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Research Article Rus

Endothelotropic activity of 4-hydroxy-3,5-di-tret-butylcinnamic acid in the conditions of experimental cerebral ischemia

Andrey V. Voronkov[1], Dmitrij I. Pozdnyakov[1]

1 *Pyatigorsk Medical and Pharmaceutical Institute, a branch of Volgograd State Medical University, 11 Kalinina Ave., Pyatigorsk 357532 Russia*

Corresponding author: *Dmitrij I. Pozdnyakov* (pozdniackow.dmitry@yandex.ru)

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Abstract

Introduction: The aim of the study was to evaluate the endothelioprotective activity of 4-hydroxy-3,5-di-tret-butylcinnamic acid in conditions of experimental cerebral ischemia.

Materials and methods: The brain ischemia was reproduced by the method of irreversible right-sided thermocoagulation of the middle cerebral artery. As comparative drugs, mexidol (30 mg/kg) and sulodexide (30 U/kg) were used. The vasodilating function of the vascular endothelium was assessed by the change in the rate of cerebral blood flow when the synthesis of nitric oxide was modified. Antithrombotic function was assessed by changes in the concentration of thromboxane A_2, fibrinogen, von Willebrand factor activity and platelet aggregation activity. Serum concentration of C-reactive protein served as a marker of the state of anti-inflammatory endothelial function. To determine the potential mechanism of endothelioprotective activity of 4-4-hydroxy-3,5-di-tret-butylcinnamic acid, the anti-radical activity of this compound toward superoxide and nitrosy-radicals was assessed; and the effect of the compound on the mitochondrial function was studied, by evaluating the functional activity of mitochondrial ATP synthetase and cytochrome-c-oxidase by ELISA.

Results and discussion: In the course of the study, a positive effect of 4-hydroxy-3,5-di-tret-butylcinnamic acid on the state of endothelial function in cerebral ischemia was established, which was expressed in the preservation of vasodilating (restoring the vascular reaction to acetylcholine, nitro-L-arginine methyl ether, L-arginine), antithrombotic (a decrease in the concentration of thromboxane A_2, fibrinogen and von Willebrand factor activity by 241.9% ($p < 0.05$), 73.5% ($p < 0.05$), 20.4% ($p < 0.05$), respectively, a decrease in the degree of aggregation and platelet aggregation rate by 56.7 % ($p < 0.05$) and 52.8% ($p < 0.05$), respectively, and anti-inflammatory vascular endothelial function (99.1% C-reactive protein reduction ($p < 0.05$)). The 4-hydroxy-3,5-di-tret-butylcinnamic acid compound *in vitro* tests suppressed generation of superoxide (IC_{50} = 1.99 mg/ml) and nitrosyl radical (IC_{50} = 1.92 mg/ml), eliminated NO-synthase uncoupling, and restored the mitochondrial function (increase in mitochondrial ATP synthase and cytochrome-c-oxidase activity by 23.5% ($p < 0.05$) and 110.8% ($p < 0.05$), respectively).

Conclusion: The study demonstrated the presence of endotheliotropic activity of 4-hydroxy-3,5-di-tret-butylcinnamic acid, which is expressed in the preservation of vasodilating, antithrombotic and anti-inflammatory functions of the vascular endothelium in conditions of cerebral ischemia. At the same time, the anti-radical properties of this compound, as well as the direct effect on the functional activity of the NO-synthase system and the improvement of the mitochondrial function, may underlie the endotheliotropic effects of 4-hydroxy-3,5-di-tret-butylcinnamic acid.

Keywords

ischemic stroke, endothelial dysfunction, cinnamic acid derivatives.

Introduction

Ischemic stroke is one of the leading causes of death and primary disability of the population. The statistical data of the World Health Organization show a steady increase in the number of cases of ischemic stroke, which annually accounts for 15 million registered facts of cerebral circulation disorders. The mortality rate also remains high: out of 15 million victims, 5 million die, 5 million get disabled, with loss of labor and social adaptation. In addition, the number of cases of ischemic stroke is projected to increase to 61 million by 2020. http://www.who.int/cardiovascular_diseases/resources/atlas/en.

Currently, the leading method of therapy for ischemic stroke is intravenous injection of thrombolytic agents (recombinant TPA, streptokinase) or endovascular intervention. However, despite the high efficiency of the measures taken, the desired result is often impossible to achieve, due to a small "therapeutic window" (less than 6 hours), the mismatch between the patients and the selection criteria, etc. (Jauch et al. 2013). Thus, the majority of patients exposed to ischemic stroke do not receive proper treatment, which dictates the need to search for new pharmacotherapeutic approaches to the therapy of this emergency condition (Yang et al. 2018). Recent advances in experimental and clinical angioneurology have significantly expanded the range of knowledge of the pathogenesis of ischemic stroke. In addition to the classic elements of the "ischemic cascade" of brain damage, which include: decreased energy production, acidosis, damage to the BBB with development of edema and local inflammation, glutamate calcium excitotoxicity, oxidative stress, cytokine cytotoxicity (Woodruff et al. 2011), some authors distinguish a relatively new link in the series of brain destruction reactions, which is endothelial dysfunction (Poggesi et al. 2016, Terpolilli et Al. 2012, Chen et al. 2012). As a labile structure of the vascular endothelium, it is constantly exposed to adverse factors: oxidative stress, estrogen deficiency, smoking, hyperhomocysteinemia, increased shear stress, hyperglycemia, etc. (Favero et al. 2014). Damage to the endothelial lining leads to failure of endothelium-mediated regulation of vascular tone, intensification of vasoconstriction, coagulation, inflammation and proliferation. The above mentioned changes create conditions for the secondary intensification of cellular damage: the permeability of the BBB increases, the calcium conductivity and acidosis increase, and the vasogen edema develops (Hu et al. 2017). Thus, damage to the vascular endothelium can be both a primary determinant of neuronal destruction, and a secondary damaging mechanism. Endothelial dysfunction is primarily associated with deficiency of NO – a key metabolite mediating all endothelial functions: vasodilating, antithrombotic, anti-inflammatory and antiproliferative (Esper et al. 2006). Reduction of the total NO can be caused by several mechanisms: disconnection of the NO synthase system with a decrease in the production of nitric oxide; intensification of biodegradation of NO in reaction with active forms of oxygen (ROS) with the formation of peroxonitrite (Radi 2013). A decrease in the activity of the NO-producing system developes for a number of reasons: a genetically determined defect, a lack of NOS substrate – L-arginine and co-enzymes tetrahydrobiopterin and NADPH, excess accumulation of ADMA, inactivation by ROS (Chen et al. 2013). Biodegradation of NO mainly occurs through its interaction with superoxide radical with the formation of cytotoxic peroxonitrite as a reaction product. ROS and superoxide radicals in particular are formed as products of reactions in the mitochondrial respiratory chain, which, without their proper inactivation by enzymes of endogenous antioxidant system, leads to the development of oxidative stress and an increase in the alteration of cells, including endotheliocytes, by the type of LPO (Kluge et al. 2013). Thus, one of the possible methods of pharmacotherapeutic correction of ischemic stroke may be endothelial targeting. To correct the endothelial dysfunction arising in conditions of cerebral circulation insufficiency, a relatively new pharmacotherapeutic group – endotheliprotective agents, including β-blockers, statins, inhibitors of phosphodiesterase 5, ivabradine – can be used. (Su 2015). However, the existing spectrum of endotheliotropic substances is limited, and there is practically no means with proven endotheliprotective activity (Radenković et al. 2013), which in turn dictates a new direction in the targeted search for pharmacologically active substances – endotheliprotective agents.

The aim of the study: to evaluate the endotheliprotective activity of 4 4-hydroxy-3,5-di-tret-butylcinnamic acid under conditions of experimental cerebral ischemia.

Materials and methods

Animals

The study was performed on 200 male rats of the Wistar line. The animals were obtained from the vivarium of the Pyatigorsk Medical and Pharmaceutical Institute, a branch of the Volgograd State Medical University of the Ministry of Health of the Russian Federation, and kept in standard conditions (ambient temperature 22±2°C, relative humidity 65±5%, with natural change of light-dark cycle). The care, and all manipulations with animals, met the requirements of the European Convention for the Protection of Vertebrates Used for Experimental and Other Scientific Purposes (Strasbourg, 22 June 1998).

Cerebral ischemia model (irreversible occlusion of the middle cerebral artery)

In rats, which were narcotized with chloral hydrate (350 mg/kg), a section of 2 cm^2 was sheared to the right and below the eye. Then an incision on the skin was made. Muscles were separated and the process of the malar bone was removed. Using a specially designed bur, a trepanation aperture was drilled above the intersection of the

middle cerebral artery with the olfactory tract (Fig. 1). Further, coagulation was performed by burning the middle cerebral artery under the site of its intersection with the olfactory tract. Soft tissues were reconstructed, bone fragments were repositioned. The wound was treated with a 5% iodine solution. Before awakening, the animals were left under a warming lamp. In the postoperative period, the animals were transferred to a soft-fodder diet (Bederson et al. 1986).

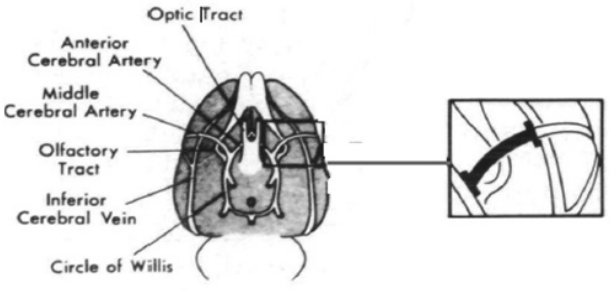

Figure 1. Schematic representation of coagulation of the middle cerebral artery.

Test-compound, formation of experimental groups. Design of the experiment

The investigated object was 4-hydroxy-3,5-di-tret-butylcinnamic acid (ATACL) in a dose of 100 mg/kg. As comparative drugs, sulodexide (Alfa Wasserman, Italy) was used in a dose of 30 U/kg and mexidol (PHARMASOFT, Russia) in a dose of 30 mg/kg. The test compound and comparative preparations were administered as a fine aqueous suspension *per os*, daily (one time per day) for 3 days from the time of the operation. The study was divided into 4 test blocks (Fig. 2), in each of which 5 experimental groups of animals (n = 10), randomized by age and weight (viripotent, weighing 200-220 grams) were formed: pseudo-operated rats (PO), to which all sequential manipulations were applied, with the exception of coagulation of the middle cerebral artery; a group of negative control animals (NC), with ischemia, but lacking pharmacological support; and groups of rats with ischemia receiving the test compound and comparative drugs. At the first, second and third stages of the experimental work, the effect of the test compound on the state of vasodilating, antithrombotic, anti-inflammatory vascular endothelial functions under conditions of cerebral ischemia, respectively, was assessed; at the fourth stage, possible aspects of the endotheliotropic effect of the studied compound were evaluated with an assessment of the change in the function of the NO-producing system, the anti-radical activity of the object under study (*in vitro*), and the functional activity of the mitochondria.

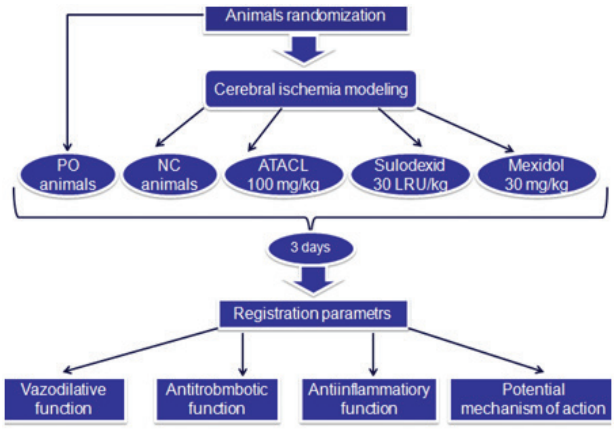

Figure 2. Design of the experiment.
Note: PO – pseudo-operated animals; NC – negative control group of animals; ATACL – 4-hydroxy-3,5-di-tret-butylcinnamic acid compound

Evaluation of vasodilating function

Evaluation of the vasodilating function of the cerebral vascular endothelium was performed using the Doppler method – recording the average systolic velocity (ASV) in the projection of the right medial cerebral artery in the modification of the synthesis of endogenous nitric oxide. The average systolic velocity was recorded with an ultrasound dopplerograph, a USOP-010-01 sensor with an operating frequency of 25 MHz, and a working computer program MM-D-K-Minimax Doppler v.1.7. (Saint-Petersburg, Russia). The modification of the release of endogenous NO was carried out by intravenous administration of test systems: acetylcholine (ACH) 0.1 mg/kg (Sigma-Aldrich), L-arginine 150 mg/kg (Panreac), nitro-L-arginine methyl ester (L-NAME) 15 mg/kg (Sigma-Aldrich). To assess endothelium-independent vasodilation, nitroglycerin (NTG, Biomed, Russia) was administered in a dose of 0.007 mg/kg. After carrying out all the necessary studies, the animals were removed from the experiment by instantaneous decapitation, before they recovered from anesthesia (Voronkov et al. 2015).

Evaluation of antithrombotic function

Antithrombotic function of the vascular endothelium was assessed by changes in platelet aggregation activity, concentration of thromboxane A_2, von Willebrand factor and fibrinogen.

The analysis of aggregation activity of platelets was carried out on a two-channel laser analyzer of platelet aggregation ALAT-2 “BIOLA” (SMF “BIOLA”, Russia), using the method of determining the relative average size of aggregates. When the experiment was set up, 0.3 ml of blood serum was put into the cuvette of the agglomerate, and incubated at 37°C for 3 minutes. After incubation, aggregation inducers were added: disodium adenosine-5-diphosphate (ADP, SMU “Renam”) in the final concentration of 5 μM. The platelet aggregation process was recorded for five minutes. The degree and rate of aggregation of the blood platelets was determined from the aggregatograms obtained (Gabbasov et al. 1989).

The concentration of fibrinogen was determined by the chronometric method on the analyzer of the hemostasis parameters of APG2-01 "MINILAB 701". During the test, standard reagent kits of SMU "Renam" were used. Lyophilized reagents were stored, meeting the conditons of temperature and prepared *ex tempore*. The progress of the analysis was strictly consistent with the instructions given in the kit.

The activity of von Willebrand factor (FW) was determined by the agglutination method. To the diluted imidazole buffer (1:5) plasma (50 µl) 50 µl Willebrand reagent was added. In transmitted light against a dark background, with a continuous rocking of the slide, the time was recorded from the addition of the Willebrand reagent until agglutination appeared and the FW activity in % was determined from the calibration plot.

The concentration of thromboxane A_2 was determined by solid-phase immunoassay. The work used species-specific sets of reagents produced by *Cloud Clone corp*. The object for the study was serum, obtained according to the instructions attached to the kit. The progress of the analysis was in accordance with the recommendations of the manufacturer of the kit. The results were read on an Infinite F 50 micro plate reader (Tecan, Austria).

Evaluation of the anti-inflammatory function

The anti-inflammatory function of the vascular endothelium was assessed by the change in the concentration of the C-reactive protein. The content of C-reactive protein (CRP) in the blood serum was measured by the latex-agglutination method using a standard set of reagents produced by the company "Arbis +".

Evaluation of potentially possible aspects of the mechanism of endotheliotropic action of 4-hydroxy-3,5-di-tret-butylcinnamic acid

The activity of the NO synthase system and the mitochondrial function was evaluated by a change in the concentration of NOS isoenzymes (eNOS, iNOS, nNOS), cytochrome-c-oxidase (COX), and mitochondrial ATP synthetase in rat brain ultracentrifugate. The work used species-specific sets of reagents produced by *Cloud Clone corp*. The sample preparation and the course of the analysis were in accordance with the instruction attached to the kit.

The antiradical activity of the compound studied was evaluated by the ability to suppress superoxide and nitrosyl radicals in the model medium.

Superoxide radical – antiradical activity

The analysis was carried out according to the method of Winterbourn (Winterbourn et al. 1975). The superoxide radical was generated in the phototreatment reaction of riboflavin. The incubation medium consisted of 0.1 ml of the solution of the test compound and comparative drugs in various concentrations (10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml) + 0.1 ml of a 1.5 mM solution of nitro-blue tetrazolium + 0.2 ml of 0.1 M solution of EDTA + 0.05 ml of 0.12 mM riboflavin solution + 2.55 ml phosphate buffer (pH 7.4). The resulting mixture was incubated for 5 minutes at 25°C. The absorbance of the samples was measured at 560 nm against air. Positive control was the incubation medium without addition of the estimated solutions. The percentage of inhibition was calculated by the formula (1):

$$\% \ inhibition = Ax / A_0 * 100, \ (1)$$ where

Ax – absorbance of the sample;

A_0 – absorbance of the positive control sample

Nitrosyl radical – antiradical activity

The course of the analysis corresponded to the method described by Marcocci (Marcocci et al. 1994): 2 ml of 10 mM sodium nitroprusside + 0.5 phosphate buffer solution (pH 7.4) + 0.5 ml of the solution of the test compound and comparative drugs were added to the cuvette. The resulting mixture was incubated at 25°C for 15 minutes. After a period of incubation, 0.5 ml of Griss reagent was added to the medium, incubated for 30 minutes at room temperature. The absorbance of the samples was measured at 546 nm. The percentage of inhibition of the nitrosyl radical was calculated according to the formula (2):

$$\% \ inhibition = Ax / A_0 * 100, \ (2)$$ where

Ax – absorbance of the sample before the addition of the Griss reagent;

A_0 – absorbance of the sample after the addition of the Griss reagent.

Statistical analysis

The results of the experiments were processed using the variational statistics method using the STATISTICA 6.0 software package (StatSoft, Inc., USA for the Windows operating system) and Microsoft Excel 2010. The average value and the standard error of the mean value were calculated, the data was expressed as M±SD. The obtained results were checked for normality by Shapiro-Wilk test. In the case of subordination to the laws of normal distribution, the Student's t-test was used to compare the averages. Otherwise, further statistical processing of the experimental results was carried out using the nonparametric Mann-Whitney U-test. The IC_{50} value was calculated by the probit analysis method.

Results

Evaluation of vasodilating function of vascular endothelium in the presence of cerebral ischaemia

In pseudo-operated rats, when administering ASH, ASV increased by 48.7% ($p < 0.01$) (Table 1) from its initial va-

lue in this group of animals. The introduction of L-arginine did not cause significant changes in cerebral hemodynamics in PO animals. At the same time, on the background of the blockade of enzyme systems for NO synthesis, by intravenous injection of nitro-L-arginine methyl ester, the rate of cerebral blood flow in PO animals decreased by 23.1% ($p < 0.05$) Evaluating the endothelium-independent vasodilation in animals of the PO groups there was an increase in ASV with nitroglycerin, relative to the background blood flow velocity, by 54.6% ($p < 0.01$).

In the NC group of rats, development of endothelial dysfunction with violation of NO-synthesizing system activity and failure of endothelium-mediated regulation of vascular tone were detected. This fact is confirmed by a small, in comparison with PO rats, insignificant response to intravenous administration of ACH and L-NAME. Thus, in the NC group of animals, an increase in (ACH) ASV and its decrease (L-NAME) from the baseline level was 12% and 11.4% (Table 1), respectively, which is 305.8% ($p < 0.05$) and 102.6% ($p < 0.05$) less that these figues in PO rats. In response to the introduction of L-arginine in NC rats, the rate of cerebral blood flow increased from its original level by 33.1% ($p < 0.05$), which in turn indicates that in this group of rats the development of the "L-arginin paradox" phenomenon. The introduction of nitroglycerin caused an increase in ASV (from its baseline level) in the group of NC rats by 55.9% ($p < 0.01$), which did not differ statistically significantly from the indices of the PO group of animals.

In animals receiving sulodexide, with the introduction of acetylcholine, ASV increased by 25.6% ($p<0.05$) from its baseline level. With the introduction of L-arginine to this group of rats, the rate of cerebral blood flow increased (in comparison with the initial value) by 18.3%, which was statistically significantly 80.9% ($p < 0.05$) less than that of the group of animals. Against the background of L-NAME in rats receiving sulodexide, the ASV decreased by 23.1% ($p<0.05$) (Table 1). Intravenous administration of NTG promoted an increase in the rate of local cerebral blood flow by 52.3% ($p < 0.01$).

When using mexidol, the rate of cerebral blood flow in rats in response to the administration of ACH, ASV increases by 21.1% ($p < 0.05$), and L-NAME decreases by 12.8%. With the intravenous administration of L-arginine to rats receiving mexidol, ASV increased from its baseline by 31.2% ($p < 0.05$). Endothelium-independent vasodilation with the introduction of NTG was not statistically significantly different from that in PO rats.

Stimulated with acetylcholine, synthesis of nitric oxide caused an increase in the rate of local cerebral blood flow by 32.9% ($p < 0.05$) from its initial level in rats receiving ATACL (Table 1). It should be noted that the vascular response to the administration of the ACH with ATACL exceeded that of the NC group of animals by 174.2% ($p < 0.05$). Under the conditions of administration of the NO donor – L-arginine, in the animals of this group ASV increased by only 13.8%, which was statistically significantly lower than the similar value in the NC group of animals by 139.9% ($p < 0.05$). When L-NAME was injected to rats treated with ATACL, ASV decreased by 17.4% from its initial value ($p < 0.05$). Intravenous introduction of NTG to this group of animals (Table 1) contributed to an increase in ASV, compared with its original value of 51.1% ($p<0.01$).

Evaluation of antithrombotic function

The degree and rate of platelet aggregation in the PO group of animals were 1.536±0.129 RU, and 0,993±0,169 RU. (Table 2), respectively. As a result of focal cerebral ischemia, an increase in the degree of platelet aggregation by 70% ($p < 0.05$) and speed by 195.8% ($p < 0.02$) was detected in the NC group of rats. The introduction of sulodexide and mexidol virtually balanced the decrease in the degree and rate of platelet aggregation. When using sulodexide, the degree of aggregation of thrombocytes in the NC group of animals decreased by 41.9% ($p < 0.05$), and with administration of mexidol it decreased by 44.8% ($p < 0.05$). The aggregation rate decreased by 74.7% ($p < 0.05$) (sulodexide) and by 65.7% ($p < 0.05$) (mexidol).

With the administration of ATACL compound, the degree of platelet aggregation decreased by 56.7% ($p < 0.05$) compared that of NC group of rats, and the rate – by 52.8% ($p < 0.05$). There were no statistically significant differences between the group of animals treated with ATACL and comparative drugs (sulodexide and mexidol) (Table 2).

In the NC group of animals, an increase in the fibrinogen concentration, activity of von Willebrand factor and thromboxane A_2 concentration was observed compared

Table 1. Effect of the test compound and comparative drugs on the change in the vasodilating function of the vascular endothelium under conditions of cerebral ischemia.

Group	ACH	L-arginine	L-NAME	NTG
PO	48.7±1.030*	-2.2±0.430	-23.1±1.235Δ	54.6±0.592*
NC	12.0±1.670α	33.1±5.482 Δ α	-11.4±0.669 α	55.9±2.167*
Sulodexide	25.6±1.062 Δ α	18.3±1.664 αμ	-23.1±1.846 Δ	52.3±1.428*
Mexidol	21.1±2.407 Δ α	31.2±3.920 Δ α	-12.8±2.805	55.1±1.018*
ATACL	32.9±2.551 Δμ	13.8±2.256μ	-17.4±1.072 Δ	51.1±0.866*

Note: statistically significant relative to background blood flow velocity (Student's t-test * – $p<0.01$; Δ-$p < 0.05$); α – statistically significant relative to the P/O group of animals (Student's t-test, $p < 0.05$); μ – statistically significant relative to the N/C of the group of animals (Student's t-test, $p<0.05$).

Table 2. Effect of the test compound and comparative drugs on the change of antithrombotic function of the vascular endothelium under conditions of cerebral ischemia.

Group	Degree of aggregation (relative units)	Rate of aggregation (relative units)	FW, %	Fibrinogen, g/l	Thromboxane A_2, ng/ml
PO	1.536±0.129	0.993±0.169	93.4±1.077	1.53±0.123	10.95±0.266
NC	2.613±0.382α	2.937±1.056#	135.8±1.2#	5.31±0.408#	43.97±1.236#
Sulodexide	1.842±0.407*	1.681±0.532*	110.4±5.144*	3.16±0.276*	14.77±0.441#
Mexidol	1.805±0.297*	1.772±0.235*	127±3.114	4.09±0.606*	14.85±0.625#
ATACL	1.668±0.148*	1.922±0.098*	112.8±4.841*	3.06±0.51*	12.86±0.124#

Note: α – is statistically significant relative to the PO group of animals (Student's t-test, $p < 0.05$); # – statistically significant relative to the NC group of animals (Mann-Whitney U test, $p < 0.02$); * – statistically significant relative to the NC of the group of animals (Student's t-test, $p < 0.05$).

to the PO group of rats by 247.1% ($p < 0.05$); 45.4% ($p < 0.05$) and 301.6% ($p < 0.05$), respectively (Table 2). Against the background of the use of sulodexide, a 68% ($p < 0.05$) decrease in the concentration of fibrinogen relative to the NC group of animals was observed, FW activity decreased by 23% ($p < 0.05$),and thromboxane A_2 concentration – by 197.7% (p <0, 05). When mexidol was used in rats, a decrease in fibrinogen and thromboxane A_2 concentration by 29.8% ($p < 0.05$) and by 196.1% ($p < 0.05$), respectively, was observed in comparison with the animals of the NC group. When animals were injected with ATACL, the concentration of fibrinogen and thromboxane A_2 decreased by 73.5% ($p < 0.05$) and 241.9% ($p < 0.05$), respectively, compared with the NC group of rats. FW activity when using ATACL compound, compared to the animals not receiving any pharmacological support, decreased by 20.4% ($p < 0.05$).

Evaluation of the anti-inflammatory

In case of focal cerebral ischemia, a 6.2-fold ($p < 0.05$) increase in the concentration of CRP in the blood serum was observed in animals, compared with the PO group of rats (Table 3).

When mexidol was used, the content of C-reactive protein (Table 3) in rats decreased by 65.5% ($p < 0.05$) compared with the NC group of animals.

In rats treated with sulodexide and ATACL, the concentration of the C-reactive protein (Table 3) in comparison with the NC group of animals decreased by 86.3% ($p < 0.05$) and 99.1% ($p < 0.05$), respectively.

Evaluation of the potential mechanisms of endotheliotropic activity of 4-hydroxy-3,5-di-tret-butylcinnamic acid

In case of focal cerebral ischemia, rats showed inhibition of endothelial NO synthase (the concentration of eNOS in the NC group of rats decreased by 65.4% ($p < 0.05$) compared with that of the PO group of animals), and an increase in the concentration of nNOS and iNOS by 63,5% ($p < 0.05$), and 30.8% ($p < 0.05$), respectively (Table 4). In addition, in the NC group of rats there was a decrease in mATP and COX relative to the PO group of animals by 129.1% ($p < 0.05$) and 130.5% ($p < 0.05$)

When mexidol was used in rats, a decrease in the concentration of nNOS and iNOS (by 14.7% and 39.3% ($p < 0.05$), respectively) was observed in comparison with

Table 3. Changes in the concentration of C-reactive protein in rats blood serum in case of focal cerebral ischemia.

Group	PO	NC	Sulodexide	Mexidol	ATACL
CRP, mg/l	2.23±0.228	13.92±1.125Δ	7.46±0.527*	8.41±0.743*	6.99±0.446*

Note: Δ – statistically significant relative to the PO group of rats (Mann-Whitney U test, $p < 0.05$); * – statistically significant relative to the NC group of rats (Mann-Whitney U test, $p < 0.05$).

Table 4. Effect of ATACL compound on the concentration of NOS isozymes and mitochondrial function markers on cerebral ischemia in rats.

Group	P/O	NC	Sulodexide	Mexidol	ATACL
nNOSng/ml	16.7±0.013	27.3±0.045#	23.8±0.012	12±0.011*	17±0.027*
iNOSng/ml	13±0.008	17±0.003#	12.2±0.013*	10.1±0.006*	10.5±0.002*
eNOSpg/ml	77.9±0.088	47.1±0.065#	53.6±0.069	60.7±0.095*	100.9±0.022*
mATPng/ml	125.5±20.894	54.77±6.583#	72.05±8.838*	56.57±2.656	67.66±4.824*
COXng/ml	48.78±0.287	21.16±2.046#	40.25±1.891*	21.24±1.961	44.6±2.235*

Note: # – statistically significant relative to the PO group of animals (Mann-Whitney U test, $p < 0.05$); * – statistically significant relative to the NC group of animals (Mann-Whitney U test, $p < 0.05$).

the NC group of animals, as well as a slight increase in the eNOS concentration by 13.8%. Also in animals when administering mexidol in comparison with NC rats, the concentration of mATP increased by 31.6% ($p < 0.05$), and the COX content increased by 90.2% ($p < 0.05$).

When administering sulodexide (Table 4), in comparison with the NC group animals, an increase in the concentration of eNOS was observed (Table 4) by 28.9% ($p < 0.05$), accompanied by a decrease in the content of nNOS and iNOS by 127.5% ($p < 0.05$) and 68.3% ($p < 0.05$), respectively. The concentration of mATP and COX when administering sulodexide, was statistically significantly different from that of the NC rat group.

In animals receiving the ATACL compound, the concentration of eNOS (compared with the NC group of rats) increased by 114.2% ($p < 0.05$), the concentration of nNOS and iNOS in this group of animals, by contrast, decreased by 60.6% ($p < 0.05$) and 61.9% ($p < 0.05$), respectively. At the same time, the administration of the ATACL compound promoted an increase in the content of mATP and COX, in comparison with that in the NC group of rats, by 23.5% ($p < 0.05$) and 110.8% ($p < 0.05$), respectively.

Assessing the antiradical activity (Fig. 3) of the test compound, it was found out that 4-hydroxy-3,5-di-tret-butylcinnamic acid exhibits pronounced "scavenging" properties with respect to the superoxide and nitrosyl radical. Thus, in a concentration of 10 mg/ml, the compound under study inhibited the generation of superoxide radical by 78.4% and of nitrosyl radical by 79.6% (Fig. 3), the IC_{50} value being 1.99 mg/ml and 1.92 mg/ml, respectively, which was comparable with the indices of mexidol (IC_{50O2-*} = 1.95 mg/ml, IC_{50NO*} = 2.35 mg/ml) and exceeded the indices of sulodexide (IC_{50O2-*} = 5.06 mg/ml, IC_{50NO*} = 6.24 mg/ml).

Discussion

Endothelium is a monolayer of cells, of mesenchymal origin, performing a number of functions aimed at maintaining vascular homeostasis (Kim et al. 2012).

Being a highly specific and highly organized system, the vascular endothelium is exposed to a number of unfavorable factors, leading to a violation of its anatomical or functional integrity – when endothelial dysfunction develops. In endothelial dysfunction, the endothelium-dependent mechanism of vascular homeostasis is disturbed, which contributes to the activation of vasoconstriction, platelet activation, oxidative stress, thrombosis, coagulation and inflammation.

Endothelial dysfunction is a universal pathophysiological process that underlies many cardiovascular and endocrine diseases such as arterial hypertension, atherosclerosis, ischemic stroke, ischemic heart disease, diabetes, etc. (Lau et al. 2015). In this connection, correction of endothelial dysfunction and the timely administration of agents with endothelioprotective activity can be considered one of the new directions in the pharmacotherapy of "vascular" diseases, including cerebrovascular disorders (Gimbrone et al. 2016). However, "endothelioprotection" in most cases refers to the number of additional (pleiotropic) effects of pharmacologically active substances, and medications with proven endotheliotropic effects currently do not exist (except for sulodexide) (Su 2015). Therefore, a targeted search for endothelioprotectors for therapy of cerebrovascular disoders is a promising modern direction of experimental and clinical pharmacology.

In the course of the study, it was found that the use of 4-hydroxy-3,5-di-tret-butylcinnamic acid contributed to the restoration of vasodilating, antithrombotic and

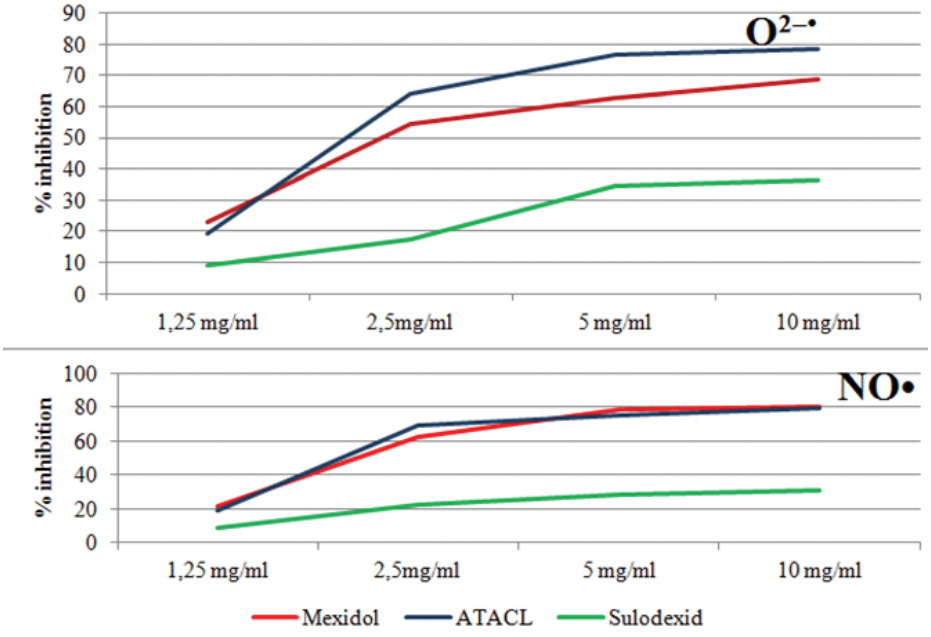

Figure 3. Antiradical properties of 4-hydroxy-3,5-di-tret-butylcinnamic acid and comparative drugs.

anti-inflammatory functions of the vascular endothelium under conditions of cerebral ischemia. The positive endotheliotropic effect of 4-hydroxy-3,5-di- tret-butylcinnamic acid is probably associated with the suppression of one of the leading pathogenetic mechanisms of endotheliocyte damage – oxidative stress. It is known that the hyperproduction of free radicals, in particular of the superoxide radical, promotes accelerated biodegradation of nitric oxide and has a direct inhibitory effect on eNOS, as a result of which the activity of iNOS increases compensatorily, with the hyperproduction of NO and the growth of the peroxonitrite amount, thereby closing the "vicious circle" of endothelial destruction (Kar et al. 2013). The study demonstrated that 4-hydroxy-3,5-di-tret-butylcinnamic acid *in vitro* exhibits pronounced antiradical activity against superoxide and nitrosyl radicals. The presence of direct antioxidant activity is probably explained by the chemical structure of 4-hydroxy-3,5-di- tret-butylcinnamic acid. The presence of a hydroxyl group in the structure of the compound increases its reducing properties, since it is the mobile proton of the O-H group that is the primary center for the binding of the free radical. In addition, a vinylenic fragment is present in the structure of 4-hydroxy-3,5-di- tret-butylcinnamic acid, which, under the influence of tret-butyl radicals of the aromatic nucleus, stabilizes the hydroxycinnamic acid radical to form a reactively inactive phenoxyl which terminates the further course of free radical reactions (Teixeira et al. 2012).

The positive effect of 4-hydroxy-3,5-di- tret-butylcinnamic acid on the mitochondrial function, expressed in the restoration of cytochrome-c-oxidase and mitochondrial ATP synthetase activity, was also established during the study, which can also underlie the antioxidant properties of 4- hydroxy-3,5-di- tret-butylcinnamic acid. It is known that the reaction of the mitochondrial respiratory chain is one of the main sources of reactive oxygen indermediates in the cell, while from 2% to 5% of the consumed oxygen goes to the production of ROS (Trinity et al. 2016). Dissociation of the function of cytochrome-c-oxidase leads to a disastrous for the cell growth of the amount of ROS, disrupting the further course of the reactions of conjugation of oxidation and phosphorylation, ie, deterioration of the function of the mATP (Cheng et al. 2012). The preservation of the optimal function of these mitochondrial enzyme complexes under the action of 4-hydroxy-3,5-di- tret-butylcinnamic acid provides to eliminate the ROS-generating ability of mitochondria. In addition, the improvement in mitochondrial function contributes to leveling the mitochondrial dependent cascade of apoptosis (Choe et al. 2015) and improving energy production (Fig. 4) (Errea et al. 2015), which requires, however, further study.

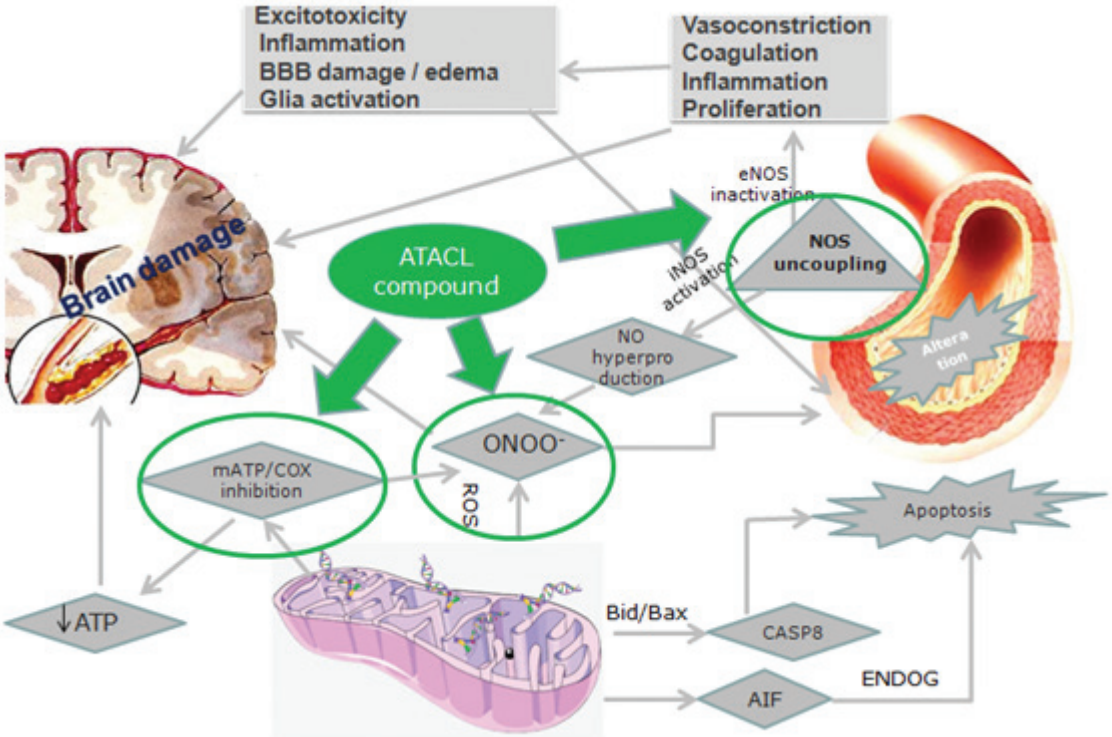

Figure 4. Mechanisms of endotheliotropic effects of 4-hydroxy-3,5-di-tret-butylcinnamic acid (ATACL)
Note: BBB – blood-brain barier; ATP – adenosine triphosphate; mATP – mitochondrial ATP-syntase; COX – cytochrome-c-oxidase; ROS – reactive oxygen radicals; NOS – nitric oxyde syntase; eNOS – endothelial nitric oxyde syntase; iNOS – inducible nitric oxyde syntase; CASP 8 – caspase 8; AIF – apoptosis inducing factor; ENDOG – endonuclease G; $ONOO^-$ – peroxonitrite; ATACL – 4-hydroxy-3,5-di-tret-butylcinnamic acid compound.

Conclusion

The study demonstrated that 4-hydroxy-3,5-di-tret-butylcinnamic acid has endothelioprotective activity in conditions of cerebral ischemia comparable to sulodexide and mexidol. The use of 4-hydroxy-3,5-di-tret-butylcinnamic acid contributed to the preservation of vasodilating, antithrombotic and anti-inflammatory functions of the vascular endothelium under conditions of cerebral ischemia in rats. Potential mechanism of endotheliotropic activity of 4-hydroxy-3,5-di-tret-butyl cinnamic acid may be its having antiradical activity, expressed in the suppression of generation of superoxide (IC_{50} = 1.99 mg/ml) and nitrosyl radicals (IC_{50} = 1.92 mg/ml), as well as an improvement in the mitochondrial function.

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Conflicts of interest

The authors state no conflict of interest with the submitted manuscript.

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Author contributions

- **Andrey V. Voronkov**, Doctor of Medical Science, Associate Professor, Head of Department of Pharmacology with course of Clinical Pharmacology, Pyatigorsk Medical and Pharmaceutical Institute; e-mail: prohor77@mail.ru. The author defined the idea of the research.
- **Dmitrij I. Pozdnyakov**, Assistant of Department of Pharmacology with course of Clinical Pharmacology, Pyatigorsk Medical and Pharmaceutical Institute; e-mail: pozdniackow.dmitry@yandex.ru. The author defined the idea of the research and conducted analysis and interpretation of the results.