

## **ВЫЯВЛЕНИЕ ВЗАИМОСВЯЗИ МЕЖДУ БИОМАРКЕРАМИ АУТОФАГИИ, АПОПТОЗА И ВОСПАЛЕНИЯ В ОСТРОМ ПЕРИОДЕ АТЕРОТРОМБОТИЧЕСКОГО ИШЕМИЧЕСКОГО ИНСУЛЬТА**

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**Резюме.** Постишемический воспалительный ответ играет существенную роль в патогенезе острого ишемического инсульта (ИИ). Установлено, что острый ИИ сопровождается асептическим воспалением, индуцирующим активацию костимулирующих молекул в процессе ответной реакции врожденного иммунитета на повреждение ткани головного мозга. Постоянно прогрессирующая деструкция нейрональных антигенов способствует увеличению объема очага ишемического поражения. Продолжают накапливаться доказательства, свидетельствующие о важной роли NLRP3-опосредованного воспаления в патогенезе ишемического инсульта, реализуемого инфламмосомами – мультипротеиновыми олигомерными комплексами. В последнее время большой интерес вызывает перспектива модулирования аутофагии при остром ИИ, являющейся мощным регулятором воспалительных реакций. Показано, что при остром ИИ аутофагия задействована в важнейших этапах воспалительного каскада, выступая либо в качестве индуктора, либо в роли ингибитора постишемического нейровоспаления. Во многих противовоспалительных механизмах, реализуемых аутофагией при остром ИИ, участвуют ключевые белки аутофагического процесса Beclin-1, LC3 и p62. Экспериментальные исследования показали, что аутофагия подавляет активацию NLRP3-опосредованного воспаления. По-прежнему противоречивыми остаются данные о перекрестных взаимодействиях между апоптозом и

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аутофагией и их отдельными медиаторами в патогенезе острого ИИ. Исследования в области нейрo-иммунологии позволили установить сигнальные белки, инициирующие как апоптотическую, так и аутофагическую гибель клеток головного мозга при остром ИИ. Целью исследования явилась оценка взаимосвязи между биомаркерами аутофагии, воспаления, и апоптоза в динамике острого периода атеротромботического ИИ. В работе представлены результаты динамического исследования сывороточной концентрации ключевых биомаркеров аутофагии Beclin-1, LC3 и p62, показателей апоптоза Bcl-2 и p53, провоспалительных цитокинов IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-18, участвующих в постишемическом нейровоспалении. Установлено статистически достоверное повышение исследуемых показателей по сравнению с группой контроля. Максимальное повышение исследуемых биомаркеров отмечается в 1-е сутки после развития ишемии у пациентов с тяжелым течением заболевания. Выявлена взаимосвязь между активностью аутофагии, биомаркерами апоптоза и некоторыми показателями системной воспалительной реакции у пациентов с атеротромботическим инсультом среднетяжелого и тяжелого течения. Полученные результаты подтверждают данные литературы об участии аутофагии в регуляции постишемического воспалительного ответа.

*Ключевые слова:* аутофагия, апоптоз, острый ишемический инсульт, биомаркеры аутофагии, биомаркеры апоптоза, Beclin-1, LC3, p62, IL-1 $\beta$ , IL-18, NLRP3-инфламмосома, постишемическое нейровоспаление

## IDENTIFICATION OF THE RELATIONSHIP BETWEEN BIOMARKERS OF AUTOPHAGY, APOPTOSIS AND INFLAMMATION IN THE ACUTE PERIOD OF ATHEROTHROMBOTIC ISCHEMIC STROKE

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**Abstract.** The postischemic inflammatory response plays a significant role in the pathogenesis of acute ischemic stroke (IS). It has been established that acute IS is accompanied by aseptic inflammation, which induces the activation of costimulatory molecules in the process of innate immunity response to brain tissue damage. The constantly progressive destruction of neuronal antigens contributes to an increase in the volume of the ischemic lesion. Evidence continues to accumulate indicating an important role of NLRP3-mediated inflammation in the pathogenesis of IS. It has been shown that autophagy is involved in the inflammatory cascade in acute IS. Many of the anti-inflammatory mechanisms mediated by autophagy in acute IS involve the key autophagic proteins Beclin-1, LC3, and p62. Experimental studies have shown that autophagy suppresses the activation of NLRP3 inflammation. Data on cross interactions between apoptosis and autophagy in the pathogenesis of IS are still controversial. The aim of the study was to evaluate the relationship between biomarkers of autophagy, inflammation, and apoptosis in the dynamics of the acute period of atherothrombotic IS. The article presents the results of a dynamic study of the serum concentration of the key autophagy biomarkers Beclin-1, LC3 and p62, apoptosis indicators Bcl-2 and p53, pro-inflammatory cytokines IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-18 which are involved in postischemic neuroinflammation. A statistically significant increase in the studied parameters was established in comparison with the control group. The maximum increase in the studied biomarkers is noted on the 1<sup>st</sup> day after the development of ischemia in patients with a severe course of the disease. The relationship between autophagy activity, apoptosis biomarkers, and some indicators of the systemic inflammatory response in patients with moderate and severe atherothrombotic stroke was revealed. The results obtained confirm the literature data on the involvement of autophagy in the regulation of the postischemic inflammatory response.

*Keywords:* autophagy, apoptosis, acute ischemic stroke, autophagy biomarkers, apoptosis biomarkers, Beclin-1, LC3, p62, IL-1 $\beta$ , IL-18, NLRP3-inflammasome, postischemic neuroinflammation

## Introduction

Postischemic neuroinflammation is a critical pathophysiological process within the framework of the entire scheme of cerebral ischemia, covering early damage and the period of tissue repair. It is characterized by microglial and astroglial activation with increased expression of inflammatory mediators and development of innate and adaptive immune responses. After the onset of acute ischemic stroke (IS), damage to the blood-brain barrier (BBB) occurs in 2 stages. The first begins within the first hours with the participation of innate immunity, and the second – 24–48 hours after an ischemic attack. During extravasation, leukocytes attracted by chemokines release matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), which cleave tight junctions between endothelial cells of cerebral vessels [12]. This leads to damage to the BBB, resulting in increased vascular permeability, which can contribute to the development of vasogenic edema, one of the most severe complications of cerebral infarction [9]. Violation of the integrity of the BBB contributes to the disintegration of brain tissue and the “leakage” of brain autoantigens (myelin basic protein, myelin oligodendrocyte glycoprotein (MOG), glial fibrillary acid protein (GFAP)) into the peripheral blood. The release of brain autoantigens into circulation is accompanied by the activation of the immune system and the influx of peripheral immunocompetent cells into the central nervous system [12]. Thus, acute cerebral ischemia triggers a local and systemic immune response [5].

According to the literature, after acute IS, microglia are polarized into the pro-inflammatory phenotype M1, characterized by the secretion of pro-inflammatory cytokines, in particular TNF $\alpha$  (tumor necrosis factor  $\alpha$ ), IL-1 $\beta$ , IL-6, IL-18, IL-21 (interleukins-1 $\beta$ , interleukins-6, interleukins-18, interleukins-21) [3]. In the acute period of IS, patients have a significant increase in the concentration of pro-inflammatory cytokines in serum. It has been shown that a high level of serum IL-1 $\beta$  correlates with the severity of acute IS and is a predictor of a poor prognosis of the disease. These results are consistent with literature data on the key role of this interleukin in ischemic injury [15]. According to the latest experimental data, autophagy is associated with postischemic neuroinflammation and is involved in its regulation [7]. In recent reports, autophagy is considered as a negative regulator of NLRP3 inflammation (nucleotide-binding oligomerization domain-like receptor [NLR] family pyrin domain-containing 3), which plays a significant role in the pathogenesis of ischemic stroke [2]. This is supported by experimental data demonstrating that the use of autophagy inhibitors promotes the activation of the NLRP3 inflammasome. It has been shown that macrophages with knockout of the Atg7 autophagy gene are characterized by a significantly increased

production of IL-1 $\beta$  in response to inflammation inducers compared to normal macrophages [10].

Numerous evidences have been provided for the involvement of autophagy in the regulation of the production of pro-inflammatory cytokines and chemokines [7, 8]. The interactions between autophagy and inflammation have been shown to work in a feedback manner. Autophagy is involved in the induction and suppression of inflammation and, in turn, can be both induced and suppressed by inflammatory mediators [4]. It has been established that basic autophagy under normal physiological conditions contributes to the maintenance of immunological tolerance, while excessive activation of autophagy in immunocompetent cells or the presence of defects in autophagy-inhibiting genes in them leads to the development of autoimmune processes [14].

A similar trend was revealed in the study of the mutual regulation of apoptosis and autophagy [6]. A number of recent studies show that basic autophagy, by stimulating the overexpression of anti-apoptotic proteins of the Bcl-2 family, inhibits apoptosis, protecting brain cells from delayed death. In contrast, activation autophagy acts in conjunction with apoptosis to exacerbate cell death. As a result of experimental studies, proteins were identified that are effectors of brain cell death both by apoptosis and by activation autophagy in acute IS. These proteins are the Bcl-2 and p53 antagonist proteins, as well as the autophagy initiator Beclin-1 (the protein encoded by the Atg6 gene initiates the initial stage of autophagy, the formation of a phagophore) [1,6]. Recent reports indicate that selective mitochondrial autophagy (mitophagy) with the participation of Beclin-1 and p62/SQSTM1 (LC3-binding adaptor p62/sequestosome 1) proteins can suppress the proapoptotic activity of the p53 protein and inhibit apoptosis [1].

Considering the latest literature data on the significant role of autophagy in the regulation of postischemic neuroinflammation and apoptosis processes in acute IS, it seems relevant to evaluate the relationship between biomarkers of autophagy, inflammation, and apoptosis in the dynamics of the acute period of IS and compare it with the severity of the clinical and neurological status of patients.

**Objective:** to quantify pro-inflammatory cytokines, C-reactive protein, autophagy biomarkers, apoptosis biomarkers in the serum of patients in the acute period of moderate and severe atherothrombotic IS. To reveal the relationship between biomarkers of autophagy, inflammation and apoptosis in the dynamics of the acute period of atherothrombotic IS.

## Materials and methods

All studies were approved by the ethical committee of the Pavlov First St. Petersburg State Medical University of Russian Federation. We examined 92 patients (63 men and 29 women) in the acute

period of newly developed atherothrombotic IS and 56 healthy donors comparable in sex and age to patients with acute IS. Inclusion criteria for the study were: informed consent to participate in the study; age from 45 to 60 years; verified by magnetic resonance imaging (MRI) for the first time identified acute IS in the system of the internal carotid artery (atherothrombotic pathogenetic variant); gender of patients: male, female; no more than 24 hours from the onset of the disease; neurological symptoms no more than 14 points on the NIHSS scale (National Institutes of Health Stroke Scale).

According to the results of clinical neurological and laboratory examinations, as well as the results of neuroimaging diagnostic methods, all patients were divided into 3 groups: with mild (n = 6), moderate (n = 59) and severe disease (n = 27). Due to the small number of observations, patients with mild acute IS were not included in further analysis. Group I (moderate course) consisted of patients with a severity of neurological symptoms of no more than 10 points on the NIHSS scale, no more than 3 points on the Rankin scale (used to assess the degree of disability after a stroke), with a brain parenchymal lesion volume of less than 50 cm<sup>3</sup>. Group II (severe course) consisted of patients with a severity of neurological symptoms of more than 10 points on the NIHSS scale, from 3 to 5 points on the Modified Rankin Scale (mRs), with a brain parenchymal lesion volume of more than 50 cm<sup>3</sup>. Group III (control) consisted of donors (n = 56).

Patients underwent a dynamic clinical and neurological examination with an assessment of the severity of neurological deficit according to the NIHSS scale, a study of the volume of the lesion by brain MRI, testing by mRs on the 1<sup>st</sup>, 7<sup>th</sup> and 14<sup>th</sup> days from the onset of the disease. At the same time intervals, blood was taken for the study.

Serum concentrations of pro-inflammatory cytokines IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-18, apoptosis biomarkers p53, Bcl-2, and autophagy biomarkers Beclin-1, LC3, and p62 were determined by enzyme-linked immunosorbent assay using appropriate test systems (ELISA Kits; Abcam, UK) and (ELISA Kits; Enzo, UK).

A highly sensitive immunoturbidimetric method (Cobas 6000, Roche Diagnostics, Switzerland) was used to quantify C-reactive protein in peripheral blood.

For statistical processing of the obtained data, the nonparametric Wilcoxon–Mann–Whitney test was used to compare the means, Spearman’s correlation analysis. The material was processed using the Statistica 10.0 software package (StatSoft, USA, Windows 10). The critical confidence level of the null hypothesis (the absence of significant differences) was taken equal to 0.05.

## Results and discussion

The results of the assessment of the studied indicators in the dynamics of the acute period of IS are presented in Tables 1 and 2.

**TABLE 1. COMPARATIVE CHARACTERISTICS OF THE CONTENT OF BIOMARKERS OF AUTOPHAGY, APOPTOSIS, AND PRO-INFLAMMATORY CYTOKINES IN THE PATIENT’S BLOOD SERUM IN THE DYNAMICS OF THE ACUTE PERIOD OF IS**

Index	Groups of examined persons								
	I group	II group	III group	I group	II group	III group	I group	II group	III group
	1 <sup>st</sup> day			7 <sup>th</sup> day			14 <sup>th</sup> day		
LC3, ng/L	180.9*	250.3**	89.3	215.6**	309.8**	–	198.5*	274.6**	–
Beclin-1, ng/L	150.6*	190.8*	75.6	199.8*	232.5**	–	170.6*	201.4**	–
p62, pg/mL	36.8*	21.9*	11.4	33.5*	40.9**	–	25.4*	37.8**	–
P53, U/mL	31.2***	19.9**	1.5	16.5**	23.5**	–	9.8*	15.9**	–
Bcl-2, ng/mL	6.6*	10.1**	1.8	11.3**	18.9**	–	19.4**	26.5***	–
TNF $\alpha$ , pg/mL	28.9**	65.3***	6.4	19.3*	28.8*	–	15.4*	21.5**	–
IL-1 $\beta$ , pg/mL	31.8**	54.6***	3.9	20.4**	30.6***	–	10.3*	19.8**	–
IL-8, pg/mL	40.5**	80.9***	12.8	31.1*	60.3**	–	25.4*	42.6***	–
IL-18, pg/mL	406.7*	894.9***	158.9	301.3*	503.9**	–	209.3*	418.4**	–

Note. I group, moderate course of the disease (n = 59); II group, severe course of the disease (n = 27); III group, control (n = 56). \*, differences in the studied indicator with the control group are statistically significant (p < 0.05); \*\*, differences in the studied indicator with the control group are statistically significant (p < 0.01); \*\*\*, differences in the studied indicator with the control group are statistically significant (p < 0.001).

The data obtained show that in the acute period of IS, there is a sharp increase in the concentration of the studied pro-inflammatory cytokines, which is most pronounced in patients with a severe course of the disease (group II), which, combined with a sharp increase in CRP, can be a criterion for a general inflammatory response. It is known that the pro-inflammatory cytokines IL-1 $\beta$ , IL-8 and TNF $\alpha$ , a sharp increase in which was revealed as a result of the study, play a key role in the development of the acute phase response to inflammation [5, 15]. Considering that the acute phase protein CRP is increased by 44.2 times in the group with severe IS and by 18.98 times in patients with a moderate course of the disease, it is probably possible to assume a systemic nature of inflammation in the acute period of IS, which is consistent with literature data [14].

As can be seen from the data presented in Tables 1 and 2, in both groups of patients there is a dynamic decrease in the concentration of inflammatory biomarkers. However, even by the 14<sup>th</sup> day, the median values of inflammation indicators are statically significantly increased compared to the control group, which indicates an ongoing inflammatory response. According to the literature, the postischemic inflammatory response, on the one hand, is aimed at removing necrotic tissue from the ischemic zone, pursuing protective goals. On the other hand, it leads to an increase in the volume of the lesion and aggravates the disease. The initial damage to neurons occurs within a few minutes after an ischemic attack, while the inflammatory response that contributes to the progression of the pathology can last from several days to several months [12].

The study found a statistically significant increase in biomarkers of apoptosis and autophagy compared to the control group, most pronounced in severe stroke (Table 1). According to the literature, this indicates that ischemic attack-induced activation autophagy acts in conjunction with apoptosis in the

early stages of the acute period of IS, contributing to the progression of the disease. Of interest is the multidirectional dynamic change in the biomarkers of autophagy and the apoptosis inducer p53 protein. As can be seen from Table 1, the maximum values of the p53 protein concentration are observed on the 1<sup>st</sup> day after a stroke, with a further clearly defined downward trend.

These results are consistent with the literature data that the apoptotic cascade is triggered in the penumbra 1-2 hours after the development of ischemia and reaches its maximum activity on the 3<sup>rd</sup> day. In the future, with properly prescribed therapy, the process of apoptosis declines [8]. Against this background, as the results show, all studied biomarkers of autophagy reach maximum values on the 7<sup>th</sup> day with a slight downward trend on the 14<sup>th</sup> day of the study (Table 1). In addition, there is a dynamic increase in the concentration of the apoptosis inhibitor Bcl-2, reaching maximum values on the 14<sup>th</sup> day. Probably, a gradual increase in the level of serum Bcl-2 is associated with the time required for the activation of compensatory anti-apoptotic processes.

To identify the relationship between autophagy activity in the dynamics of the acute period of IS, indicators of apoptosis and inflammation, the microtubule-associated protein light chain 3 (LC3) protein, which is involved in the formation of autophagosome, was chosen as the studied parameter of autophagy. It is a reliable marker of autophagy, since its content in the studied biological material positively correlates with the number of active autophagosomes, the most important components of the autophagy process [1]. The results of the study of the relationship between the LC3 protein and the studied parameters are presented in Table 3.

A strong direct correlation was found, more pronounced in the group with severe stroke, between the level of autophagy and the concentration of serum protein p53 ( $r = 0.74$ ;  $p < 0.05$ ), CRP ( $r = 0.81$ ;

**TABLE 2. CONCENTRATION OF C-REACTIVE PROTEIN (CRP) IN THE PATIENT'S PERIPHERAL BLOOD IN THE DYNAMICS OF THE ACUTE PERIOD OF IS (HIGHLY SENSITIVE METHOD)**

Groups of examined persons	Time from onset of stroke		
	1 <sup>st</sup> day	7 <sup>th</sup> day	14 <sup>th</sup> day
	CRP concentration (mg/L)		
I (n = 59)	12.34**	7.65*	5.11*
II (n = 27)	28.75***	15.11*	10.89**
III (n = 56)	0.65	–	–
RI	0.00-1.00		

Note. As for Table 1. RI, abbreviation for the reference interval.

**TABLE 3. CORRELATIONS BETWEEN THE CONCENTRATION OF THE KEY AUTOPHAGY BIOMARKER LC3 (ng/L) AND THE LEVEL OF INDICATORS OF APOPTOSIS AND INFLAMMATION IN THE DYNAMICS OF THE ACUTE PERIOD OF IS IN PATIENTS WITH DIFFERENT SEVERITY OF THE DISEASE**

Index	Spearman's coefficient (r), p < 0.05					
	Groups of examined patients					
	I	II	I	II	I	II
	1 <sup>st</sup> day		7 <sup>th</sup> day		14 <sup>th</sup> day	
p53, U/mL	0.67	0.74	0.51	0.63	0.39	0.31
Bcl-2, ng/mL	-0.59	-0.68	0.31	0.45	0.58	0.69
TNF $\alpha$ , pg/mL	0.69	0.73	0.42	0.34	0.44	0.36
IL-1 $\beta$ , pg/mL	0.79	0.83	0.31	0.23	-0.39	-0.45
IL-8, pg/mL	0.44	0.56	0.35	0.29	0.36	0.26
IL-18, pg/mL	0.71	0.79	0.33	0.21	-0.33	-0.53
CRP, mg/L	0.65	0.81	0.61	0.74	0.59	0.76

Note. I group, moderate course of the disease (n = 59); II group, severe course of the disease (n = 27). The revealed correlations between the concentration of the LC3 biomarker and the studied parameters are indicated in the table with a lowercase letter "r" (Spearman's coefficient).

p < 0.05), and IL-1 $\beta$  (r = 0.83; p < 0.05), respectively. At the same time, there is a pronounced inverse correlation between the key autophagy biomarker and the apoptosis inhibitor Bcl-2 (r = -0.68; p < 0.05). These results confirm the literature data on the synergistic destructive effect of autophagy and activation apoptosis at the beginning of the acute period of IS. Of particular interest is the dynamic change in the relationship between autophagy and the level of Bcl-2, which by the end of the observation acquired a pronounced positive character (r = 0.69; p < 0.05). These results may indicate that timely prescribed therapy inhibits activation and triggers basic autophagy, which performs a neuroprotective function and has a beneficial effect on the outcome of the disease.

The revealed relationship between the autophagy biomarker LC3 and the concentration of cytokines IL-1 $\beta$  and IL-18 draws attention (Table 3). As is known, IL-1 $\beta$  is a universal mediator of post-stroke inflammation [15]. In acute IS, this proinflammatory cytokine activates almost all inflammatory processes: it polarizes immune cells according to the pro-inflammatory phenotype, causes recruitment of leukocytes from the bloodstream, stimulates excessive activation of the NLRP3-inflammasome, leading to the death of neurons and microglia by the pyroptosis mechanism [7, 13]. Experimental animal studies have shown that the use of recombinant human IL-1RA (an antagonist of the IL-1 receptor) reduces the area of brain damage in ischemic stroke and its

accompanying symptoms, and also helps to restore lost motor functions [14, 15]. Blocking of IL-1 $\beta$  and NLRP3 receptors is currently considered as a promising approach to limiting inflammation in ischemic stroke [8, 13]. The negative correlations between LC3, IL-1 $\beta$  and IL-18 (r = -0.45; p < 0.05 and r = -0.53; p < 0.05, respectively) revealed in our study indirectly confirm the literature data. that autophagy in acute IS plays the role of a negative regulator of NLRP3 inflammation [7].

## Conclusion

Thus, the obtained results indicate a clear relationship between autophagy, apoptosis, and neuroinflammation in the pathogenesis of acute IS. Considering the numerous mechanisms by which autophagy affects individual stages of postischemic neuroinflammation, it is reasonable to consider possible ways of its modulation in order to influence certain targets of the inflammatory process. Such targets can be autoreactive T lymphocytes, NLRP3-inflammasome, proteins of signaling complexes that activate NLRP3-inflammation, receptors of some pro-inflammatory cytokines.

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