ORIGINAL ARTICLE

CONTENTS

Molecular markers of endogenous neuroprotection in the brain of rats with experimental Parkinson's disease treated with various pharmacotherapy regimens

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ABSTRACT

Aim: To study apoptotic processes and their role in the formation of premature dopaminergic neurodegeneration, to identify key biomarkers for early diagnosis and implementation of complex measures towards braking the progression of PD in the early stages, to develop possible treatment regimens with a specific neuroprotective effect on the dopaminergic system.

Materials and Methods: The experiment involved 90 Wistar rats (6 months old, 220–290 g). Parkinsonism was induced by the neurotoxin MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Intact animals received saline (1 ml/100 g, i.p.); the control group received MPTP followed by saline.

Rats were divided into nine groups: I – intact; II – PD control; III – PD + Amantadine (AM); IV – PD + AM + Cerebrocurin; V – PD + AM + Pramistar; VI – PD + AM + Gliatilin; VII – PD + AM + Noophen; VIII – PD + AM + Pronoran; IX – PD + AM + Melatonin.

Results: The data obtained indicate that neuroprotective therapy of PD with drugs such as melatonin, cerebrocurin, pronoran, and gliatilin in combination with amantadine leads to an increase in the expression of the HIF-1α, HIF-3α, and HSP70 genes, and can also serve as a molecular marker for the activation of endogenous neuroprotection mechanisms under experimental PD conditions.

Conclusions: We have experimentally demonstrated a new target of neuroprotection in PD conditions – apoptosis of dopamine-producing neurons and substantiated modulators of this process – drugs for combined therapy with amantadine (melatonin, cerebrocurin, pronoran, and gliatilin) as promising drugs for PD treatment.

KEY WORDS: caspase-3, HIF-1α, HSP70, BCL-2, c-Fos, HIF-3α

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INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases among the elderly. Motor symptoms are primarily due to significant dopamine depletion caused by degeneration of dopaminergic neurons in the compact substantia nigra. Apoptosis, considered one of the main mechanisms of neuronal death in PD, is mediated by a number of initiator and executioner caspases, occurring either intrinsically or extrinsically. The activation of initiator caspase-9 mediates the intrinsic pathway, which is also called the mitochondrial-mediated pathway [1]. Alternatively, the activation of initiator caspase-8 mediates the extrinsic pathway of apoptosis, which is the pathway mediated by the cell death receptor. Both initiator caspases converge on a common pathway of executioner caspases that include caspase-3 and caspase-6. The activation of "caspase killers" leads to the development of morphological features characteristic of apoptosis, such as DNA cleavage and its subsequent fragmentation. Pro-apoptotic factors, i.e. Bax, are involved in neuronal cell death in PD, and there is evidence that both intrinsic and extrinsic apoptotic pathways can play a significant role in this process [2].

Neuronal death occurs during normal development and in response to a variety of pathological factors,

such as traumatic injury, ischemia, infectious agents, or genetic aberrations. The main mechanisms of neuronal death are apoptosis and necrosis. Apoptosis is the predominant mode of neuronal death in many neurodegenerative diseases, including PD. While the pathogenic processes in PD are not fully understood, convergent mechanisms result in neuronal death via apoptosis, making the apoptosis pathway an interesting potential therapeutic target. Cell death via apoptosis is observed in cell culture models of animal with PD, as well as in the nigrostriatal regions of the brains of PD patients at autopsy [3].

Apoptosis, the main pathway of programmed cell death, can be initiated by a number of broad classes of cell death stimuli, including abnormal intracellular calcium concentrations (exitotoxicity), deprivation of afferent or efferent trophic factors, activation of death receptors, and stress. Neuronal apoptosis, which is common observed during development and maturation, is essential for the formation of the nervous system and the development of appropriate neural circuits. This process involves a certain sequence of events that are energy-dependent [4]. It is characterized by specific morphological and biochemical changes, including cell contraction, chromatin compaction, nuclear DNA fragmentation, and the formation of apoptotic bodies containing nuclear material. The cell membrane retains its integrity. Apoptotic bodies are removed by phagocytosis without further inflammatory response. Biochemically, apoptosis is characterized by an increased rate of protein degradation and increased caspase activity. These biochemical components of apoptosis are a group of molecules called the B-cell lymphoma family (BCL-2), apoptotic peptidase activation factor (Apaf-1) and caspases [5].

Evidence from in vitro, in vivo and postmortem studies in humans suggests that apoptotic cell death is involved in PD pathogenesis. Identification of the main triggers of the apoptotic process in PD may contribute to a better understanding of the sequence of events leading to programmed cell death. Consequently, it would be possible to determine potential factors that could be targeted therapeutically to stop or slow down the progression of the disease as well as to recognize people who are predisposed to develop Parkinson's disease at the early and preclinical stages.

AIM

Aim of the study: to study apoptotic processes and their role in the formation of premature dopaminergic neurodegeneration, to identify key biomarkers for early diagnosis and implementation of complex measures towards braking the progression of PD at the early stages, to develop possible treatment regimens with a specific neuroprotective effect on the dopaminergic system.

MATERIALS AND METHODS

The study was conducted in accordance with Directive 2010/63EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes, as well as with the national regulations, such as "General Ethical Principles for Animal Experimentation" (Ukraine, 2001) and "Basic Principles for Studying the Toxicity of Potential Pharmacological Agents" (the State Pharmacopoeia of Ukraine (SPhU), Kyiv, 2000). The experiment was approved by the Bioethics Committee of Zaporizhzhia State Medical University (ZSMU). This study is an expanded presentation and continuation of the study DOI:10.21303/2585-663.2020.001491.

Experimental groups of animals were formed after a 2-week quarantine period. The experimental groups were created based on the age and weight of the rodents. All experiments were conducted at the Educational Medical and Laboratory Center of ZSMU, certified by the Ministry of Health of Ukraine (certificate No. 039/14). The experiments were carried out in a well-lit room in complete silence. During the experiments, the influence of external and internal visual, olfactory and auditory stimuli was excluded.

The study was conducted on 90 Wistar rats aged 6 months and weighing 220-290 grams. The animals were kept in standard vivarium conditions (12-hour light cycle, temperature 22^{0}C). To run the experiments, the animals were subjected to food deprivation, described below in detail. In order to tame the rats, before the experiment, they were hand-handled for 2-3 minutes for 5 days, which facilitated further experimental studies.

Parkinsonism was induced by injection of the neurotoxin MFTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) into experimental rats. The intact group received a single intraperitoneal (parenteral) injection of saline 1 ml per 100 g of body weight, whereas the control group received a single intraperitoneal injection of saline in a similar dosage after the administration of MFTP.

We have developed a concept for PD treatment, which is based on selective effects on the leading neurodegeneration targets: nitrosative stress, deprivation of endogenous neuroprotective factors (HSP70), disruption of the thiol-disulfide system,

mitochondrial dysfunction, inhibition of dopamine transmission, neuroapoptosis, and activation of IL-1b pathways involved in neurodegeneration. To confirm the prospects of the selected target pathways, we selected drugs whose mechanism of action impacts these particular target pathways. Cerebrocurin contains neurotrophic factors and rilin protein, reduces signs of primary and secondary mitochondrial dysfunction, regulates transcriptional processes; Noophen can increase dopamine levels, activates Roberts' compensatory shunt; Pramistar is a nootropic of the racetam class, exhibits dopamine agonist properties, increases the density of nicotinic cholinergic receptors, stimulates energy metabolism; Pronoran has the properties of a dopamine agonist; Melatonin in therapeutic doses stimulates HSP70 expression, normalizes the thiol-disulfide system, modulates the mitochondrial pore, activates the compensatory malate-aspartate shunt, regulates NO \ SN mechanisms of gene transcription; Gliatilin is a nootropic with specific mitoprotective properties and cholinomimetic effects.

The rationale for a PD therapy strategy was based on the study of drug activity in the following groups of animals: I - intact (passive control); II – animals with experimental Parkinson's disease (PD, active control); III – PD + Amantadine (AM); IV – PD + AM + Cerebrocurin; V – PD + AM + Pramistar; VI – PD + AM + Gliatilin; VII – PD + AM + Noophen; VIII – PD + AM + Pronoran; IX – PD + AM + Melatonin.

The treatment course with the investigated drugs in experimentally substantiated doses was as follows: Amantadine Sulfate – 1 mg/kg, Cerebrocurin - 150 µl/kg, Pramistar – 10 mg/kg, Gliatilin – 250 mg/kg, Noophen – 100 mg/kg, Pronoran – 2 mg/kg, Melatonin – 10 µg/kg in animals with experimental PD [4]. All of the above drugs were administered intragastrically, and Cerebrocurin was administered intraperitoneally (parenterally).

All steps in the preparation of brain homogenate were carried out in the cold at +2°C, using the Silent Crusher S homogenizer (Heidolph). The medium used for homogenization of brain tissue was characterized by neutral pH value and the required osmotic pressure which protect the particles from swelling and rupture.

The concentration of HSP70 protein in the cytoplasmic fraction of the brain was determined by Western blot analysis. The proteins were separated in a 10% polyacrylamide gel (PAGE). The separation of protein fractions was performed by electrophoresis: initially at 100 V (to allow stacking), then at 200 V after the samples reached the separating gel, continuing until the dye front reached the end of the gel.

The real-time reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the expression of HSP70, HIF-1α, HIF-3α, and BCL-2 genes. The object of the study was brain homogenate. The molecular genetic study consisted of several stages.

Total RNA was isolated from rodent brain tissue using the TRIzol RNA Prep 100 kit. RNA was isolated according to the kit protocol. Reverse transcription (cDNA synthesis) was performed using the Reverse Transcription Reagent Kit (RT-1). To determine the expression level of the studied genes, the CFX96™-Real-Time PCR Detection Systems amplifier (Bio-Rad Laboratories, Inc., USA) and a set of reagents for PCR-RV in the presence of SYBR Green R-402 were used. The final reaction mixture for amplification included SYBR Green dye, SynTaq DNA polymerase with enzyme-inhibiting antibodies, forward and reverse specific primers, dNTPs, and a cDNA template. Specific primer pairs (5'-3') for the analysis of the studied and reference genes were selected by using PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast) as well as manufactured by Thermo-Scientific (USA). Amplification was performed under the following conditions: initiating denaturation at 95°C for 10 min; then 50 cycles: denaturation at 95°C for 15 s, primer annealing at 58-63°C for 30 s, elongation at 72°C for 30 s. The fluorescence intensity was recorded automatically at the end of the elongation stage of each cycle using the SybrGreen channel. The beta-actin (Actb) gene was used as a reference gene to determine the relative value of changes in the expression level of the studied genes. The comparative Ct method ($_{\Delta\Delta}$Ct method) was used to quantify the relative level of gene expression. Negative controls were included in the experiment: no addition of cDNA matrix in the PCR reaction, no addition of mRNA matrix in cDNA synthesis, no addition of enzyme in cDNA synthesis. Each amplification reaction was performed in triplicate on individual samples.

The beta-actin (Actb) gene was used as a reference gene to determine the relative value of changes in the expression level of the studied genes. The comparative Ct method ($_{\Delta\Delta}$Ct method) was used to quantify the relative level of gene expression. The statistical analysis of real-time PCR data was carried out with the help of CFX Manager™ software (Bio-Rad, USA). Each amplification reaction was performed in triplicate on individual samples. The results of the real-time PCR analysis of Hif-1α, Hif-3α, and HSP70 were presented as the relative normalized expression of these mRNAs.

To detect the expression of c-Fos and VCL-2 proteins in the brain of the animals, the immunohistochemical method of indirect immunofluorescence was used

Table 1. Influence of the studied drugs on the expression of endogenous neuroprotection genes under conditions of PD formation in rats (M ± m; Q50,(Q25;Q75), n = 20)

Marker	HIF1a mRNA expression, a.u./g protein	HIF-3α mRNA expression, a.u./g protein	HSP_{70} mRNA expression, a.u./g protein	Number of Fos-positive neurons per 1 mm²	c-Fos mRNA expression, a.u./g protein
Intact	45.37±4.51	6.26±0.57	39.51±4.48	42.14±2.85	26.81±0.45
Control (PD)	33.70±2.61	1.62±0.12	30.69±2.26	89.18±6.72	48.33±0.39
PD+AM	32.11±2.52*	5.54±4.92	45.04±4.25*	82.71±8.43*	38.75±1.49
PD + Cerebrocurin + AM	32.76±2.77	9.18±7.31*	36.14±4.01*	61.32±6.19*	31.09±1.27
PD+Pramistar +AM	32.88±2.62	5.02±3.19	44.12±3.02	71.63±10.11	35.48±0.56
PD + Gliatilin +AM	32.16±2.81*	7.88±7.11*	36.65±3.56	57.21±7.83	30.15±3.09*
PD +Noophen +AM	34.41±2.95	4.98±3.23	40.26±3.53	65.37±7.02	32.50±1.22
PD +Pronoran+AM	34.07±3.07	8.83±7.65	40.36±4.72*	70.15±9.14*	32.62±0.53*
PD +Melatonin +AM	35.89±3.75*	9.49±3.41	46.97±3.48*	55.43±8.12*	28.89±4.27

Notes: p – the level of statistical significance when comparing samples using ANOVA (Kruskal-Wallis test), *–p≤0.05 relative to the control group
Source: compiled by the authors of this study

after preparation of 15 μm tissue sections. First, primary antibodies to c-Fos and VSL-2 protein (Sigma Chemical, USA) were applied to the sections and incubated at +400°C for 24 hours. After the incubation, the sections were washed three times with 0.1 M phosphate buffer. Then, the samples were coated with secondary antibodies (fluorescein-conjugated goat IgG) (Sigma Chemical, USA) and incubated at room temperature for 60 min. After the incubation, the sections were washed with 0.1 M phosphate buffer. Fos-immunopositive neurons and BCL-2-immunopositive neurons were examined using an Axioskop fluorescence microscope (Ziess, Germany). Images of Fos- and BCL-2-immunopositive neurons of the CA-1 region of the hippocampus were acquired using a fluorescence microscope and captured with a high-sensitivity video camera COHU-4922 (COHU Inc., USA). The images were then processed with the VIDAS digital image analysis system.

IL-4, TNF-α, and caspase-3 were determined by ELISA, an enzyme-linked immunosorbent assay of a sandwich type, based on the specific binding of an antibody to an antigen, with one of the components being conjugated to an enzyme. Samples and standards are incubated in microplate wells coated with antibodies up to the component to be detected. The biotinylated indicator antibody binds to the captured component which is to be detected. The unbound sample material is removed by washing. The streptavidin-peroxidase conjugate binds to the biotinylated indicator antibody. The streptavidin-peroxidase conjugate reacts with the substrate tetramethylbenzidine (TMB). As a result of the reaction with the chromogenic substrate, a colored, detectable product is formed. The enzymatic reaction is stopped by the addition of acid, and the absorbance is measured using a Sirio-S microplate immunoassay reader (seac Radim Company, Italy) at 450nm/630nm. The concentration of the components is calculated from the calibration curve based on the absorbance of known standards. IL-4 was determined using the eBioscience™ IL-4 Platinum ELISA Kit (USA) according to the instructions in the kit.

Statistical processing of the results was performed using Microsoft Excel 2016 with the statistical processing package AtteStat 12. To assess the significance of differences in the study groups, the Kruskal-Wallis test with Dunn's correction was used. Differences at $p < 0.05$ were considered significant.

RESULTS

HIFs are key regulators of neuroprotection and neuroplasticity under the conditions of nervous system pathology, in particular in PD. They stimulate reparative processes in nervous tissue, increasing the pool of free radical scavengers and angiogenesis factors. Additionally, under conditions of PD formation, HIFs increase the energy resources of the neuron, participating in glucose and key glycolysis enzymes metabolism, thereby increasing ATP synthesis. HIFs have pronounced antiapoptotic effects that lead to a decrease in neuronal death in PD.

In the control group of our study, in the rats with PD, under conditions of increased reactions of oxidative, nitrosative stress and deficiency of energy resources in brain tissues, a 25.72% decrease in the synthesis of HIF1a was observed compared to the intact group

of animals, whereas HIF3a synthesis decreased by 74.12%. These processes are associated with the activation of the ubiquitin-independent degradation pathway of oxidatively modified HIF-1α and HIF3a, and the suppression of its synthesis at the stage of translation.

The administration of Amantadine, as well as its combination with Cerebrocurin, Gliatilin, Noophen, Pronoran, and Pramistar, under conditions of PD, had almost no effect on the level of HIF1a mRNA expression compared with the control group, except for the Melatonin group, where a statistically significant increase in the expression of this gene was observed (6.10%). The increase in HIF3a mRNA expression compared with the control group in the Amantadine group was 70.76%, in the Cerebrocurin group – 82.35% ($p \leq 0.05$), Pramistar – 67.72%, Gliatilin – 79.44% ($p \leq 0.05$), Noophen – 67.47%, Pronoran – 81.65%, Melatonin – 82.92% (Table 1).

Protein quality control is an important component of neuronal survival under conditions of PD. We investigated the neuroprotective activity of small chaperones, heat shock proteins HSP70, whose synthesis is increased in response to hypoxia, ischemia, metabolic disorders, and stress. HSP70 has an antiapoptotic effect by inhibiting the action of apoptosis activator protease-1, Bax family proteins; prevents translocation of apoptosis-inducing factor (AIF) to the nucleus and capture of procaspase-3 by the apoptosome; and increases the activity of the antiapoptotic protein BCL-2.

The anti-inflammatory mechanisms of HSP70 in PD involve limiting the local inflammatory response by reducing the synthesis of pro-inflammatory cytokines, inhibiting the release of matrix metalloproteinases and inducible nitric oxide synthase, decreasing NADPH oxidase activity while simultaneously increasing SOD activity, and suppressing the activity of the transcription factor NF-kB along with the kinases, i.e. IkB, JNK, and p38. HSP70 also functions as a signaling molecule, interacting with Toll-like receptors 2 and 4 as well as promoting the activity of immunocompetent cells.

The discovered abnormalities that occur in neurons during PD are accompanied by a deficit in the functioning of the endogenous cytoprotection factor, the HSP70 protein. All this is the result of structural changes and a decrease in the synthesis of the latter under conditions of severe energy deficiency and in the context of reduced levels of neuronal antioxidant defense enzymes. HSP70 deficiency in a neuron against the background of systemic glutathione deprivation is associated with hyperproduction of reactive oxygen species and cytotoxic forms of nitric oxide, which leads not only to modification of various molecules, including HSP70 itself, but also to a reduced activity of genes encoding the synthesis of the latter. HSP70 deficiency is one of the causes of mitochondrial dysfunction. Thus, we found a 22.32% decrease in HSP70 mRNA expression in the control group, compared to the intact group.

The neuroprotective effects of the drugs under study are linked to the normalization of endogenous HSP70-mediated neuroprotection against the background of activated expression of antioxidant enzyme genes, compared to the control group: in the Amantadine group – by 31.86% ($p \leq 0.05$), in the Cerebrocurin group – by 15.08% ($p \leq 0.05$), in the Pramistar group – by 30.44%, in the Gliatilin group – by 16.26%, in the Noophen group – by 23.77%, in the Pronoran group – by 23.96% ($p \leq 0.05$), in the Melatonin group – by 34.66% ($p \leq 0.05$). The antioxidant properties of the studied drugs, combined with the ability to activate the transcription of HSP70 genes and antioxidant enzymes, enhances the resistance of neurons to PD, which indicates an important contribution of these drugs to neuroprotection.

The brains of rats of the control group in the PD model revealed an increased expression of c-Fos (c-Fos-positive neuronal cells) by almost 52.75%, compared to the intact group of the animals, which reflects the progressive activation of apoptotic processes and the death of dopamine-producing neurons. The expression of c-Fos mRNA in the control group grew by 44.53% compared to the intact group. The increased c-Fos gene expression does not always have a protective physiological function. In case of this gene overexpression, c-Fos already plays a negative role, significantly raising the amount of c-Fos protein in neurons, where it is directly involved in DNA fragmentation and initiation of apoptotic cell death.

Given the extremely important role of c-Fos gene expression in physiological and pathological processes, an urgent task of experimental medicine is to find ways of pharmacological correction of various pathological conditions, which will be aimed at correcting hyper- or insufficient c-Fos gene expression. In our study, the following dynamics of Fos-positive neurons was observed: Amantadine administration decreased expression by 7.25% ($p \leq 0.05$), Cerebrocurin – by 31.24% ($p \leq 0.05$), Pramistar – by .68%, Gliatilin – by 35.85%, Noophen – by 26.70%, Proran – by 21.34% ($p \leq 0.05$), Melatonin – by 37.84% ($p \leq 0.05$).

A similar statistically reliable dynamics was also observed in the study of c-Fos mRNA expression against the background of therapeutic effects in rats with PD: a decrease in c-Fos mRNA expression in all groups, but

Table 2. Influence of the studied drugs on the expression of proapoptotic factors and the level of inflammatory process markers under conditions of PD formation in rats (M ± m; Q50,(Q25;Q75), n = 20)

Marker	Density of BCL-2-positive neurons per 1 mm²	BCL-2 mRNA expression, a.u./g protein	Caspase-3, ng/ml	IL-1β, pg/ml	TNF-α, pg/ml
Intact	310.20±9.43	0.23±0.01	2.56±0.12	6.75±0.54	0.15±0.02
Control (PD)	223.80±17.87	0.17±0.01	5.15±0.19	11.35±0.95	0.89±0.09
PD+AM	249.16±22.41*	0.18±0.01*	4.84±0.22*	10.03±0.99*	0.82±0.07*
PD + Cerebrocurin + AM	293.37±2.55*	0.21±0.006*	3.18±0.30	8.15±0.74	0.54±0.07
PD+Pramistar +AM	271.41±3.16	0.20±0.01	4.73±0.41*	9.47±1.31*	0.76±0.05*
PD + Gliatilin +AM	285.87±1.99*	0.22±0.02*	3.05±0.24*	7.54±0.75	0.48±0.03
PD +Noophen +AM	274.94±3.11	0.19±0.01	4.09±0.35	8.89±0.90	0.65±0.05
PD +Pronoran+AM	278.82±3.64	0.21±0.009*	3.69±0.38	7.94±0.72*	0.52±0.05*
PD +Melatonin +AM	302.96±3.42	0.22±0.02	2.83±0.40	7.18±1.03*	0.39±0.06*

Notes: p – the level of statistical significance when comparing samples using ANOVA (Kruskal-Wallis test), *p≤0.05 relative to the control group
Source: compiled by the authors of this study

a particularly pronounced depression of the apoptotic activity of this early response gene was observed when treated with Cerebrocurin, Gliatilin, Noophen, Pronoran, and, especially, Melatonin, with the values being close to the intact group.

We also investigated the changes in the activity of specific regulatory proteins that prevent the implementation of the apoptotic program. One of them, the BCL-2 protein, has a protective effect on many types of cells that are under unfavorable conditions. BCL-2 promotes cell survival, and the antiapoptotic effect of this protein is associated with the normalization of mitochondrial function, which is involved in the initiation of apoptosis. First of all, BCL-2 blocks the release of cytochrome *c* from mitochondria, forms transmembrane mitochondrial pores, which determines the transmembrane potential, as well as the release of various active compounds and ions from mitochondria.

In the control group during PD, the density of BCL-2-positive neurons decreased by 27.85%, compared to the intact group, and BCL-2 mRNA expression decreased by 26.09%. After rats with PD had been treated with Amantadine, the density of BCL-2-positive neurons increased by 10.18% (p≤0.05), compared with the control; with Cerebrocurin – by 23.71% (p≤0.05); with Pramistar – by 17.54%; with Gliatilin – by 21.71% (p≤0.05); with Noophen – by 18.60%; with Pronoran – by 19.73%; and with Melatonin – by 26.13%. There was a synchronous increase in BCL-2 mRNA expression in all experimental groups compared with the control group, especially with the administration of Melatonin, Cerebrocurin, Gliatilin, and Pronoran within the range of statistical significance Table 2.

A 50.29% increase in caspase-3 activity in the control group, compared with the control group, was also a sign of neuronal cell apoptosis activation during PD. The therapeutic effect of Amantadine led to a decrease in this marker by only 6.01% (p≤0.05), while the combination of Amantadine with Cerebrocurin reduced caspase-3 by 38.25%, with Gliatilin – by 40.78% (p≤0.05), with Noophen – by 20.58%, with Pronoran – by 28.35%, and with Melatonin – by 45.05%.

Localized inflammation in brain tissue is an unavoidable consequence of oxidative and nitrosative stress during PD, and results in pathological activation of microglia structures. The following markers served as signs of inflammatory process in our experimental study: IL-1β and TNF-α with a stable elevation in the control group, compared to the intact group, by 40.53 and 83.15%, respectively. The resultant vector of the changes described is a cytokine-mediated increase in the activity of inducible NOS, which leads to an even greater growth in the production of free radicals, as well as to the induction of an apoptotic scenario of neuronal death in experimental PD.

When treating rats with PD with Amantadine, the levels of IL-1β and TNF-α decreased, compared with the control group, by 11.63 and 7.87% (p≤0.05), with Cerebrocurin – by 28,19 and 39.33% (p≤0.05), with Pramistar – by 16.56 and 14.61%, with Gliatilin – by 33.57 and 46.07% (p≤0.05), with Noophen – by 21.76 and 26.97%, with Pronoran – by 30.04 and 41.57%, with Melatonin – by 36.74 and 56.18%.

DISCUSSION

Apoptosis is the main mechanism of neuronal loss in PD, as evidenced by the identification of DNA

fragmentation and apoptotic chromatin changes in dopaminergic neurons of PD patients in pathological studies [6]. Similar approaches to clinical and instrumental evaluation of degenerative and neurofunctional changes have been presented in studies of athletes with intervertebral disc pathology [7]. In addition, the role of apoptosis in PD pathogenesis has been confirmed via in vitro studies showing increased caspase-3 activity and increased expression of active caspase-3 in the substantia nigra pars compacta. Moreover, dopaminergic neuronal death is characterized by a decrease in the expression of antiapoptotic proteins, such as BCL-2, in cellular models of PD [8]. It has also been revealed that caspase inhibitors rescue neurons from death in cellular models of PD, which further supports the idea that apoptosis is the main mechanism of neuronal death in PD. Increased levels of pro-apoptotic proteins, such as Bax, have also been observed in postmortem brain tissue of patients with PD [9].

Although there are some suggestions that the extrinsic apoptotic pathway may be active in PD, its role remains unclear. It is believed that the predominant mechanism of neuronal death is the intrinsic apoptosis pathway [10]. Mitochondria-mediated apoptosis has been extensively studied in PD. It involves a sequence of events: increased formation of reactive oxygen species, cytochrome *c* release and ATP depletion, as well as activation of caspase-9 and caspase-3. It remains unclear how the multiple pathogenic processes of PD, such as alpha-synuclein aggregation and mitochondrial dysfunction, for example, interact with each other to cause apoptotic cell death.

Alpha-synuclein is widely expressed in the central nervous system, especially presynaptically. It is prone to aggregation of fibrils that form the main component of Lewy bodies, which are a pathological feature of Parkinson's disease. During PD, aggregation and inclusion of α-synuclein occurs in the brain, as well as in rodent cells and cells treated with mitochondrial toxins [11]. The accumulation of wild-type α-synuclein in dopaminergic neurons leads to a decrease in mitochondrial complex I activity and an increase in the generation of reactive oxygen species, an effect that is more pronounced during the expression of the aggregation-prone α-synuclein A53T mutant. It has also been shown that α-synuclein localizes on the mitochondrial membrane in SHSY cells and in isolated rat brain mitochondria. This interaction was assumed to lead to oxidative stress and release of cytochrome *c* into the cytosol in in vitro systems. After the release into the cytoplasm, cytochrome *c* interacts with antiapoptotic proteins that promote survival by triggering mitochondrial-mediated apoptosis [12].

Mitochondrial dysfunction can undoubtedly be an early phenomenon in humans and in animal PD models. Impaired mitochondrial complex I activity has been found in the substantia nigra of patients with PD. Dopamine metabolism leads to the formation of reactive oxygen species, which can lower the threshold for apoptotic cell death. Dopamine is enzymatically metabolized by monoamine oxidase (MAO), which triggers the production of H^2O^2, which subsequently results in the formation of reactive oxygen species. The breakdown products of dopamine undergo auto-oxidation, which causes an increase in the formation of reactive oxygen species. Thus, dopaminergic neurons are particularly susceptible to dysfunction of mitochondrial complex I, which is considered one of the main sources of reactive oxygen species in PD. Therefore, the production of reactive oxygen species may be a potentially important mechanism contributing to the death of dopaminergic neurons through apoptosis. It is assumed that impaired mitochondrial complex I activity increases the susceptibility of dopaminergic neurons to degeneration by lowering the threshold for activation of the intrinsic apoptotic pathway [13].

Given that the end point of PD pathogenesis is apoptotic neuronal death, treatments targeting the molecular and biochemical events that allow apoptosis to progress may protect against the loss of dopaminergic neurons. As already discussed, apoptosis depends on the activation of caspases. Thus, inhibition of caspases is considered to be a new therapeutic approach to neurodegenerative diseases that arise due to apoptosis. Indeed, caspase inhibition prevents the death of dopaminergic neurons in the substantia nigra pars compacta induced by MPTP or its active metabolite MPP+ in vitro and in vivo [14].

MPTP is a neurotoxin that selectively damages dopaminergic neurons in the compact part of the substantia nigra. MPTP is a lipophilic substance that actively penetrates the blood-brain barrier and enters the central nervous system, where it is converted into its active metabolite, the so-called MPP+ (1-methyl-4-phenylpyridinium). This conversion is carried out with the help of MAO, which is present in glial cells. After being reuptaken by the dopamine carrier, MPP+ accumulates in the mitochondria of dopaminergic neurons, inhibiting mitochondrial complex I, which leads to ATP depletion and increased generation of reactive oxygen species. As a result, nigrostriatal dopaminergic neurons die via a caspase-dependent apoptotic pathway. Apoptosis induced by MPTP

is characterized by the generation of reactive oxygen species, cytochrome *c* release, p53 expression, caspase-3 cleavage and DNA fragmentation, as well as other morphological features characteristic of apoptosis. The apoptosis induced by MPTP is attenuated by BCL-2 overexpression [15].

However, although dopaminergic neurons could be rescued, nigrostriatal terminals were already damaged, suggesting that this approach may simply ensure the survival of dysfunctional neurons, which means that apoptosis inhibition alone may not be that promising for the treatment of PD. At the same time, the simultaneous administration of glial cell line-derived neurotrophic factor (GDNF) allowed us to circumvent this problem by restoring dopamine concentration in the striatum. Thus, the literature suggests that caspase inhibition in combination with specific growth factors may play a notable role in treating PD in the future [16].

Before caspase activation, intervention in the induction phase of the apoptosis has been considered as a strategy to prevent the death of dopaminergic neurons and restore their function. For example, Bax is activated in dopaminergic neurons to treat MPTP. In addition, genetic deletion of Bax prevented dopaminergic neurodegeneration in a mouse model of MPTP-induced nigrostriatal degeneration. Furthermore, Bax inhibition could reduce the loss of nigral dopaminergic neurons induced by intrastriatal injection of 6-OHDA (6-hydroxydopamine), suggesting that Bax-inhibitory peptides are a possible therapeutic agent for the treatment of PD [17].

The propargylamine derivative CGP 3466 (dibenzo[b,f]oxepin-10-ylmethyl-methyl-prop-2-ynylamine) has been shown to have neuroprotective and antiapoptotic characteristics. CGP 3466 B inhibits neuronal apoptosis by activating protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1, protein-L-isoaspartate (D-aspartate) O-methyltransferase), an enzyme that repairs damaged L-isoaspartyl residues in intracellular proteins. Up-regulation of PCMT1 leads to overexpression of the anti-apoptotic protein BCL-2, as well as under-expression of the pro-apoptotic Bax and active caspase-3, inhibiting mitochondrial-dependent apoptosis, as a result. At the same time, it prevents the death of dopaminergic cells in vitro in PD models of rodents, and thus suppresses the development of motor symptoms caused by MPTP and 6-OHDA. Therefore, therapies that prevent the development of apoptotic pathways may represent promising therapeutic strategies aimed to protect against the loss of dopaminergic neurons and the subsequent pathogenesis of PD in patients [18]. Similar physiological and biochemical adaptation patterns have been observed in studies of vascular and respiratory regulation in athletes [19, 20].

Having discussed these approaches, it should be noted that there are concerns regarding apoptosis being a specific target in neurodegenerative diseases. It is believed that apoptosis in PD is triggered by a number of intracellular pathological processes, with mitochondrial dysfunction being particularly important. So, inhibition of apoptosis can prevent the programmed removal of dysfunctional, non-viable neurons, which can ultimately lead to necrosis and the potential development of an inflammatory response. In cell culture models of PD, treatment with caspase inhibitors did trigger a shift from neuronal apoptosis to necrosis [21]. In addition, despite the fact that genetic deletion of Bax inhibited the death of dopaminergic neurons in response to 6-OHDA in transgenic mice, it failed to improve the behavioral deficits associated with PD, whereas the surviving dopaminergic neurons showed severe neuronal atrophy. In addition, the systemic administration of antiapoptotic drugs can promote prolonged survival and accumulation of dysfunctional and potentially neoplastic cells in many tissues, which will undoubtedly be detrimental to the body as a whole [22].

The site of HSP70 action in this complex cascade of reactions initiating neuroapoptosis is not precisely determined. An alternative pathway for triggering apoptosis via Fas/Apo1 includes the Daxx protein. The mechanism of action of this protein is poorly understood. Normally, Daxx is localized in the nucleus, where it is associated with certain proteins, but it is able to move into the cytoplasm and play the role of an adaptor protein responsible for triggering the JNK kinase cascade by activating Fas/Apo [23]. It is assumed that HSP70 is able to move to the nucleus, where it interacts with Daxx, preventing its release into the cytoplasm and activation of the receptor. It has been previously stated that HSP70 may be involved in the regulation of apoptosis not only at the level of the Fas/Apo1 receptor, but also at the level of certain intracellular target proteins. HSP70 has been shown to prevent mitochondria-induced apoptosis [24]. We have experimentally substantiated for the first time the pathogenetic effect of complex neuroprotection on the damaged brain, which is the ability to modulate the expression of 70kDa heat shock proteins in neurons. An increase in the concentration of HSP proteins in brain tissues was found. Due to chaperone activity and stabilization of actin filaments, these proteins prevent the development of neuroapoptosis and progression of PD.

Cerebrocurin has a complex neuroprotective effect due to its ability to stabilize the functional state of mitochondria and suppress the development of mitochondrial dysfunction; prevent the formation of energy deficiency; block the development of lactate acidosis against the background of activated compensatory mitochondrial-cytosolic shunts of energy production, particularly the malate-aspartate shuttle; reduce the manifestations of oxidative and nitrosative stress; modulate the expression of all NOS isoforms, as well as HIF and HSP proteins; enhance the activity of the antioxidant and thiol-disulfide system enzymes; morphologically stabilize neuronal and glial cells with parallel activation of RNA synthesis in them, as well as restore the morphological ultrastructure of mitochondria; and affect the processes of apoptosis / necrosis, also through regulatory influence on c-Fos expression. Cerebrocurin increases HSP70 expression by activating NFkB. Melatonin in PD increases the level of HSP70 by activating the melatonin receptors MT1 and MT2 [25]. In addition, the unique structure of the melatonin molecule makes it an effective scavenger of ROS / RNS and prevents total damage to polypeptide bonds, inactivation of enzyme systems, and antioxidant links of endogenous protection, including HSP70. Melatonin is also known to have cytoprotective effects by supporting the activity of glutathione peroxidase, Cu, Zn- and Mn-superoxide dismutase, and γ-glutamylcysteine ligase. At the same time, the drug is also capable of inhibiting a number of prooxidant enzymes, such as lipoxygenase and NO synthase, which reduces the production of ROS during PD. The positive effect of melatonin on energy metabolism is explained by its ability to prevent damage to aconitate hydrolase and thus maintain the Krebs cycle at the citrate-isocitrate stage. Pramistar restores thiol-disulfide balance in the brain during PD and limits iNOS expression. By increasing the level of reduced glutathione, Pramistar is able to upregulate the expression of HSP70. Gliatilin promotes synaptogenesis in cholinergic structures, has a mitoprotective effect, increases intra-mitochondrial glutathione levels and is able to elevate HSP70 levels [26].

Thus, although apoptosis is the final stage of the pathogenetic pathway in PD, it remains to be seen whether apoptosis inhibition in PD can be effective and safe. Additionally, a thorough evaluation of the literature data and experimental findings is needed.

CONCLUSIONS

1. The results obtained indicate that neuroprotective therapy of PD with such drugs as Melatonin, Cerebrocurin, Proran, and Gliatilin in combination with Amantadine leads to an increase in the expression of HIF-1α, HIF-3α and HSP70 genes and can serve as a molecular marker indicating activation of endogenous neuroprotection mechanisms in experimental PD.
2. The study of the mechanisms of programmed neuronal death via apoptosis in PD under oxidative stress conditions and pharmacological correction of apoptosis execution mechanisms is a pathogenetically justified target for the treatment of socially important diseases. We propose c-Fos and BCL-2 proteins, along with effector caspase-3, as markers of apoptosis.
3. We have experimentally demonstrated a new target of neuroprotection in PD, i.e. apoptosis of dopamine-producing neurons, and substantiated this process modulators – Amantadine therapy combined with other drugs (Melatonin, Cerebrocurin, Pronoran, and Gliatilin), which can be seen as promising treatment of PD.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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