INTRODUCTION

Stroke is the leading cause of death and disability across the world. Mortality of those who have suffered a stroke is up to 40% during the first 12 months after the disease. Half of patients who have suffered an acute cerebrovascular accident (ACVA) lose their social adaptation and need outside help. Among all ACVAs, ischemic stroke (IS) is the most common type, making approximately 85% of the total number of ACVAs [1]. Hemorrhagic stroke (HS), in its turn, makes approximately 10-15% of all ACVAs [2]. About 50% of patients who have suffered HS fail to return to their previous place of work, and 95% of patients have cognitive disorders and low quality of life [3].

In case of IS, acute ischemia-reperfusion and excitotoxicity, and in case of HS, a mechanical pressure of the hematoma and the influence of hemoglobin degradation products upon the perifocal tissue of the brain [4] cause the increased permeability of the blood-brain barrier (BBB), vasogenic and cytotoxic cerebral edema. Consequently, it leads to the necrotic neurons [5], microglia and inflammation [6, 7]. Glial cells play an ambiguous role in a post-stroke period. On the one hand, they produce trophic factors and energetically support neurons, and on the other hand, they produce inflammatory mediators, stimulate inflammation and slow down healing [8, 9]. In case of acute cerebral ischemia, together with acute changes in the critical area of the brain due to ejection of proangiogenic growth factors [10] stimulation of angiogenesis and vasculogenesis takes place. Angiogenesis and vasculogenesis are considered the main processes making it possible to restore the integrity of connections between neuron, astrocyte and pericyte (vessel microcirculatory bloodstream) owing to remodeling and formation of new vessels after injury in the perifocal area of the brain [11].

Apart from acute changes in neurons, some of these cells, as a result of acute ischemia, and in case of HS under the influence of ferritin, there is hyperphosphorylation and accumulation of microtubule-associated τ-protein of neurons, with its accumulation in the cell as paired spiral filaments and neurophytes. [12]. These changes cause the loss of neural connections, which then clinically manifests itself as cognitive deficit [13]. As the problem of quality of life of patients who have suffered ACVA is up-to-date, in order to improve the diagnosis, prevention and treatment, pathomorphological changes of the critical area of the brain in case of strokes of various geneses, has been thoroughly studied.

THE AIM

To study the manifestations of the resolution of the perifocal tissues of the brain in case of strokes of various types based on a comprehensive immunohistochemistry of neurons, astrocytic glia and angiogenesis depending on the period of the disease.

ABSTRACT

The aim: The study is to research the resolution of perifocal brain tissue at various type strokes using immunomorphology

Materials and methods: The immunohistochemical study of perifocal brain tissue in 21 cases of various strokes types was conducted

Results: When comparing the GFAP + astrocytes detection area at IS, HS and IS with HT, no significant difference was found. At the 1st degree of GFAP + astrocytes were in the border around the necrosis nucleus at IS and IS with HT, and at HS GFAP + astrocytes accumulated along the hematoma edge. CD34 + cells were found in most cases of strokes. Over time, cases with a larger CD34 + cells detection area increased (Kendal’s Tau = 0.512, p = 0.001) in all groups. The capillary network at HS was around the hematoma and formed a gliomesodermal capsule with microglia and inflammation. 1st degree τ-protein accumulation was detected in 2/3 of cases (66.7%) of all strokes without significant difference. If compared in different stroke periods, τ-protein detection frequency increased and accumulated in brain structures – Kendal’s Tau = 0.359; p = 0.023.

Conclusions: With the development of the disease, the number of cases with a larger area of detection of GFAP + astrocytes and CD34 + cells increased in strokes of various types. τ-protein was detected in neurons in all variants of ACVA in the first period.

KEY WORDS: stroke, GFAP + astrocytes, CD34 + cells, τ-protein
MATERIALS AND METHODS

Samples of perifocal tissue of the brain of 21 deceased patients were taken for immunohistochemistry, of which 7 (33.33%) cases were IS, 8 (38.09%) were HS, and 6 (28.57%) were the cases of ischemic stroke with hemorrhagic transformation (IS with HT). The test material was grouped according to the classification of the stages of cerebral infarction Mena H et al. [14] and clinical and radiological classification of stages of organization of hematoma Bradley W. [15]. Based on these classifications, the time intervals corresponded to three periods: the first period – 1–3 days from the occurrence of stroke, the second – 4–7 days from the onset of the disease, and the third period > 7 days from the onset of ACVA (table I). The frequency of cases with various stages did not differ between groups (p>0.1, Mann-Whitney test).

In the course of the study, in each case, pieces of brain tissue 2.0x2.0 cm were sampled in the projection of the cortex and subcortical nuclei of the temporal lobe, which bordered on the area of necrosis or hematoma, then the brain tissue was fixed in 10% neutral formalin solution and dehydrated in alcohols with growing concentrations according to the standard method, poured into paraffin and as visual preparations stained sections with hematoxylin and eosin. For immunohistochemistry of neurons τ-protein marker was used (NeoMarkers, polyclonal), to study the state of astrocytic glia GFAP – glial fibrillar acidic protein (DAKO, polyclonal). To verify angiogenesis in the perifocal areas marker CD34 (DAKO, QBEnd10) marker was used. The expression of the antigens studied in the brain tissue appeared to be of a clear brown intracellular and extracellular staining in the course of the study under a light optical microscope Zeiss Primo Star (Germany) at a magnification of × 100, × 200 and × 400. Microphotographs were taken using a Leica DM 750/4 microscope (Germany) with a Leica DFC 420 digital camera (Germany) and Leica Application Suit Vers software 3.8. Immunohistochemistry was performed on the basis of the laboratory of the diagnostic and consulting center CSD Health care, Ukraine, Kyiv.

The work was approved by the Commission on Bioethics (excerpt from Protocol No.2 as of February 26, 2018), all moral, ethical and professional requirements and norms in the study of cadaver material were in line with the principles of the Declaration of Helsinki, the Council of Europe Convention on Human Rights and Biomedicine and relevant laws of Ukraine.

Ranking scales were used to objectify and semiquantitatively compare the expression of the studied markers depending on the groups and periods of the disease. For the expression of GFAP + astrocytes and CD34 + cells, it was filling by them 0-30% (1st degree of distribution), 31-60% (2nd degree of distribution) and > 61% (3rd degree of distribution) of the field of vision. 9 fields of vision, which corresponded to an area of 1 mm² were assessed; and to increase the reliability of the assessment the average value of the detected changes in 9 fields of vision was used. For the accumulation of τ-protein, the scale included 4 gradations of the degree of distribution – none; presence only in neurons; neurons and interstitium; neurons, interstitium and astrocytes. Therefore, the degree was determined taking into account the worst of the results in 9 fields of vision.

The expression of the studied markers between groups was compared using the unpaired Mann-Whitney test. To assess the dynamics of expression according to the periods of the disease, the Kendall rank correlation coefficient (Kendal’s Tau) was used, and pairwise comparison of periods was performed using the Mann-Whitney test. Statistical processing was performed using the software package STATISTICA for WINDOWS 6.0 (StatSoft, USA).

RESULTS

COMPARISON OF GFAP + ASTROCYTES BETWEEN ALL STROKE GROUPS

When comparing the area of detection of GFAP + astrocytes between all groups of strokes, no significant difference was identified (table I). In case of the 1st degree of GFAP expression +astrocytes were placed mainly in the border area around the necrosis nucleus, concentrated either around blood vessels or as single cells in the neuropile in case of IS and IS with HT, while in case of HS GFAP + astrocytes accumulated in brain tissue along the edge of the hematoma. There was a relative minority of such cases – 6 (28.6%, 25-33% depending on the group). To the contrary, most often in all groups there were cases with GFAP + cells of the 2nd degree of distribution: 12 (57.1%), and in HS and IS with HT groups they made the vast majority (62.5% and 66.7%, respectively). In such cases, GFAP + astrocytes distribution from the border area of the penumbra or perigematomata and were located diffusely, except for vessels, around hyperchormic neurons, neurons with vacuolated cytoplasm, and around neurons with chronic changes. GFAP + astrocytes, which in total occupied more than 60% (Figs. 1, 2) of the area, were detected in 2 (28.57%) cases in IS group and in 1 (12.5%) case in HS group.

COMPARISON OF ANGIGENESIS BETWEEN ALL GROUPS OF STROKE

Angiogenesis in the critical area with activated CD34 + cells was detected in most cases of strokes of all types (Table II). When comparing the severity of this feature between IS, HS and IS with HT no significant difference was identified, although this result may be caused by insufficient statistical sampling, as the proportion of cases with the 1st degree of angiogenesis (0-30%) in HS group was 62, 5% (Fig. 3), in IS with HT group it was -50%, and in IS group it was only 28.6%. To the contrary, the 3rd degree angiogenesis (> 61% of CD34 + cell distribution area) was detected only in IS group – 28.6% (marginal significance when compared with the sum of the other two groups, p = 0.1).
COMPREHENSIVE STUDY OF MANIFESTATIONS OF BRAIN TISSUE RESOLUTION IN CASE OF VARIOUS TYPES OF STROKE

Comparison of τ-protein detection between all stroke groups

In addition to ‘red’ and ‘shadows’ of neurons, neurons with chronic changes, activated microglia and newly formed vessels, immunohistochemistry of the perifocal tissue of the brain revealed acute hypoxia-stimulated processes of neurodegeneration with intracytoplasmic neuroprotective accumulation and hyperthyroidism. Accumulation of τ-protein of the 1st degree was detected in a total of 2/3 of cases (66.7%) of all stroke variants without significant differences between groups (Table II). The statistical sampling was insufficient to detect significant differences depending on the type of stroke in the

Fig. 1. Perifocal area of the brain with GFAP + astrocytes, some of which are elongated, with branched processes, occupying > 60% of the study area in case of ischemic stroke (IS).

Fig. 2. GFAP + > 60% of the occupied area of perifocal tissue of the brain in case of hemorrhagic stroke (HS).

Fig. 3. Perifocal tissue of the brain with CD34 + cells that form single thin-walled capillaries and occupy up to 30% of the study area in case of ischemic stroke with hemorrhagic transformation (IS with HT).

Fig. 4. CD34 + cells 0-30% of the occupied area of perifocal tissue of the brain in case of ischemic stroke (IS).

Fig. 5. CD34 + form a thin-walled densely located capillary network of the gliomesodermal capsule in the perigematos tissue of the brain in case of hemorrhagic stroke (HS).

Fig. 6. CD34 + form a thin-walled densely located capillary network of the gliomesodermal capsule in the perigematos tissue of the brain in case of hemorrhagic stroke (HS).

Fig. 7. Detection of τ-protein depending on the period of stroke.
Notes: First period (1-3 days), Second period (4-7 days), Third period (> 7 days)
severity of the accumulation of this protein – its presence in different structures (only neurons, neurons and neuropil; neurons, neuropil and astrocytes). However, in almost half of the cases – 3 (42.86%) in IS group the accumulation of pathological τ-protein was of the 2nd degree, in contrast when we talk about HS and IS with HT such cases made a third or less of the total number of each group. All cases with the presence of hyperphosphorylated τ-protein of the 3rd degree were detected only in HS group I – about 1/3 of the group – 3 (37.50%), but the difference becomes significant only if we compare HS group with other cases (Fisher’s exact test).

COMPARISON OF THE DETECTION OF GFAP + ASTROCYTES, CD34 + CELLS AND T-PROTEIN BETWEEN STROKE PERIODS

The assessment of the intensity of expression of markers of injury and resolution of perifocal tissue of the brain (table III) in the temporal aspect revealed a clear increase in the expression of GFAP + astrocytes with the course of the disease – Kendall’s Tau = 0.774, p <0.001. In the first period, GFAP + astrocytes were localized around the border zone with the area of 1st degree distribution, and in the second period – in more than 1/3 of cases (37.50%) – expressed astrocytes showed an area> 61% of the studied portion of the brain, and in other cases – 31-60% of the studied brain tissue.

The critical area of the brain occupied by CD34 + cells in the first period, mainly did not go beyond the 1st degree of the entire perifocal area – 6 (85.71%) (Fig. 4), only in 1 (14.29%) case the detection area of CD34 + cells was 31-60%. In the second period, CD34 + cells created newly...
Table III. Damage markers (τ-protein) and resolution (GFAP + and CD34 +) in different periods of stroke

<table>
<thead>
<tr>
<th></th>
<th>First period (1-days) (n=7)</th>
<th>Second period (4-days) (n=6)</th>
<th>Third period (&gt;7 days) (n=8)</th>
<th>R (Kendal’s Tau), ***p</th>
</tr>
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<tbody>
<tr>
<td><strong>GFAP: n (%)</strong></td>
<td></td>
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<tr>
<td>0 degree (none)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1 degree</td>
<td>6 (85.71%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2 degree</td>
<td>1 (14.29%)</td>
<td>6 (100%)</td>
<td>5 (62.50%)</td>
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<tr>
<td>3 degree</td>
<td>0</td>
<td>0</td>
<td>3 (37.50%)</td>
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<td><strong>CD34: n (%)</strong></td>
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<tr>
<td>0 degree (none)</td>
<td>0</td>
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<td>0</td>
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</tr>
<tr>
<td>1 degree</td>
<td>6 (85.71%)</td>
<td>2 (33.33%)</td>
<td>2 (25%)</td>
<td></td>
</tr>
<tr>
<td>2 degree</td>
<td>1 (14.29%)</td>
<td>4 (66.67%)</td>
<td>4 (50%)</td>
<td></td>
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<tr>
<td>3 degree</td>
<td>0</td>
<td>0</td>
<td>2 (25%)</td>
<td></td>
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<tr>
<td><strong>τ-protein: n (%)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0 degree (none)</td>
<td>4 (57.14%)</td>
<td>1 (16.67%)</td>
<td>2 (25.0%)</td>
<td></td>
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<tr>
<td>1 degree</td>
<td>2 (28.57%)</td>
<td>3 (50%)</td>
<td>0</td>
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<tr>
<td>2 degree</td>
<td>1 (14.29%)</td>
<td>0</td>
<td>5 (62.50%)</td>
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<tr>
<td>3 degree</td>
<td>0</td>
<td>2 (33.33%)</td>
<td>1 (12.50%)</td>
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</tbody>
</table>

n is the absolute number of cases; % – the relative number of cases

* The value of the relative area of brain tissue with positive expression of the studied cell markers (GFAP and CD34): 1st degree – 0-30% of the area; 2nd degree – 31-60% of the area; 3rd degree -> 61% of the area

** Presence of τ-protein in brain tissue structures: 0 degree – absent; 1st degree – in neurons; 2nd degree – in neurons and neuropil; 3rd degree – in neurons, neuropiles and astrocytes

**** Kendal’s Tau.

formed thin-walled capillaries and in more than half of the cases – in 4 (66.67%) the area of distribution corresponded to the 2nd degree of distribution. In the third period, the intensity of angiogenesis increased: in half of the cases CD34 + cells (Fig. 5), which inhabited 31-60% of the study area were observed, and in 2 (25%) cases they occupied > 61% of the perifocal area. Only in ¼ of all cases of the third period the area of detection of CD34 + cells did not go beyond the 1st degree. Thus, as the disease develops, the number of cases with a larger area of detection of CD34 + cells naturally increases (Kendal's Tau = 0.512, p = 0.001).

Having compared changes in different periods of stroke, it has been identified that over time, the frequency of detection of τ-protein (from 42.86% in the first period and to 75% in the third period) increased, and it was accumulating in certain brain structures – Kendal’s Tau = 0.359; p = 0.023.

In the first period of the disease hyperphosphorylated τ-protein of the 1st degree was found in 2 (28.7%) cases, in the second period it was found in 3 (50%) cases, also in the second period pathological τ-protein of the 3rd degree was observed in a third of cases (coming from the intracellular space of neurons, accumulated in neuropil and astrocytes). Over time, in the third period, hyperphosphorylated τ-protein of the 2nd degree was detected in more than half of the cases of strokes, and in one case this protein was diagnosed in all three studied structures of brain tissue (Fig. 6, 7).

Thus, all three study markers (GFAP +, CD34 +, τ-protein) showed a significant increase depending on the post-stroke period, mainly due to the difference between the first period and the third period (p = 0.06 for τ-protein, p < 0.05 for the area of detection of GFAP + astrocytes and CD34 + cells, no significant difference between the second period and the third period was found, between the first period and the second period the difference was significant only for the area of detection of GFAP + astrocytes). Consequently, there was also a direct correlation between τ-protein and GFAP + (Kendal’s Tau 0.484, p = 0.002) GFAP + and CD 34+ (0.740, p < 0.001), but no correlation was found between τ-protein accumulation and CD34 + cell detection area. In the first period, it increased the most in all 3 types of strokes.

DISCUSSION

Astrogliosis is a stereotypical reaction of astrocytes to brain injury [16 – 18]. In case of acute injury, activated astrocytes were located in the border area of the penumbra/perigematomata in some cases on the 2nd day from the onset of the disease. In subsequent post-stroke periods, in our study and according to other authors [19] as a compensatory-adaptive response, the frequency and area of detection of GFAP + astrocytes increased. Cells were located diffusely in the perifocal area, astrocytes were elongated, the processes of some of these cells went beyond their own domain, branched and intertwined with each other, establishing contacts with neurons and vessels, and
subsequently formed a glial scar. Post-stroke repair under the influence of growth factors [20 – 22] developed during the first 12-24 hours after stroke and lasted for 4 weeks. According to the results of our study early angiogenesis began with the germination of endothelial cells, the formation of tubular vascular branches and anastomoses in the perifocal parts of the brain. In all variants of ACVA, around the area of necrosis/hematoma, CD34 + cells were detected, the expression of which increased as the stroke continued [23, 24]. Less noticable angiogenesis in case of IS with HT may be associated with secondary hemorrhage into the necrosis area. We believe that such changes are similar to the changes in case of HS, in which the rate of repair was influenced by the products of degradation of erythrocytes [25, 26]. In case of HS, a dense newly formed capillary network was located around the hematoma and in combination with microglia and inflammatory infiltrate separated the hematoma from the perifocal area and formed a gliomesodermal capsule.

In this study, τ-protein in brain neurons was observed in the acute period of stroke. This meant induced cell damage, destabilization of neuronal microtubules caused by impaired glutamate transport in the brain. The presence of this protein in the extracellular space after the neurons disappearance, its internalization by other brain cells, in particular astrocytes and distribution in the CNS ‘contribute’ to the pathogenesis of neurodegenerative proteinopathies, affecting both neurodegeneration [27 – 29] and possibly neuroprotection, as well as act as potential mediators in the spread, and the elimination of protein-associated diseases [30].

CONCLUSIONS
1. Early healing processes in the perifocal tissue of the brain began in the first three days of the disease, GFAP + astrocytes were located in the border area around the nucleus of necrosis/hematoma, and over time the intensity of GFAP + astrocytes increased, most in case of IS and IS with HT.

2. The least noticable neoangiogenesis was found in IS with HT group. CD34 + cells in the acute period in case of HS were found in the area of the brain directly adjacent to the hematoma. CD34 + cells subsequently in combination with activated microglia and inflammatory cells became the basis for the formation of gliomesodermal capsule. With the development of the disease, the number of cases with a larger area of detection of CD34 + cells increased in strokes of various types.

3. τ-protein was detected in neurons in all variants of ACVA in the first period. With excessive hyperphosphorylation, in the second and third periods of stroke τ-protein came out of the periaxonal part of the neurons into the neurofied. In case of HS, this protein was captured and found in astrocytes, which may indicate the distribution of τ-protein between the structural components of the brain and subsequently clinically manifest itself in the disorder of the higher integrative function of the brain.

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Conflict of interest:
The Authors declare no conflict of interest.

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