ISCHEMIC STROKE SUBTYPES: SOME ASPECTS OF APOPTOSIS IN ACUTE PHASE OF BRAIN INFARCTION

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ABSTRACT

Objectives. The aim of the given research is to analyze the peculiarities of apoptosis and intracellular oxidative protection in acute phase of different ischemic stroke subtypes.

Material and methods. The study included 366 patients. We determined the number of white blood cells in the stage of apoptosis and necrosis, with a high content of reactive oxygen spices, with reduced mitochondrial potential, the activity of Cu, Zn-SOD (superoxide dismutase), Mn-SOD and cathepsin D.

Outcomes. We noticed mitochondrial dysfunction, intracellular oxidative stress, apoptosis and necrosis of white blood cells at all subtypes of brain infarction on the 1st day. A direct impact of mitochondrial dysfunction on the course of large arteries atherosclerosis (LAAS) in acute phase was established. In acute phase of LAAS we detect activation of both ways of apoptosis: lysosomal one and apoptosis associated with mitochondrial dysfunction. At LACunar stroke (LAC) we observed activation of mainly lysosomal way of apoptosis, due to the apoptosis of endothelial cells, proved by significant correlation between the content of white blood cells in peripheral blood in the stage of apoptosis (ANV+-cells) and total and free activity of cathepsin D. All ischemic stroke subtypes are associated with significantly ($p<0,01$) decreased activity of intracellular SOD-dependent antioxidant protective system (total SOD, Mn-SOD and Cu, Zn-SOD), that along with the increased number of intracellular reactive oxygen species (ROS) indicates misbalance between ROS formation process and the possibility of its elimination. At cardioembolic infarct (CEI) neutralization of intracellular ROS is due to mitochondrial SOD activity, while at LAC and LAAS – due to the intracellular one.

Conclusions. The peculiarities of apoptosis activity and intracellular antioxidant protection in acute phase of brain infarction depend on its subtype.

Keywords: ischemic stroke subtypes, apoptosis, mitochondrial dysfunction, cathepsin D, SOD-dependent antioxidant protective system

INTRODUCTION

Stroke is a leading cause of disability in adults that has a heavy social burden worldwide. This disease is the third highest cause of mortality, resulting in approximately six million deaths annually (1). Ischemic stroke may occur as a consequence of a wide range of vascular diseases that lead to thromboembolism in the brain. The concept of ischemic stroke pathogenic heterogeneity provides a variety of causes and mechanisms of acute focal brain damage. The common stages for all ischemic stroke subtypes are only the final ones that refer to the destructive process, while the pathogenesis of various ischemic stroke subtypes is different.

The results of recent studies have demonstrated that in acute phase of brain infarction cell death occurs via three main mechanisms: necrosis, apoptosis and autophagy (2). An important role in cell death play oxidative stress, decreased activity of antioxidant protective system (AOPS), mitochondrial dysfunction, inflammation, changes of NO metabolites content (3).

One of the main sources of oxidative stress is free radicals: superoxide anion (O2 • -), hydroxyl radical (HO •), lipid radicals (POO) and some types of reactive nitrogen (RNS) (4). Factories for the formation of free radicals are leukocytes whose impact on the pathogenesis of cerebral ischemia includes the initiation of thrombosis, reduction of cerebral perfusion through occlusion, and damage of the blood-brain barrier (5). Cerebral ischemia initiates two main ways of apoptosis: internal and ex-

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ternal (6). Caspase-3 is considered the main effector apoptotic protease, which is involved in brain endothelial cytotoxicity during hypoxia followed by reoxygenation (7). Besides caspase-dependent apoptosis, there are numerous evidences that point to the existence of other mechanisms of cell death without activation of caspases and absence of other classic markers of apoptosis (8). Many non-caspase proteases, such as lysosomal cathepsin (including cathepsin D) play an important role in stroke-induced cell death (9). Increased number of free radicals simultaneously with reduced antioxidant activity causes oxidative stress associated with damage of neurons at cerebral ischemia (10). Several studies strongly suggested a specific role of superoxide dismutase (SOD) in cerebral ischemia because of its ability to scavenging super-oxide anions. An important role in antioxidant protective system play two main types: SOD1 (Cu, Zn-SOD), which is present in the cell cytoplasm and SOD2 (Mn-SOD), located in the mitochondrial matrix. It is believed that intracellular SOD and mitochondrial one play dominant role in destruction of free radicals at brain infarction compared with extracellular fractions (11).

Despite the large number of recent clinical and experimental studies, pathogenetic mechanisms at different ischemic stroke subtypes that affect the course of acute phase are not completely clarified. The intracellular antioxidant protection, disorders of lysosomal membrane permeability and its relationship to apoptosis, mitochondrial dysfunction are not completely studied. The situation is complicated by the fact that in the experiment, it is not always possible to simulate all pathogenic variants of ischemic stroke subtypes and therefore positive experimental data often cannot be used in clinical practice. Therefore, the necessity to solve these issues determines the relevance of this study.

OBJECTIVES

To study the peculiarities of apoptosis and intracellular oxidative protection in acute phase of different ischemic stroke subtypes.

MATERIAL AND METHODS

We select subjects for the present analysis (366 patients) from an ongoing prospectively collected cohort of patients with ischemic stroke subtypes, admitted to the neurological departments in Ternopil regional communal clinical psychoneurological hospital. Among them there were 212 (57.9%) male and 154 (42.1%) female patients. Diagnosis of ischemic stroke subtype was put according to the TOAST criteria (12). Totally, there were 125 patients with cardioembolic infarct (CEI), 119 patients – with large artery atherosclerosis (LAAS) and 122 patients with lacunar stroke (LAC).

Patients were included if they presented with verified diagnosis of ischemic stroke on non-contrast computed tomographic scan (NCCT) or magneto-resonance imaging (MRI). The patients were followed in acute period after hospitalization on the 1st, 7th and 14th day of the disease. Exclusion criteria were as follows: (1) the presence of repeated ischemic strokes and impaired consciousness deeper than sopor (according to Glasgow scale less than 9-10 points), (2) chronic heart failure II B - III stages, III or higher functional class by New York Heart Association (NYHA), (3) chronic kidney disease (filtration rate $\leq$ 60 ml/min).

Two age categories of patients were studied. The first one included 164 (44.8%) patients at the age 45-59 years old (the first age group). The second one consists of 202 (55.2%) patients at the age 60-74 years old (the II-nd age group). All the patients were performed general clinical and clinical neurological examination (with determination of the degree of neurological deficit according to the National Institutes of Health Stroke Scale (NIHSS) (Odderson I.R., 1999). Based on NIHSS all strokes were divided into: minor (score 1-4) – 105 (28.7%), moderate (score 5-15) – 177 (48.4%), moderate\severe (score 15-20) – 71 (19.4%) and severe stroke (score 21-42) – 13 (3.5%) patients.

The clinical characteristics of patients with different ischemic strokes subtypes is presented in Table 1.

In order to establish the subtype of ischemic stroke ultrasound scanning of extra cranial and intracranial vessels by means of Philips Human Development Index (HDI) and Echo Doppler cardiography by means of «Biomedica» (Japan) were performed. Examination of indexes of apoptosis, mitochondrial dysfunction, and intracellular oxidative stress indexes was performed by means of cytofluorometric method (flow cytofluorometry EpicsXL (Beckman Coulter, USA)). The number of white blood cells in peripheral

TABLE 1. *The clinical characteristics of patients with different ischemic stroke subtypes*

Index	LAAS	CEI	LAC
General group	119	125	122
Female/ male,%	33.6/ 66.4	51.2 / 48.8	41.8 /58.2
Age of the patients, years	67.24 ± 1.27	69.61 ±1.40	57.46 ± 1.64
Hypertension, abs. (%)	83 (69.7%)	74 (59.2%)	116 (95.1%)
DM, abs. (%)	27 (22.7%)	19 (15.2%)	32 (25.5%)
Hypercholesterolemia	50 (42.0%)	35 (28.0%)	32 (26.2%)
Patients at the age 45–59 years old, n (%)	51 (42.9%)	47 (37.6%)	65 (53.3%)
Patients at the age 60-74 years old, n (%)	68 (57.1%)	79 (62.4%)	57 (46.7%)
NIHSS, the 1-rst day, points	11.27±0.57	11.42±0.54	6.72±0.42
NIHSS, the 7-th day, points	12.26±0.69	10.20±0.48	5.80±0.41
NIHSS, the 14-th day, points	10.65±0.60	8.95±0.59	4.27±0.50

blood in the stage of apoptosis (ANV+-cells) and necrosis (PI+-cells) was defined by a set of reagents ANNEXIN V-FITC-kit (Bender Medsystems, Austria). The number of white blood cells in peripheral blood with enhanced level of intracellular reactive oxygen species ROS (ROS+-cells) was defined by means of Dichlorofluorescein diacetate («SigmaAldrich», USA), the number of white blood cells with reduced level of mitochondrial membrane potential (Mito+-cells) – by means of set of reagents Mito Capture TM mitochondrial Apoptosis Detection Fluorometric Kit («Biovision», USA). The intensity of apoptosis was determined via activity of caspase-3 (Bonovini, 2004). The state of intracellular antioxidant activity was studied via activity of Cu, Zn-SOD and Mn-SOD according to the reducing rate of recovery of Nitroblue tetrazolium in the presence of Phenazynmetasulfas and NADH (Beauchamp C., Fridovich I., 1971). Instability of lysosomal membranes was studied by analyzing activity of cathepsin D: total and free by means of modified method by Dingle J.T. (1971). The control group (CG) consist of 42 people, representative by age and sex in relation to the patients with brain infarction: 23 males and 19 females at the age of 45 up to 71 years old. The average age of patients in CG was (60.7 ± 2.1) years. Statistical analysis of the results was made by means of IBMSPSS Statistics using Wilcoxon-Mann-Whitney U-criterion (for independent samples), Wilcoxon t-test (for dependent samples) and factor of pair linear correlation (r) by Spearman.

OUTCOMES

In the result of studying apoptosis and necrosis in white blood cells we found that on the 1st day of brain infarction significantly increases the content of ANV+-, PI+-, ROS+- and Mito+-cells in relation to control group (CG) ($p < 0.05$) (table 2).

On the 7th day of brain infarction the quantity of PI+-cells in general cohort of the patients significantly decreased. However we observed high activity of intracellular oxidative stress, apoptotic processes and mitochondrial dysfunction.

In 31 patients with CEI, we detect hemorrhagic transformation (HT) on CT. It was found that the level of circulating leukocytes in the stage of apoptosis (33.52 ± 1.09)% and ROS+ cells (42.60±1.17)% was significantly increased in patients with HT compared to other patients with CEI: (27.31 ± 1.28)% and (32.60 ± 2.27) ($p <0.001$). The level of PI+ cells and Mito+ cells at hemorrhagic transformation was significantly different from the values of patients without HT.

While studying the activity of caspase-3 we found that it was significantly ($p<0.05$) increased in the 1st day compared to CG. On the 7th day of stroke, the highest values of caspase-3 were observed at LAAS.

We found multidirectional changes of total activity and free activity of cathepsin D and its dynamics in patients with different ischemic stroke subtypes.

While studying the state of intracellular antioxidant protective system we found that the on the 1st day of brain infarction total activity of SOD, Cu, Zn-SOD and Mn-SOD significantly reduced compared to the CG ($p<0.05$). On the 7th day of stroke in spite of its subtype compared to the first day, we observed significantly reduced total SOD activity.

The obtained results indicate that apoptosis, necrosis, and oxidative stress are highly active, as indi-

cated by the increased number of cells in the apoptosis, necrosis and high content of reactive oxygen species. On the content of ANV+, PI+-, ROS+-cells a great impact have patients' age, severity of stroke, the stroke volume, the presence of edema, localization of brain infarction. Significantly increased number of ANV+-, PI+- and ROS+-cells was observed in elderly patients compared to the patients of middle age, at large brain infarction (> 100 cm^3) compared to the patients with the volume of infarct up to 10 cm^3, at severe and very severe infarction compared to minor brain infarction, at cortical localization of infarction.

The processes of apoptosis and necrosis in white blood cells in acute phase of brain infarction depended on pathogenic subtype of stroke. Significantly higher content of ANV+-cells was observed at LAAS ($p < 0.05$) compared to other ischemic stroke subtype. Quantity of PI+-cells at LAAS was significantly ($p < 0.05$) higher compared to LAC.

Expression of intracellular oxidative stress that manifested as high content of ROS in leukocytes, was the highest at CEI, especially significant - in hemorrhagic transformation (42.60 ± 1.17)%. This is probably an evidence of early post ischemic reperfusion at cerebral embolism that contributes to ROS production. At non cardiogenic brain infarction intracellular oxidative stress was less expressed compared to CEI. There were no significant difference between the quantity of ROS+-cells in the peripheral blood at LAAS and LAC.

On the 1st day of brain infarction we found a direct correlation between the content of ROS+- and ANV+- cells (r=0.65, p=0.001), PI+- cells (r=0.58, p=0.002), Mito+-cells (r=0.87, p=0.001); as well as between the amount of Mito+-cells and ANV+-cells (r=0.62, p=0.002) and PI+-cells (r=0.78, p=0.001). Particularly strong relationship was established between the quantity of Mito+- and ANV+-cells at LAAS (r=0.84, p=0.004). These data suggest that mitochondrial way of apoptosis initiating in acute phase of stroke is mainly expressed at LAAS. The processes of mitochondrial dysfunction and apoptosis were not well expressed at LAC (table 2).

We found the correlation between the severity of brain infarction and quantity of PI+-cells on the 1st (r = 0.53, p = 0.010), the 7th (r = 0.42, p = 0.012) and the 14th day (r = 0.47, p = 0.010). A reliable connection between stroke severity and content of ANV+-cells in the peripheral blood was observed on the 7th (r = 0.60, p = 0.015) and the 14th day (r = 0.67, p

TABLE 2. *The content of ANV$^+$-, PI$^+$-, ROS$^+$-, Mito$^+$-cells, activity of caspase-3, cathepsine D at ischemic stroke subtypes on the 1st and the 7th day (M±m)*

Index	CG	Day	Ischemic stroke subtype		
			CEI (n=60)	LAAS (n=62)	LAC (n=54)
ANV$^+$- cells,%	5.12 ± 1.31	1-st	29.24±1.12	32.51±2.35*	24.25±1.93*
		7-th	31.75±1.99	29.42±1.39	25.10±0.12*
PI$^+$- cells,%	0.13 ± 0.03	1-st	1.92±0.11	2.08±0.17	1.61±0.12
		7-th	1.76±0.16	1.61±0.17	1.67±0.10
ROS$^+$- cells,%	12.15±2.04	1-st	39.12±2.86*	35.21±3.11	30.71±2.55
		7-th	34.65±2.64	28.62±1.15	31.45±1.25
Mito$^+$- cells,%	4.52±0.25	1-st	13.23±0.53	15.33±0.74*	12.00±0.35*
		7-th	12.75±1.25	12.00±0.27	11.71±0.34
Caspase-3, mcmol/kg protein	2.42±0.33	1-st	6.14±1.45	11.47±1.22	15.10±0.27*
		7-th	5.75±0.82	6.71±0.70	5.42±0.31
Total activity of cathepsine D, nmol tyrosine/ (min.*mg protein)	0.42±0.08	1-st	3.66±0.66	4.46±0.87	1.10±0.04
		7-th	2.69±0.29	3.91 ±0.36	2.81±0.13
Free activity of cathepsine D, nmol tyrosine/ (min.*mg protein)	0.17±0.05	1-st	2.25±0.44	3.34±0.37*	0.20±0.08*
		7-th	1.32±0.20	1.94±0.42	1.88±0.07
SOD total, standard units mg^{-1} protein	10.74±0.86	1-st	5.16±0.22	7.12±0.44	4.42±0.32
		7-th	2.28±0.29	4.36±0.51*	3.21±0.38
Cu,Zn-SOD, standard units mg^{-1} protein	5.25±0.29	1-st	1.63±0.18	1.87±0.34	2.76±0.29*
		7-th	1.28±0.24	2.13±0.30*	0.95±0.27
Mn-SOD, standard units mg^{-1} protein	5.61±0.74	1-st	3.55±0.33*	5.12±0.12*	1.82±0.21*
		7-th	0.98±0.30*	2.26±0.14	2.05±0.21

Notes:
1 * - reliable performance in relation to the values of patients with other stroke subtypes ($p < 0.05$);
2. The underlined figures are significantly different compared with those on the 1st day ($p < 0.05$).

= 0.007), between the severity of brain infarction and content of ROS+-cells (r = 0.51, p = 0.012) on the 1st day of the disease. Thus, high levels of PI+-cells and ANV+-cells on the 1st day of brain infarction is considered unfavorable for the regress of neurological deficits in acute phase.

We found significantly increased level of circulating leukocytes in the stage of apoptosis and ROS+ cells in patients with HT compared to other patients with CEI. The content of leukocytes with increased intracellular ROS in the peripheral blood was more than 40% in all patients with HT. The obtained data indicate the prevalence of apoptotic mechanisms of cell death over necrosis at HT. We also assume that intracellular ROS play an important role in enhancing BBB permeability and the emergence of HT. HT is usually caused by reperfusion into the foci of necrosis, followed by swelling around the affected area. We did not find a significant difference between the number of Mito + cells in patients with HT and without it on the 1st day that can be explained by the fact that the above mentioned changes do not have time to develop within this time interval.

The results obtained by us coincide with the data of experimental studies. At large cerebral infarctions, massive secretion of ROS causes endothelial dysfunction by increasing BBB permeability and increased metalloprotease output, thereby increasing infarct size due to cell death in the penumbral region (13). HT is caused by reperfusion into the foci of necrosis followed by swelling around the affected area. Studies on animals have shown that uncontrolled reperfusion leads to mitochondrial swelling, which peaks 24 hours after reperfusion (14). Mitochondrial swelling in turn is associated with the opening of mitochondrial pores through which calcium ions pass into the cell and the concentration of Ca2 + increases in proportion to the degree of opening of these pores (15). As a result, the oxidative stress is increased by increasing the synthesis of intracellular (primarily mitochondrial) ROS, activated by different signaling pathways.

On the 7th day of brain infarction the quantity of PI+-cells in general cohort of the patients significantly decreased. However, we observed high activity of intracellular oxidative stress, apoptotic processes and mitochondrial dysfunction. Significant reduction of ANV+- and ROS+-cells was found in patients of middle age, with small volume of brain infarction and absence of cerebral edema according to NCCT and/or MRI. The obtained results regarding the dynamics of PI+-cells indexes demonstrated that the processes of cell death via necrosis at all ischemic stroke subtypes are mainly expressed at the first day of stroke.

Positive dynamics in quantity of ROS+-, PI+- and Mito+-cells on the 7th day was observed at LAAS. At CEI quantity of PI+-cells was the highest compared to other ischemic stroke subtypes. At LAC the quantity of PI+-cells does not change compared to the 1st day. Perhaps LAC contributes to other mechanisms of cell death via necrosis. It was established that the number of ANV+-cells slightly increased at CEI on the 7th day of brain infarction. We assume that a significant role in this fact play a high content of intracellular ROS on the 1st day of the disease, which contributes to the activation of apoptotic processes.

The obtained results regarding the dynamics of PI+ cells indicate that the processes of cell death by necrosis at all subtypes of brain infarction are more expressed in the first days of stroke. Subsequently, necrotic cell death is reduced. At CEI, the number of PI + leukocytes was on the top at the 7th day compared to the other stroke types. At LAC the number of PI + cells was virtually unchanged compared with the 1st day. The fact can be explained by the other mechanisms of cell death by necrosis, other than mitochondrial-induced ones, that are triggered at LAC. The higher rates of PI + leukocytes were detected in case of the presence of two or three lacunar foci on CT compared with the patients with one foci. It was found that the number of ANV + cells increased at the 7th day. We assume that a significant role in this fact is played by the high content of intracellular ROS on the 1st day of the disease, which promotes the activation of apoptotic processes, as well as the presence of a group of patients with hemorrhagic transformation, in which the number of ROS + cells is especially increased.

While studying the activity of caspase-3 we found that it was significantly ($p < 0.05$) increased in the 1st day compared to CG. There was no significant difference in the activity of caspase-3 in patients of different ages. However, its activity depended on the severity of brain infarction and its volume. The highest activity of caspase-3 was ob-

served at moderate stroke and volume of the infarct 10-100 cm3 (respectively (10.05 ± 1.15) and (11.40 ± 0.92) pmol / mg protein).

Numerous studies prove the important role of caspase-3 in the pathogenesis of brain infarction. In particular, the neuroprotective effect of a caspase-3 inhibitor at ischemia was demonstrated (16). It is also known that proteases (including caspase-3) are involved in ischemic damage of brain endothelial cells (17). Clinical results have shown that increased serum caspase-3 levels are an indicator for size of infarction and worse outcome after stroke (18). Clinical research also demonstrated that treatment with minocycline, a drug with many anti-apoptotic effects, which include inhibition of caspase-1 and caspase-3, improves the functional effects of stroke (19).

The activity of caspase-3 depended on ischemic stroke subtype and on the 1st day was the highest at LAC and LAAS. At LAC activity of caspase-3 was significantly higher compared to other ischemic stroke subtypes, along with the least amount of ANV+- and PI+-cells. In patients with LAC we noted the relationship between the activity of caspase-3 and the number of ischemic lesions. Experimental studies have demonstrated that zone of infarction contains inactivated caspase due to the rapid exhausting of adenosine triphosphate (ATP), damage of intracellular ionic composition, massive production of nitric oxide or ROS via calpain activation (20). Probably because of that fact on the 1st day of stroke with large volume, we not always diagnosed high activity of caspase-3. On the 7th day of stroke activity of caspase-3 significantly ($p < 0.05$) decreased compared to the first day. However, despite the above-mentioned results, the activity of caspase-3 remained significantly higher than in CG. The activity of caspase-3 did not depend on the age of patients. Significant decrease of caspase-3 activity was observed at mild and moderate brain infarction, as well as at small and medium brain infarction. The most demonstrative changes were observed at medium brain infarctions.

The analysis of caspase-3 activity at various ischemic stroke subtypes detects significant decline of it at LAC. On the 7th day of stroke, the highest values of caspase-3 were observed at LAAS. We found a reliable connection between the activity of caspase-3, content of ANV+-cells ($r = -0.58$; $p = 0.006$) and the number of ROS+-cells ($r = -0.61$, $p = 0.009$). These correlations may indicate that the high activity of caspase-3 contributes to apoptosis of white blood cells and declining in numbers of cells with increased intracellular oxidative stress.

In acute phase of brain infarction, we observed disorders of lysosomal membranes stability, that manifest as cathepsin D release in cellular cytoplasm followed by significant growth in blood of its total and free activity compared to the CG. The activity of cathepsin D depended on the age of the patients and stroke severity. In middle-aged patients on the 1st day, we found significantly higher total activity and free activity of cathepsin D compared to elderly patients. On the 7th day, free activity of cathepsin D in patients of the first age group was significantly lower than in patients at the age 60-74 years old and correlated with age. The greatest impact on the course of acute phase of brain infarction has free activity of cathepsin D, which was proved by the presence of significant connections between free activity and severity of cerebral infarction ($r = 0.63$, $p = 0.002$) and age of the patients ($r = 0.61$, $p = 0.004$) on the 7th day of the disease. The lowest total and free activity of cathepsin D on the 1st day was found at mild (respectively (1.66 ± 0.44) and (1.38 ± 0.17), the highest – at moderate stroke (3.98 ± 0.22 and (2.92 ± 0.28) nmol protein tyrosine /(min*mg protein), that coincides with the results of other studies showing the limiting role of cathepsins in neuronal damage at cerebral ischemia (21). It is reported that cathepsins play a critical role in the injury associated with moderate stroke (after 5-minute oxygen-glucose deprivation) but not because of more severe stroke (after 10-minute occlusion). Other processes are likely to be more damaging than cathepsin proteolysis at severe stroke.

We found multidirectional changes of total activity and free activity of cathepsin D and its dynamics in patients with different ischemic stroke subtypes. We recorded significantly higher indexes of total and free activity of cathepsin D at CEI compared to other patients. We observed declining in these indexes on the 7th day. At LAAS and LAC we observed a significant growth of total and free activity of cathepsin D on the 7th day compared to the first day. Particularly noteworthy is increasing of free activity of cathepsin D in patients with LAC

- in 9.4 times, indicating increased permeability of lysosomal membranes. To our point of view, LAC on the background of systemic vascular lesions at hypertensive disease and diabetes mellitus can be a trigger factor for the release of proteolytic enzymes from lysosomal cells whose activity increases during the 1st week of stroke.

We found a direct relationship between the activity of cathepsin D and the activity of caspase-3 on the 1st day of stroke (r=0.43, p = 0.012). This may indicate that caspase and lysosomal way of apoptosis are simultaneously being activated in acute phase of brain infarction. On the 1st day of LAC we observed correlation between the content of ANV+-cells and total activity of cathepsin D (r = 0.57, p = 0.017) also with free activity of cathepsin D (r = 0.59, p = 0.009). As mentioned above, the number of ANV+-cells at LAC is the lowest and it does not depend on the volume of infarction. Severity of apoptosis may be related to endothelial apoptosis and caused mainly via lysosomal way of apoptosis at LAC. Growth of free and total activity of cathepsin D on the 7th day of LAC did not correlate with the number of white blood cells in the stage of apoptosis. Such growth of free and total activity without regard to other indicators of apoptosis may be due to other mechanisms of damaging action of cathepsin D, including direct action on the connective fibers of blood brain barrier (BBB), which increases the permeability of the BBB. On the 1st day of LAC we also observed correlation between the content of ROS+-cells and free activity of cathepsin D (r = 0.36, p = 0.014). On the 7th day of stroke the relationship between free activity of cathepsin D and content of ROS+-cells become stronger (r = 0.61, p = 0.003), indicating the effect of ROS on increased permeability of the lysosomal membranes. We found a relation between the number of ANV+-cells with total activity of cathepsin D on the 1st day of LAAS (r = 0.52, p = 0.014). These data may indicate that lysosomal way of apoptosis at LAAS started simultaneously with mitochondrial one mentioned above.

While studying the state of intracellular antioxidant protective system we found that the on the 1st day of brain infarction total activity of SOD, Cu, Zn-SOD and Mn-SOD significantly reduced compared to the CG ($p < 0.05$). It was established that the activity of all subtypes of SOD depends on the severity and volume of brain infarction. The highest activity of SOD was observed at moderate and severe stroke and stroke volume 10-100 cm^3. In case of brain edema, the activity of various SOD increases, as a compensatory response to increased ROS content. Analysis in dynamics of activity of various SOD subtypes on the 7th day of stroke demonstrated its further decline in 88.4% of patients. There was no significant difference between the activity of SOD in patients of different age groups. There was a different dynamics of SOD activity depending on the severity of stroke: at mild brain infarction activity of SOD increased, at moderate and severe – it decreased. Activity of all types of SOD (especially mitochondrial one) decreased at medium and large volumes infarcts and in patients with cerebral edema.

Activity of total SOD at different ischemic stroke subtypes on the 1st day was significantly ($p < 0.05$) lower than in CG. Significantly lower activity of total SOD was observed in patients with LAAS and CEI compared to other patients ($p < 0.05$). The activity of Cu, Zn-SOD at LAC was significantly ($p<0.05$) higher than at other stroke subtypes. At LAAS activity of intracellular Cu, Zn-SOD, although was lower compared to LAC, was significantly higher than in patients with CEI. Thus, Cu, Zn-SOD, which is responsible for the growth and remodeling of blood vessels, almost does not respond at microvascular stroke. This is probably due to the small volume of infarction and less severity of LAC.

The highest activity of Mn-SOD was found in patients with CEI. The lowest mitochondrial SOD activity was observed at LAC and LAAS.

A number of studies indicate that Mn-SOD protects vascular mitochondrial DNA from damage and prevents the development of atherosclerosis in the aorta (22). In addition, it is the endothelium that produces high levels of Mn-SOD and its sharply reduced activity may indicate endothelial cell death and marked endothelial dysfunction in patients with LAC and LAAS.

On the 7th day of stroke in spite of its subtype compared to the first day, we observed significantly reduced total SOD activity. We established that CEI is associated with decline of total SOD activity, Mn-SOD activity and activity of Cu, Zn-SOD, which on the 1st day of stroke was significantly re-

duced compared to the CG. Simultaneously we notice increased number of ROS+-cells in these patients. As a result, we noticed inadequate disposal of intracellular ROS production. At LAC significant decrease of total activity of SOD and Cu, Zn-SOD was found. Mn-SOD activity increased unreliably. A significant increase of white blood cells with a high content of intracellular ROS was observed. At LAC we established inverse correlation between the amount of ROS+-cells with total SOD activity ($r = -0.71$, $p = 0.007$) and Mn-SOD activity ($r = -0.68$, $p = 0.009$) on the 7th day of stroke. We found significant inverse correlations between total SOD activity, Mn-SOD activity and volume of infarct, severity of LAC on the 7th day of the disease. These data suggest that neutralization of intracellular ROS is mainly due to Mn-SOD activity in the acute phase of CEI, while at LAC and LAAS it is due to Cu, Zn-SOD activity.

On the 7th day of stroke we found correlation between the activity of caspase-3 and total SOD activity ($r = 0.79$, $p = 0.004$) and Mn-SOD activity ($r = 0.84$, $p = 0.001$). That means, in response to the increased number of effectors of apoptotic cell death, intracellular antioxidant defense mechanisms are being activated. We noticed a significant relationship between free activity of cathepsin D with activity of Mn-SOD on the 1st ($r = -0.47$, $p = 0.020$) and 7th ($r = -0.50$, $p = 0.018$) days of brain infarction. We found reliable inverse relationship between the content of ROS+-cells and total SOD activity ($r = -0.55$, $p = 0.014$) and Mn-SOD activity ($r = -0.63$, $p = 0.005$) on the 1st day of stroke.

The highest activity of Mn-SOD was found in patients with CEI, which may be a compensatory response to increased release of free radicals during ischemia. At CEI we observed a significant imbalance between the processes of formation of intracellular ROS with activity of SOD-dependent antioxidant protective system, especially intracellular. Mn-SOD activity in patients with CEI was reduced to a smaller extent than Cu, Zn-SOD. However, on the background of early reperfusion, which is mainly expressed at CEI compared to other ischemic stroke subtypes, Mn-SOD activity does not have enough time to neutralize hyper production of intracellular ROS generated mainly in mitochondria. The lowest mitochondrial SOD activity was observed at LAC and LAAS, indicating a lack of synthesis of Mn-SOD by mitochondria in conditions of ischemic-hypoxic damage. Furthermore, mitochondrial SOD in the brain is primarily synthesized by endotheliocytes. That is why its lowest activity at LAC can indicate their dysfunction.

CONCLUSIONS

1. In acute phase of all ischemic stroke subtypes on the 1st day we noticed mitochondrial dysfunction, intracellular oxidative stress, apoptosis and necrosis of white blood cells, proved by significant growth of Mito+-, ROS+-, ANV+- and PI+ cells.
2. We established direct impact of mitochondrial dysfunction and mitochondrial-induced apoptosis on the course of LAAS in acute phase. This was confirmed by the presence of correlations between the severity of LAAS and quantity of Mito+-cells on the 1st day ($r = 0.74$, $p = 0.009$) and 7th day ($r = 0.72$, $p = 0.005$) and quantity of ANV+-cells on the 7th day ($r = 0.85$, $p = 0.002$). In acute phase of LAAS we detect activation of both ways of apoptosis: lysosomal one and apoptosis associated with mitochondrial dysfunction.
3. At LAC we observed activation of mainly lysosomal way of apoptosis, due to the apoptosis of endothelial cells, proved by significant correlation between the content of ANV+-cells and total ($r = 0.57$, $p = 0.017$) and free activity of cathepsin D ($r = 0.59$, $p = 0.009$).
4. All ischemic stroke subtypes are associated with significantly ($p < 0.01$) decreased activity of intracellular SOD-dependent antioxidant protective system (total SOD, Mn-SOD and Cu, Zn-SOD), that along with the increased number of intracellular ROS indicates misbalance between ROS formation process and the possibility of its elimination. At CEI neutralization of intracellular ROS is due to mitochondrial SOD activity, while at LAC and LAAS – due to the intracellular one.

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