Research Paper: The Protective Effects of Citrus Aurantium Extract on a 6-Hydroxydopamine-Induced Model of Parkinson's Disease in Male Rats

Leila Elyasi[1*], Hamed Ghazvini[2]

1. Department of Anatomy, Neuroscience Research Center, Faculty of Medicine,Golestan University of Medical Sciences, Golestan, Iran.
2. Department of Neurosience, Immunogenetics Research Center, School of Advanced Technologies in Medicine, Mazandaran University of Medical Sciencess, Mazandaran, Iran.

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ABSTRACT

Introduction: Parkinson's Disease (PD) is a prevalent neurodegenerative condition among the elderly. Considering the limited symptomatic improvement associated with PD treatments, introducing more effective agents is necessary. Citrus Aurantium flower Extract (CAE) is recognized as a neuroprotective and hepatoprotective agent with bioactive compounds, such as flavonoids, phenolics, and vitamins. Considering the mitigating role of CAE against oxidative damage, the present study examined the neuroprotective effects of CAE in a PD model.

Methods: Overall, 60 male rats were classified into 6 groups, as follows: Sham (SH); Control (C); Lesion (L); and CAE-treated lesion (200, 400, and 600 mg/mL CAE+L). For the hemi-PD model, 6-hydroxydopamine (12.5 g/L of saline ascorbate) was injected intrastriatally. Intraperitoneal pretreatment with hydroalcoholic CAE (200, 400, and 600 mg/kg) was applied in the E+SH and E+L groups at one week pre-surgery. At two weeks post-surgery, rotational behaviors were examined by apomorphine hydrochloride. Moreover, stained neurons were measured in the Substantia Nigra pars compacta (SNc).

Results: In comparison with the C group, significant contralateral turning was reported due to apomorphine, in the L group at two weeks post-surgery ($P<0.0001$); while the neuron count reduced on the left SNc ($P<0.05$). The rotational behaviors reduced using alcoholic CAE, and reduction in the neuron count of SNc was attenuated in the lesion groups ($P<0.05$). However, in the SH group, CAE caused no significant effects on apomorphine-induced rotation and neuron count in the SNc. CAE could decrease the number of degenerated neurons in the SNc. Cell count assessment revealed that neural cell count significantly increased in 200, 400, and 600 mg/mL CAE groups.

Conclusion: According to the present findings, CAE can be suitable for preventing PD in rats.

*** Corresponding Author:**
Leila Elyasi, PhD.
Address: *Department of Anatomy, Neuroscience Research Center, Faculty of Medicine,Golestan University of Medical Sciences, Golestan, Iran.*
Tel: *+98 (911) 3932235*
E-mail: *elyasy_leila@yahoo.com*

1. Introduction

Parkinson's Disease (PD) is a neurodegenerative condition, which damages dopaminergic neurons in the Substantia Nigra pars compacta (SNc). There is inadequate information on the etiology of PD; however, various pathological mechanisms, including lysosomal and mitochondrial dysfunctions, oxidative stress, pathological inclusions, and neuroinflammatory processes have been mentioned [1].

PD can be prevented through regulating intracellular Reactive Oxygen Species (ROS), besides the inhibition of protein aggregation and neuroinflammation [2]. Overall, 6-hydroxydopamine (6-OHDA) is recognized as a common neurotoxin, used to selectively damage dopaminergic neurons [3]. Citrus species are used as medical herbs and food seasoning. These species contain active compounds, such as coumarin, flavonoids, and hesperidins [4]. Among different species, Citrus aurantium (C. aurantium) is a frequently used medicinal plant, endemic to Iran. In traditional medicine, this herb is used to treat and manage neurodegenerative conditions, including seizures, weakness, sleep disorders, pain, and migraine [5].

According to previous studies, C. aurantium has antioxidant, anti-cancer, and anti-inflammatory properties. Moreover, flavonoid phytochemical studies have indicated the antioxidant capacity of this plant and its effects on the free-radical reduction of neuronal support metabolism in Alzheimer's disease [6]. Therefore, we examined the efficacy of C. aurantium extract in protecting against 6-OHDA-mediated oxidative stress [7].

2. Materials and Methods

In this study, 60 male Wistar rats (250-300 g) were obtained from Kerman Animal House. They were kept based on the principles of the Iranian Council of Animal Care. The Ethics Committee of Golestan University approved this study [8]. The animals' rotational responses to Intraperitoneal (IP) apomorphine injections (2.5 mg/kg in normal saline) were examined. For all the experiments, rats with <30 contralatral turns within 1 h were included. The studied rats were assigned into 6 groups (10 rats per group), as follows: Sham (SH); Control (C); Lesion (L); and CAE-treated lesion (200, 400, and 600 mg/mL CAE+L).

To obtain the extract, the plant was first soaked in methanol. A part of the plant flower was washed and air-dried in a dark place and at room temperature (22-25°C) for 1 week. Briefly, 150 g of the air-dried parts of flower was ground into 50 g of fine powder. Next, 100 g of aerial parts of the C. aurantium powder was mixed with 80% methanol.

Furthermore, to prevent chemical changes under the influence of chemical interactions induced by sunlight radiation on the plant constituents and the evaporation of solvent, the Erlenmeyer flask was covered with foil and its lid with Parafilm. The flask was placed on a shaker at 120 rpm at room temperature for 48 h. The resulting extract was filtered several times with Whatman filter papers to obtain a transparent solution. To remove the organic solvent and increase the concentration of the extract, a distillatory was used in vacuum at 40°C. The required amount of the extract was kept at 4°C; while the remaining extract was driedin an oven at 40°C for 48 h for long-term storage. The latter portion was then stored at 4°C. Figure 1 shows the C. aurantium plant [9].

Ketamine and xylazine (80 and 10 mg/kg, respectively) were injected to anesthetize the rats; then, they were put in a Kopf Instrument (Stoelting Instruments, USA). The right side of SNc was determined at the coordinates from the interaural line; the injection site was 3.7, 2.2, and 7.7 mm codal to the interaural line, lateral to the midline, and ventral to the dura, respectively [10]. A Hamilton syringe was used after opening the dura mater. Then, 6-OHDA in 0.2% vitamin C (8 µg/ 2.5 µL) was unilaterally injected to each rat.

The 6-OHDA solution injection was performed gradually at 1 µL/min. The wounds were sutured after the needles remained in place for 5 min before slow removal. The animals received 0.25 mg/kg of apomorphine (Sigma Co, USA) at 2 weeks post-surgery. At 10 min following the injection, the number of rotations was measured for 1 h. The lesion was confirmed if the animal rotated >7 times/min. Additionally, a vehicle (0.9% saline solution with 0.2 mg/mL of l-ascorbate) was administered to the sham group. CAE (200 mg/kg; Tishcon, Osaka, Japan) was orally administered to the L+E group at 1 week before neurotoxin injection, which continued for 2 months [11].

For conducting the apomorphine-induced rotational test, apomorphine hydrochloride (0.5 mg/kg) was used to assess rotational behaviors at one week pre-surgery, as well as two weeks post-surgery. The rotations were determined using a previously introduced method [12]. The rats were accustomed to the experiment during 10 min. Then, complete rotations were measured in a container (height: 35 cm, diameter: 33 cm) at 1 min after

the injection in an isolated room for 1 h within 10 min intervals. The positive score minus the negative score indicated the net number of rotations.

For histological analysis, we randomly selected half of the rats from each group. After completing the behavioral experiments, they were deeply anesthetized with 150 mg/kg of ketamine and perfused from the ascending aorta using 0.9% saline (50-100 mL), fixative solution (100-200 mL, 4% paraformaldehyde in phosphate buffer, pH: 7.4), then 100 mL of 0.1 ml phosphate buffer (with 10% sucrose). After removing the animals' brains following perfusion, brainstem and forebrain blocks were prepared. A freezing microtome (Leica, Germany) was used to cut the sections (50 µm) after preparation in 30% sucrose for 2 to 3 days. Then, they were collected in phosphate buffer (0.1 ml). In addition, 0.1% cresyl violet (Sigma) was used for Nissl staining on every second section.

For mesencephalic sections analysis (2.9-4.2 mm), a method suggested in the literature was applied. Furthermore, stained neurons were manually measured in SNc via light microscopy with a superimposed grid (magnification ×400). At least 2 sections of 4 Paxinos-Watson planes (2.97, 3.2, 3.7, and 4.2) were assessed by scanning each side. The received treatment was not considered in the process of counting [13]. The obtained data are expressed as Mean±SEM. Comparisons were made using t-test and one-way Analysis of Variance (ANOVA). P<0.01 was considered as statistically significant.

3. Results

Based on apomorphine-induced rotations analysis, the groups were not significantly different before conducting the surgery. However, the 6-OHDA-lesion group revealed major contralateral rotations in comparison with the sham group at 1 week post-surgery (P<0.001). However, in the first behavioral assessment conducted before surgery, no significant difference was found between the groups (Figure 1A). The C and SH groups were not significantly different in terms of rotation in the second assessment at 2 weeks after surgery. Accordingly, comparisons were made between group C and other groups. In the L and L+E200 mg/kg groups, apomorphine majorly increased contralateral turning (P<0.001). Moreover, it significantly declined the L+E400 mg/kg and L+E600 mg/kg groups versus the L group (Figure 1B).

According to the histochemical analyses (Figure 2) and counting (Figure 3), the sham and control groups were not significantly different, considering the stained neuron count on the left side of SNc. Additionally, a significant decline was reported in the 6-OHDA group (lesion = L, P<0.01); while a less prominent decline was observed in the L+E200, L+E400, and L+E600 groups (P<0.05).

4. Discussion

There are no effective treatments for PD as a neurodegenerative disorder. Unilateral 6-OHDA microinjection in the neostriatum of rats produces a reliable and reproducible model of PD for exploring the molecular mecha-

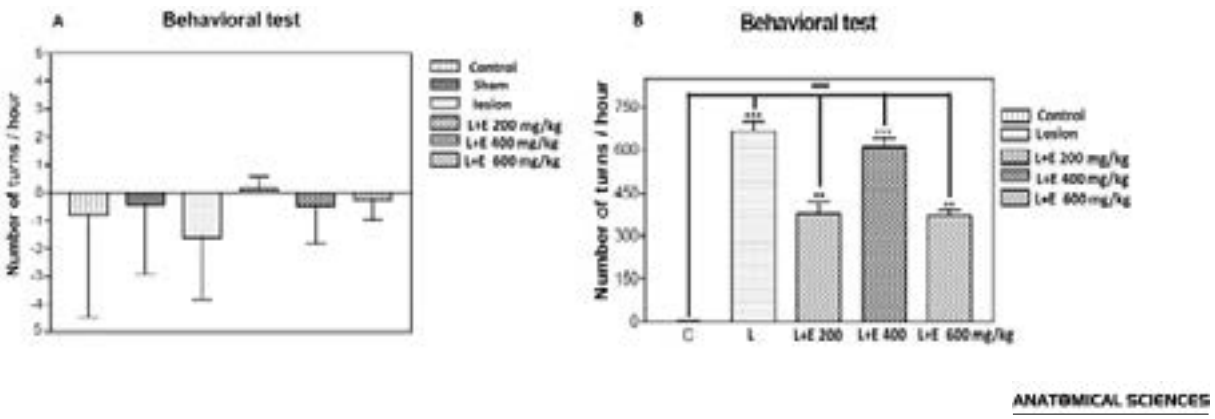

Figure 1. The total net number of rotations (Mean±SEM) induced by apomorphine (0.5 mg/kg, IP) over a period of 60 min pre-surgery.

There was no significant difference (P<0.01) between the groups

A and B. The average number of contralateral turns in different groups caused by apomorphine administration 2 weeks after lesions in rats.

P<0.001

** P<0.01 (B)

*** P<0.001

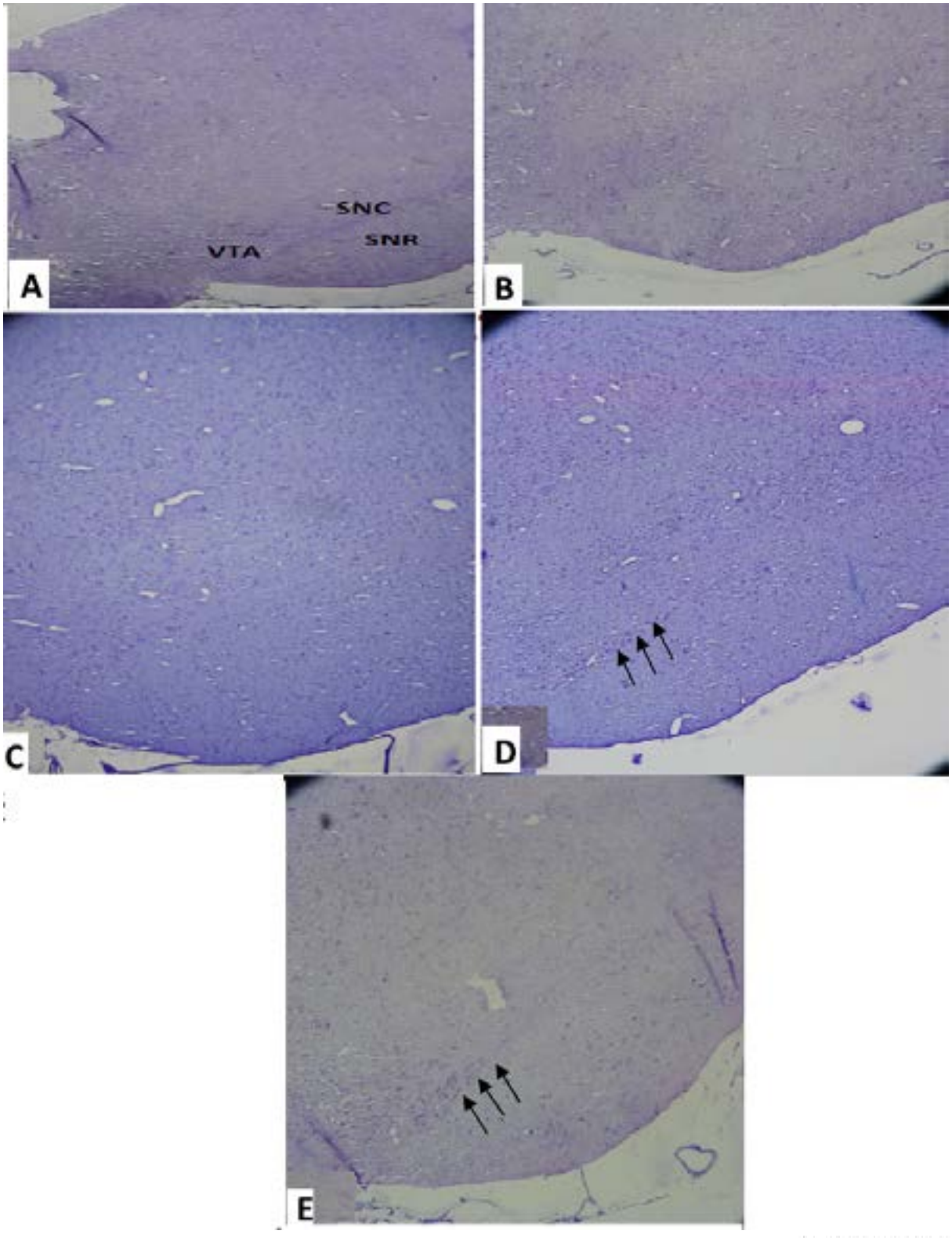

Figure 2. Photomicrograph of coronal sections through the midbrain showing Nissl-stained neurons in the experimental groups. Control group without reduction in the number of neurons

A. severe reduction in the number of neurons in SNc was observed in the lesion group = L; B. and L+E200; C. and L+E400; D. L+E600; F. however, an increase was noted in the E400 treated l groups in comparison with Lesion group. Scale bar = 250 µm (100X) SNc and Subtantia Nigra Region (SNR), substantianigra pars compacta and pars reticulate, respectively)

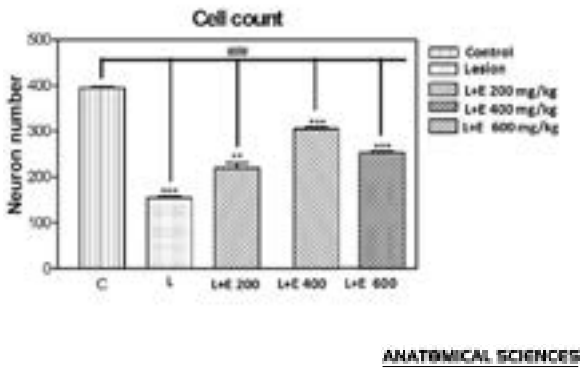

Figure 3. Quantification of neuron numbers, in the striatum in different groups

The average neuronal count in the lesion group was significantly less than in other experimental groups.

** P<0.01

*** P<0.001

P<0.001

nisms of dopaminergic neuron degeneration and evaluating the efficacy of promising therapeutic candidates [14]. Unilateral disruption (due to intrastriatal 6-OHDA injection) of the nigrostriatal dopaminergic system reduces dopamine inside the striatum and upregulates postsynaptic dopaminergic receptors. According to the present study, these changes in 6-OHDA-lesion rats cause significant motor asymmetry due to contralateral rotations [15]. The lower prevalence of rotational behaviors in the current study might be attributed to the CAE potential in preserving dopaminergic neurons of SNc and keeping striatal dopamine at a suitable level (unrelated to robust motor asymmetry).

C. aurantium seems to produce neuroprotective effects in the primary neurons of mice at concentrations where antioxidant effects are less likely to dominate [16]. Despite its lower estrogenic and antioxidant activity, hesperetin, a vital component of C. aurantium, exerts neuroprotective effects through different pathways (e.g. stimulating tyrosine kinases and estrogenic receptors) [17].

The narrow beam test on 6-OHDA-lesion rats suggested that the time on the beam and latency increased, in comparison with the sham group. Therefore, the depletion of dopamine in the striatum increased the delay in initiating the task and reduced the speed of crossing; i.e. indicative of akinesia or bradykinesia [18]. The frequency of rotations attributed to apomorphine injection reduced in the group. The extract demonstrated protective capacity, which helps keep the dopamine level without causing rotation [19]. CAE, through its antioxidant properties, leads to the functional return of nigroestriatal system [20].

The flavonoids in the extract, including hesperetin, due to free radicals' collection feature (with anion hydroxyl as a secondary propagulant) activate signaling molecules (e.g. C-protease, and other nuclear factors). By inhibiting ROS production, hesperetin reduces signaling molecules' activities [21]. The present findings confirmed the therapeutic use of C. aurantium in vivo. This compound was effective in the reversal of behavioral disorders in a PD model. The current findings may provide a new clinical insight into progressive neurodegenerative diseases, like PD.

Ethical Considerations

Compliance with ethical guidelines

The study was performed in accordance with the guidelines of National Institute of Health for the care and use of laboratory animals. The animals were kept based on the principles of the Iranian Council of Animal Care. The Ethics Committee of Golestan University approved this study.

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Authors contributions

All authors have read and approved the manuscript and fulfilled the criteria for authorship according to the ICMJE recommendations for authorship.

Conflict of interest

The authors declared no conflicts of interest.

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