Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-4-387-399

SYNERGIC EFFECT OF PREPARATION WITH COORDINATION COMPLEX "TRIMETHYDRAZINIUM PROPIONATE+ETHYMTH METHYLHYDROXYPIRIDINE SUCCINATE" ON ENERGY METABOLISM AND CELL RESPIRATION

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Received 08 Jule 2022 After peer review 12 Aug 2022 Accepted 22 Aug 2022

The article presents the results of an *in vitro* study of the synergetic effect evaluation of the combined preparation based on coordination complex ethylmethylhydroxypyridine succinate and trimethylhydrazinium propionate on energy metabolism and cell respiration.

The aim of the study was to evaluate the mitochondria-directed action of the metabolic and antioxidant preparation based on succinic acid coordination complex with trimethylhydrazinium in relation to optimizing the energy metabolism in the cells under the oxidative stress conditions, as well as against the background of ischemic processes.

Materials and methods. The study of the hydroxysuccinate complex effect of the drug Brainmax® components was carried out on isolated mouse liver mitochondria. In the course of the study, the potential of mitochondria, the generation rate of hydrogen peroxide during the respiration, the respiration rate were evaluated in the following positions: a) unstimulated by malate and pyruvate, b) stimulated by malate and pyruvate (complex I substrates), by succinate (complex II substrates), c) against the background of the initial section of the electron transport chain blockade by rotenone, d) in phosphorylation blockade by oligomycin, e) against the background of the FCCP-induced uncoupling, and f) in cyanide-blocked complex IV (cytochrome C oxidase).

Results. It has been shown that the succinic acid coordination complex with trimethylhydrazinium, which is the active principle of the Brainmax® drug, significantly reduced the transmembrane potential of mitochondria (IC_{50}=197±5 μM), compared with the widely used preparations of ethylmethylhydroxypyridine succinate and trimethylhydrazinium propionate, which facilitates the transfer of the produced ATP into the cell and preserves a vital activity of mitochondria even under stress. In the study of

For citation: M.V. Zhuravleva, M.V. Granovskaya, K.Ya. Zaslavskaya, Yu.G. Kazaishvili, V.S. Shcherbakova, A.A. Andreev-Andrievsky, D.I. Pozdnyakov, M.Yu. Vysokikh. Synergic effect of preparation with coordination complex "trimethydrazinium propionate+ethymth methylhydroxypiridine succinate" on energy metabolism and cell respiration. *Pharmacy & Pharmacology.* 2022;10(4):387-399. **DOI:** 10.19163/2307-9266-2022-10-4-387-399

Для цитирования: М.В. Журавлева, М.В. Грановская, К.Я. Заславская, Ю.Г. Казаишвили, В.С. Щербакова, А.А. Андреев-Андриевский, Д.И. Поздняков, М.Ю. Высоких. Синергическое действие препарата с координационным комплексом триметилгидразиния пропионата и этилметилгидроксипиридина сукцината на энергетический обмен и дыхание клетки. *Фармация и фармакология.* 2022;10(4):387-399. **DOI:** 10.19163/2307-9266-2022-10-4-387-399

RESEARCH ARTICLE

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

the mitochondrial respiration stimulated by the substrates of complex I (NADP-coenzyme Q-oxidoreductase), pyruvate and malate, the studied drug led to a more pronounced increase in the oxygen consumption with IC_{50}=75±6 μM. When evaluating the effect of the complex on the production of ATP by mitochondria, the most pronounced effect was observed with the addition of studied complex, which indicated to the uncoupling of respiration and oxidative phosphorylation at the given concentrations of the studied compounds. When assessing the effect of the complex on the production of hydrogen peroxide by isolated mitochondria, a significant decrease in the peroxide production was shown in the samples containing the complex of trimethylhydrazinium propionate and EMHPS.

Conclusion. Based on totality of the results obtained, it can be assumed that a favorable conformation of the pharmacophore groups of ethylmethylhydroxypyridine succinate and trimethylhydrozinium propionate coordination complex included in the composition of Brainmax® leads to a synergetic interaction and more pronounced pharmacological effects on target cells. This complex provides stabilization of a mitochondrial function, intensification of the adenosine triphosphate energy production and the optimization of energy processes in the cell, reduces the severity of the oxidative stress and eliminates undesirable effects of an ischemic-hypoxic tissue damage.

Keywords: ethylmethylhydroxypyridine succinate; trimethylhydrazinium propionate; succinic acid coordination complex with trimethylhydrazinium; hydroxysuccinate complex with trimethylhydrazinium; mitochondria; breath; oxidative stress; peroxide production.

Abbreviations: EMHPS – ethylmethylhydroxypyridine succinate; TMHP – trimethylhydrozinium propionate; FCCP – carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; ATP – adenosine triphosphate; ATPase – adenosine triphosphatase; ROS – reactive oxygen species; DAMPs – damage associated molecular patterns; ADP – adenosine diphosphate; NADP – nicotinamide adenine dinucleotide phosphate; MUPs – mitochondrial uncoupling proteins.

СИНЕРГИЧЕСКОЕ ДЕЙСТВИЕ ПРЕПАРАТА С КООРДИНАЦИОННЫМ КОМПЛЕКСОМ ТРИМЕТИЛГИДРАЗИНИЯ ПРОПИОНАТА И ЭТИЛМЕТИЛГИДРОКСИПИРИДИНА СУКЦИНАТА НА ЭНЕРГЕТИЧЕСКИЙ ОБМЕН И ДЫХАНИЕ КЛЕТКИ

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Получена 08.07.2022 После рецензирования 12.08.2022 Принята к печати 22.08.2022

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ

DOI: 10.19163/2307-9266-2022-10-4-387-399

В статье представлены результаты *in vitro* исследования оценки синергического действия препарата лекарственного препарата на основе координационного комплекса этилметилгидроксипиридина сукцината и триметилгидразиния пропионата на энергетический обмен и дыхание клетки.

Целью данного исследования являлась оценка митохондриально-направленного действия метаболического и антиоксидантного лекарственного средства на основе янтарно-кислого координационного комплекса с триметилгидразинием в отношении оптимизации энергообмена в клетках в условиях оксидативного стресса, а также на фоне ишемических процессов.

Материалы и методы. Исследование действия гидросукцинатного комплекса компонентов препарата Брейнмакс® проводили на изолированных митохондриях печени мыши. В процессе исследования оценивали потенциал митохондрий, скорость генерации в ходе дыхания перекиси водорода, а также скорость дыхания: а) нестимулированного малатом и пируватом, б) стимулированного малатом и пируватом (субстраты комплекса I), сукцинатом (субстрат комплекса II), в) на фоне блокады начального участка электрон-транспортной цепи ротеноном, г) при блокаде фосфорилирования олигомицином, д) на фоне вызванного FCCP разобщения и е) при заблокированном цианидом комплексе IV (цитохром С оксидазе).

Результаты. Было показано, что янтарно-кислый координационный комплекс с триметилгидразинием, являющийся действующим началом лекарственного препарата Брейнмакс®, значимо снижал трансмембранный потенциал митохондрий (IC_{50}=197±5 μM), по сравнению с широко применяемыми препаратами этилметилгидроксипиридина сукцинатом и мельдонием, что облегчает перенос продуцируемых АТФ в клетку и сохраняет жизнедеятельность митохондрий даже в условиях стресса. При исследовании дыхания митохондрий, стимулированном субстратами комплекса I (НАДФ-коэнзимQ-оксидоредуктазы), пирувата и малата, изучаемый препарат приводил к более выраженному росту потребления кислорода с IC_{50}=75±6 μM. При оценке влияния комплекса на продукцию митохондриями АТФ, наиболее выраженное действие наблюдалось при добавлении изучаемого комплекса, что свидетельствовало о разобщении дыхания и окислительного фосфорилирования при данных концентрациях исследуемых соединений. При оценке влияния комплекса на продукцию изолированными митохондриями перекиси водорода, было показано значимое снижение продукции перекиси в пробах, содержащих комплекс триметилгидразиния пропионата и ЭМГПС.

Заключение. По совокупности полученных результатов можно предполагать, что выгодная конформация фармакофорных групп координационного комплекса этилметилгидроксипиридина сукцината и триметилгидрозиния пропионата в составе лекарственного препарата Брейнмакс® приводит к синергетическому взаимодействию и более выраженному фармакологическому воздействию на клетки-мишени. Данный комплекс обеспечивает стабилизацию митохондриальной функции, интенсификацию выработки энергии аденозинтрифосфата и оптимизацию энергетических процессов в клетке, снижает выраженность оксидативного стресса и устраняет нежелательные эффекты ишемически-гипоксического повреждения тканей.

Ключевые слова: этилметилгидроксипиридина сукцинат; триметилгидразиния пропионат; янтарно-кислый координационный комплекс с триметилгидразинием; гидроксисукцинатный комплекс с триметилгидразинием; митохондрии; дыхание; оксидативный стресс; продукция перекиси

Список сокращений: ЭМГПС – этилметилгидроксипиридинна сукцинат; ТМГП – триметилгидразиния пропионат; FCCP – карбонилцианид-n-трифторметокси-фенилгидразон; АТФ – аденозинтрифосфат; АТФ-азы – аденозинтрифосфатаза; АФК – активные формы кислорода; DAMPs – молекулярные паттерны клеточного повреждения; АДФ – аденозиндифосфат; НАДФ – никотинамидадениндинуклеотидфосфа́т; МРБ – митохондриальные разобщающие белки.

INTRODUCTION

Pathological conditions associated with dyscirculatory disorders and tissue ischemia are the most common causes of death and a primary disability in the population. According to WHO Health Estimates[1], a coronary heart disease (terminal myocardial infarction) and an ischemic stroke occupy dominant positions in the list of leading non-communicable diseases with a high risk of death.

The pathogenesis of any ischemic tissue damage is based on an imbalance between the cells metabolic activity, expressed in the consumption of oxygen and substrates of biological oxidation, and an adequate delivery of essential nutrients [1].

Modern studies show: the key pathogenetic aspect that determines the severity of this imbalance is a violation of the cell mitochondria functional activity. Mitochondria are two-membrane organelles that perform many functions in cells. First of all, mitochondria are assigned the role of “energy stations” that provide an optimal pool of intracellular energy [2].

Mitochondria also regulate oxidation-reduction processes and apoptosis reactions. In this regard, disruption of a mitochondrial activity can lead to a deficiency of macroergic compounds, an increase in the generation of reactive oxygen species (ROS), and a premature cell death *via* the programmed pathway [3]. The main trigger initiating these processes is the lack of oxygen and oxidation substrates [4].

Vascular occlusion and subsequent hypoxia causes a number of severe biochemical and metabolic disorders that mediate a failure of the mitochondria functional activity. Cell metabolism switches from mitochondrial oxidative phosphorylation to anaerobic glycolysis, which leads to the intracellular accumulation of lactate and protons, lowering the pH with further activation adenosine triphosphatases (ATPases), primarily the Na^+/H^+ exchanger, but due to the rapid depletion of the energy resources in the form of ATP, there is an overload

[1] World Health Organization. The top 10 causes of death. Available from: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death

of the cell with sodium ions and, as a result, calcium (under the conditions of sodium overload, Na^+/Ca^{2+} ATPase is activated) [5].

A high intracellular calcium content in an ischemic cell disrupts the buffer capacity of mitochondria, and therefore the entry of calcium ions into the mitochondrial matrix is activated. [6]. Calcium ions entering the mitochondria cause the respiratory chain dysfunction, contributing to the hyperproduction of reactive oxygen species and activation of the cell death mechanism (necrosis, apoptosis). As a result of the calcium ions influx, mitochondria also "swell" (the phenomenon of mitochondrial blebbing) and are destroyed, releasing compounds into the cytosol increasing the degree of cellular damage [7].

First, these substances present damage associated molecular patterns (DAMPs) which activate the AGE/RAGE pathway, enhancing immunological reactivity in the ischemic focus [8].

Taking into account the peculiarities of the pathogenetic pathways of the ischemic cell damage described above and the central role of mitochondria in these processes, it is not surprising that the "energy stations" of cells have become the main target for the motivated cytoprotection. In order to correct mitochondrial dysfunction in ischemia, a number of chemically modified substances with a benz-γ-pyrone scaffold, ubiquinone, and a triphenylphosphonium linker [9], as well as the substances of a protein nature, are currently used. An example of them are peptides of the *Szeto-Schiller* group (SS-31) [9, 10]. However, a number of studies show that native, unmodified molecules can act as means of correcting a mitochondrial dysfunction. For example, succinates [11] or agents that bypass metabolic processes (trimethylhydrazinium propionate, trimetazidine) can prevent an irreversible damage to cell mitochondria [12, 13].

In the Russian Federation in 2022, a new original drug complex from the group of neuroprotectors and antioxidants, Brainmax®, was registered. This is an original fixed combination of ethylmethylhydroxypyridine succinate (EMHPS) and trimethylhydrazinium propionate (TMHP) in the form of capsules or a solution for intravenous and intramuscular injections. Trimethylhydrazinium propionate usually exists as a zwitterion (dihydrate) that has a positive charge on the hydrazine fragment and a negative charge on the carboxylate group [14]. It is described in the literature that salts of some polybasic acids (acid salts of fumaric and maleic acids, dihydrogen phosphate, acid salt of oxalic acid, mono- or disubstituted salt of mucic acid, salts of pamoic and orotic acids) in combination with trimethylhydrazinium propionate demonstrated special pharmacokinetic and pharmacodynamic properties [15]. A specific feature of the drug under consideration is the formation of a hydrosuccinate complex with trimethylhydrazinium during the preparation of the finished dosage form. The components of the complex are interconnected by hydrogen bonds and an electrostatic intermolecular interaction, which provides an advantageous conformation of pharmacophore fragments for better binding to receptors and a more pronounced effect. At the same time, it is important that the components of the complex have different action points of application, as a result of which, a synergistic effect can develop when they are used combined.

THE AIM of the study was to evaluate the mitochondria-directed action of the complex ethylmethylhydroxypyridine succinate and trimethylhydrazinium propionate included in composition of Brainmax®.

MATERIALS AND METHODS

Animals

The study included 50 CBA´B6 male mice aged 4-5 months, obtained from the Center for Genetic Resources of Laboratory Animals (Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences). The studies met the requirements of the Law of the Russian Federation "On the Protection of Animals from Cruelty" dated June 24, 1998, the rules of laboratory practice for preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), and the directives of the European Community (86/ 609 EU), the rules of the International Recommendations of the European Convention for the Protection of Vertebrate Animals used in experimental studies (1997) and the Rules of Laboratory Practice adopted in the Russian Federation (Order of the Ministry of Health of the Russian Federation No. 708 dated 29.08.2010). The study protocol has undergone expert appraisal by the bioethics commission of the Scientific Research Institute of Mitoengineering of Moscow State University (Conclusion No. 171 dated 13 Jan 2022).

Study design

The study was carried out on isolated mouse liver mitochondria. The potential of mitochondria, the generation rate of hydrogen peroxide, the respiration rate were evaluated in the following positions: a) unstimulated by malate and pyruvate, b) stimulated by malate and pyruvate (complex I substrates), by succinate (complex II substrates), c) against the background of the initial section of the electron transport chain blockade by rotenone, d) in phosphorylation blockade by oligomycin, e) against the background of the FCCP-induced uncoupling, and f) in cyanide-blocked complex IV (cytochrome C oxidase).

For each of the indicators in the three experiments, the following were recorded: 1) a reaction to meldonium, 2) a reaction to EMHPS, and 3) a reaction to the succinic acid coordination complex of trimethylhydrazinium propionate and EMHPS. For each of the experiments, 7 independent repetitions of the experiment were performed.

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DOI: 10.19163/2307-9266-2022-10-4-387-399

Isolation of liver mitochondria

To obtain the liver, mice were euthanized by a cervical dislocation, after which the abdominal cavity of the animals was immediately opened and the liver was excised. The liver was placed in an ice-cold phosphate-buffered saline (pH=7.0) and kept on ice until mitochondria were isolated, but not longer than 3 min.

After homogenization in approximately 20 volumes of an isotonic solution, the suspension of liver tissue was transferred into test tubes and the remnants of intact tissues were precipitated by centrifugation at 1000 g and 4°C for 10 min. The supernatant was collected, avoiding the milky suspension being pipetted, on the surface and centrifuged at 14 000 g and 4°C for 10 min. The resulting dark precipitate of mitochondria was washed free from the upper light loose layer, collecting the latter with a pipette and washing the mitochondria with a buffer of the following composition: 250 mM sucrose, 20 mM Hepes/NaOH, pH 7.5, 0.5 mM EGTA, 0.1% BSA. The pellet was suspended in 0.5 ml of this buffer and carefully homogenized with 5–10 passes of a Teflon pestle in a 1 ml glass homogenizer (G-Biosciences, USA). The homogenate was quantitatively transferred into a new tube and mitochondria were precipitated by centrifugation at 12 000 g and 4°C for 10 min. The pellet was suspended on ice in 70–100 µl of the isolation buffer with a plastic pestle until a homogeneous suspension was obtained.

Measurement of mitochondrial respiratory function

An aliquot of the obtained mitochondria preparation in the amount of 50 µg for the protein determined by the method with bicinchoninic acid (Pierce, USA) was used to determine the rate of respiration, the intensity of the oxidative phosphorylation, the degree of conjugation, and the calcium load of mitochondria. To determine the rate of respiration, the method of a direct registration of oxygen uptake using a highly sensitive oxigraph (Hansatech, England) was used. To do this, an aliquot of mitochondria was placed in a glass cuvette filled with 0.5 ml of a buffer containing: 120 mM sucrose, 75 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 20 mM HEPES, pH=7.5 (titration with NaOH). The measurement of the oxygen consumption was carried out in a closed system at 37°C and constant stirring on a magnetic stirrer at the speed of 500 rpm.

The obtained values (a change in O_2 over time, dO_2/dt) were normalized to the protein content. Mitochondria were energized using substrates I and II of the respiratory chain complexes. After the registration of the respiration rate activated by adding 4 mM pyruvate to the system in the presence of 10 mM malate, the effect of the rotenone (2 µM) blocking complex I, was studied. To record the respiration rate of mitochondria with activated complex II, after the addition of rotenone, 1 mM potassium succinate was added to the measurement system.

The study of the conjugation degree of the obtained preparations was carried out in the presence of adenosine diphosphate (ADP) after the respiration stimulation by introducing 0.1 mM ADP into the system of 1 µg of oligomycin and the inhibition of the stimulated respiration was recorded. The ratio of the stimulated and unstimulated respiration rates was used as a characteristic value (respiratory control coefficient), which makes it possible to assess the quality of the mitochondria obtained preparation and their state in the tissues under study. The maximum rate of the uncoupled respiration was determined in the presence of 20–50 nM protonophore FCCP. When analyzing the respiration rates, the value corresponding to the oxygen consumption rate in the presence of 0.5 mM KCN was subtracted from all values.

To do this, 25 µl of mitochondrial protein was added to the microcuvette with a volume of 250 µl, the kinetics of changes in the 555/523 nm ratio were recorded in a two-wave mode on an Aminco DW2000 spectrophotometer (Olis Inc., USA) before and after the addition of the respiratory substrates and specific inhibitors of the electron transfer in the respiratory mitochondria chain such as rotenone, antimycin, malonate and myxothiazol. Dissipation of the transmembrane potential was achieved using FCCP.

Measurement of calcium capacity

The calcium capacity of mitochondria was determined by titration while measuring the light scattering at 575 nm, spectrophotometrically in Cary Varian 300 (Agilent, США and in the medium of 250 mM sucrose, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 20 mM HEPES, pH=7.4 (NaOH titration). At the same time, both the total amount of calcium, which induces a drop in absorption, corresponding to the maximum swelling of mitochondria in the iso-osmotic system (calcium capacity), and the kinetics of swelling, which characterizes the ability of mitochondria to transport calcium, were studied.

Assessment of ATP synthesis

The level of adenosine triphosphate (ATP) synthesis was determined by the ATP-dependent luminescence of mitochondrial suspension in various states, when various respiratory chain complexes were energized by substrates. Since the level of the ATP production by mitochondria during aerobic oxidation of the substrates is determined by the activity of the ATP synthetase sensitive to oligomycin; the use of this inhibitor makes it possible to calculate the total maximum amount of the ATP synthesized in mitochondria, thus characterizing the differences in the ability of mitochondria to maintain energy metabolism.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

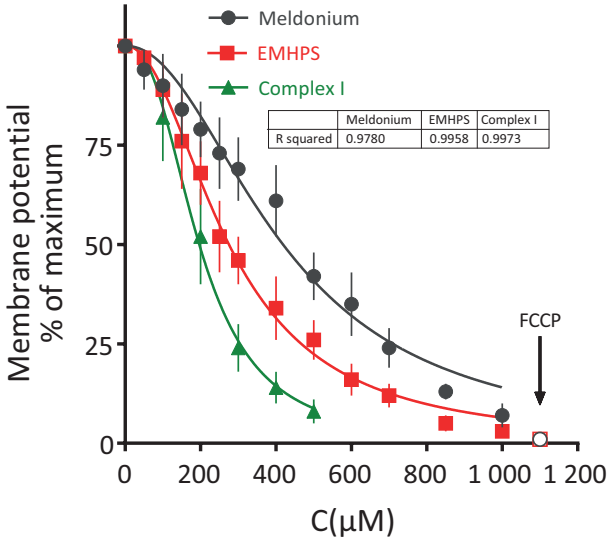

Figure 1 – Dependence of mitochondria membrane potential on a meldonium dose, EMHPS or a complex of trimethylhydrazinium propionate and EMHPS (Complex I)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

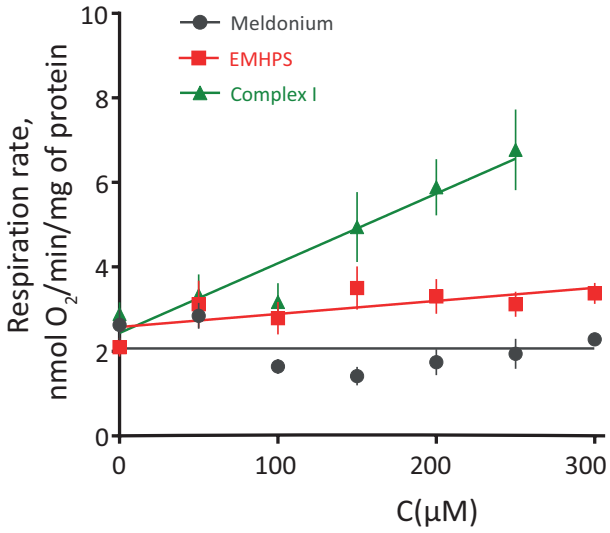

Figure 2 – Basal respiration rate (without addition of exogenous substrates) of mouse liver mitochondria in presence of meldonium, EMHPS, or complex of components (Complex I)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

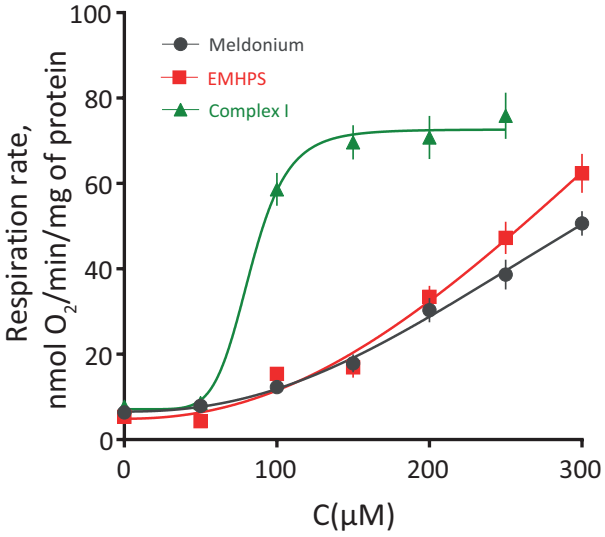

Figure 3 – Respiration rate of mouse liver mitochondria stimulated with 5 mM pyruvate and 1 mM malate in presence of meldonium, EMHPS or a complex of components (Complex I)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

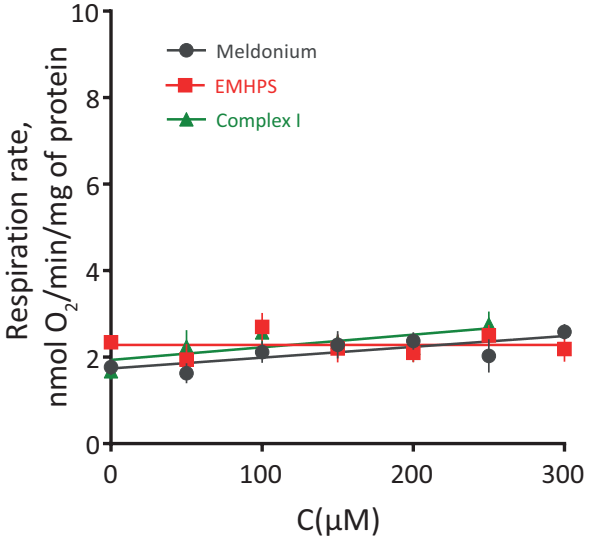

Figure 4 – Respiratory rate of mouse liver mitochondria stimulated with 5 mM pyruvate and 1 mM malate in presence of meldonium, EMHPS or complex components (Complex I) and I inhibitor rotenone (2 μM)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

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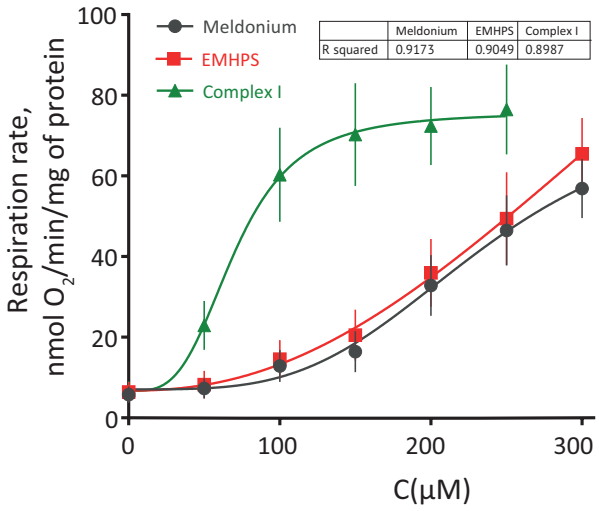

Figure 5 – Respiratory rate of mouse liver mitochondria stimulated with 1 mM succinate in presence of meldonium, EMHPS, or studied complex (Complex I) and complex I inhibitor rotenone (2 μM)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

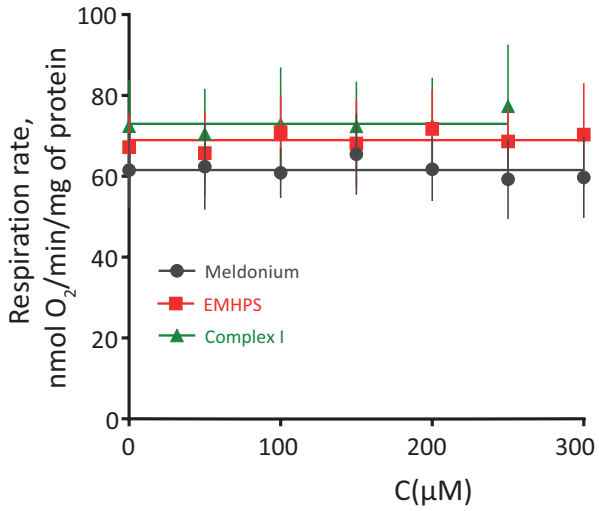

Figure 6 – Respiration rate of mouse liver mitochondria stimulated by 1 mM ADP in presence of meldonium, EMHPS, or trimethylhydrazinium complex (Complex I)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

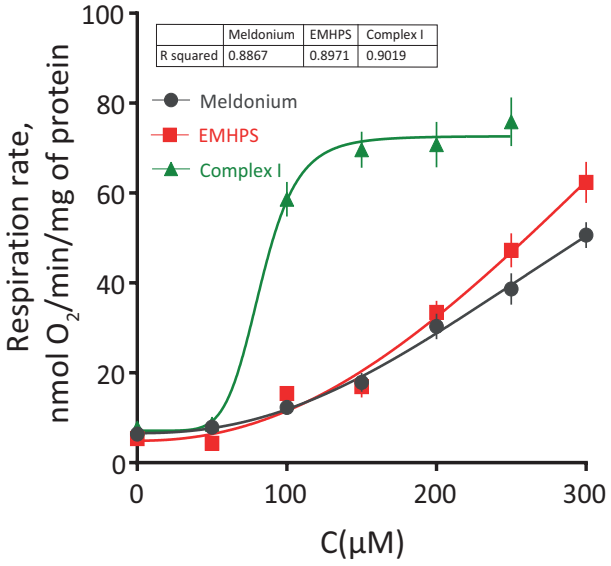

Figure 7 – Respiratory rate of mouse liver mitochondria stimulated with 1 mM ADP in presence of meldonium, EMHPS, or complex under consideration (Complex I) and ATP synthase blocker oligomycin (1 μM)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

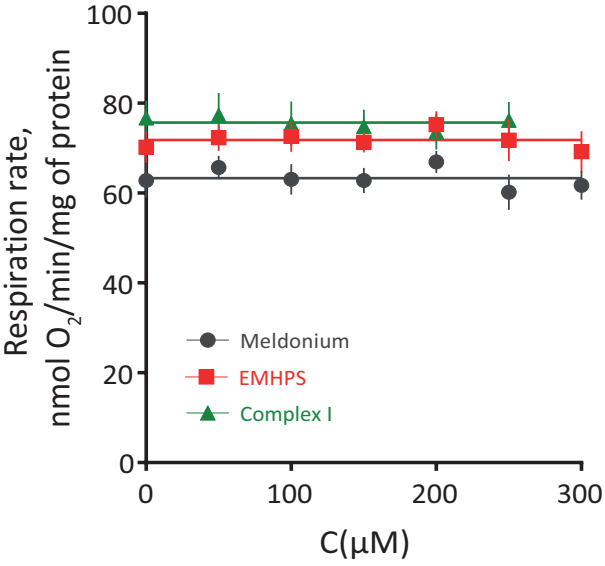

Figure 8 – Respiration rate of mouse liver mitochondria stimulated with 1 mM ADP in presence of meldonium, EMHPS or complex under consideration (Complex I) and FCCP protonophore (1 μM)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

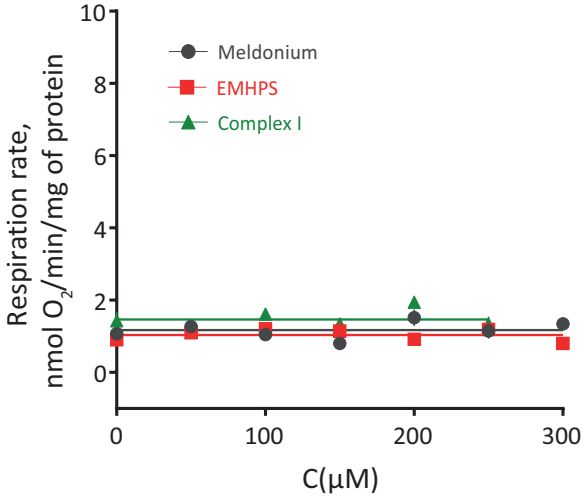

Figure 9 – Respiration rate of mouse liver mitochondria stimulated by 1 mM ADP in presence of meldonium, EMHPS or studied complex (Complex I) and cyanide (1 μM)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

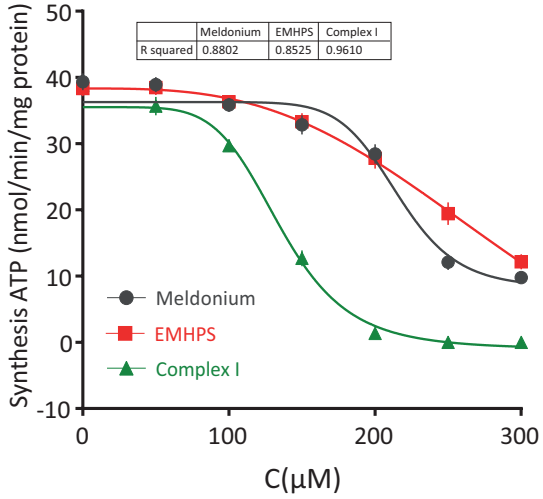

Figure 10 – The ATP synthesis rate by mouse liver mitochondria in presence of meldonium, EMHPS or studied complex (Complex I)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

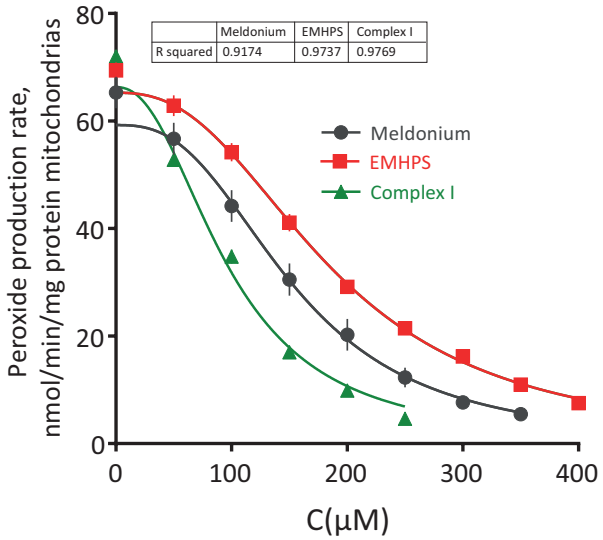

Figure 11 – H_2O_2 production rate of mouse liver mitochondria in presence of meldonium, EMHPS or complex EMHPS with trimethylhydrazinium (Complex I)

Note: experimental data (M±SEM) are shown by dots, logistic regression is shown by lines; EMHPS – ethylmethylhydroxypyridine succinate.

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Measurement of hydrogen peroxide generation

The generation of hydrogen peroxide by mitochondria was assessed using horseradish peroxidase (Thermo Scientific, USA) and its fluorogenic substrate Amplex Red reagent (Thermo Scientific, USA), on a Cary Eclipse fluorescent spectrophotometer (Agilent, USA) in the presence of a catalase inhibitor with a specific inhibitor of 3-amino-1,2,4-triazole.

Statistical data analysis

For the primary analysis, the data were tabulated and descriptive statistics were calculated: mean (M), standard deviation (SD), a standard error of the mean (SEM). The data obtained submitted the Gaussian distribution law, on the basis of which parametric methods of statistic processing were chosen. Statistical analysis of the data was performed using non-linear regression methods, the Student's test for the comparison and a one-way variance analysis (for the comparison of several samples). The differences were considered significant at $p < 0.05$. The data were analyzed using Microsoft Excel 2016 (Microsoft, USA) and Prism 5.0 (Graphpad, USA) software.

RESULTS

Measurement of the transmembrane potential of mitochondria

When evaluating the effect of the studied drugs on the indicators of the mitochondria transmembrane potential, it was shown that when the increasing drugs concentrations were added, it decreased. The least pronounced effect was exerted meldonium, for which the value of the semi-effective concentration (IC_{50}) was 419±17 μM. EMHPS had a significantly ($F(1.22) = 82.90$; $p < 0.0001$) more pronounced effect on the potential (IC_{50}=275±6 μM).

The complex of trimethylhydrazinium propionate and EMHPS components reduced the potential of mitochondria with IC50=197±5 μM, which is significantly less compared to only meldonium ($F(1.15) = 166.5$; $p < 0.0001$) or only with EMHPS ($F(1.22) = 107.5$; $p < 0.0001$). When the uncoupler FCCP was added, the mitochondrial potential decreased to zero, regardless of the presence of meldonium, EMHPS, or the studied complex in the incubation mixture (Fig. 1).

Mitochondrial respiration

In the absence of the exogenous respiration, the background respiration of mitochondria did not change with the addition of increasing concentrations of meldonium. EMHPS also insignificantly increased the rate of mitochondrial basal respiration from 2.2±0.2 to 3.4±0.25 nmol O_2/min/mg protein ($F(1.47) = 4.34$; $p = 0.0426$), and when the complex of the components was added, the rate of basal respiration increased from 2.9±0.3 to 6.8±1.0 nmol O_2/min/mg of protein ($F(1.40) = 28.95$; $p < 0.0001$), which may indicate the uncoupling of mitochondrial respiration by the action of the TMHP and EMHPS complex (Fig. 2). This effect makes it possible for protons to translocate into the intermembrane space *via* specific respiratory complexes of the electron transport chain and return to the mitochondrial matrix independently of ATP synthase. The established proton "leakage" is an important mechanism for the distribution of energy in the cell and accounts for up to 25% of the basal metabolism. Uncoupling of mitochondria against the background of the action of the components of the complex under study can be considered as a cytoprotective strategy mediated by mitochondrial uncoupling proteins (MUPs) under the conditions of the oxidative stress in any ischemic injury, including aging processes, as well as diabetes and resistance to anticancer drugs.

Then, the mitochondrial respiration stimulated by the substrates of complex I (NADP coenzyme Q oxidoreductases), pyruvate, and malate, was studied (Fig. 3). An increase in the respiration rate stimulated by the substrates of complex I, was found out with an increase in the concentration of the succinic acid complex of TMHP and EMHPS in the cell. Semi-effective concentrations of meldonium and EMHPS were 273±67 and 350±204 μM, respectively, and did not differ significantly ($F(1.90) = 0.21$; $p = 0.6470$). The addition of the studied complex led to a more pronounced increase in the oxygen consumption with IC_{50}=75±6 μM, which is significantly less compared to meldonium ($F(1.83) = 34.37$; $p < 0.0001$) and EMHPS ($F(1.83) = 30.17$; $p < 0.0001$).

After the addition of the complex inhibitor rotenone (2 μM), the rate of pyruvate-malate-stimulated mitochondrial respiration decreased almost to zero and did not change depending on the concentration of meldonium, EMHPS, or studied complex (Fig. 4).

Succinate is a complex II substrate of the electron transport chain; thus, in the presence of rotenone, the respiration stimulated by succinate makes it possible to assess the state of complexes II, III, and IV of the mitochondrial electron transport chain. As shown in Fig. 5, the studied complex effect on the rate of the oxygen consumption during the respiration supported by the complex II substrate of the electron transport chain (ETC) was similar. A semi-effective concentration for these two substances was 237±81 μM and 453±1059 μM and did not differ significantly ($F(1.90) = 0.20$; $p = 0.6596$). An equimolar mixture of the components in the complex stimulated the respiration with IC_{50} = 141±27 μM. The differences with meldonium ($F(1.83) = 3.55$; $p = 0.0632$) and EMHPS ($F(1.83) = 3.90$; $p = 0.0516$) approached the statistical significance level.

To assess the mitochondrial conjugation, the respiration rate was measured in the presence of excess ADP and phosphate (Fig. 6), and then in the presence of 1 μM oligomycin, the ATP synthase inhibitor (Fig. 7). It was found out that in the presence of excess ADP, meldonium

and EMHPS did not affect the respiration rate, either alone or in the complex. Against the background of ATP synthase blockade by oligomycin meldonium, EMHPS and succinic acid complex with trimethylhydrazinium increased the respiratory rate from IC_{50}=380±699, 536±1578 and 165±40 μM. The significant differences in IC_{50} did not reach the statistical significance.

The rate of the FCCP-uncoupled respiration did not change with the addition of meldonium, EMHPS, or the complex under consideration (Fig. 8).

Finally, the respiration rate did not change during the inhibition of cytochrome C oxidase by cyanide after the addition of meldonium, EMHPS, or component complex (Fig. 9).

Thus, the data on the effect of the studied complex on the mitochondrial respiration indicate the restoration of the oxygen exchange in the cells to ensure normal life and modulation of cellular metabolism under conditions of cardiovascular risks.

Assessment of ATP synthesis

According to the results of the experiment, it was found out that meldonium, EPGMS and succinic acid coordination complex with trimethylhydrazinium have a pronounced effect on the ATP production by mitochondria (Fig. 10). Thus, the least pronounced effect on the rate of the ATP production was exerted by EMHPS, for which the concentration of the half-maximal inhibition was 321±168 μM. A half-maximal decrease in the ATP production with the addition of meldonium was observed at the substance concentration of 216±6 μM. The most pronounced decrease in the ATP production was observed with the addition of the TMHP coordination complex and EMHPS with IC_{50}=136±4 μM.

Peroxide production rate

The peroxide production rate was evaluated fluorometrically. As shown in Fig. 11, TMHP, EMHPS and their complex reduced the hydrogen peroxide production by isolated mitochondria. EMHPS had the least effect on the peroxide generation (EC_{50}=186±6 μM), which is significantly less than that of TMHP (IC_{50}=153±11 μM, $F(1.113) = 16.36$, $p < 0.0001$). The greatest suppression of the peroxide production was observed when complex of meldonium and EMHPS were added to the isolated mouse liver mitochondria (IC_{50}=96±10 μM), which was significantly less than for meldonium alone ($F(1.92) = 68.94$, $p < 0.0001$) or for EMHPS alone ($F(1.99) = 310.2$, $p < 0.0001$). These results indicate a pronounced decrease in the production of reactive oxygen species and the antioxidant effect of the complex under consideration, which determines its protective effect on cells under conditions of ischemia and hypoxia.

DISCUSSION

Means of metabolic therapy are increasingly used in practical medicine. The representatives of this pharmacotherapeutic group are trimethylhydrazinium propionate and ethylmethylhydroxypyridine succinate, widely known in the domestic pharmaceutical market. Meldonium is the tool that makes it possible you to "shunt" bioenergetic processes, switching the cell to a more energetically favorable mode of functioning. As a rule, this is reflected in a decrease in the intensity of fatty acid β-oxidation reactions and the predominance of carbohydrate metabolism reactions in the energy production. It is important that meldonium has a selective effect on the ischemic tissue, with virtually no effect on intact tissue areas. That makes it possible to avoid the "steal" effect [17].

The action of ethylmethylhydroxypyridine succinate is primarily aimed at suppressing the processes of lipid peroxidation and reducing the total ROS pool in the cell, as well as stimulating the energy production. The use of ethylmethylhydroxypyridine succinate limits the production of reactive oxygen and nitrogen species, eliminates negative endothelial effects in the form of an increase in the activity of inducible nitric oxide synthase, and increases the activity of endogenous antioxidant defense enzymes (superoxide dismutase, catalase). The presence of a succinate fragment in the structure of the molecule allows this compound to act not only as an antioxidant, but also as a direct substrate of mitochondrial complex II, which, given a high bioavailability, can contribute to the high metabolic activity. The variability of the targeted effects of complex TMHP and EMHPS may underlie their synergy with respect to the energy-producing function of cells. Currently, the domestic pharmaceutical market presents a neuro- and cytoprotector based on the coordination complex of trimethylhydrazinium propionate and ethylmethylhydroxypyridine succinate – Brainmax® – which has antiamnestic, antihypoxic, antioxidant and antiischemic effects [18].

In this regard, an investigation on the study of the mitochondria-directed action of the complex under consideration was conducted. As a result, it was shown that in the culture of mitochondria, compounds with *pK* of about 4 (trimethylhydrazinium propionate and ethylmethylhydroxypyridine succinate) act as moderate uncouplers of a mitochondrial respiration. This fact can be associated with the presence of a positively charged atom of tertiary nitrogen (trimethylhydrazinium) and heterocyclic nitrogen (ethylmethylhydroxypyridine succinate) in the structure of these compounds. In this connection, a formation of ion pairs between these compounds under physiological conditions and the corresponding pH value can be assumed. It is of interest to note that nitrogen with a positive charge closed by methyl groups can play the role of a penetrating cation and, upon the formation of an ion pair, increase the efficiency of the counterion delivery (in this case, succinate), including with subsequent proton dissociation. Thus, the coordination complex

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under consideration can be a donor of additional protons (protonophores), which are so necessary for the respiratory chain.

The use of respiration uncouplers (the complex under study), which moderately increase the proton conductivity of mitochondria, can eliminate the negative effects caused by an increase in the ROS generation by mitochondria [19].

This assumption is confirmed, first, by the activation of the endogenous substrates (pyruvate and malate) utilization, but to a much greater extent, by the combined action of the studied substances in the concentration range of the tens of nmol order on the parameters of energized mitochondria. The obtained data on the dissipation of the membrane potential correlate well with the data on the decrease in the peroxide generation during a reverse transfer, while the kinetics of the peroxide generation suppression overtakes the kinetics of the ATP synthesis suppression, which makes it possible to attribute the observed phenomenon to the so-called soft depolarization, when the potential is below the threshold value for the formation of peroxides, but ATP synthesis is still possible.

In order for protonophores not to have a toxic effect and not to show their activity in cases where the cell needs ATP synthesis, it is necessary for their activity to depend on the functional state of mitochondria, e.g., on the potential on its inner membrane. In the state of hyperpolarization, the protonophore should remove only the excess potential, but not reduce it excessively, which will inevitably lead to the inhibition of the respiration process. An ideal protonophore should not inhibit the mitochondrial respiration even at relatively high concentrations. Previous attempts were made to synthesize substances with the properties of “soft” mitochondrial uncouplers, but they failed [20].

Thus, the considered hydrosuccinate complex probably acts as such a “soft” uncoupler, that it reduces the intensity of the ROS formation and optimizes ATP synthesis. As a result of the work, a high effect of the complex on the respiratory function of mitochondria has been shown. This study showed that a component complex of metabolic and antioxidant actions increases the basal level of the mitochondrial respiration, which may be relevant for increasing the initially normal respirometric function of mitochondria, for example, in the prevention of hypoxic conditions in the absence of pathology. An increase in the intensity of the stimulated respiration is also an interesting aspect of the metabolic action of the complex.

It was shown that the studied complex increased the intensity of the substrate respiration, and pronounced changes were obtained throughout the mitochondrial respiratory chain, which is an important therapeutic advantage in conditions of oxidation substrates deficiency – in ischemic-hypoxic damages.

The universal metabolic pattern of ischemia is the accumulation of the succinate precursor, cyclic citric acid, which is responsible for the mitochondrial production of reactive oxygen species. Excess succinate is re-oxidized by succinate dehydrogenase, which leads to a rapid accumulation of reactive oxygen species. Transferring the cell into an anaerobic cycle, the trimethylhydrazine component of the studied complex reduces the availability of molecular oxygen species for succinate oxidation, thus interrupting the pathological cascade of the formation of destructive free radicals and exerting a pronounced antihypoxic effect [21].

In addition, taking into account the metabolic profile of the trimethylhydrazinium propionate action, i.e., the restriction of oxygen-demanding processes of fatty acid oxidation with the cell transfer to the intensive carbohydrate metabolism and the shunting effect of ethylmethylhydroxypyridine succinate, an increase in the resistance of cells to oxygen deficiency at different conjugated metabolic levels can be assumed. Thus, by increasing the transport of carbohydrates into the cell and limiting glycolysis mediated by the inhibition of phosphofructokinase, the trimethylhydrazinium fragment can increase the efficiency of the influence degree of ethylmethylhydroxypyridine succinate on electron transport processes, thereby modulating the optimal production of ATP under conditions of ischemia, which is sufficient to maintain the cell normal functioning [22].

Under the prevailing conditions of tissue ischemia, modulation of ATP synthesis may be important for the cell survival. It is known that under conditions of an ischemic stroke, a decrease in the concentration of the intracellular ATP pool to a critical level mediates the activation of caspase-dependent apoptosis reactions, leading to the cell death and increased neuroinflammation reactions [23, 24]. During the manifestation of Alzheimer’s disease, one of the most common neurodegenerative diseases, an increase in the formation of ATP due to the activation of substrate-dependent respiration, i.e., the switching of bioenergetic processes from one used substrate to another, can also prevent spontaneous self-aggregation of the tau protein, thereby suppressing the main pathogenetic cascade of Alzheimer’s disease (in this case, ATP acts as a natural hydrotrope that stabilizes protein molecules) [25].

A significant increase in the succinate-dependent respiration under the influence of the complex (trimethylhydrazinium propionate + ethylmethylhydroxypyridine succinate) under conditions of the activity blockade of the mitochondrial complex I by rotenone, associated with the ROS-inhibiting activity, is likely to achieve certain therapeutic benefits in Parkinson’s disease. It has been established that one of the pathogenetic triggers of this disease is the mitochondrial complex I dysfunction, followed by an increase in electron leakage from the mitochondrial respiratory chain and the development of the oxidative

damage to substantia nigra neurons [26]. Under these conditions, the use of the considered components complex will make possible achieving the effect of "metabolic bypass" of complex I, which will reduce the retrograde current and electron leakage, reducing the formation of ROS.

CONCLUSION

Based on the totality of the results obtained, it can be assumed that the drug Brainmax® leads to the stabilization of mitochondrial function, rationalization of cell function under stress, normalization of energy metabolism in the cell even under conditions of hypoxia, and the elimination of undesirable effects of the ischemic-hypoxic tissue damage. Moreover, for complex of active components with a synergistic interaction, these effects are more pronounced than when used separately. The spectrum of biochemical reactions occurring in the cell under the action of a succinic acid complex with trimethylhydrazinium and the corresponding pharmacological effects may be the subject of further, more detailed studies on the corresponding experimental models of pathological processes.

FUNDING

The study was conducted with the support of PROMOMED RUS LLC. The sponsor had no influence on the choice of material for publication, analysis and interpretation of the data.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

MVZ – analysis of results, text editing; MVG – interpretation of results;
KYaZ – research concept development, text editing; YuGK – data statistical processing, text writing;
VSSh – text writing, references searching; AAAA – organization and conduct of study, results interpretation;
DIP – data analysis, text writing; MYuV – development of design and writing of the research program.

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