# Natural Complexes Are More Effective in Neuroprotection than Single Antioxidants

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**Abstract.** Antioxidants can delay or prevent apoptosis of neurons in animal Parkinson's disease models. The aim of this study was to find out which of known antioxidants is the most effective in neuroprotection. Using morphological and behavioral tests in rats treated with different antioxidants, correlations between extension of dopaminergic neurons lesions in the mesolimbic and nigrostriatal midbrain after 6-hydroxydopamine (6-OHDA) neurotoxin injections were investigated. We found that some antioxidants were capable in preventing 6-OHDA-induced apoptosis of neurons to a considerable extent. The efficacy of the tested antioxidants corresponded to the following sequence: wheat malt>Melatonin>Trolox>$Q_{10}$. We conclude that though Melatonin has a significant protective effect preventing the 6-OHDA-induced apoptosis of neurons in the midbrain structures, the natural complexes derived from food, such as wheat malt, are more effective in neuroprotection than commercial preparations of separate pure antioxidants. Natural extracts of antioxidants may be beneficial to prevent or delay age-related neurodegenerative diseases in humans.

**Keywords:** Parkinsonism, 6-hydroxydopamine, rat model, antioxidants.

## 1 Introduction

Parkinson's disease (PD) is one of the most widespread neurodegenerative diseases. The basis of this pathology is selective loss of dopaminergic (DA-ergic) neurons of the *substancia nigra* (*SN*) and the *ventral tegmental area* (*VTA*) and, as a consequence, dopamine (DA) deficiency in the mesostriatal system of the brain. DA, in comparison with other catecholamines, most easily succumbs to enzymatic and auto-oxidation, that leads to the formation of endogenous toxins and reinforcement of the generation of active forms of the oxygen radicals [1, 2]. Intensified production of free radicals in the brain tissues facilitates the death of DA-ergic neurons; thus, the radicals are additional damaging factors [3, 4]. However, the antioxidant system to a certain extent successfully withstands the negative effect of free radicals. The malfunction of the antioxidant system in the brain tissues is one of the important reasons for the neuronal death during the development of PD in humans. In postmortem investigations of the brains of PD patients significant decrease in the glutathione level in the striatum and the *SN* was observed [5]. The development of oxidative stress in PD and its role in damage of the cell membranes, organelles and nucleic acids of the DA-ergic neurons has been shown earlier on the MPTP-induced model of Parkinsonism in animals [6]. Though, there are no reasons to suppose that the products of free-radical oxidation induce apoptosis of nigral DA-ergic neurons, other damaging agents also exist [7]. Oxidative stress is associated with DA deficiency in the mesostriatal system and also with abnormalities of the L-arginine-NO synthesis pathways in various structures of the brain [8, 9].

Unilateral lesion of DA-ergic neurons in the *SN* is usually performed on rats using intracerebral injections of a selective neurotoxin, 6-hydroxydopamine (6-OHDA). It is generally agreed that this neurotoxin, which was found in the brain tissues [10] and in the blood [11] of parkinsonian patients, can be produced in the organism under natural conditions due to auto-oxidation of DA. So, 6-OHDA can be considered as an important endogenous pathogenic factor responsible for the development of PD. It is also known that free-radical oxidation underlies the loss of DA-ergic neurons caused by 6-OHDA injections into the brain [12]. We have shown previously that the level of unilateral degeneration of DA-

 

ergic mesostriatal neurons in experimental hemi-parkinsonian model in rats can be easily detected by a behavioural apomorfine (Apo) test [9]. The apoptosis of more than 90% of DA-ergic nigral neurons, which is characteristic for patients with the bright clinical picture of PD, can be revealed in rats by intense rotational movements caused by systematic injections of the DA receptor agonist Apo into animals with unilateral destruction of DA-ergic cells in the *SN* and *VTA* [13]. In our numerous previous experiments on animals, we have found that injections of 6-OHDA into the brain were accompanied by the development of unilateral degeneration of a significant number of DA-ergic neurons in the *SN* and *VTA*, and the manifestation of the respective behavioural asymmetry in about 40% of the operated animals [8, 9, 14]. We suppose that the decrease in this parameter caused by some antioxidants can be considered as a proven mark of their protective effects. Therefore, in the present study we aimed to investigate the efficacy of some well-known antioxidants, in particular Trolox (vitamin E), Melatonin, $Q_{10}$, and wheat malt in attenuation of behavioural asymmetry in rats with experimental hemi-parkinsonism.

## 2 Methods

### 2.1 Animals

All experiments were carried out on male Wistar rats weighing 200 – 250 g before surgery. Animal care and all experimental procedures were performed in accordance with the European Communities Council Directive (1986, 86/609/EEC) and in conformity with the guidelines of the Ministry of Public Health of Ukraine. Animals were divided into five groups (Grs). Gr I included 197 rats, which were gathered during many years of work at various projects, without any additional treatment before or after intracerebral injections of 6-OHDA and served as the control. Gr II consisted of 28 rats fed *per os* with $Q_{10}$ (10 mg/kg, Nature's Sunshine Products, Inc, USA) during 30 days before and 7 days after injections of 6-OHDA. 25 rats of Gr III were subjected to *i.p.* injections of an antioxidant Trolox (10 and 50 mg/kg, Sigma, USA) 10 min before and 4 h after 6-OHDA injections, respectively. Gr IV included 16 animals, which were subjected to *i.p.* injections of Melatonin (10 mg/kg, Sigma, USA) 10 min before injections of 6-OHDA. Gr V included 19 animals, which were fed *per os* with wheat malt during 30 days before and 7 days after 6-OHDA injections. Animals of all groups were housed under natural illumination conditions with *ad libitum* access to food and water.

### 2.2 Stereotaxic Surgery

Unilateral lesions of mesolimbic and nigrostriatal DA-ergic neurons were made under general Nembutal anesthesia (50 mg/kg, *i.p.*, Sigma, USA) by stereotaxic injection of 6-OHDA (8 μg, Sigma, USA) into the left medial forebrain bundle using the following stereotaxic coordinates: A -2.2, L +1.5 with respect of the *bregma*, V -8.0 with respect to the *dura mater*; the tooth bar was localized 4.5 mm above the interaural line [15]. The neurotoxin was dissolved in 4 μl of 0.9% ice-cold saline with 0.1% ascorbic acid, added to prevent oxidation of neurotoxin, and injected using glass micropipettes (tip diameter 80–100 μm) attached to the micro-syringe. Administration of pargyline (40 mg/kg, *i.p.,* Sigma, USA) was made 30 min before the neurotoxin injection to inhibit metabolic transformation of the neurotoxin by monoamine oxidase. In addition, desipramine (25 mg/kg, *i.p.*, Sigma, USA) was injected to block the uptake of 6-OHDA by noradrenergic neurons [9].

### 2.3 Behavioral Assessment

Seven days after the surgery all animals were screened with the DA receptor agonist Apo (0.5 mg/kg, *i.p.*, Sigma, USA) for verification of the efficiency of the lesion. It is known that systemic injections of Apo into the rats with unilateral lesions of the nigrostriatal DA system induce rotational contralateral movements of animals [16]. According to the mean frequency of rotations per minute (rpm) within a 30-min-long observation period, experimental animals in each group were divided into three subgroups: rats without behavioral asymmetry, 0 rpm (subgroup a), animals with less than 6 rpm (subgroup b) and rats with more than 6 rpm (subgroup c). The intensity of movements depends on the extension of unilateral

 

lesion of the *SN* and *VTA*. Therefore, this hemi-parkinsonian rat model can be considered as a convenient way to obtain forced movement sessions of various intensities. Such patterns of asymmetry of the motor behavior of animals in subgroups clearly correlated with the level of destruction of the *SN* and *VTA* neurons, which was estimated using morphological control.

### 2.4 Morphology

Three animals in each subgroup (a, b, c) of Gr I were used in morphological experiments. Rats were decapitated and their brains were fixed in 4% paraformaldehyde before embedding in paraffin wax. Serial of frontal 20- μ m-thick sections, including the *SN* and *VTA* in the rostro-caudal direction, were prepared. The sections were taken at the levels of –5.2 to –6.0 mm from the *bregma* according to the atlas of the rat brain [17]. The obtained sections were stained by the Klüver–Barrera technique using 0.5% aqueous suspension of Cresyl Violet (Fluka, Switzerland); 0.4 ml of 10% acetic acid was added to 10 ml of dye suspension (pH $< 5.0$). The cells of the *SN* and *VTA* stained by the Nissl technique were easily identified under a light microscope by their cytological characteristics. The *SN* and *VTA* neurons were large and fusiform and displayed intense staining by Cresyl Violet.

### 2.5 Statistical Analysis

In Apo test we used the $x^2$ criterion for statistical analysis in order to divide animals into subgroups. The numbers of stained (undegenerated) neurons per section (mean ± s.e.m.) were calculated in 10 frontal 20-μm-thick sections of the *SN* and *VTA* at the levels –5.2 to –6.0 caudal to the *bregma,* obtained from each rat on the 6-OHDA-lesioned (left) and opposite (right) sides. The morphological data were subjected to two-way statistical analysis of variance (ANOVA) and displayed graphically. Newman-Keuls' *post hoc* analysis was used when significant differences were found. Values of $p < 0.05$ were considered to be significant.

## 3 Results

### 3.1 Morphological and Behavioral Assessment in Control Rats (GrI)

In all control animals (Gr I) subjected to morphological examination, 6-OHDA injections caused loss of the stained *SN* and *VTA* neurons on the lesioned side. We found a direct correlation between the number of rotational movements and the intensity of the loss of neurons stained by Nissl within sections of the *SN* and *VTA* on the lesioned (left) side (in rats per subgroup, $p < 0.05$). In animals of Gr I subgroup c, the numbers of DA-ergic cells (stained by Nissl) in 20 μ m-thick section were the following: 198.9 ± 6.1 units of the *SN* and 156.0 ± 10.1 units of the *VTA* on the intact (right) side. On the contrary, only 6.7 ± 0.7 units of the *SN* and 12.3 ± 1.8 units of the *VTA* on the lesioned side were observed (Fig. 1). All these animals demonstrated more than 180 contralateral rotations within 30-min-long intervals (or >6 rpm) after Apo injections. Thus, in the animals that manifested >6 rpm, about 96% of neurons in the *SN* and 92% of cells in the *VTA* were lost on the lesioned side. However, in rats of Gr I subgroup a, which demonstrated no motor asymmetry, only 44% of neurons of the *SN* and 38% of cells in the *VTA* were lost on the lesioned side. Since each rat is characterized by some individual anatomical peculiarities, (even if the body mass of the animals is the same), sites of injections of the neurotoxin in various rats can differ slightly from each other. This factor determines certain variability in the intensity of degeneration of DA-ergic cells within the *SN* and *VTA* in individual animals (Fig. 2).

 

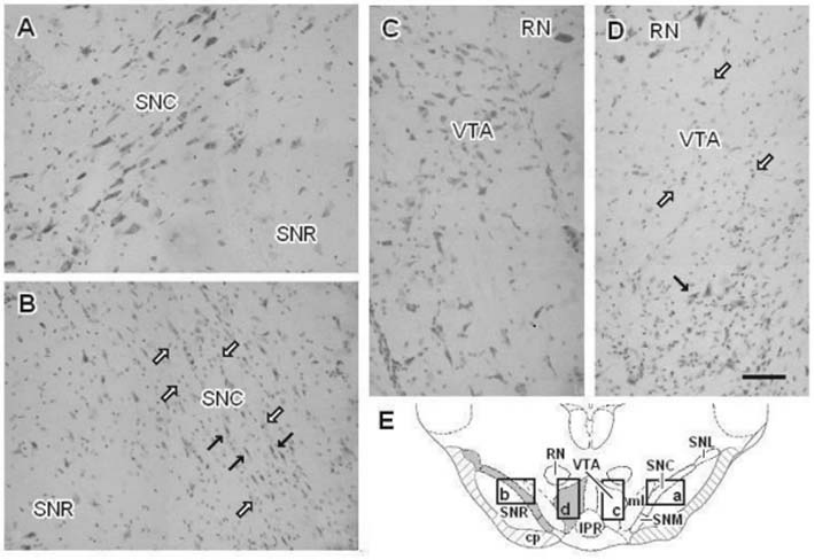


**Figure1.** Photomicrographs of neurons stained by the Nisll technique within the substantia nigra compact and reticular parts (SNC and SNR, respectively), and ventral tegmental area (VTA) in rats with unilateral (left) degeneration of dopaminergic cells induced by intracerebral injections of 6-hydroxydopamine on lesioned (B,D) and intact (A, C) sides of the rat brain; cp – cerebral peduncle, IPR – interpeduncular nucleus, RN – red nucleus, SNL – substantia nigra lateral part, SNM – substantia nigra medial part. Prevalence of degenerated neurons (black arrows) and aggregation of intensively stained glial nuclei (white arrows) are indicated in B and D. The scale bar for A, B, C, D is 100 μm. (E) The scheme of the midbrain structures at the level -6.0 mm caudal to the bregma; rectangles (a, b, c, d) corresponds to A, B, C, D. The DA-ergic structures, which were destroyed by 6- hydroxydopamine injections are colored in grey.

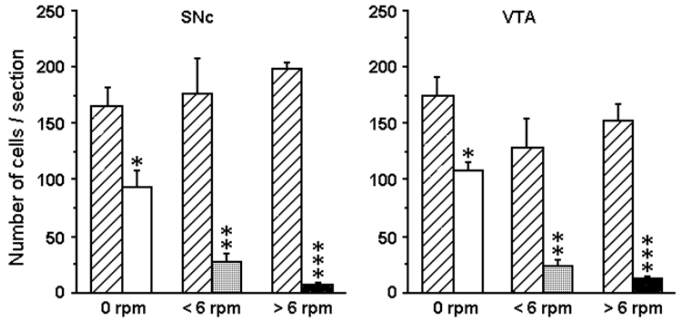


**Figure2.** Dependence of intensity of the motor asymmetry of rats subjected to systemic injections of apomorfine on the level of the unilateral degeneration of the substantia nigra compact part (SNc) and ventral tegmental area (VTA) induced by unilateral intracerebral injections of 6-hydroxydopamine. Vertical scales – number (mean ± s.e.m.) of neurons stained by the Nissl technique in 20 μm-thick sections; dashed and open columns correspond to the number of stained cells in intact and 6-hydroxydopamine-lesioned hemispheres, respectively in animals that demonstrated no motor asymmetry (white columns), mild (<6 rpm) asymmetry (grey columns) and intense (>6 rpm) rotational movements (black columns). * $p<0.05$; ** $p<0.01$; *** $p<0.001$, as compared with the data obtained for the intact hemisphere (dashed columns).

Under conditions of chronic DA deficiency typical of PD, one of the compensatory mechanisms is the intensified unilateral synthesis of DA receptors in neurons of the neostriatum, which results in hypersensitivity of these cells to Apo injections. This phenomenon is manifested in behavioral motor asymmetry (contralateral rotations), whose intensity depends directly on the degree of degeneration of the nigrostriatal DA-ergic system in the rat brain. Taking into account obtained data, we believe that

 

results of the behavioral Apo test in Gr I can serve as an adequate index of the degree of unilateral destruction of DA-ergic cells of the *SN* and *VTA* caused by 6-OHDA in rats of different experimental groups used in further investigations (Gr II – Gr V). We must emphasize that in our experimental series of behavioral investigations only 42.6% of rats in Gr I (subgroup *c*) manifested on average of >6 rpm within 30-min-long intervals after injections of Apo and 3.6% of rats (subgroup *b*) manifested <6 rpm (Fig. 3).

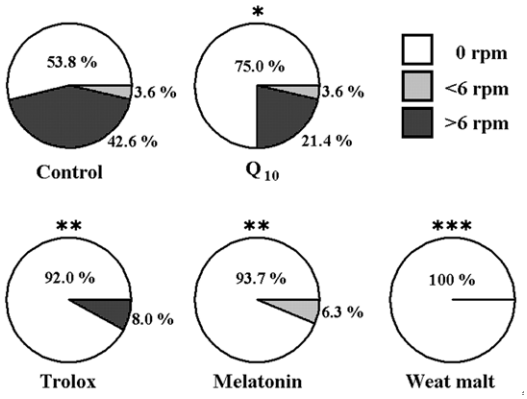


**Figure3.** Various degrees of the development of experimental hemi-parkinsonism in rats after intracerebral injections of 6-hydroxydopamine without any additional treatment – control (Gr I), fed by coenzyme $Q_{10}$ (Gr II), with introduction of Trolox (Gr III), Melatonin (Gr IV) and fed with wheat malt (Gr V). Black sector – number of animals (%) with intensive (> 6 rpm) rotational movements after apomorfine injection (corresponds to the unilateral degeneration of dopaminergic neurons of the SN and VTA on the average of 96 %); grey sector – number of animals with mild behavioral asymmetry (<6 rpm) (corresponds to degeneration of 86 % of dopaminergic neurons); white sector – number of animals without rotational movements after apomorfine injections (about 44 % of dopaminergic neurons were lost); * $p<0.05$; ** $p<0.01$; *** $p<0.001$, as compared with the data obtained for control.

### 3.2 Behavioral Assessment in Experimental Rats (GrII-GrIV)

In Gr II, six rats (21.4%) were observed with intensive rotational movements (>6 rpm) and only one animal (3.6%) with rotational movements <6 rpm. The number of rats with intense rotational movements (>6 rpm) in the animals of Gr III receiving double *i.p.* injections of the antioxidant Trolox decreased to 8%. Single preliminary *i.p.* injections of Melatonin (rats of Gr IV) also led to significant decrease (to 6.3%) in the number of animals that demonstrated rotational movements (<6 rpm) after Apo administration. It is important to note that rats from Gr V (which were fed with wheat malt) demonstrated no rotational movements, and therefore, intracerebral injections of 6-OHDA did not lead to degeneration (at least within no more than 45%) of DA-ergic neurons of the *SN* and *VTA* of the animals in this group. It should be noted that wheat malt exerted greater neuroprotective effect than all minor antioxidants used in our experiments. Thus, the efficacy of the studied antioxidants corresponded to the following sequence: wheat malt > Melatonin > Trolox > $Q_{10}$.

## 4 Discussion

This study confirms and extends our previous results indicative of various degrees of 6-OHDA-induced damage of DA-ergic neurons of the *SN* and *VTA* in the rat brain [9,14]. It is obvious that stereotaxic injections of 6-OHDA cannot be strictly identical in all cases; in addition, the animals demonstrate different individual response to this neurotoxin. In the first approximation, two main subgroups should be classified within rat groups. These are rats demonstrating practically no rotation movements after Apo injections and animals with intensive motor asymmetry. We must emphasize that the results of behavioral Apo test can serve as an adequate index of the degree of unilateral 6-OHDA-caused destruction of DA-ergic cells of the *SN* and *VTA* of the rats in Gr II–V.

 

Among rats of Gr II (fed with coenzyme $Q_{10}$) 21.4% of animals demonstrated intensive rotational movements and only one rat showed insignificant circulations after injections of Apo. Since coenzyme $Q_{10}$ is a fat-soluble antioxidant, special enzyme systems are present in the organisms of animals and humans for its regeneration [18]. Less neuroprotective effect of $Q_{10}$, as compared to other antioxidants used in our study, is probably related to a long time to achieve adequate composition of this coenzyme in the brain tissues. The initial effect of $Q_{10}$ is usually observed after a month of the coenzyme intake, while its maximum effect is revealed after six months [19].

In rats of Gr III, which were subjected to *i.p.* injections of Trolox, intensive lateral movements after Apo injections were observed in two animals. Other rats did not demonstrate any behavioral asymmetry. It is known that Trolox – a water-soluble form of vitamin E, fulfills many functions in the organism: it suppresses the activity of proteinkinase C, 5-lipooxygenase and inhibits cell proliferation [20]. It is also known that it increases the resistance of organisms to toxins and tissue injuries. The deficiency of this vitamin leads to hypersensitivity of the cell membranes to oxidative stress [21, 22]. Moreover, vitamin E is one of the most important inhibitors of free-radical reactions responsible for antioxidant defense of the membranes [23]. All these factors explain the effective neuroprotective action of Trolox in our study.

In Gr IV (animals receiving Melatonin), only one rat (6.3%) demonstrated insignificant rotations after Apo injections. Melatonin is considered to be one of the strongest absorbers of endogenous free radicals. It also increases the expression of mRNA of the antioxidative enzymes [24–26]. It has been proven that it is localized not only in the cytoplasm but also in the cell nuclei. It effectively prevented 6-OHDA-induced degeneration of DA-ergic neurons in the rat brain in our experiments.

It is important to note that in Gr V (receiving wheat malt) none of the animals demonstrated Apo-induced rotation movements, and therefore, injections of 6-OHDA did not result in massive degeneration of DA-ergic neurons. Thus, wheat malt exerted the more considerable neuroprotective effect than all minor antioxidants used in our study.

It has been hypothesized that the natural complexes of antioxidants derived from food may be more effective in delaying or preventing apoptosis of DA-ergic neurons in the brain. It is known that, during the germination of grains, the maintenance of antioxidant and vitamin levels, in particular vitamin E and B-group vitamins, rises sharply. Each vitamin, antioxidant, and bioactive component interacts with analogs, synergists and stabilizers in a complex manner, mutually strengthening and prolonging the effects of each other. Attempts to select concrete antioxidants from this mixture inevitably lead to the decline of their activity; a minor substance appears to be less active. Medicinal plant preparations can be used protractedly without the risk of toxic complications, addiction, or incidental reactions. Due to the antiradical, antioxidative fundamental mechanisms underlying physiological and pharmacological effects of plant preparations, these antioxidants are one of the most important compounds of the vegetable world [27, 28].

## 5 Conclusion

Thus, our findings allow us to conclude that the commercial antioxidant Melatonin and especially a natural complex of antioxidants, such as wheat malt, are capable of exerting a significant protective effect preventing the 6-OHDA-induced apoptosis of the mesolimbic and nigrostriatal DA-ergic neurons in the rat brain. Based on the results of this study we hypothesize that natural extracts of antioxidants may be beneficial to prevent or delay the occurrence of age-related neurodegenerative diseases, and especially PD, in human.

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