the least significant difference test was applied.

We have compared postmortem brains of deceased individuals with and without evidence of head trauma. The test results for the two means (Student's *t*-test) showed no statistically significant differences between these two kinds of controls, both for the average of the NF area fraction ($p = 0.185$), as well as to NF number ($p = 0.517$), so the distant (non-contused) area from the same brain may be a sufficient control for the analysis of changes in the NF architecture following brain contusion.

Results

Examples of neurofilament structures after application of the Met-Ilo method are presented in Figure 6.

The results of statistical analysis of morphometric studies of NF in the researched groups are presented in Figures 4 and 5. The results of the application of the least significance difference test (value of *p* significance level) are presented in the aforementioned figures at the base of 3D bar charts. Statistically significant differences are bolded (in Figs. 4 and 5, values 0.001 actually mean $p \leq 0.001$).

Figure 4 presents the relationship between the area fraction (i.e. the density of NF) and the time

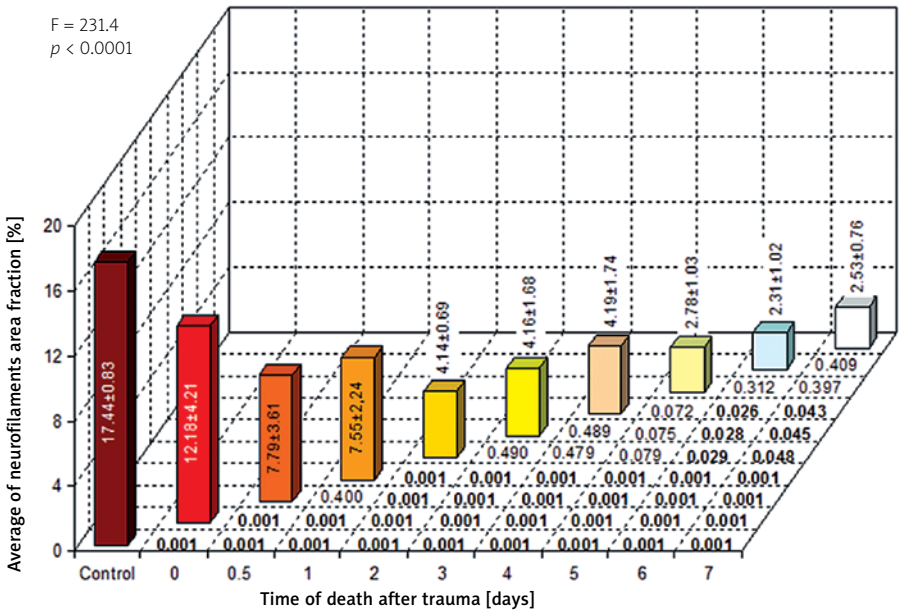

Fig. 4. The relationship between the area fraction (i.e. the density of neurofilaments) and the time lapsed after head trauma.

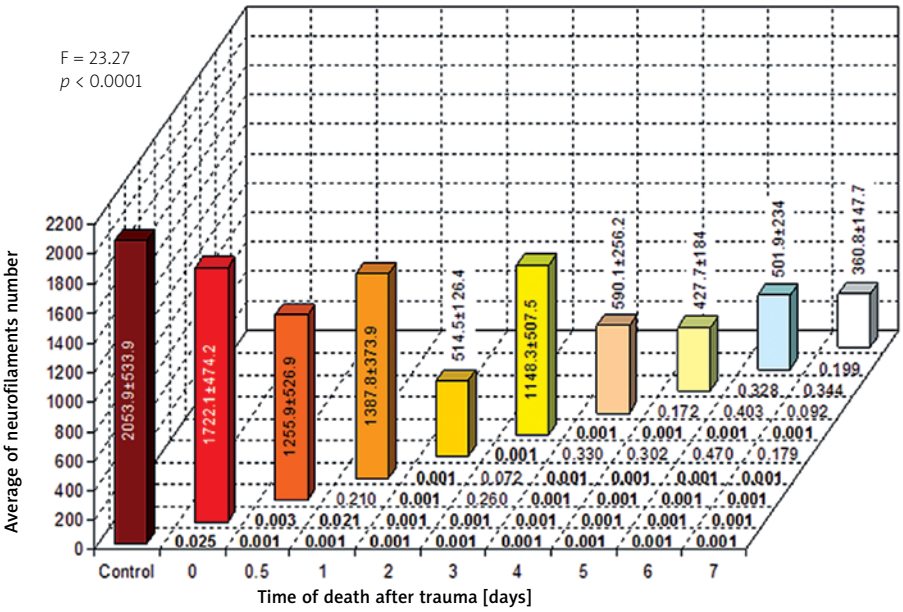

Fig. 5. The relationship between the number of neurofilaments and the time lapsed after head trauma.

lapsed after head trauma. The results of one way analysis of variance (Fig. 4) have shown that the average area fraction of NF is significantly different ($F = 231.4$; $p < 0.001$) within the period of seven days after head trauma. Calculations carried out with the least significant different method have yielded the following results:

- in the researched groups there was a statistically significant constant decrease of the area fraction of NF when compared to the control group,
- average values of the area fraction of NF after 12 and 24 hours (there were no statistically significant differences between these two) have significantly lower values than the values in the con-

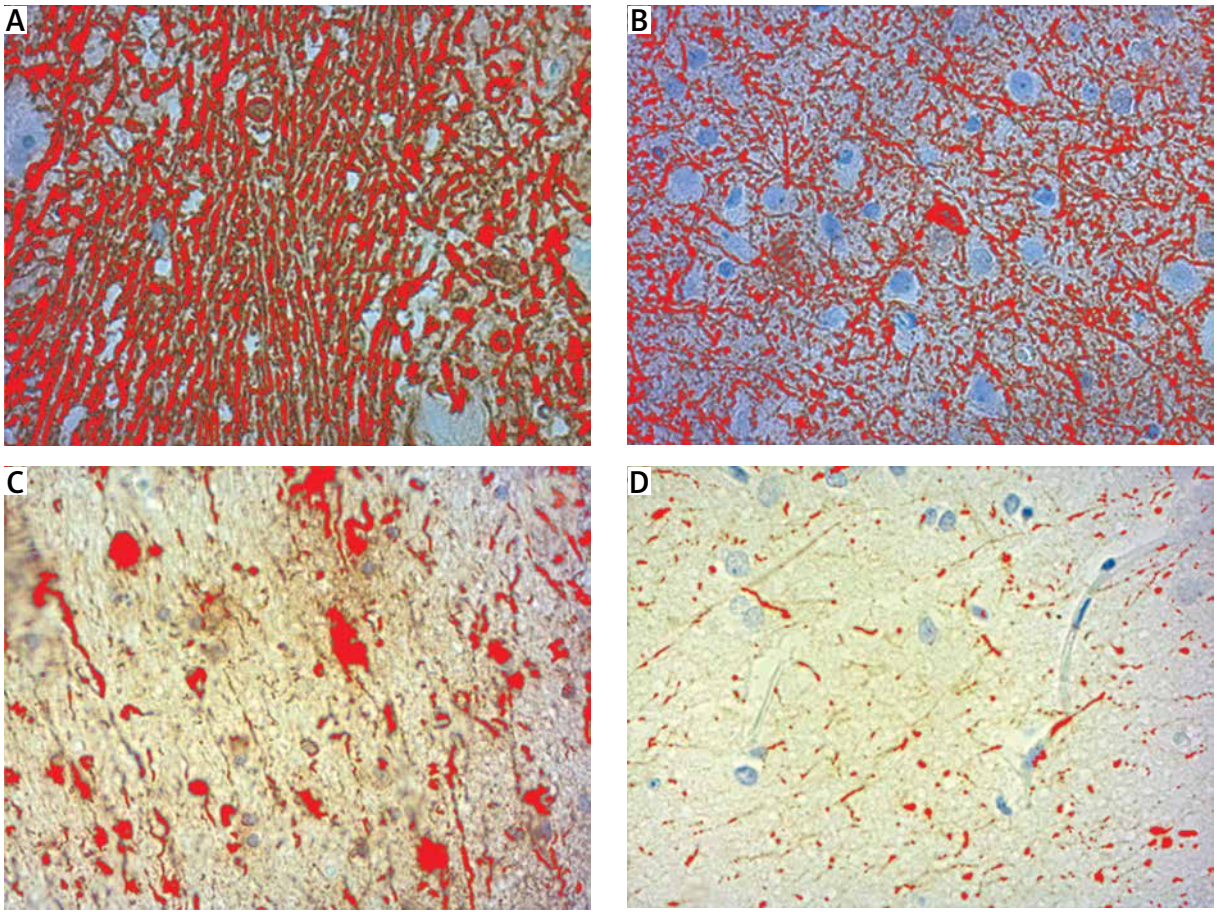

Fig. 6. Neurofilament structures: **A**) brain region remote from trauma with no damage, immediate death; **B**) contusion site, immediate death; **C**) contusion site, death 2 days after head trauma; **D**) contusion site, death 7 days after head trauma.

trol group and the initial values in the researched groups,

- from 2 to 4 days after head trauma, the average values are not significantly different, but they are significantly lower than the values in the preceding periods,
- from 5 to 7 days after trauma, there is a further decrease in the average area fraction. The average values are not significantly different, but they are lower than in the preceding periods. In spite of the lack of significance with regard to the period of 2 to 4 days after trauma, the differences are so large that they are almost statistically significant.

Figure 5 presents the relationship between the number of NF and the time lapsed after head trauma.

The results of one way analysis of variance (Fig. 5) have revealed that the average numbers of NF are statistically different ($F = 23.27$; $p < 0.001$) within the period of seven days after head trauma. Levels of significance, as calculated from the least significant different method, show that:

- the distribution of differences of average numbers of NF, when related to time after trauma, is similar to the distribution of the area fraction,
- a distinct deviation from this regularity occurs on day 3 after trauma – there is a statistically significant increase in the average number which is probably due to defragmentation,
- the average number of neurofilaments in later stages of observation decreases to a lesser extent than the area fraction.

Discussion

Neurofilaments are structural proteins which comprise the cytoskeleton of the nerve cell. The cytoskeleton is present in the perikaryon and extends into the dendrite and axons. Three kinds of proteins of different molecular weights form low-weight (NF-L),

medium-weight (NF-M) and high-weight (NF-H) neurofilaments. Neurofilaments together with cytokeratins, lamins, vimentin and vimentin-like filaments constitute intermediate filaments which scaffold the nerve cell [6,26]. NF-L, NF-M and NF-H are uniformly dispersed in the perikaryon and processes both in the central and peripheral nervous systems [28]. In the brain NF can be shown by means of immunohistochemical methods with labelled antibodies, in the cerebrospinal fluid with ELISA or Western-blotting [6,28].

Traumatic brain injuries (TBI) cause mechanical deformation of the nerve tissue, including the NF cytoskeleton, which may play a key role in the posttraumatic cessation of axonal transport [7]. Experiments have shown that TBI results in the loss of cytoskeleton proteins, including NF, such as NF68, NF200, NF300, spectrin and microtubule-associated protein 2 (MAP2). In the rat nerve tissue those changes are observed as soon as three hours after trauma. Correct reconstruction of NF and, more broadly speaking, of cytoarchitecture facilitates convalescence, but increased disarrangement of NF can cause the death of neurons, often preceded by the dysfunction of the central nervous system. Changes in NF were observed both in acute ischemia and TBI in studies involving animal experiments [1,18]. In humans as well as in animals morphologic changes in NF occur in DAI following TBI. In animals those changes are observed in sites of contusions, accompanied by selective necrosis of neurons in the brain cortex, especially in the hippocampus which is exceptionally susceptible to hypoxia and several other damaging factors [3,5,10,16,19,20,22-26].

Evaluation of quantitative alterations in NF in the site of TBI is in fact impossible due to their specific structure, size and number which can be observed only after specially-targeted staining with labelled antibodies. The morphometric method the authors have employed allows for thorough evaluation of changes in NF in the sites of contusion with respect to the time flow. Two parameters were used: the number and area fraction of NF (see above), but it appears that the relationship between the area fraction of NF and the time after injury seems more valuable.

It has been reported previously that dexamethasone – the drug given to the patients who did not die immediately at the site and were treated in hospital – has been used to prevent NF degradation and suppress vasogenic edema and inflammatory responses following brain insult [9]. There are also additional data that dexamethasone treatment maintains NF integrity following intracerebral hemorrhage damage [14]. However even with this potential protective influence of dexamethasone authors were able to see significant differences (decrease) in numbers and area fractions of NF between the studied groups.

As already mentioned, the available literature suggests that all previous studies of posttraumatic changes in NF have involved animal experimental models (rats, mice, pigs). In majority of studies, NF-H and NF-L were researched [3-5,7,10,12,15,16, 19,20,23,28]. Neurofilaments at the site of the brain contusion disintegrate, their complete disappearance was observed 1 to 2 weeks following trauma [10,20,22,26]. These results are not inconsistent with ours. In our study the last group of the deceased consists of patients who died on day 7 after trauma and at that point of time both the number and area fraction of NF have the lowest values.

Our results are consistent with results of a Czech team [29] who stained S-100B, GFAP and hyperphosphorylated NF with immunohistochemical (tissues) and biochemical (blood plasma) methods. The material they worked on had been collected from living patients with DAI who survived 10 days after trauma and from patients who died. NF-H concentration in the blood plasma was increasing with the lapse of time, which implies there is a continuous disintegration of the cytoskeleton and leakage of NF-H to the blood. Throughout the whole ten-day period the blood plasma NF-H concentration was higher in patients with DAI than in patients with limited brain injuries.

It must be noted, however, that some works have shown an increase in immunoreactivity of NF after trauma. Li et al. observed a statistically significant gradual time-dependent increase in immunoreactivity (average density) of NF-L (up to 72 hours) in certain structures of the rat brain: the corpus callosum, internal and external capsule and pyramidal tracts [15].

To sum up, we think that the morphometric analysis of NF in the sites of the brain contusion can become a valuable method for determination of the age of brain contusions. However, this promising method should be further verified by prospective studies of specimens collected from human corpses.

Disclosure

Authors report no conflict of interest.

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