ABSTRACT

Background and Purpose: Ischemic stroke remains one of the leading causes of death in the population. In addition, mitochondrial dysfunction is an essential part of the pathogenesis of cerebral ischemia and is a promising pharmacotherapeutic target.

Experimental Approach: the work was performed on male *Wistar* rats, which were simulated cerebral ischemia by irreversible occlusion of the middle cerebral artery. 4-hydroxy-3,5-di-tret-butyl cinnamic acid (25 mg/kg, 50 mg/kg and 100 mg/kg) was injected intraperitoneally for 3 days after ischemia (daily). On the 4th day of the experiment, the changes of rat's cognitive functions in the Morris water maze test, cellular respiration processes, the activity of the mitochondrial respiratory chain complexes and citrate synthase activity, the intensity of oxidative stress and apoptosis reactions were assessed.

Key Results: it was found that the administration of 4-hydroxy-3,5-di-tret-butyl cinnamic acid at doses of 25 mg/kg and 50 mg/kg practically equivalently promotes the restoration of aerobic metabolism reactions and the activity of the mitochondrial respiratory chain complexes, decrease of the intensity of oxidative stress reactions and apoptosis, as well as an increase in the activity of citrate synthase. As a result, the restoration of mitochondrial function in the hippocampal cells contributed to the restoration of the animal's spatial memory.

Conclusion and Implications: a study showed that 4-hydroxy-3,5-di-tret-butyl cinnamic acid at doses of 25 mg/kg and 50 mg/kg has a neuroprotective effect on hippocampal neurons under conditions of permanent occlusion of the middle cerebral artery, realized by restoration of mitochondrial function.

Key words: mitochondrial dysfunction, cerebral ischemia, neuroprotection, cinnamic acid derivatives, apoptosis, oxidative stress.

ABBREVIATIONS

DALYs – disability-adjusted life days

PSD – post-stroke dementia

rt-PA – recombinant plasminogen activator

ATACL - 4- hydroxy-3,5-di-tret-butyl cinnamic acid

PBS – phosphate buffer solution

EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

BSA – bovine serum albumin

OCR – oxygen consumption rate

FCCP - carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

TMPD - N, N, N ', N' tetramethyl-1,4-phenylenediamine

mPTP - mitochondrial permeability transition pore

ROS – reactive oxygen species

TBARS - thiobarbituric acid reactive substances

SOD - superoxidedismutase

GPx - glutathioneperoxidase

ATP – adenosinetriphosphate

AIF – apoptosis-induced factor

SO- sham-operated rats

NC- negative control rats

1. INTRODUCTION

Ischemic stroke is one of the leading causes of death and primary disability in the population (Kimura, 2020). Global statistics show that despite the progress achieved in the prevention, diagnosis and treatment of ischemic stroke, this pathological condition is still a rather serious problem in modern medicine. More than 80 million cases of ischemic stroke are reported annually, and more than a 25 million are fatal in the acute phase of the disease (Guzik & Bushnell, 2017). The percentage of primary disability due to stroke also remains high - more than 40% of stroke survivors are unable to return to work and loss social adaptation skills, which inevitably leads to a decreasing of the DALYs (Virani, et al., 2020). According to a study by *Johnson, et al., 2016* published in *The Lancet Neurology*, 116.4 million DALYs were lost due to ischemic stroke in 2016 alone (Johnson, et al., 2016). In many ways, the high level of disability in stroke patients mediated by damage of the hippocampus and the development of cognitive impairment, combined by the term that called post-stroke dementia (PSD). *Renjen, et al, 2015*, was shown that ischemic stroke increases the risk of developing dementia by 4-12 times, and PSD *per se* is observed in a fairly wide range - from 6% to 55% of cases and, which is very important, PSD increases the probability re-stroke almost twice as much (Renjen, et al., 2015). In this regard, numerous attempts have been made to reduce the risk of developing PSD by developing and implementing appropriate pharmacotherapeutic strategies in clinical practice. It was found that the use of recombinant plasminogen activator (rt-PA) preparations in patients with mild ischemic stroke reduced the probability of cognitive impairment developing (Rosenbaum, et al., 2019). However, the administration of rt-PA drugs, the "gold standard" of the ischemic stroke treatment, has a significant number of limitations to its use with strict patient selection, a small "therapeutic window" and the risk of developing hemorrhagic complications (Stroke Study Group, 1995). Alternative approaches to PSD correction were also developed. *Zhang et al, 2019* demonstrated that an appropriate rehabilitation program, without active pharmacological intervention, could effectively reduce post-stroke cognitive impairment (Zhang, et al., 2019). At the same time, neuroprotection may be one of the promising areas of PSD treatment (Mijajlović, et al., 2017). Recently, there has been a growing scientific interest in the targeted search of neuroprotective agents as adjuvant therapy of ischemic stroke, which can reduce the risks associated with the use of tr-PA drugs, as well as increase the effectiveness of thrombolytic therapy (Savitz, et.al., 2017).

Among potential neuroprotective agents, antioxidant agents stand out, which, through a number of mechanisms, can significantly reduce the degree of brain neurons damage during ischemia (Angeloni & Vauzour, 2019). The multifunctional mechanism of action of antioxidants includes the effect on changes in mitochondrial function, which in turn can contribute to both the enhancement of the neuroprotective effect of antioxidants and an independent aspect of neuroprotection, which was shown by the example of KoQ_{10} (Yang, et al., 2016). In previous studies, it was found that the administration of 4-hydroxy-3,5-di-tert-butyl cinnamic acid to ischemic animals contributed to a decrease of the brain necrosis area, restoration of cerebral hemodynamics and a decrease in blood thrombogenicity (Voronkov & Pozdnyakov, 2018). However, despite the potentially high neuroprotective potential of this compound, the mechanism

by which the neuroprotective effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid is realized has not been established. Also, the effect of this compound on the change in post-stroke syndrome, including the development of cognitive impairments, was not evaluated, which was the aim of this study - to assess the effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on the activity of mitochondria in the hippocampus of rats under conditions of cerebral ischemia.

2. METHODS

2.1 Animals

The work was performed on 120 male *Wistar* rats weighing 200-220 grams, 3 months old. The animals were obtained from the «Rappolovo» laboratory animal nursery (Russia, Leningrad region) and during the experiment were kept under controlled conditions in the laboratory of living systems of the Pyatigorsk Medical and Pharmaceutical Institute. Conditions of detention: ambient temperature - 22 ± 2^0C, relative humidity - 60 ± 5%, with a 12-hour change in the daily cycle. The rats were kept by 5 animals in macrolon cages on a granular hardwood bedding with free access to water and food. The study was carried out in accordance with the recommendations of Directive 2010/63 / EU of the European Parliament and of the council on the protection of animals used for scientific purposes, September 22, 2010 and ARRIVE guidelines (Percie du Sert, et al., 2020). The work was approved by the local ethics committee (protocol No. 20 dated 05.16.2020)

2.2. Chemicals

The test compound, 4-hydroxy-3,5-di-tret-butyl cinnamic acid (laboratory code ATACL), was obtained at the Department of Organic Chemistry of the Pyatigorsk Medical and Pharmaceutical Institute. The structure was confirmed by NMR spectroscopy. The reference drug was ethylmethylhydroxypyridine succinate (Mexidol®, Pharmasoft, Russia). The reagents used in this work (unless otherwise indicated) were obtained from Sigma-Aldrich (Darmstadt, Germany).

2.3. Study design

When setting up the experiment, the following experimental groups of animals were formed: SO - sham-operated rats (all consecutive surgical procedures without arterial coagulation were applied to this group); NC - negative control group (not receiving pharmacological support); a group of rats that received the test compound at doses of 25 mg/ kg; 50 mg/kg and 100 mg/kg; a group of animals that received a reference drug at a dose of 100 mg/kg (Voronkov & Pozdnyakov, 2018). The number of animals in each group was equal to 20 individuals. The study design provided for the therapeutic administration of the test compound and the reference drug. The administration was carried out immediately after awakening the animals and then once a day for three days, intraperitoneally (the test compound was dissolved in water for injection). On the 4^{th} day, the experiment of the spatial memory of rats was assessed in the Morris water maze test. After that, rats were decapitated, and the brain was removed. Further, the hippocampus was isolated, which was used as an analyzed biomaterial. In 10 animals from the group, the parameters characterizing the change of the cellular respiration processes were determined, and in the 10 remaining animals, the change in the pro / antioxidant balance and the activity of apoptosis were assessed. The study design is shown on Figure 1.

2.4 Cerebral ischemia model

Cerebral ischemia was modeled by the method of irreversible middle cerebral artery occlusion. The course of the operation: in anesthetized animals (chloral hydrate (Acros Organics) 350 mg / kg, intraperitoneally), on the depilated area below and to the right of the eye, the skin was dissected and the muscles were moved apart. Then the process of the zygomatic bone was removed and the skull was exposed. Next, a trepanation hole above the intersection of the middle cerebral artery and the olfactory tract was made, the dura mater was removed, and the artery was coagulated by electric coagulator. The suture was treated by an antiseptic solution - benzyldimethyl [3-myristoilamine) propyl] ammonium chloride monohydrate); 0.01% solution, INFAMED corp.). Animals were placed under a heating lamp until awakening (Tamura, et al., 1981).

2.5 Morris water maze test

The Morris water maze device is a water arena with a diameter of 150 cm with a wall height of 60 cm and a movable platform with a diameter of 10 cm. During the study, the arena was filled by water to the 50 cm level, after which the water was tinted by blue dye. Before cerebral ischemia modeling, rats were trained in the testing procedure: within 2 minutes the animals were allowed to find a platform, provided no execution of the task, rats were moved to the platform for 10 sec. after that, the testing was repeated. The training lasted for 5 days. After reproducing of the ischemia in similar conditions, the test was repeated. The latency period of reaching the platform in seconds, the distance that the animal spent on finding the platform in meters and average speed was recorded. The experiment was recorded and processed using the «Minotaur software» (Neurobiotics, Russia) with infrared monitoring of activity (Vorhees & Williams, 2006).

2.6 Biomaterial sampling. Mitochondrial isolation

In this work, the rat hippocampus was used as a biomaterial. The rats were decapitated and the brain was removed, the hippocampus was isolated. All procedures were performed at 4^0C. In 10 animals from group the hippocampus was homogenized in PBS with a pH of 7.4 (a ratio of 1: 7) in a Potter mechanical homogenizer and centrifuged in mode 10000g for 5 min. The resulting supernatant was used in an ELISA study and for antioxydant activity determination. In the remaining 10 rats, the hippocampus was homogenized in an isolation medium (1 mmol EGTA + 215 mmol mannitol + 75 mmol sucrose + 0.1% BSA solution + 20 mmol HEPES, with a pH of 7.2). For mitochondrial isolation, the resulting homogenate was centrifuged at 1100g for 2 minutes. The resulting supernatant in the amount of 700 µl was transferred into Eppendorf tubes and mixed with 75 µl of 10% percol solution and centrifuged at 18000g for 10 minutes. The precipitate was resuspended in 1 ml of the isolation medium and centrifuged for 5 minutes at 10,000g. The resulting fraction was removed for respirometric analysis and measurement of latent time of mitochondrial permeability transition pore opening (Voronkov, et al., 2019).

2.7 Respirometric analysis

Respirometric analysis was carried out on a laboratory respirometer AKPM 1-01L (Alfa Bassens, Russia) with the determination of the change in oxygen consumption (OCR) against the background of the injection of mitochondrial respiration uncouplers into the analyzed medium. As uncouplers in this work, oligomycin - 1 µg/ml; FCCP -1 µmol/ml; rotenone -1 µmol/ml; sodium azide –20 mmol/ml was used. Based on the obtained values of the change in oxygen consumption, the following parameters were calculated: ATP-generating capacity, maximum respiration rate, and respiratory capacity. The activity of glycolysis processes was assessed by the change of OCR in the medium against the background of the addition of an oxidation substrate - glucose at a concentration of 15 mmol/ml, as well as oligomycin and sodium azide.

The intensity of glycolysis, glycolytic capacity and glycolytic reserve were determined. The activity of the complexes of the mitochondrial respiratory chain I, II, IV, and V was studied by adding the corresponding oxidation substrates to the analyzed medium: 10 mmol/ml; malic acid - 1 mmol/ml; succinic acid - 10 mmol/ml; ascorbate - 2 mmol/ml; adenosinediphosphate - 1 mmol/ml; N, N, N ', N' tetramethyl-1,4-phenylenediamine (TMPD) - 0.5 mmol/ml as described by *Connolly et al., 2018* (Connolly, et al., 2018). The complex I activity was investigated by the difference in oxygen consumption when malate/pyruvate was used as oxidation substrates. The complex II activity was investigated by the difference in OCR when succinate was used as an oxidation substrate and adding of oligomycin to the medium. The activity of complex IV was defined by the difference in OCR after adding the mixture of rotenone/TMPD/ascorbate as an oxidation substrate. The activity of complex V was defined by the difference in OCR after the injection of rotenone and ADP to the analyze medium. The activity of complex III was assessed spectrophotometrically by increasing in the absorbance of the incubation medium with 1 M succinate solution + 0.5 M cytochrome C solution + 0.2 M KCN solution + 10 mmol rotenone solution and 10 µl of the analyzed sample at 550 nm (Spinazzi, et al ., 2012). Oxygen consumption was determined in ppm and expressed in terms of the protein concentration in the analyzed sample. Protein content was estimated using the Bradford method (Carlsson, et.al., 2011)

2.8 Measurement of latent time of mitochondrial permeability transition pore (mPTP) opening

The latent time of mPTP opening was studied using a spectrometric method based on the ability of cyclosporin A to inhibit the opening of mPTP. The incubation medium contained 0.05 ml of supernatant, 0.05 ml of 1 µmoles/ml solution of cyclosporin A and 20 µl of 200 mmoles of potassium chloride solution. The resulting mixture was adjusted to 200 µl by a HEPES solution with a pH of 7.4. Then the optical density was recorded at 540 nm for 25 min at room temperature. The latent time of mPTP opening was determined in seconds required to decrease the absorbance of the medium by 0.2 units. (Zhyliuk, et al., 2015)

2.9 Measurement of citrate synthase activity

Citrate synthase activity was determined according to the method described by *Shepherd & Garland*. The method is based on the determination of the colored degradation products of 5.5'-di-thiobis- (2-nitrobenzoic acid) in the presence of acetyl-CoA and oxaloacetate. The reaction mixture contained: 100 mM solution 5.5 '- di-thiobis- (2-nitrobenzoic acid); 100mM acetyl CoA solution; 0.1% Triton-X solution; 4 µl of the analyzed supernatant and Tris-HCl buffer solution with pH 7.8 to 100 µl. The optical density of the mixture was recorded at 412 nm for 3 minutes. The citrate synthase activity was determined by the change in the absorbance of the medium and was expressed in U / mg of protein. Protein concentration was determined according to the Bradford method (Shepherd & Garland, 1969).

2.10 Measurement of the calcium ions concentration

The calcium content in the samples was determined by the fluorescence method using Fura-2/AM as a reporter. The incubation medium contained 100 µl of the analyzed sample and Fura-2/AM in equal content. The intensity of the fluorescent signal was determined at excitation / emission wavelengths of 340 nm/380 nm and a 510 nm filter. Fluorescence was recorded on a Hitachi MPF-4 spectrofluorometer. The calcium content in the samples was

determined in terms of the protein concentration. Calcium concentration was estimated in fresh samples of biomaterial without freezing (Field, et al., 1994).

2.11 Measurement of the H_2O_2 concentration

The concentration of H_2O_2, was determined by fluorescence analysis using a standard Amplex Red kit (*Thermo Fisher Scientific*). The Amplex Red reagent is a colorless substrate that reacts with hydrogen peroxide with a stoichiometry of 1:1 to form a highly fluorescent resorufin (excitation/emission = 570/585 nm). The analysis process followed the manufacturer's instructions. The content of hydrogen peroxide was estimated in nmol/ml.

2.12 Measurement of thiobarbituric acid reactive substances (TBARS)

The TBARS concentration was determined spectrophotometrically by the detection of condensation products of acylaldehydes with 2-thiobarbituric acid at 532 nm. 100 µl of the analyzed biomaterial was mixed with an equivalent amount of 1.7% trichloroacetic acid solution and incubated for 5 minutes. The resulting mixture was centrifuged in the 1000g for 10 min. The supernatant was transferred into Eppendorf tubes and a 0.88% solution of 2-thiobarbituric acid was added, incubated in a boiling water bath for 15 minutes. The absorbance of the obtained colored solutions was recorded at 532 nm. The concentration of TBARS was calculated by the value of the molar extinction coefficient of malonildialdehyde ($1.56 * 10^5$ l * mol-1 * cm-1), the results were expressed in nmol/mg protein (Stalnaya & Garishvili, 1977).

2.13 Measurement of catalase activity

Catalase activity was determined spectrophotometrically by the rate of hydrogen peroxide destruction. The amount of hydrogen peroxide was determined in reaction with a 4% solution of ammonium molybdate. The color intensity of the reaction product was evaluated at 410 nm. Catalase activity was calculated by the difference between the extinctions of the experimental and blank samples, using the molar extinction coefficient of hydrogen peroxide equal to $22.2 * 10^3$ mm^{-1} cm^{-1} and expressed in U/ mg of protein (Korolyuk, 1988)

2.14 Measurement of superoxidedismutase activity

The activity of superoxidedismutase (SOD) was evaluated by the xanthine-xanthine oxidase method based on the reaction of the dismutation of the superoxide radical that formed during the oxidation of xanthine and reduction of 2- (4-iodophenyl) -3- (4-nitrophenol) -5-phenyltetrazolium chloride. The incubation medium contained: xanthine 0.05 mmol/L; 2- (4-iodophenyl) -3- (4-nitrophenol) -5-phenyltetrazolium chloride 0.025 mmol/L; EDTA 0.94 mmol/ L, xanthine oxidase 80 U/L, CAPS - 40 mmol/L. The optical density of the mixture was recorded at 505 nm. SOD activity was expressed in units/protein mg. (Woolliams, et al., 1983).

2.15 Measurement of glutathioneperoxidase activity

Glutathioneperoxidase (GPx) activity was determined spectrophotometrically in the conjugated glutathione reductase reaction according to the decrease NADPH concentration. The incubation medium contained: 1 mmol/L EDTA, 50 mM PBS buffer, pH 7.4; 1 U/ml glutathionreductase; 20 mmol/L NADPH; 1 mmol/L GSH. The optical density of the mixture was recorded at 340 nm. The reaction was started by adding a substrate (cumene hydroperoxide - 1.5 mmol/L) and was carried out at a temperature of 25^0C. GPx activity was expressed in U/protein mg (Pierce &Tappel, 1978).

2.16 ELISA – study

In this study, the change in the concentration of adenosine triphosphate (ATP), apoptosis-inducing factor (AIF) and caspase-3 was assessed by the method of enzyme-linked immunosorbent assay in the supernatant of the animal's hippocampus. Assay kits were obtained from *Cloud Clone corp*. (Houston, USA). The determination process, sample preparation and processing of the obtained spectrophotometric signal corresponded to the manufacturer's recommendations.

2.17 Statistical analysis

The analysis of the obtained data was carried out using the STATISTICA 6.0 software package. Results were expressed as M ± SEM (mean ± standard error of the mean). Comparison of the means was carried out by the ANOVA method with the Newman-Keuls post-test in the case of data obeying the law of normal distribution and the Kruskell-Wallis test when the data was distributed other than normal. The normality of the distribution was checked using the Shapiro-Wilk test. Differences between the study groups were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on behavior change of rats in the Morris water maze test

The results are shown in Figure 2. When carrying out this experimental block, it was found that in the NC group of animals, in comparison with SO rats, an increase in the latent time of task performance by 4.3 times, swimming distance - by 3.0 times, with a decrease in the average speed of movement by 58.9%. The use of the ATACL compound contributed to a decrease in the latent time of the task and the covered distance relative to the NC group of animals at a dose of 25 mg/kg by 24.5% and 33.0%, respectively, at a dose of 50 mg/kg by 23.1% and 30.1% respectively. Also, in rats treated by ATACL at doses of 25 mg/kg and 50 mg/kg, there was an increase in average swimming speed by 71.2% and 65.6%, respectively. At the same time, the administration of the reference drug to the animals contributed to a decrease in the latent time of the task and the distance covered, with an increase in the average speed by 19.5%; 20.6% and 44.8% respectively. It should be noted that the use of the ATACL compound at a dose of 100 mg/kg did not have a significant effect on the change in the behavior of animals in the Morris water maze test. In addition, in rats that were treated by ATACL at doses of 25 mg/kg and 50 mg/kg, the latency time for finding the platform and the covered distance were lower, and the average swimming speed was correspondingly higher than in animals that received the reference drug.

3.2 Effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes of cellular respiration in the supernatant of the animals hippocampus

The results of this research block are shown in Figure 3. During the analysis of changes in the processes of cellular respiration in ischemic rats, it was found that the NC group of animals showed a decrease in ATP-generating capacity, maximum respiration level and respiratory capacity by 72%; 67.2% and 65.7%, respectively (data are shown in comparison with SO rats). Against the background of the use of the reference drug, there was an increase in the ATP-generating capacity by 125.5%; the maximum level of respiration - by 109.6% and the respiratory capacity by 66.7% in relation to the rats of the NC group. The use of the ATACL compound at doses of 25 mg/kg and 50 mg/kg contributed to a significant increase in the parameters of cellular respiration - the ATP-

generating capacity increased by 109.8% and 89.2%, the maximum respiration rate - by 83.3% and 116.7% ; respiratory capacity - by 100.8% and 65.0%, respectively. At the same time, the maximum level of respiration in rats treated by ATACL at a dose of 50 mg/kg and the respiratory capacity in animals that received the test compound at a dose of 25 mg/kg were higher than those in the group of rats receiving the reference. It is significant that at a dose of 100 mg/kg with intraperitoneal administration, the ATACL compound did not have a significant effect on the change in the processes of cellular respiration.

3.3 Effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes in the activity of anaerobic processes in the supernatant of the animals hippocampus

The results of assessing the activity of anaerobic processes in the hippocampus of ischemic animals are shown in Figure 4. For example, in animals that did not undergo pharmacological intervention, the intensity of glycolysis was 8.4 times higher than that in SO of rats, with a decrease of glycolytic capacity and glycolytic reserve by 76.4% and 68.2%. With the administration of the referent, a decrease in the intensity of glycolysis in relation to the NC group of animals by 38.1% was noted, accompanied by an increase in the glycolytic capacity by 138.9% and the glycolytic reserve by 43.6%. The use of the ATACL compound at a dose of 25 mg/kg led to a decrease in the intensity of glycolysis, an increase in glycolytic capacity and glycolytic reserve by 41.8%; 161.1% and 74.3% respectively. At the same time, in rats that were treated by the test compound at a dose of 50 mg/kg, a decrease in the intensity of glycolysis by 43.6%, with an increase in glycolytic capacity and glycolytic reserve by 198.4% and 68.2%, respectively was noted (indicators are given in comparison with the NC group of animals). It should be noted that statistically significant differences between the NC group of rats and animals receiving ATACL at a dose of 100 mg/kg were not found.

3.4 Influence of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes of the mitochondrial respiratory chain complexes activity in the supernatant of the animals hippocampus

The results of this experimental block are shown in Figure 5. It was found that in the NC group of animals relative to the SO rats, a decrease of the activity of mitochondrial complexes I, II, III, IV and V by 68.6%; 48.8%; 53.7%; 84.8% and 42.2% respectively was observed. At the same time, the use of the reference drug promoted an increase of the respiratory complexes activity in comparison with the NC group of animals by 69.2% - complex I; 31.7% - complex II; 73.1% - complex III; 143.3% - complex IV and 24.3% - complex V. In animals that were treated by the test compound at doses of 25 mg/kg and 50 mg/kg, an increase in the activity of mitochondrial respiratory chain complexes was also observed (in relation to the NC group of rats), while the activity of complex I increased by 107.7% and 87.2%; complex II - by 50.4% and 48.0%; complex III - by 97.9% and 104.3%; complex IV - by 145.0% and 155.0%; complex V - by 40.0% and 30.0%, respectively. It should be noted that the activity of complexes I, II and V in animals treated by the ATACL compound at doses of 25 mg/kg and 50 mg/kg were higher than those in rats that were treated by the reference. Administration of the test compound at a dose of 100 mg/kg did not lead to a significant change in the activity of mitochondrial complexes (no statistically significant differences relative to the NC group of rats were found).

3.5 Effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes in citrate synthase activity and ATP concentration in the supernatant of the animals hippocampus

The change of the citrate synthase activity is shown in Figure 6, the concentration of ATP is shown in Figure 7. In the course of assessing the change of the citrate synthase activity, it was found that the catalytic properties of this enzyme in the NC group of animals decreased by 45.8% in relation to the same indicator of SO rats, also in NC the group of rats showed decreasing of ATP concentration by 39.6%. The use of a reference drug contributed to increase of citrate synthase activity and ATP content by 39.4% and 17.9%, respectively. At the same time, against the background of the administration of the ATACL compound to animals at doses of 25 mg/kg and 50 mg/kg, an increase of the citrate synthase activity by 55.1% and 43.4% was observed, as well as the level of ATP increased by 25.7% and 23.9%, respectively. The activity of citrate synthase was higher in animals that received the test compound at doses of 25 mg/kg and 50 mg/kg than in rats that were treated by the reference. It should be noted that there was no statistically significant change of citrate synthase activity (relative to the NC group of rats) in rats that were treated by ATACL at a dose of 100 mg/kg.

3.6 Effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes in the pro/antioxidant balance in the supernatant of the animals hippocampus

The results of evaluating the effect of the test compound and the reference drug on the change in the pro/antioxidant state are presented in Table 1. In the course of this experimental block, it was found that in the NC group of rats, a decrease (relative to the SO animals) of the endogenous antioxidant enzymes activity SOD - by 57, 6%, catalase - by 52.2% and GPx - by 38.7%, with an increase in the concentration of H_2O_2 and TBARS by 2.4 and 3.6 times, respectively was noted. The use of the referent increased the activity of SOD, catalase and GPx in relation to the NC group of rats by 22.4%; 14.3% and 40.2%, while the content of H_2O_2 and TBARS decreased by 32.7% and 39.0%, respectively. At the same time, the administration of the test compound at a dose of 25 mg/kg promoted an increase of SOD activity by 38.6%; catalase - by 47.0% and GPx - by 24.9%, as well as a decrease of the prooxidants concentration - H_2O_2 - by 36.0% and TBARS - by 32.2%. Similarly, the use of ATACL compound at a dose of 50 mg/kg led to the restoration of pro/antioxidant balance, which was expressed in an increase of the SOD, catalase and GPx activity by 46.1%; 47.0% and 27.8%, respectively, with a decrease of the H_2O_2 and TBARS content by 35.4% and 17.5%. It should be noted that the activity of SOD and GPx in rats treated by the ATACL compound was higher than in animals treated by the reference. In addition, administration of the ATACL compound at a dose of 100 mg/kg did not have a significant effect on the change in the activity of endogenous antioxidant defense enzymes, but statistically significant in relation to the NC group of rats increased the content of H_2O_2 - by 31.0% and TBARS - by 41.0%

3.7 Effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on the change of apoptosis activity in the supernatant of the animals hippocampus

The activity of apoptosis reactions in the supernatant of the hippocampus in rats was assessed by changes in the concentration of ionized calcium (Figure 8), the latent time of opening of the mPTP (Figure 9) and the content of AIF and caspase-3 (Figure 10). As a result, it was found that in the NC group of animals, in comparison with SO rats, an increase in the concentration of ionized calcium - 2.1 times; a decrease in the latent time

of mPTP opening - by 44.2%, as well as an increase in the content of AIF and caspase-3 by 4.8 and 3.8 times, respectively was noted. Against the background of the administration of the referent, there was a decrease (in relation to the NC group of animals) in the concentration of calcium, AIF and caspase-3 by 36.6%; 31.4%; and 35.0%, respectively, with an increase of the latent time of mPTP opening by 35.0%. The use of ATACL compound at a dose of 25 mg/kg also led to a decrease in the intensity of apoptotic reactions, which was expressed in a decrease of calcium content - by 25.4%, AIF - by 40.5%, caspase-3 by 42.0% and an increase in latency time of mPTP opening by 50.0%. At the same time, in animals that receiving ATACL compound at a dose of 50 mg/kg, relative to the NC group of rats, a decrease in the concentration of AIF and caspase-3 by 37.2% and 36.9%, respectively, ionized calcium - by 28.2%, with an increase in latent time of opening of mPTP by 58.4%was noted. It should be noted that the use of the ATACL compound at a dose of 100 mg/kg promoted an increase in the calcium content in the supernatant of the hippocampus of animals by 29.8%, as well as an insignificant increase in the concentration of AIF and caspase-3 in relation to the a same indexes of the NC group.

4. DISCUSSION

Neuroprotection involves the protection of neurons from the action of a damaging factor through the elimination of certain pathogenetic mechanisms of neuronal damage and represents a promising direction in the adjuvant therapy of ischemic stroke (Griauzde, et al., 2019). It should be noted that, despite the lack of translational success, the scientific and practical interest in neuroprotective agents remains at a sufficiently high level, which determines the relevance of the search for new strategies for neuroprotection, as well as the neuroprotectors themselves. (Neuhaus et al., 2017). New areas of neuronal protection include suppression of excitotoxicity, oxidative stress, and "repair of neurons", which can be achieved by restoring metabolic reactions in the ischemic penumbra zone. (Antonelli et al., 2012). It is known that a decisive role in the cells metabolism, including neurons, is played by a change in mitochondrial function, which is reflected at once in several leading cellular processes - ATP synthesis in oxidative phosphorylation reactions, redox state and regulation of apoptotic signal (MacDougall, et al., 2019). To date, it has been established that mitochondrial dysfunction is an integral part of the ischemic brain damage pathogenesis, while mitochondrial alteration can negatively affect on several cell functions at once, namely, a decrease in the synthesis of high-energy phosphates, an apoptotic cascade is activated and lipid peroxidation processes are enhanced (Sorrentino et. Al. , 2018). As a rule, mitochondrial damage is observed when the level of cerebral blood flow reaches less than 23 ml/100 g tissue/min and is mediated by a decrease in oxidative metabolism due to a lack of oxygen. At the same time, the electronic flux at the level of complexes I and III is directed not along the main, but along the secondary path, which leads to an increase in ROS production (Angelova & Abramov, 2018). At the same time, a decrease in the intensity of oxidative phosphorylation reactions and, accordingly, ATP synthesis, leads to the activation of the proapoptotic signal, an increase in the level of intracellular calcium and the opening of mPTP, promoting AIF release and activation of caspase-associated apoptosis (Jeong & Seol, 2008). In this regard, this study were carried out on a promising neuroprotective molecule - 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes in mitochondrial function in rats under conditions of cerebral ischemia. In previous studies, it was found that the use of this compound helps to reduce the zone of ischemic necrosis of the brain and restore the level of cerebral blood flow in experimental permanent ischemia. However, the mechanism by which 4-hydroxy-3,5-di-

tret-butylcinnamic acid reduces the extent of neuronal damage has not been established. Also, given that the most formidable complication of ischemic stroke is PSD, which is associated with damage to the hippocampus, this study focused on assessing the effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on changes in mitochondrial function in the hippocampus of animals under permanent brain ischemia. In this study, preference was given to the model of permanent middle cerebral artery occlusion because, as *McBride & Zhang, 2017* points out, this experimental pathology most fully reflects the pathogenetic mechanisms of brain damage observed on the clinic in humans (McBride & Zhang, 2017). The STAIR working group (Recommendations for standards regarding preclinical neuroprotective and restorative drug development) also recommends a model of permanent middle cerebral artery occlusion for the study of neuroprotective agents. In addition, one of the STAIR recommendations that allows translational success is to compare potential neuroprotective agents with drugs already introduced into medical practice. In this regard, the reference drug in this work was ethylmethylhydroxypyridine succinate (Mexidol®), which demonstrates a high level of efficacy, both in experimental pharmacology (Voronina, 2012) and in clinical conditions, when used as a neuroprotective adjuvant to the based therapy of ischemic stroke (Gromova, et al., 2018). As a result of the study, it was found that the use of the test compound in doses of 25 mg/kg and 50 mg/kg with parenteral administration contributed to the restoration of energy-producing mitochondrial function in the hippocampus of ischemic animals, which was reflected in the normalization of the processes of aerobic/anaerobic metabolism and the maintenance of the mitochondrial respiratory chain complexes activity. Besides, with the administration of 4-hydroxy-3,5-di-tret-butyl cinnamic acid in the indicated doses, a decrease in the intensity of apoptosis reactions was noted, as evidenced by a decrease in the content of pro-apoptotic molecules - AIF and caspase-3. At the same time, the antiapoptotic effect of the test compound may be associated with a decrease in the concentration of intracellular calcium and an increase in the latent time of mPTP opening, as a main predictors of an apoptotic event (Parks, et al., 2018). It is known that mPTP is a channel of the inner mitochondrial membrane, which is also formed by the c-ring residues F_1F_0 of ATP-synthase, leading to the release of apoptotic proteins and induction of apoptosis (Bonora, et al., 2017). In many ways, the formation of mPTP is a consequence of the dissipation of mitochondrial bioenergetic processes and an increase in the concentration of calcium, which is supported by ROS. As a rule, the formation of mPTP is a no-return point, after which the process of cell death is irreversible (Šileikytė & Forte, 2019). The decrease in the latent time of mPTP opening observed against the background of the administration of 4-hydroxy-3,5-di-tret-butyl cinnamic acid may be associated with a decrease in the concentration of ionized calcium, as well as with the stabilization of F_1F_0 ATP-synthase, which prevents its dimerization into one of the mPTP components. As a result, mitochondria remain viable, ATP synthesis is stabilized, and the concentration of AIF and caspase-3, respectively, decreases, preventing cell death through apoptosis.

In addition, when the test compound was used in doses of 25 mg/kg and 50 mg/kg (intraperitoneally), it reduced the intensity of oxidative stress reactions. So, against the background of the administration of 4-hydroxy-3,5-di-tret-butyl cinnamic acid in the indicated doses, a decrease intensity of lipid oxidation processes and ROS generation was noted, as evidenced by a decrease in the concentration of TBARS and H_2O_2. Also, the use of the test compound led to an increase in the activity of the main antioxidant enzymes - SOD, catalase and GPx. At the same time, the nature of the antioxidant action

of 4-hydroxy-3,5-di-tret-butyl cinnamic acid is interesting: in low and medium doses (25 mg/kg and 50 mg/kg), this compound prevents the development of oxidative stress, probably due to the presence of scavenger properties. In high doses (100 mg/kg) (Voronkov & Pozdnyakov, 2018), on the contrary, it intensifies free radical processes, which is an example of the classic "parabolic" nature of antioxidant action. Also of particular interest is the effect of 4-hydroxy-3,5-di-tret-butyl cinnamic acid on the change in citrate synthase activity. Cytrasynthase is an enzymatic complex localized on the inner mitochondrial membrane that catalyzes the condensation reaction of acetyl-CoA and oxaloacetate with the formation of citrate and CoA, i.e. the first stage of the Krebs cycle (Agostinho, et al., 2011). In addition to the metabolic role, citrate synthase is also a quantitative measure of the presence of intact mitochondria and the integrity of both outer and inner mitochondrial membranes (Valenti, et al., 2014). Thus, the increase of citrate synthase activity observed with the use of 4-hydroxy-3,5 -di-tret-butyl cinnamic acid may result from an increase in mitochondrial biogenesis *de novo*. As a result, the observed changes contributed to an increase of the ATP concentration in the hippocampus of ischemic rats. The consequence of maintaining optimal bioenergetic metabolism when the test compound are administered to animals is the restoration of cognitive functions was noted, which is confirmed by the results obtained in the Morris water maze test. At the same time, the rats, which were treated by 4-hydroxy-3,5-di-tret-butyl cinnamic acid, spent much less time searching for a "rescue platform" than animals without pharmacological intervention. Also, the distance covered and the average speed of movement in rats receiving the test compound were higher than those in the NC group, which may indicate an increase in motivation and the speed of decision-making in animals (Deng-Bryant, et al., 2016). As a result, the study showed that the use of 4-hydroxy-3,5-di-tret-butyl cinnamic acid at doses of 25 mg/kg and 50 mg/kg (equivalent) with intraperitoneal administration, the difference from the dose of 100 mg/kg, promotes recovery mitochondrial function and spatial memory of animals under cerebral ischemia conditions, while parenteral administration of the test compound can reduce the effective dose from 100 mg/kg (*per os*, previously obtained data) to 25 mg/kg.

5. CONCLUSION

Based on the obtained results, it is assumed that the use of 4-hydroxy-3,5-di-tret-butyl cinnamic acid at a dose of 25 mg/kg with parenteral administration is capable of providing the development of a neuroprotective effect realized by restoring mitochondrial function in the hippocampus of animals, which had a favorable effect on cognitive functions of ischemic animals. At the same time, the pharmacological effect from the administration of the test compound in most cases was comparable, and in some cases (the effect on the processes of cellular respiration, the activity of respiratory complexes and citrate synthase) exceeded that from the use of the referent - ethylmethylhydroxypyridine succinate. Thus, the study showed that 4-hydroxy-3,5-di-tret-butyl cinnamic acid is a promising neuroprotective agent of complex action, the use of which is able to restore the optimal course of bioenergetic reactions and eliminate the PSD phenomenon, as the most serious complication of ischemic stroke.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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