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Original Contribution

OPTIONS FOR REDUCING OXIDATIVE TOXICITY OF L-DOPA BY COMBINATION WITH SYNTHETIC OR NATURAL RADICAL PROTECTORS

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ABSTRACT

Parkinson disease (PD) is a multifactorial disease that is not well-established. It takes a leading place among contemporary frequent diseases of the central nervous system (CNS). The levodopa (L-dopa) clinical effect is diminished by motor complications resulting from prolonged treatment. Due to the L-dopa neurotoxic effect in the disease treatment, the L-dopa administration is delayed as long as possible in order to avoid side effects. In addition, combining L-dopa therapy with antioxidants (from natural or synthetic origin), may decrease side-effects and provide symptomatic relief. The aim of the current research is through experimental model of healthy mice to explore the possibility to reduce the oxidative stress (OS) induced by the L-dopa drug after its combining with: an essential oil isolated from Rosa damascena Mill., the vitamin C and synthetic antioxidant 1-ethyl-3- [4- (2,2,6,6-tetramethylpiperidine-1-oxyl)] - 1-nitrosourea SLENU. The antioxidants protective effects against the L-dopa oxidative toxicity were evaluated through the oxidative stress indicators - the lipid and protein oxidation end products – measured as MDA, protein carbonyl content, and advanced glycation end products (AGEs) in blood plasma of experimental mice.

Key words: Parkinson disease, L-dopa, natural antioxidants, synthetic antioxidants, PCC, AGEs

ITRODUCTION

Parkinson disease (PD) is a multifactorial disease that is not well-established. It takes a leading place among contemporary frequent diseases of the central nervous system (CNS) [1]. The major clinical disturbances in PD are dopamine depletion consequence in the neostriatum, due to dopaminergic neurons' degeneration [2]. The agent that initiates the disease is unknown. The drug therapy depends on disease severity, remains relatively nonspecific and limited long term efficacy. In the early disease stages, is used a monoamine oxidase β-inhibitor. Initially, the drug is used to inhibit the dopamine degradation. In later disease phases, the patients are treated with Levodopa (L-dopa) a dopamine precursor [3]. The dihydroxyphenylalanine (Levodopa, L-dopa) - is a "gold standard" and the most effective symptomatic treatment for PD, against which new drugs are compared [4]. A number of therapies have been developed in an attempt to improve the disease treatment, such as dopaminergic agonists and inhibitors COMT and MAO-B, but most patients still depend on L-dopa because it is the most able to control the PD symptoms [5]. The L-dopa clinical effect is diminished by motor complications resulting from prolonged treatment. Due to the L-dopa neurotoxic effect in the disease treatment, the L-dopa administration is delayed as long as possible in order to avoid side effects. In addition, combining L-dopa therapy with antioxidants (from natural or synthetic origin), may decrease side-effects and provide symptomatic relief.

Natural antioxidants are isolated from different parts of plants, like flowers, seeds, leaves, roots, bark and their phenolic extracts can slow the lipid oxidation [6]. Rose oil from Rosa damascena *mill* has a wide range of medicinal properties. It contains various substances with antioxidant activity as anthocyanins, flavonoids, terpenes and glycosides [7, 8], kaempferol and quercetin [9]. Moreover, essential rose oil has useful medical properties such as antifungal and antiviral effects,

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improves memory, tones up, and depressions relieves [10].

The vitamin C possesses strong antioxidant properties, and interacts with hydrogen peroxide (H_2O_2), hydroxyl ($\cdot OH$) and superoxide ($\cdot O_2^-$) radicals, and turning them into non-radical products, providing cells with a free radical "neutralizer" [12]. Moreover, the Vitamin C exhibit pro- oxidant properties in the presence of free transition metals, because it reduces ferrions to ferro-ions in a Fenton-like reaction and in the H_2O_2 presence stimulate the hydroxyl radicals formation [13]. Whether the ultimate effect will be a pro-oxidant or an antioxidant depends on the ratio of the ascorbic acid concentration to the available ferrions [14]. At sufficiently high concentrations, ascorbic acid reduces and destroys the formed radicals [15]. Plasma antioxidants traceability under oxidative stress indicates that the ascorbic acid level has been lowered at the earliest, and after depletion, lipid peroxidation has developed, even if plasma tocopherol is in normal concentrations [15].

Over the last decade, many researchers have focused their attention on creating and researching new, more effective synthetic antioxidants to inhibit free-radical reactions mediating specific lesions in biomolecules [16]. The new class discovery of paramagnetic compounds from the sterically hindered N-oxides series, called nitroxyl or iminoxyl free radicals, is an important contribution to medicine. In medical studies they are used as spin-markers, spin-probes, spin traps and paramagnetic models of biologically active nuclear magnetic resonance imaging (MRI) [17] they are generally broad-spectrum compounds [18]. There are three basic conditions for the stability of the nitroxyl radicals, namely: a) delocalization of free valence; b) spatial shielding of the paramagnetic reaction center; (c) Resistance to disproportionation. The most commonly used nitroxyls today are derivatives of the following stable nitroxyl radicals **(Figure 1):**

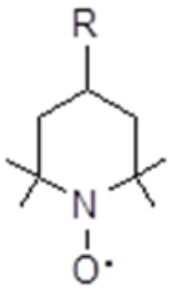

Figure 1. Nitroxyl radical structure

Where: **R = H, TEMPO** (2,2,6,6-tetramethyl-4-oxo-piperidine-1-oxyl); **R = O, O –TEMPO** (2,2,6,6-tetramethyl-4-amino-piperidine-1-oxyl); **R = NH_2, A –TEMPO** (2,2,6,6-tetramethyl-4-oxo-piperidine-1-oxyl). It is believed that by its free valence, nitroxyls have an effect on the oxidative-reductive processes in the cell that are formed by the free radicals formation. The nitroxyl radicals having only one functional group are substances with relatively low acute toxicity. It has been shown that nitroxides at non-toxic concentrations are effective both *in vitro* and *in vivo* [19, 20].

There are several possible explanations for the nitroxyl radicals' protective effect: 1) due to SOD-mimetic action; 2) due to the reduction of metals having the potential to generate specific OH radicals; 3) termination of the free-radical chain reaction induced by alkyl, alkoxyl, alkylperoxyl radical species, and detoxifying drug-derived radicals, and 4) detoxification of toxic metals such as ferric and cupric ions. *In vitro* was detected the synergistic effect of the spin-labeled nitrosourea 1-ethyl-3- [4- (2,2,6,6-tetramethylpiperidine-1-oxyl)] - 1-nitrosourea (SLENU) on the cytotoxicity of bleomycin and farmorubicin in human lymphoid leukemic tumor cells [21]. Recent studies have demonstrated the protective effect of SLENU against drug-induced oxidative stress in liver and blood in experimental animals [22, 23].

The aim of the current research is through experimental model of healthy mice to explore the possibility to reduce the oxidative stress (OS) induced by the L-dopa drug after its combining with: an essential oil isolated from Rosa damascena Mill., the vitamin C and synthetic antioxidant 1-ethyl-3- [4- (2,2,6,6-tetramethylpiperidine-1-oxyl)] - 1-nitrosourea SLENU.

The antioxidants protective effects against the L-dopa oxidative toxicity were evaluated through the oxidative stress indicators - the lipid and protein oxidation end products – measured as MDA, protein carbonyl content, and advanced glycation end products (AGEs) blood plasma of experimental mice.

MATERIALS AND METHODS

CHEMICALS

Spin-labeled drug SLENU was previously synthesized by Gadzheva V et al 2001. Stannous Chloride dehydrated ($SnCl_2.\ 2H_2O$), the spin- trapping agent, PBS and $K_3[Fe(CN_6)]$, 2-Thiobarbituric acid (TBA), L-3,4 dihydroxyphenyl alanine (L-dopa), L-Ascorbic acid were purchased from Sigma-Aldrich Chemie GmbH (Germany). All other

chemicals used in this study were analytical grade. Deionized and distillated water were used for all experiments. Essential Rose oil was provided from the Institute for Roses and Aromatic Plants, Kazanluk, Bulgaria.

ANIMALS

In the experiment were used male non-inbred albino mice (25-40 g). The experimental animals were housed in polycarbonate cages in controlled conditions: 12 h light/dark cycles, 18-23^0 C and humidity 55%, with free access to tap water, provided by the Trakia University vivarium, Stara Zagora, Bulgaria. The animal procedures were in accordance with *Directive 2010/63/EU* on the protection of animals used for experimental and other scientific work, and approved by the *Ethical Committee for Animals of BABH and Trakia University, Stara Zagora, Bulgaria (131/ 6000-0333/ 09.12.2015).*

Mice were divided into five groups (12 animals in each group). The control group of mice was inoculated two i.p. injections with solvent, only. The second injection was administered 45 min after the first. To study the L-dopa effect we used the acute model of Bottiglieri et al., [24]. The mice from all tested groups (except controls) received either two i.p. injections of L-dopa (100 mg/kg) followed by benserazide (10 mg/kg). The second injection was administered 45 min after the first. The groups undergoing combination therapy were pre-treated first for one hour with i.p. injections in doses of 400 mg/kg of Ascorbic acid, Rose oil and after that received L-dopa and benserazide. The group with synthetic antioxidant SLENU was pretreated first for one hour with i.p. injections in doses of 40mg/kg SLCNU [16] and after that received L-dopa and benserazide. All mice were sacrificed by light anesthesia (Nembutal 50 mg/kg i.p.) after 30 min. Fresh blood (1-2 cm^3) was collected directly from the heart in cold EDTA-containers (5 cm^3 Monovette, Germany), and centrifuged at 4000 rpm, 10 min, 4°C and plasma samples were carefully separated.

Ex vivo SPECTROPHOTOMETRY ASSAY FOR EVALUATION THE LEVELS OF MDA

To evaluate the levels of lipid peroxidation, Thiobarbituric acid reactive substances (TBARS) assay was used, which measures MDA reactive substances [25]. The spectrophotometric measurements were performed on a Thermo Scientific spectrophotometer.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Preliminarily were determined the total protein amount in the test sample with the Total Protein Kit, Human. Each protein sample were diluted to 10 μg/mL in 1X PBS, pH =7.4 prior to use in the assay. Protein carbonyl content was measured using an OxiSelect protein carbonyl ELISA kit (Cell Biolabs). Briefly, the BSA standard (reduced/oxidized) and the assayed samples (10μg/mL) were adsorbed on a 96-well plate for 2 hours at 37°C. Protein carbonyl found in samples and standard reacted with DNPH to form DNP hydrazone, treated with anti-DNP antibody and HRP conjugated secondary antibody. Protein carbon content in the samples was determined by a standard curve prepared from the absorbance obtained on the basis of the oxidized/reduced BSA standards and the protein carbonyl content in the samples were calculated in nmol/mg.

The AGEs level was similarly assessed with an OxiSelect AGE competitive ELISA kit (Cell Biolabs). an AGE conjugate is coated on an ELISA plate. The unknown AGE protein samples or AGEBSA standards are then added to the AGE conjugate preabsorbed ELISA plate. After a brief incubation, an anti-AGE polyclonal antibody is added, followed by an HRP conjugated secondary antibody. The content of AGE protein adducts in unknown samples is determined by comparison with a predetermined AGE-BSA standard curve.

STATISTICAL ANALYSIS

Statistical analysis was performed with Statistica 7, StaSoft, Inc. and the results were expressed as means ± S.E. All data were expressed as mean ± SE and obtained by one-way ANOVA. $p>0.05$ was considered statistically significant. To define which groups are different from each other were used LSD post hoc test.

RESULTS

In the current manuscript was investigated the possibilities of reducing the oxidative stress induced by L-dopa by combining with liposoluble Rose oil and spin-labeled nitrosourea SLENU that cross the blood-brain barrier. The results were compared with animals treated with L-dopa alone and with a reference antioxidant –vitamin C (ascorbic acid). Spin-labeled nitrosourea SLENU was administered at a dose exhibiting *in vivo* maximum antioxidant activity (maximum "curative" dose) 40mg/kg [21]. Based on literature data [26], we selected the 400mg /kg dose as "protector" for essential oils and the referent antioxidants –ascorbic acid.

MDA LEVELS IN BLOOD PLASMA

Figure 2 shows the plasma MDA level in healthy untreated mice and mice treated with L-dopa and combinations of L-dopa with synthetic and natural antioxidants.

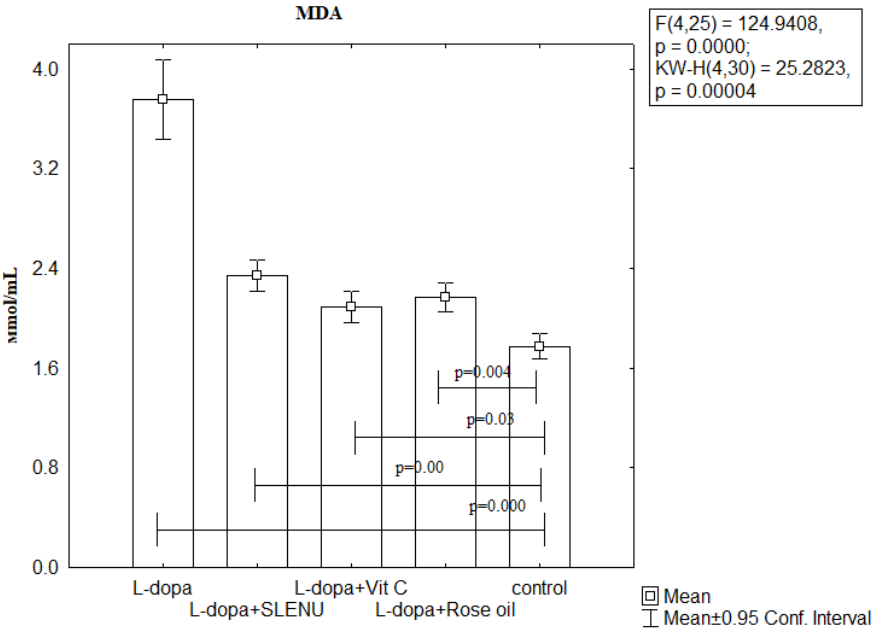

Figure 2. The lipid peroxidation products measured as MDA level in mice treated with L-dopa alone and in combination of L-dopa + SLENU/ Ascorbic acid/ Rose oil were statistically significant higher than controls, p<0.00, t-test; (F (4,25) = 2316.3257, p = 0.0000; KW-H(4,30) = 27.8834, p = 0.00001). According the LSD post hoc test L-dopa vs controls p=0.000130; L-dopa + SLENU vs controls p= 0.000173; L-dopa + Ascorbic acid vs controls p=0.028226; L-dopa + Rose oil vs controls p= 0.004231). The results are presented as mean ± S.E. p <0.01; (*) relative to controls; (**) relative to L-dopa.

The lipid peroxidation products measured as MDA level from mice treated with L-dopa alone was statistically higher compared to controls (mean 3.75 ± 0.12µmol/mL, vs mean 1.77 ± 0.03µmol/mL, p<0.00, t –test). The L-dopa +SLENU combination the plasma MDA is also statistically higher than controls (mean 2.34 ± 0.04µmol/mL, vs mean 1.77 ± 0.03µmol/mL, p<0.00, t –test) and statistically lower than samples treated with L-dopa alone (mean 2.34 ± 0.04µmol/mL, vs mean 3.75 ± 0.12µmol/mL, p<0.00, t–test). The MDA level in the combination L-dopa+ Asc. acid is statistically significantly higher than the controls (mean 2.08 ±0.04µmol/mL vs mean 1.77 ± 0.03µmol/mL, p<0.00, t –test) and statistically significantly lower than those of mice treated with L-dopa alone (mean 2.08 ± 0.04µmol/mL vs mean3.75 ± 0.12µmol/mL, p <0.00, t–test). The MDA level in combination L-dopa+Rose oil is statistically significantly higher than controls (mean 2.16 ±0.05µmol/mL vs mean 1.77 ± 0.03µmol/mL, p< 0.00, t-test), and statistically lower than samples treated with L-dopa alone (mean 2.16 ± 0.05µmol/mL vs mean 23.75 ± 0.12µmol/mL, p <0.00, t-test).

PROTEIN CARBONYL CONTENT (PCC) DETERMINATION IN BLOOD PLASMA

Figure 3 shows the protein carbonyl content in blood plasma from mice treated with L-dopa alone and in combination with antioxidants. Compared to controls the PCC in L-dopa group is statistically significant higher then controls (mean 5.23 ± 0.01nmol/mg, vs mean 1.55 ± 0.04 nmol/mg, p<0.000, t –test), and all group pretreated with combination L-dopa + antioxidants.

The PCC measured in group treated with L-dopa + SLENU is statistically significantly higher compared to controls (mean 4.51 ± 0.03 nmol/mg vs. mean 1.55 ± 0.04 nmol/mg, p <0.000, t-test). Against the group treated with L-dopa alone the PCC is statistically lower (mean 4.51 ± 0.03 nmol/mg vs. mean 5.23 ± 0.01 nmol/mg, p <0.000, t-test). Statistically significant increase in PCC is observed in group treated with L-dopa +Asc acid versus controls (mean 3.18 ± 0.09 nmol /mg vs. mean 1.55 ± 0.04 nmol /mg, p <0.000, t-test), but compared with group treated with L-dopa alone, the PCC level was statistically significant lower (mean 3.18 ± 0.09 nmol/mg

vs. 5.23 ± 0.01nmol/mg, p <0.00, t-test). The L-dopa + Rose oil combination versus controls was statistically significantly higher (mean 3.07 ± 0.02 nmol/mg vs. mean 1.55 ± 0.04nmol/mg, p <0.000, t-test), and statistically significantly lower in samples treated with L-dopa alone (mean 3.07 ± 0.02 nmol/mg vs. mean 5.23 ± 0.35nmol / mg, p <0.000, t-test).

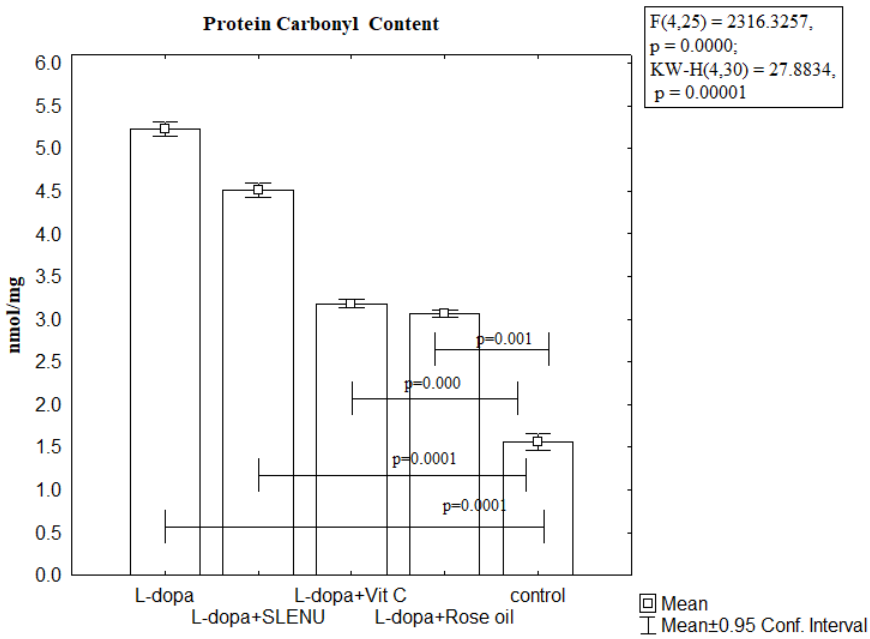

Figure 3. The protein carbonyl content (PCC) in mice treated with L-dopa alone and in combination of L-dopa + SLENU/ Ascorbic acid/ Rose oil were statistically significant higher than controls, p<0.00, t-test; (F(4,25) = 124.9408, p = 0.0000; KW-H(4,30) = 25.2823, p = 0.00004). According the LSD post hoc test L-dopa vs controls p=0.0001; L-dopa + SLENU vs controls p= 0.0001; L-dopa + Ascorbic acid vs controls p=0.000; L-dopa + Rose oil vs controls p= 0.001). The results are presented as mean ± S.E. p <0.05; (*) relative to controls; (**) relative to L-dopa.

ADVANCED GLYCATION END PRODUCTS (AGES) BLOOD PLASMA

The AGEs **(Figure 4)** in blood plasma in group treated with L-dopa alone was statistically significantly higher compared to control group (981.36 ± 5.99 µg/ml vs 431.45 ± 7.77 µg/ml, p <0.000, t-test). Statistically significantly increase was observed in pretreated with antioxidant groups compared to controls: L-dopa + SLENU versus controls 706.91 ± 10.1 µg/ml vs 431.45 ± 7.77 µg/ml, p <0.000, t-test; L-dopa + Ascorbic acid versus controls 685.3 ± 8.8 µg/ml vs 981.36 ± 5.99 µg/ml, p <0.000, t-test; and versus L-dopa alone L-dopa + SLENU versus L-dopa 706.91 ± 10.1 µg/ml vs 981.36 ± 5.99 µg/ml, p <0.000, t-test; L-dopa + Ascorbic acid versus controls 685.3 ± 8.8 µg/ml vs 981.36 ± 5.99 µg/ml, p <0.000, t-test.

CORRELATIONS BETWEEN PARAMETERS

All parameters show positive correlation MDA vs AGEs show r=0.886, p=0.000; MDA vs PCC r=0.823, p=0.000; PCC vs AGEs show r=0.899, p=0.000.

DISCUSSION

Protein carbonyl formation is an important marker for protein oxidation that results from free radical attack on amino acid side chains [27]. The PCC increases in stress and the accumulating carbonyls cause protein damage and dysfunction. Another oxidative stress–related biomarker was AGEs. Due to their synergism with oxidative stress, AGEs have been associated with all major neurodegenerative diseases. Augmented accumulation of AGEs may indicate an accelerated process of aging-related deterioration that likely accompanies neurodegeneration. However, it should be noted that AGEs mainly accumulate on long-lived proteins. The analysis of AGEs in blood may therefore not necessarily reflect their tissue levels, yet circulating AGEs keep their biomarker potential [27].

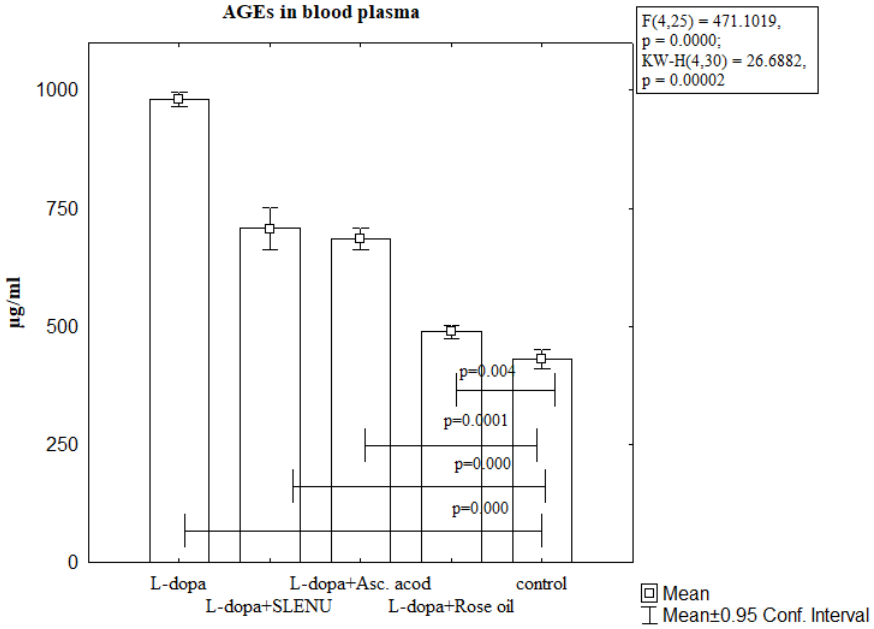

Figure 4. The Advanced glycation end products (AGEs) blood plasma in mice treated with L-dopa alone and in combination of L-dopa + SLENU/Ascorbic acid/Rose oil were statistically significant higher than controls, p<0.00, t-test; (F(4,25) = 471.1019, p = 0.0000; KW-H(4,30) = 26.6882, p = 0.00002). According the LSD post hoc test L-dopa vs controls p=0.000; L-dopa + SLENU vs controls p= 0.000; L-dopa + Ascorbic acid vs controls p=0.0001; L-dopa + Rose oil vs controls p= 0.004). The results are presented as mean ± S.E. p <0.05; (*) relative to controls; (**) relative to L-dopa.

The obtained from our study results in mice blood after treatment with L-dopa compared to controls showed a significant increase in lipid oxidation end products (MDA, p <0.00) of proteins (PCC, p <0.00) and advanced glycation end products (AGEs, p<0.00). These results can be related to the presence of oxidative stress after treatment with L-dopa. The effect of spin-labeled SLENU nitrosourea on induced oxidative stress under L-dopa in experimental models of healthy mice showed that after L-dopa + SLENU administration, the MDA levels p <0.00, PCC p <0.00 and AGEs p <0.00 are declining. A similar statistically significant decrease in the levels of oxidative stress is also observed in the combination of essential oil + L-dopa and referent vitamin C + L-dopa. The results obtained from treatment with L-dopa and combination of L-dopa and synthetic or natural antioxidants can summarize as *in vivo* successfully neutralizing the ongoing oxidative processes in blood resulting in oxidative status normalization in the test animals. Natural polyphenols and terpenes contained in rose oils may have a protective effect on a number of pathological conditions, including neurodegenerative diseases [28]. Phenols and flavonoids are the main ingredients noted in most of the ethereal plants (as well as rose oil) reported by many researchers to have antioxidant and free radical activity [6, 29, 30]. The neuroprotective effects of many polyphenols and terpenes have the ability to pass through the blood-brain barrier and directly capture the pathological concentrations of ROS and RNS and chelated transient metal ions and exert antioxidant activity directly in brain cells [28].

Essential oils as antioxidants have been studied in detail to investigate their protective role for highly unsaturated lipids in animal tissues [31]. They shown their actions as hepatoprotective agents [32] and coincide with our results. Furthermore, oils possess antioxidant properties at extremely low dilution rates administered either by inhalation or by lipophilic fractions, interacting with the lipid parts of the cell membranes, thereby altering the calcium ion channels activity and in some dosas saturated the membranes [33]. The results obtained from a blood MDA after treatment with the combinations L-dopa + ascorbic acid showed levels commensurate with the controls and statistically decreased from the L-dopa-only group. This appears to confirm that the endogenous antioxidant - ascorbic acid can successfully limit peroxidation processes in lipids under the described experimental conditions. Combinations with ascorbic acid showed a statistically significant decrease in PCC compared to L-dopa treated only mice and a statistically significant increase compared to control mice (p =0.00). Studies have shown that vitamin C can slowly migrate proteins

under oxidative conditions and protect them from UV oxidation [15]. In plasma, vitamin C is not able to protect proteins from the action of ROS and oxidation-generating systems but is able to protect isolated lipoproteins from oxidation [15].

CONCLUSION

Antioxidants - polyamines, flavonoids, ascorbic acid, plant phenols, vitamin C, vitamin E, etc. can stabilize the membranes by reducing their permeability and are able to bind to the free fatty acids. It is believed that essential oils can act as antioxidant agents. The use combinations of L-dopa with natural or synthetic antioxidants, may be a necessary approach in modern therapy of Parkinson's disease. The combination of L-dopa+Rose oil improves oxidative status in experimental animals and could be applied in medical practice.

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