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The Effect of FTY720 on Brain Lipids, Ceramide, and TNF-α in Acute Cerebral Ischemia in Rats

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Summary

Modulation of sphingosine-1-phosphate receptors exerts various neuroprotective effects under conditions of cerebral ischemia. In this study, we investigated the relationship between the lipid composition of brain tissue, lipid signaling, and the content of the proinflammatory cytokine TNFα, as well as the expression of its receptor, TNFR1.

Objective. In a rat model of acute cerebral ischemia, to evaluate the effects of FTY720 on the lipid composition of brain tissue, ceramide concentration, and the expression of key enzymes involved in its synthesis, as well as on the contents of TNF-α and its receptor TNFR1.

Materials and Methods. The study was conducted on 37 male white non-linear rats weighing 180–230 g. Acute cerebral ischemia was induced by a combined procedure involving irreversible ligation of the left common carotid artery and reversible ligation of the right common carotid artery. The animals were divided into three groups: sham-operated, rats with acute cerebral ischemia, and rats with ischemia following prior administration of FTY720 (fingolimod). On the third day of observation, neurological deficits in surviving animals were assessed using the Garcia scale. The sphingolipid and phospholipid composition of brain tissue was examined using thin-layer chromatography. Ceramide concentration, the expression of enzymes involved in its biosynthetic, TNFα, and TNFR1 concentrations were evaluated using immunofluorescent microscopy.

Results. Pretreatment with fingolimod was associated with better survival rates: 31% in the FTY720 group vs. 20% in the Ischemia group ($p=0.043$). Functional impairments on the Garcia scale were significantly less severe in the FTY720 group than in the Ischemia group: 14 [13.5; 15] vs. 11 [10; 12.5] scores (*Me* [*Q1*; *Q3*]), $p<0.01$). FTY720 group also demonstrated a decrease in ceramide concentration in the brain tissue compared to the Ischemia group ($p=0.0005$), along with downregulated expression of aSMase ($p=0.0012$), nSMase ($p=0.0003$) enzymes involved in its synthesis, of SPT ($p=0.0002$), and CerS ($p=0.0001$), a decrease in pro-inflammatory cytokine TNFα ($p=0.0003$) concentration and normalized expression of its receptor TNFR1.

Conclusion. Preservation of phospholipid composition and reduction in the excessive production of ceramide and pro-inflammatory cytokines in the brain tissue are associated with less severe neurological deficits and improved survival rates in rats during the acute phase of cerebral ischemia.

Keywords: FTY720; ceramide; TNFα; TNFR1; cerebral ischemia; phospholipids; sphingolipid signaling

Conflict of interest. The authors declare no conflict of interest.

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Introduction

Neuronal inflammation in acute cerebral ischemia develops within minutes of the onset of injury and is characterized by activation of resident immune cells (astrocytes and microglia), infiltration of brain tissue by peripheral immune cells, release of pro-inflammatory cytokines, chemokines, and reactive oxygen species, and by migration of effector T cells [1].

There is evidence regarding the role of the sphingosine-1-phosphate (S1P) pathway in neuroprotection during ischemia. S1P synthesis involves sphingosine kinases SphK1 and SphK2, and the non-selective S1P receptor modulator fingolimod (FTY720) exhibits neuroprotective activity by influencing neuronal inflammation [2]. The S1P receptor family includes five subtypes (S1P1–S1P5) with different effects. S1P1 is the most highly expressed; its activation increases intracellular calcium concentration, triggers anti-apoptotic mechanisms, cytoskeletal reorganization, and cell migration [3]. FTY720 has high affinity for S1P1, and studies in rats show that its use reduces infarct volume, the severity of neurological deficits, and neuronal apoptosis [4]. However, some data suggest that the neuroprotective properties of FTY720 may be associated with the inhibition of S1P1 [5].

The pathophysiological role of S1P1 in ischemia is primarily attributed to its modulation of neuronal inflammation: it reduces the production of pro-in-

flammatory cytokines, microglial activity, and the expression of MAPK and NF-κB, while decreasing blood-brain barrier (BBB) permeability [6]. Inhibition of S1P2 also reduces infarct volume, cerebral edema, and BBB permeability, likely through its effects on matrix metalloproteinase-9 and the migration of neuronal progenitors [7]. S1P3 inhibition is accompanied by reduction in cellular infiltration and production of pro-inflammatory mediators, by synthesis of anti-inflammatory cytokines, and a decrease in microglial activation [8]. The role of S1P4 and S1P5 has been studied to a limited extent, although it is known that activation of S1P4 stimulates neutrophil migration, and inhibition of S1P5 may increase levels of pro-inflammatory cytokines [9].

Ceramide is a known biochemical precursor of S1P. Pro-inflammatory mediators, hypoxia, and oxidative stress stimulate synthesis of ceramide, which acts as a pro-apoptotic signal and regulates autophagy, the inflammatory response, and cell differentiation [10].

Three main mechanisms of FTY720-mediated neuroprotection have been described: interaction with S1P receptors on lymphocytes, leading to lymphopenia and reduced lymphocytic infiltration; interaction with S1P receptors on neurons, microglial cells, astrocytes, oligodendrocytes, and endothelial cells; and a receptor-independent mechanism in which FTY720 binds to intracellular proteins, modulating signaling pathways and epigenetic transcription [11].

Objective of the study: to evaluate the effect of FTY720 on the survival and neurological deficits in rats, and assess the phospholipid and sphingolipid composition of brain tissue, concentration of ceramide and expression of enzymes involved in its synthesis in the catabolic and de novo pathways, as well as levels of TNFα and its receptor TNFR1 in a model of acute cerebral ischemia.

Materials and Methods

The study was conducted on 37 male white nonlinear rats weighing 180–230 g. All procedures involving the animals were approved by the local ethics committee of Izhevsk State Medical University (Protocol No. 736/1 dated May 11, 2022). The study was conducted in accordance with international standards (Recommendation No. 33 of the Board of the Eurasian Economic Commission dated November 14, 2023, «On the Guidelines for the Care and Use of Laboratory (Experimental) Animals in Preclinical (Nonclinical) Research»), the ARRIVE 2.0 guidelines, the Guide for the Care and Use of Laboratory Animals (NRC, 2011), and Directive 2010/63/EU, in compliance with the 3R principles. Adequate anesthesia was used, and humane endpoints were determined.

Modeling of acute cerebral ischemia. The animals were fasted the day before surgery. The procedure was performed under strict aseptic conditions. Anesthesia was induced with a combination of zolazepam and tiletamine (Zoletil 100, Virbac, France) at a dose of 30 mg/kg body weight. The surgical site was shaved and treated with a 5% alcohol solution of iodine. A midline incision was made on the ventral surface of the neck, after which the carotid arteries were dissected layer by layer from the surrounding soft tissues and neural structures. Loose ligatures were applied to both carotid arteries. The left carotid artery was ligated, and a bulldog clamp was applied to the right carotid artery for 30 minutes. Upon completion of the procedure, the wound was sutured layer by layer after being treated with a 0.05% aqueous solution of chlorhexidine digluconate [12].

The animals were randomly assigned to three groups. The first group (Ischemia) consisted of rats in which acute cerebral ischemia was induced. Of the 85 animals that underwent surgery in this group, 17 survived, corresponding to a 20% survival rate. The second group (FTY720) consisted of animals that were administered fingolimod (FTY720) at a dose of 1 mg/kg body weight prior to ischemia induction. Of the 35 animals in the second group, 11 survived (31% survival rate). The third group consisted of sham-operated animals (SO) that underwent all stages of the surgical procedure without carotid artery ligation ($n=9$).

Fingolimod was administered at a dose of 1 mg/kg body weight immediately prior to the induction of acute ischemia. The choice of dose and timing of administration was based on data from systematic reviews demonstrating a reduction in the volume of ischemic lesion, the severity of cerebral edema, and the degree of behavioral deficits when using this dosing regimen [13].

On day 3, survival and neurological status were assessed using the Garcia scale [14], after which the animals were euthanized under general anesthesia.

Macroscopic visualization of the ischemic lesion. The animal was anesthetized with a combination of zolazepam and tiletamine. Once the desired depth of anesthesia was achieved, access to the heart was established, and a cannula was inserted into the left ventricular cavity. Systemic perfusion was performed with a 4% solution of 2,3,5-triphenyltetrazolium chloride prepared in phosphate-buffered saline (PBS; composition, mM: 3.2 NaH_2PO_4, 0.5 K_2HPO_4, 1.3 KCl, 135 NaCl; pH 7.4). After decapitation, the brain was removed and fixed in a 10% formalin solution, and after 24 hours, 1-mm-thick coronal sections were prepared.

Identification of the phospholipid and sphingolipid composition of brain tissue. Brain tissue was homogenized, after which the homogenate was incubated in a 2:1 chloroform/methanol mixture using the Folch method to ensure complete lipid extraction. Lipid fractions were separated by thin-layer chromatography in chambers containing the

appropriate solvent systems. A chloroform/methanol/glacial acetic acid/distilled water system (60:50:1:4) was used to isolate phospholipid fractions, and a butanol/glacial acetic acid/distilled water system (3:1:1) was used to separate sphingolipids. Samples were applied to Merck UV-labeled chromatographic plates (TLC Silica gel 60 F254, 20×20 cm). Lipids were visualized in iodine vapor, and quantitative analysis was performed using a «Sorbfil» densitometer [16].

Immunofluorescence microscopy study. Under general anesthesia with zolazepam/tiletamine, transcardial systemic perfusion was performed using phosphate-buffered saline, followed by a 4% paraformaldehyde fixative solution prepared on a PBS base. The brain was removed, placed in 4% paraformaldehyde for 2 hours for additional fixation, then kept in a 30% sucrose solution for 24 hours and frozen using dry ice.

In the next step, 14-micrometer-thick coronal sections of the brain were prepared using an HM525 NX Cryostat (Thermo Fisher Scientific, USA). The resulting sections were mounted on adhesive-coated slides (Thermo Fisher Scientific, Waltham, MA, USA).

The sections were incubated in a permeabilization solution (1% Triton X-100 in PBS containing 5% bovine serum albumin) for 20 minutes. They were then incubated in a solution of primary antibodies: anti-TNFR1, anti-TNFα, anti-aSMase, anti-nSMase (monoclonal rabbit IgG, 1:200 dilution, Affinity Biosciences), and anti-Cer (monoclonal mouse IgG, 1:200, ALX-804-196-T050, Enzo Life Sciences, USA), anti-SPT2, and anti-CerS1 (rabbit IgG, 1:200, Abcam) in the presence of 5% bovine serum albumin. Incubation was performed overnight. After washing in PBS, the sections were incubated in secondary antibody solutions: anti-rabbit Alexa Fluor 488 (goat IgG, 1:300, Abcam) and anti-mouse Alexa Fluor 647 (goat IgG, 1:300, Abcam) for 1 hour at room temperature under light-protected conditions [17].

Immunofluorescence in brain sections was analyzed using the DS-Fi3 EF-2E attachment for the Nikon Eclipse E200 microscope, NIS-Elements D software (Nikon Instruments Inc., USA), and ImageJ. The intensity of expression was calculated based on the fluorescence intensity over a standard area of 0.1 mm².

Statistical data were analyzed using IBM SPSS Statistics 23 and reported as the median and interquartile range (*Me* [*Q1*; *Q3*]). The normality of the distribution was assessed using the Shapiro–Wilk test. Due to deviation of indicators distribution from the normal, nonparametric tests were used for intergroup comparisons. For intergroup comparisons of three independent groups, the Kruskal–Wallis *H*-test was used. When statistically significant differences were identified, post-hoc analysis was performed via pairwise comparisons of groups using the Mann–Whitney *U*-test with Bonferroni correction for multiple comparisons. Fisher's exact test was used to analyze differences in frequency characteristics (proportions of surviving animals) between the groups. Differences were considered statistically significant at a p-level < 0.05. The results were presented graphically as box-and-whisker plots with statistical significance levels indicated.

Results

Animals that received FTY720 prior to the induction of acute cerebral ischemia showed a statistically significantly higher survival rate compared to animals in the Ischemia group: 31% and 20%, respectively, $p = 0.043$.

Pretreatment with FTY720 also contributed to a reduction in neurological deficits. Functional impairments on the Garcia scale were significantly less obvious in the FTY720 group with the median of 14 scores (*Me* [*Q1*; *Q3*] = 14 [13.5; 15]) vs. 11 scores (*Me* [*Q1*; *Q3*] = 11 [10; 12.5]) in the Ischemia group. A comparison of the three groups using the Kruskal–Wallis test revealed statistically significant

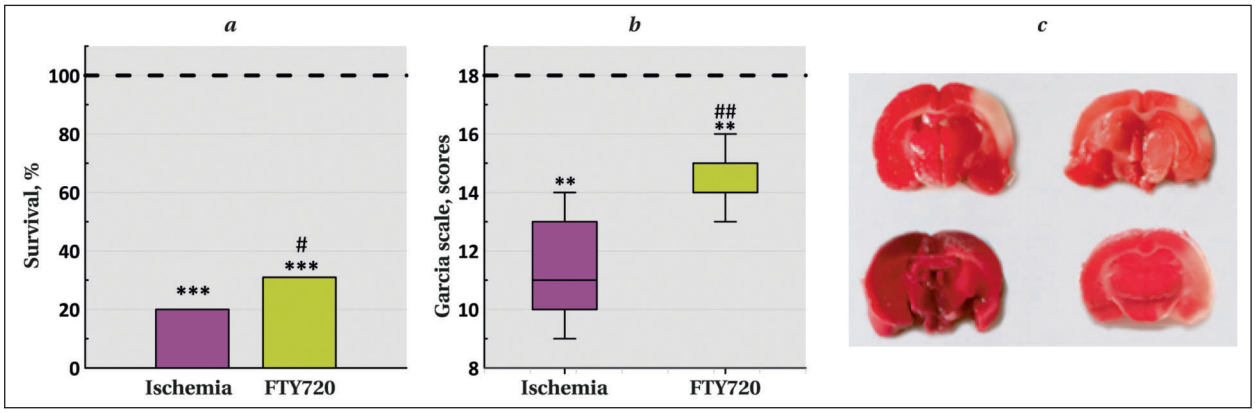

Fig. 1. Survival (*a*), functional deficit (*b*), and visualization of the ischemic lesion using 2,3,5-triphenyltetrazolium chloride staining (*c*) in a model of acute cerebral ischemia and ischemia after pretreatment with FTY720.
Note. The dotted line represents the SO group. ** — $p < 0.01$; *** — $p < 0.001$ — compared to SO group animals; # — $p < 0.05$; ## — $p < 0.01$ — compared to the Ischemia group animals.

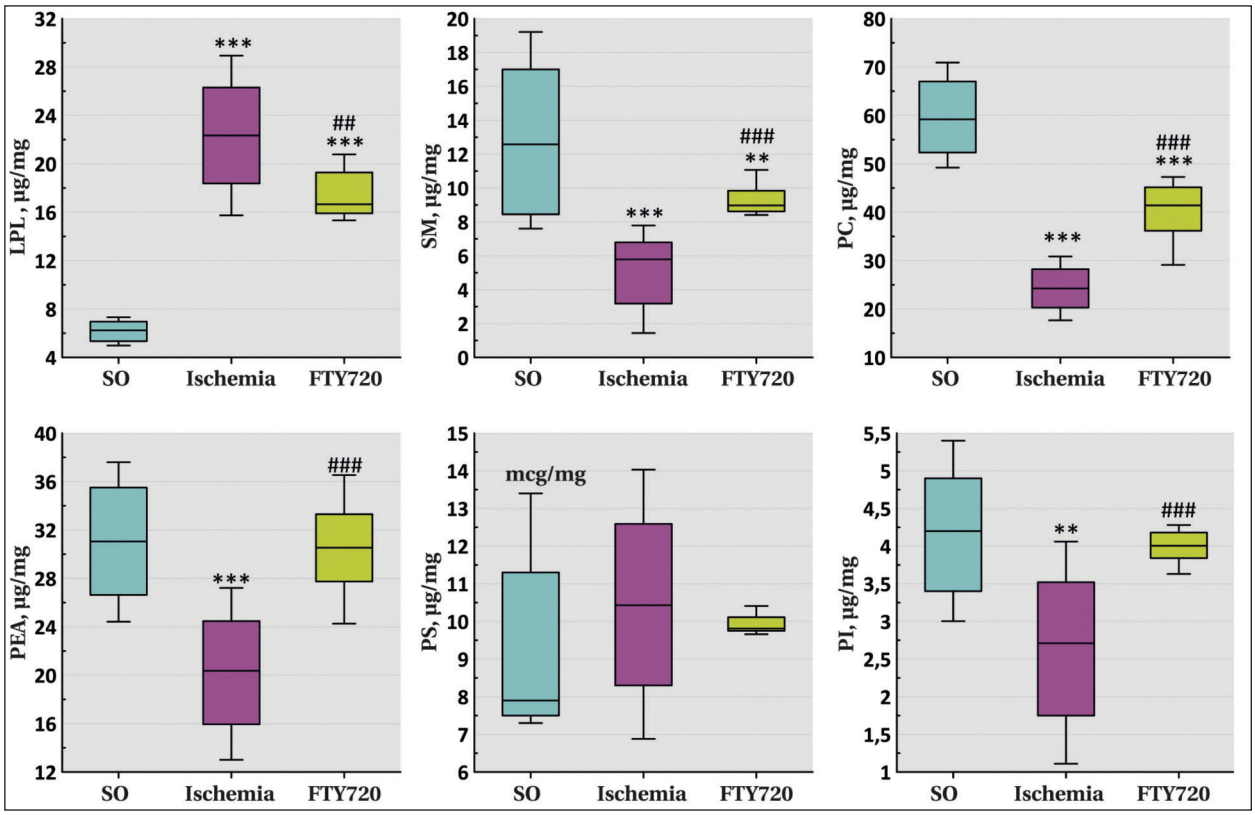

Fig. 2. Concentrations of major phospholipid fractions in the study groups.
Note: LPL — lysophospholipids; SM — sphingomyelin; PC — phosphatidylcholine; PEA — phosphatidylethanolamine; PS — phosphatidylserine; PI — phosphatidylinositol. ** — $p<0.01$; *** — $p<0.001$ — compared to SO animals; ## — $p<0.01$; ### — $p<0.001$ — compared to the group of animals that did not receive FTY720.

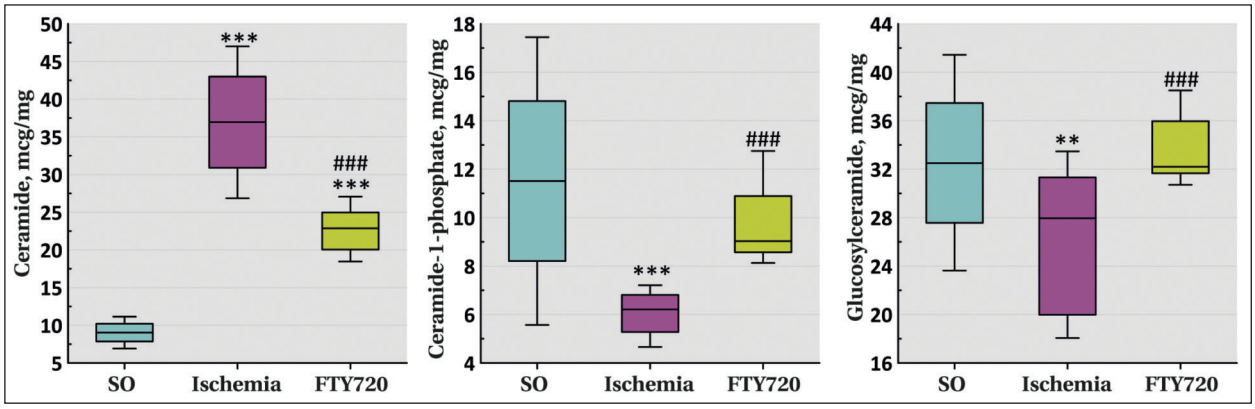

Fig. 3. Concentrations of ceramide and its metabolites in the study groups.
Note. ** — $p<0.01$; *** — $p<0.001$ — compared with SO group; ### — $p<0.001$ — compared with Ischemia group.

differences ($p=0.000008$). The subsequent pairwise comparison of the groups using the Mann–Whitney test with Bonferroni correction revealed higher Garcia scale scores in the FTY720 group compared to the Ischemia group ($p=0.0019$). No neurological deficits were found in the SO group.

Fig. 1, *a* shows survival rates, while Fig. 1, *b* presents data on neurological deficits according to the Garcia scale. Fig. 1, *c* shows coronal sections of the forebrain of rats stained with 2,3,5-triphenyltetrazolium chloride (TTC). The bottom row on the left shows a brain section from a sham-operated (SO) animal: the tissue is uniformly stained red, indicating its viability and preservation of dehydrogenase activity. The remaining sections (top row and bottom row on the right) show white, unstained areas corresponding to the zone of ischemic damage, where enzymatic activity has been lost.

Fig. 2 shows changes in the phospholipid composition of brain tissue during acute cerebral ischemia. Three-day ischemia resulted in a significant decrease in concentrations of the main phospholipid

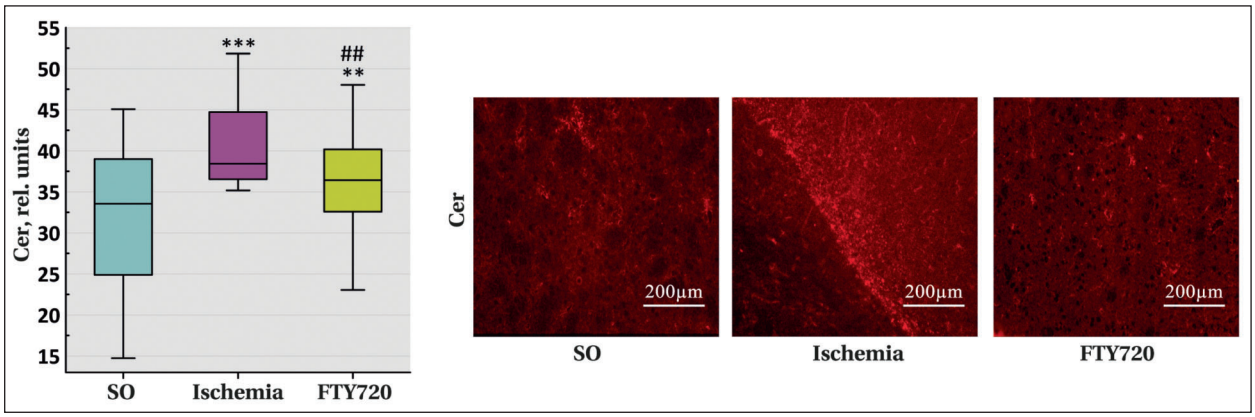

Fig. 4. Ceramide expression in the study groups.
Note. ** — $p<0,01$; *** — $p<0,001$ — по сравнению с ЛО животными; ## — $p<0,01$ — по сравнению с группой животных с ишемией.

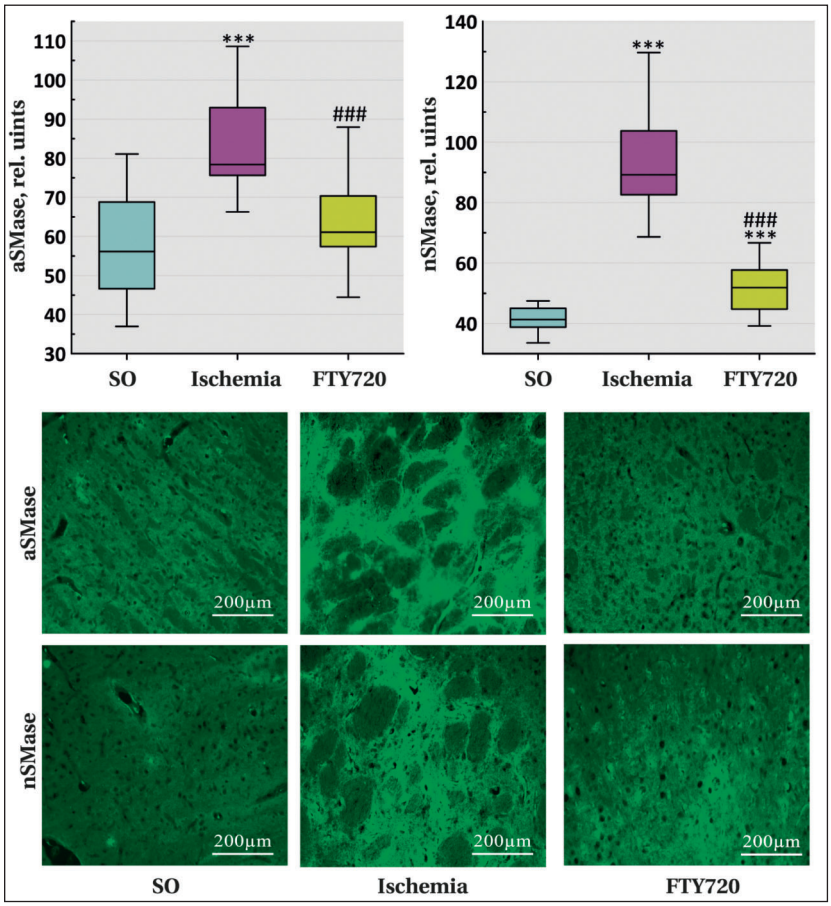

Fig. 5. Expression of aSMase and nSMase in the studied groups.
Note. *** — $p<0.001$ — compared to SO animals; ### — $p<0.001$ — compared to the group of animals with ischemia.

fractions: phosphatidylethanolamine (PEA) by 34.9% ($p=0.0002$), phosphatidylcholine (PC) — by 59.3% ($p=0.0000012$). Concentration of sphingomyelin (SM) dropped by 60.2% ($p=0.0003$), and of phosphatidylinositol (PI) — by 37% ($p=0.00012$). There was a concomitant accumulation of lysophospholipids (LPL) with 3.79-fold increase of LPL concentration ($p=0.0002$).

In animals pretreated with FTY720, SM concentration was 1.9-fold higher ($p=0.0004$), and concentration of PC — 1.7-fold higher ($p=0.0001$) compared to the Ischemia group; however, these values did not reach the levels observed in the SO group. PEA and PI concentrations in the FTY720 group increased 1.5-fold ($p=0.0002$ and $p=0.001$, respectively) and were comparable to those in the SO group.

In the Ischemia group ceramide concentration in brain tissue increased 4.2-fold ($p=0.0003$) compared to the SO group, while ceramide-1-phosphate concentration decreased by 47.4% ($p=0.0008$), and that of glucosylceramide decreased by 14.1% ($p=0.009$).

Pretreatment with FTY720 mitigated these effects, and ceramide concentration was 1.6 times lower in the FTY720 group vs the Ischemia group ($p=0.0005$), while concentrations of ceramide-1-phosphate and glucosylceramide were comparable to those in the SO group. Fig. 3 presents obtained data on concentrations of ceramide and its metabolites.

Immunofluorescence microscopy findings confirmed that pretreatment with FTY720 downregulated ceramide synthesis in the ischemic brain.

Ceramide concentration was 1.3 times higher in Ischemia group compared to the SO group ($p=0.0006$), while in rats pretreated with FTY720, it was 1.3 times lower than in the Ischemia group ($p=0.002$), although remained significantly higher

than in the SO group animals. Data on ceramide content in brain tissue of all studied groups is presented in Fig. 3, 4.

In the sphingomyelinase pathway, ceramide is formed by hydrolysis reaction, catalyzed by acidic and neutral sphingomyelinases aSMase and nSMase. In acute cerebral ischemia, the expression of aSMase and nSMase significantly increased: by 1.4 times (p=0.0001) and by 1.8 times, respectively (p=0.0004). Pretreatment with FTY720 resulted in a decrease of aSMase expression to a level comparable with SO group, and 1,2-fold decrease in nSMase, although, not reaching the values in the SO group. Sphingomyelinases aSMase and nSMase expression in all studied groups is shown in Fig. 5.

Serine palmitoyltransferase (SPT) and ceramide synthase (CerS) enzymes expression in acute cerebral ischemia was evaluated to characterize «de novo» synthesis of ceramide. SPT expression in the Ischemia group was increased by 1.5 times (p=0.0001), and CerS expression — by 1.9 times (p=0.0001) compared to the SO group. Pretreatment with FTY720 prevented an increase in the expression of both enzymes, bringing it to values comparable to those in the SO animals. The results are presented in Fig. 6.

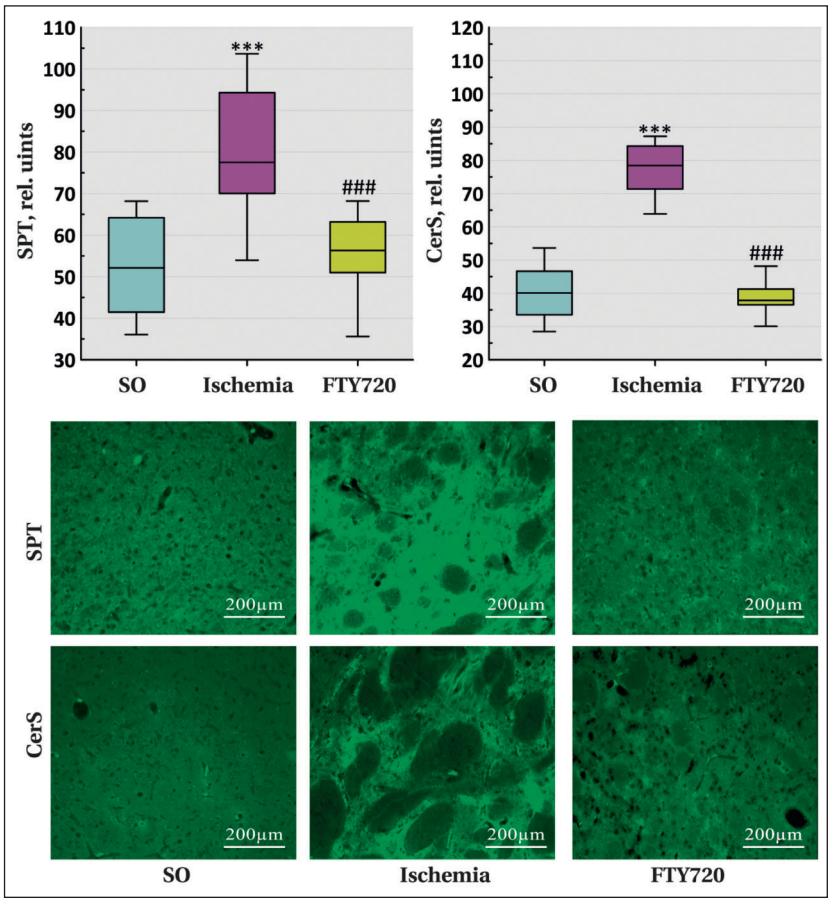

Fig. 6. Expression of SPT and CerS in the studied groups.
Note. *** — $p<0.001$ — compared to SO animals; ### — $p<0.001$ — compared to the group of animals with ischemia.

Evaluation of pro-inflammatory TNFα cytokine and its receptor TNFR1 revealed 1.6-fold increase in TNFα concentration (p=0.0001), and 1.9-fold increased expression of TNFR1 (p=0.0001) in the Ischemia group compared to the SO group. Pretreatment with FTY720 led to a 1.3-fold decrease in TNFα production compared to the Ischemia group (p=0.0003), although its concentration was higher than in controls. TNFR1 density in the FTY720 group also decreased to the levels close to the SO group. The effect of FTY720 on TNFα and TNFR1 expression is shown in Fig. 7.

Discussion

Membrane lipids maintain cellular structural and functional integrity. Degradation of phospholipids contributes to neuronal death in ischemic damage. In ischemia, enhanced hydrolysis of phospholipids is caused by increased activity of phospholipase A_2, which is induced by reactive oxygen species and pro-inflammatory cytokines, primarily IL-1, TNFα, and IFNγ [18].

Further in this continuum oxylipins and lysophospholipids with potent biological signaling functions are generated following activation of cyclooxygenase, lipoxygenase, and cytochrome-P450 monooxygenase. Established effects of lysophosphatidic acid and sphingosine-1-phosphate include stimulation of neuronal inflammation and leukocyte infiltration. Production of these compounds is downregulated by FTY720, mainly due to a decrease in the inflammatory response, a reduction in T-cell and neutrophil infiltration, and a decrease in microglia activation [19].

The experiment of G. Payne et al. (2007) on a mast cells culture demonstrated FTY720 potential to inhibit phospholipase A_2 activity, thereby reducing secretion of prostaglandins and thromboxanes, which led to a decrease in inflammation and hydrolysis of cell membranes [20].

In our experiments a decrease in SPT expression was documented. Published data does not support a direct inhibitory effect of FTY720 on this enzyme, contrary to its natural precursor, myriocin, which is a known SPT inhibitor [21]. The myocardial is-

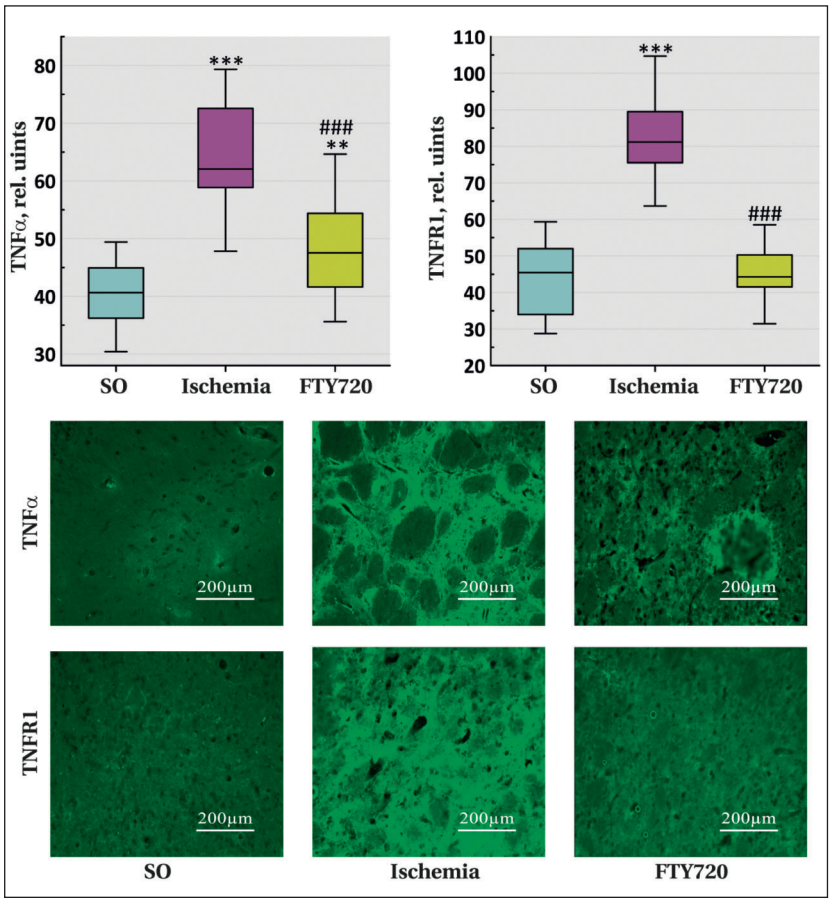

Fig 7. Dynamics of TNFa and TNFR-1 expression in the studied groups.
Note. ** — $p<0.01$; *** — $p<0.001$ — compared to SO animals; ### — $p<0.001$ — compared to the group of animals with ischemia.

chemia model described by Reforgiato et al. (2016) demonstrated, that SPT activity was associated with increased levels of IL-6, IL-1β, TNF-α, and increased production of reactive oxygen species. This suggests that inhibition of inflammatory responses by FTY720 may decrease SPT stimulation [22].

According to Henryk Jęśko et al. (2019) data obtained on the Alzheimer's disease model, FTY720 is able to suppress the expression of ceramide synthase mRNA [23]. Of interest is the lowered concentration of sphinganine, a precursor of sphinganine-1-phosphate, in cerebral ischemia. Established anti-ischemic effects of alkaloid nuciferin are accompanied by increasing levels of sphinganine-1-phosphate, while suppression of CerS activity leads to accumulation of sphinganine [24].

It has been reported that FTY720 reduces aSMase activity by causing proteolytic degradation of the enzyme complex, via a mechanism similar to that of tricyclic antidepressants [25]. In our study, FTY720 also reduced nSMase expression. Since the direct effect of FTY720 on this enzyme has not been previously described, most likely this reduction is associated with a decrease in inflammatory infiltration and downregulated production of pro-inflammatory cytokines.

Conclusion

Acute cerebral ischemia in rats is associated with significant disruptions in lipid homeostasis, characterized by degradation of membrane phospholipids, accumulation of lysophospholipids, and activation of both the sphingomyelinase and «*de novo*» pathways of ceramide synthesis. These changes are accompanied by an increase in pro-inflammatory cytokine TNFα levels and expression of TNFR1, i. e., activation of the neuro-inflammatory response, and in entirety lead to development of severe neurological deficits and increased mortality.

Pretreatment with FTY720 produced a modifying effect on these pathological processes. This resulted in partial optimization of brain tissue phospholipid composition, a decrease in sphingomyelin degradation, and a reduction in ceramide accumulation. The latter came off inhibited expression of enzymes involved in catabolic and synthetic pathways of ceramide biosynthesis, such as acid and neutral sphingomyelinases, serine palmitoyltransferase, and ceramide synthase.

Along with its effect on lipid metabolism, FTY720 reduces TNFα production and normalizes TNFR1 expression in ischemic brain tissue, downregulating the pro-inflammatory response, thereby mediating a reduction in neurological deficits and an increase in animal survival during the acute phase of ischemic injury.

The data obtained indicate a significant role of lipid signaling disruptions, particularly ceramide-dependent mechanisms, in the pathogenesis of neuronal inflammation in acute cerebral ischemia. Modulation of sphingolipid metabolism and TNFα/TNFR1 signaling pathways by FTY720 can be considered as one of the key directions of neuroprotective strategy in ischemic brain damage.

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