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# Neuroprotective and Antiapoptotic Potential of Trigonelline in a Striatal 6-Hydroxydopamine Rat Model of Parkinson's Disease

**M. Mirzaie,[1,2] M. Khalili,[1] Z. Kiasalari,[1] and M. Roghani[1]**

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Considering neuroprotective and antioxidant effects of trigonelline, our study was undertaken to evaluate its protective effect in a 6-hydroxydopamine-induced model of Parkinson's disease (PD) in rats. Unilateral intrastriatal 6-OHDA-lesioned rats were pretreated with trigonelline at doses of 50 and 100 mg/kg. Significant rotational behavior, a significant reduction in the number of Nissl-stained neurons on the left side of the *substantia nigra pars compacta* (SNC), increased apoptosis, enhanced levels of malondialdehyde (MDA) and nitrite, and a lower level of glutathione (GSH) were observed in 6-OHDA-lesioned rats. Trigonelline at a dose of 100 mg/kg significantly reduced rotations, prevented reduction of SNC neurons, prevented apoptosis, and restored the MDA level. These results suggest that pre-lesion trigonelline treatment exerts dose-dependent neuroprotective and antiapoptotic effects under conditions of 6-OHDA toxicity and may be, henceforth, advantageous for the management of early PD.

**Keywords:** Parkinson's disease, trigonelline, 6-hydroxydopamine, apoptosis, oxidative stress.

## INTRODUCTION

Parkinson's disease (PD) is a rather widespread neurodegenerative disorder with such cardinal clinical symptoms, as bradykinesia, muscle rigidity, resting-state tremor, postural imbalance, and gait impairment [1]. These motor disabilities inevitably lead to a lower quality of life [2]. The global occurrence of PD is about 0.3% in the general population and 1–2% in subjects older than 60–65 years [2]. Behavioral and cognitive problems, including dementia, depression, anxiety, and sleep disturbances, also appear in the late stages of PD [3, 4]. At present, treatment for PD includes mainly symptomatics and usually starts with Levodopa and/or dopamine agonists [5]. Nevertheless, after some time, most PD patients experience significant side effects, including motor and nonmotor fluctuations and dyskinesia [6].

Oxidative stress-mediated neuronal death plays a key role in the pathogenesis of PD [7]. A neurotoxin, 6-hydroxydopamine (6-OHDA), is routinely used to damage mesencephalic dopaminergic neurons and to provide a widely used animal model of PD [8]. Because environmental factors are engaged in the majority of PD cases, it is important to assess the effectiveness of natural products in neuroprotective interventions for PD [9]. Meanwhile, patients with PD often turn to complementary and alternative medicine [10].

The alkaloid trigonelline (N-methylnicotinic acid) is a metabolite of nicotinic acid. It is mainly found in fenugreek seeds and coffee beans. Trigonelline demonstrates a potent antioxidant property and strong free-radical scavenging activity [11, 12]. This agent was shown to attenuate diabetes-induced auditory neuropathy [13] and, in general, was reported to induce neurite outgrowth and reconstruction of neuronal networks in the damaged brain, which is exclusively important for the therapeutic treatment of neurodegenerative diseases [14]. In addition, a standardized extract of fenugreek seeds, which contain high amounts of trigonelline, was capable of reversing motor dysfunction in rats with 6-OHDA-induced unilateral cerebral lesion [15]. For these reasons, trigonelline may be regarded as a prospective effective weapon

[1,2]Shahed University, Tehran, Iran
([1]Neurophysiology Research Center; [2]Student Research Committee)
Correspondence should be addressed to: M. Roghani (mehjour@yahoo.com)

against PD. Our study was undertaken to assess the possible neuroprotective potential of pre-lesion trigonelline treatment in the 6-OHDA-induced early model of PD in rats.

## METHODS

**Chemicals and reagents**. Trigonelline, 6-OHDA, apomorphine, cresyl violet, and oxidative stress kits were obtained from Sigma-Aldrich (Germany). All other chemicals were procured from Merck (Germany).

**Animals**. Adult male Wistar rats (180–210 g, $n$ = 60) were provided by the Pasteur's Institute (Tehran, Iran). The animals were housed in a temperature-controlled colony room under 12/12 h light/dark cycle with free access to food and water and held in the above room for at least one week before being tested. Only rats showing no rotational behavior (net rotations less frequent than 30 $h^{-1}$) were subjected to i.p. injections of apomorphine hydrochloride (2 mg/kg) in our study. The animals were randomly divided into five groups: (i) sham-operated; (ii) sham-operated trigonelline(100); (iii) 6-OHDA-lesioned; (iv) lesioned + trigonelline(50), and (v) lesioned + + trigonelline(100), see below. Stereotaxic unilateral intrastriatal 6-OHDA injections (left side) were performed using a 5 ml Hamilton syringe into anesthetized rats (ketamine 80 mg/kg and xylazine 10 mg/kg, i.p.). A stereotaxic apparatus (Stoelting, USA) was used (coordinates: L –3 mm, AP +9.2 mm, and V +5 mm from the center of the interaural line, according to the atlas of Paxinos and Watson [16]). After injection, the needle was left in place for an additional 5 min and then withdrawn at a rate of 1 mm/min. The lesioned groups received single injections of 2.5 mg/ml of 6-hydroxydopamine HCl in 5 ml of 0.9% saline containing 0.2% ascorbic acid (w/v) at a rate of 1 ml/min. The sham group received an identical volume of the ascorbate-saline solution. The 6-OHDA+trigonelline(50) and 6-OHDA+trigonelline (100) groups received the neurotoxin in addition to trigonelline i.p. dissolved in normal saline at doses of 50 and 100 mg/kg. Trigonelline was administered daily three times before the surgery, with the last injection made 1 h pre-surgery. The doses of trigonelline were chosen according to our preliminary study.

**Behavioral evaluation**. The animals ($n$ = 9 in each group) were tested for apomorphine hydrochloride (2 mg/kg, i.p.)-induced rotational behavior one week before surgery (baseline) and at the 1st week post-surgery. The rotations were measured according to a method described previously [8]. Briefly, the animals were allowed to habituate for 10 min; then, 1 min after the injection, full rotations were counted in a cylindrical container (diameter 33 cm and height 35 cm) within 10-min-long intervals for 60 min (in a dimly-light and quiet room). Net number of rotations was defined as the positive score minus the negative score.

**Assessment of oxidative stress**. Midbrain samples ($n$ = 5 for each group) were dissected from the rat brains and washed off in cold normal saline. Their anterior one-thirds were blotted dry, weighed, homogenized (5% of tissue) in ice-cold normal saline, and centrifuged at 4°C. The obtained supernatant was aliquotted and stored at –70°C until being assayed.

**Estimation of the MDA content in the midbrain**. The malondialdehyde (MDA) concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured as described before [8]. Briefly, trichloroacetic acid and a TBARS reagent were added to the supernatant, then mixed and incubated in boiling water for 90 min. After cooling on ice, the samples were centrifuged at 1000g for 10 min, and the absorbance of the supernatant was read at 532 nm. The TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

**Midbrain nitrite assay.** The nitrite content in the supernatant was assayed by the Griess method according to previous studies [17]. Because NO is a compound with a short half-life and is rapidly converted into stable end products, nitrate ($NO_3^-$) and nitrite ($NO_2^-$), the principle of the assay is the conversion of nitrate into nitrite by cadmium. This is followed by color development with the Griess reagent (containing sulfanilamide and N-naphthylethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was measured at 540 nm with a spectrophotometer.

**Catalase activity asssay**. For this purpose, Claiborne's method was used [18]. Briefly, $H_2O_2$ was added to a mixture of 50 mM potassium phosphate buffer (pH 7.0) and supernatant, and the rate of $H_2O_2$ decomposition was assessed by measuring the absorbance changes at 240 nm for 2 min. One unit of the catalase activity was defined as 1 μmol of $H_2O_2$ decomposed within 1 min.

**Measurement of the reduced glutathione (GSH) level**. The GSH level was measured spectrophotometrically as described before [19, 20]. Briefly, the supernatant was centrifuged with 5% trichloroacetic acid to eliminate proteins. Two milliliters of phosphate buffer (pH 8.4), 0.5 ml of 5.5′-dithiobis(2-nitrobenzoic acid, DTNB), and 0.4 ml of bidistilled water were added to 0.1 ml of the homogenate. The mixture was vortexed, and the absorbance was read at 412 nm within 15 min.

**Protein assay**. The protein content in the supernatant was measured with the Bradford method using bovine serum albumin as the standard [21].

**Estimation of DNA fragmentation (apoptosis)**. In this experiment, three rats from each group were euthanized one day after intrastriatal 6-OHDA injection, and the midbrain tissue supernatant was prepared as above. The determination of histone-associated DNA fragments was performed using the Cell Death Detection ELISA kit (Roche Diagnostics, Germany) as an indicator of apoptosis according to the protocol from the company and the procedure as described before [22]. The assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies against DNA and histones, respectively. This makes it possible to specifically determine mono- and oligonucleosomes (histone-associated DNA fragments) in the fraction of tissue lysates. The amount of nucleosomes demonstrating DNA degradation was quantified by peroxidase retained in the immunocomplex. The peroxidase content was determined photometrically at 405 nm with 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid as a substrate by a microplate reader (BioTek, USA) after 15 min of the substrate reaction time. The results were expressed as the optical density (OD).

**Histochemistry**. At the end of the study, the rats ($n$ = 4 from each group) were deeply anesthetized with a high dose of ketamine (150 mg/kg) and perfused through the ascending aorta with 50-100 ml of 0.9% saline followed by 100–150 ml of the fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 ml of 0.1 M PB. After perfusion, the brains were removed from the skull; blocks of the forebrain and brainstem were prepared, and, after the final steps of preparation (immersion in 30% sucrose solution for 1–2 days), 30-mm-thick sections were cut on a freezing microtome and collected in PB (0.1 M). Every second section was Nissl-stained with 0.1% cresyl violet.

**Histology**. For each animal, mesencephalic sections (interaural 2.9-4.2 mm) were examined by a method described previously [7]. Nissl-stained neurons in the SNC were manually counted (light microscopy, ×400) using a superimposed grid to facilitate the procedure. At least two sections representative of each of four Paxinos-Watson planes (4.2, 3.7, 3.2, 2.97, interaural) were examined by scanning the entire extent. Counting was done blind to the treatments received.

**Statistical analysis**. All numerical data are expressed as means ± s.e.m. For statistical evaluation of the data, the parametric one-way ANOVA followed by Tukey's *post-hoc* test was used. In all analyses, the null hypothesis was rejected at $P < 0.05$.

## RESULTS

The beneficial effect of trigonelline at doses of 50 and 100 mg/kg was evaluated according to apomorphine-induced rotations for a period of 1 h (Fig. 1). There were no significant differences among

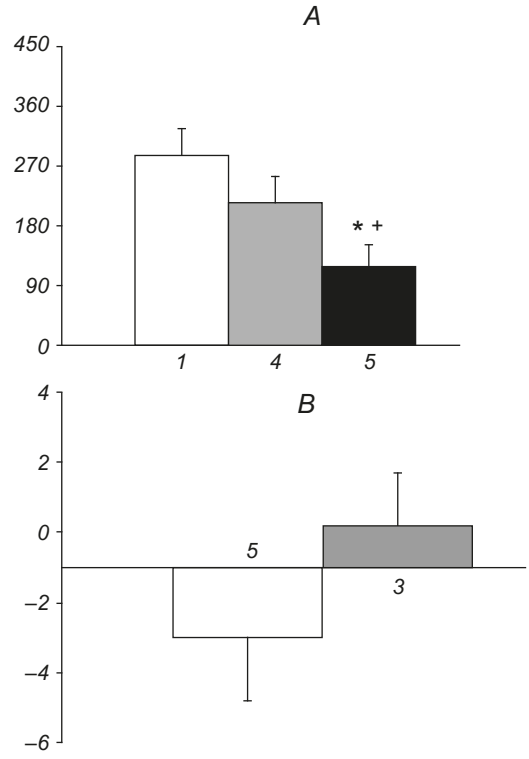


**Fig. 1.** Intensity of apomorphine-induced rotations in the 6-OHDA model of Parkinson's disease. Mean numbers of rotations within 1 h in the 6-OHDA-lesioned, lesioned + trigonelline(50) (4), and lesioned + trigonelline(100) (5) groups.

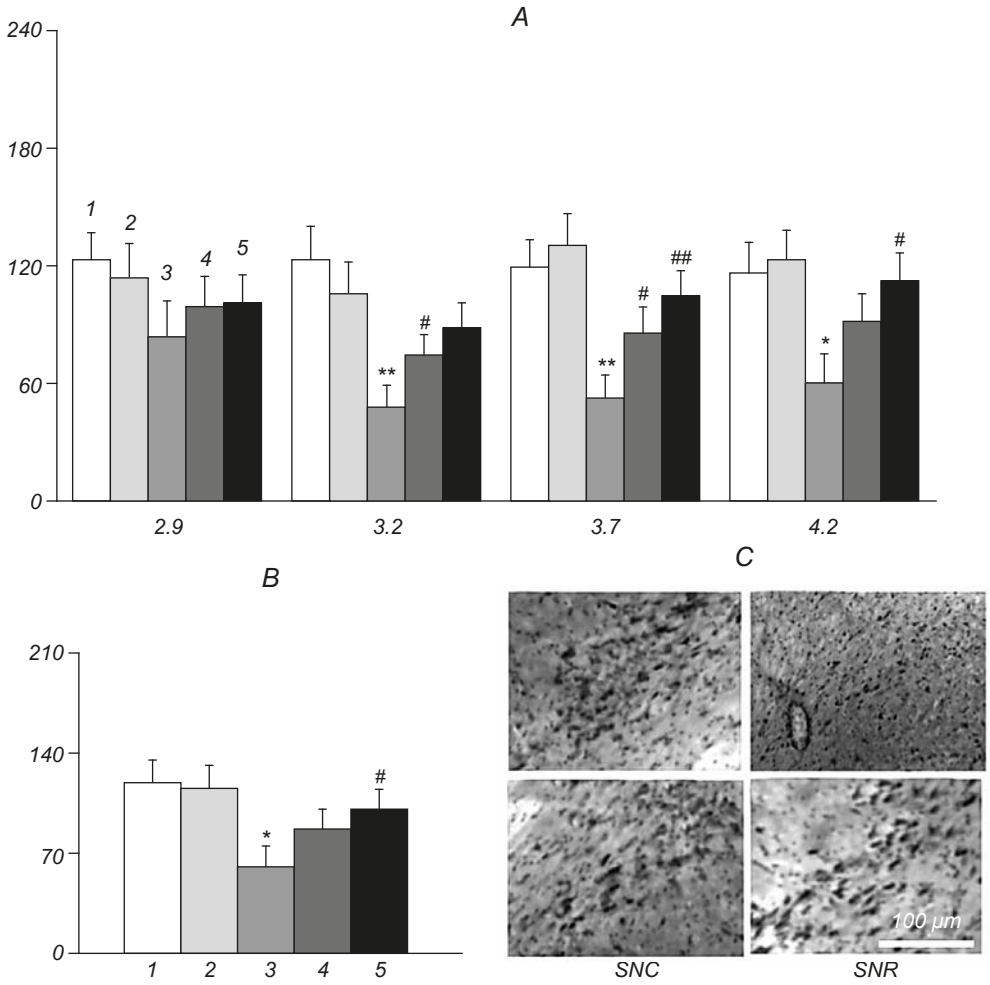


**Fig. 2.** Numbers of Nissl-stained neurons in the left side of the *substantia nigra* (SN) in different experimental groups. A) Numbers of stained neurons at different frontal levels; B) averaged numbers of stained neurons; C) photomicrographs of SN *pars compacta* (SNC) and SN *pars reticulata* (SNR). Indications of the groups, see the text.

the groups at baseline (before surgery). Statistical analysis of the total net number of rotations at the first week post-surgery showed that apomorphine injections caused very significant contralateral turning in the rats of the 6-OHDA-lesioned group ($P < 0.001$) and induced significant but less intense rotations in the 6-OHDA+trigonelline(50) ($P < 0.05$) and 6-OHDA+trigonelline(100) groups ($P < 0.01$) in comparison with the sham group. Moreover, the group 6-OHDA+trigonelline(100) showed a significantly smaller number of rotations ($P < 0.05$) when compared to 6-OHDA-lesioned rats.

Findings of Nissl staining (Fig. 2) showed that there was no significant difference between the sham and sham+trigonelline(100) groups regarding the number of Nissl-stained neurons within the left SNC side. In addition, a significant reduction was noticed in the 6-OHDA-lesioned group ($P < 0.05$). There were no significant reductions in the 6-OHDA+trigonelline(50) and 6-OHDA+trigonelline(100) groups when compared to the sham group. In this respect, the number of Nissl-stained neurons on the left side of SNC was significantly higher in the 6-OHDA+trigonelline(100) group vs. 6-OHDA-lesioned group ($P < 0.05$).

With respect to biochemical markers of oxidative stress (Fig. 3), 6-OHDA-lesioned rats demonstrated a significant elevation of the MDA content ($P < 0.05$) and nitrite amount ($P < 0.05$), as well as reduction of the GSH level ($P < 0.05$). There was also significant reduction of the activity of the defensive enzyme (catalase) vs. the respective index in the sham group. The treatment of lesioned

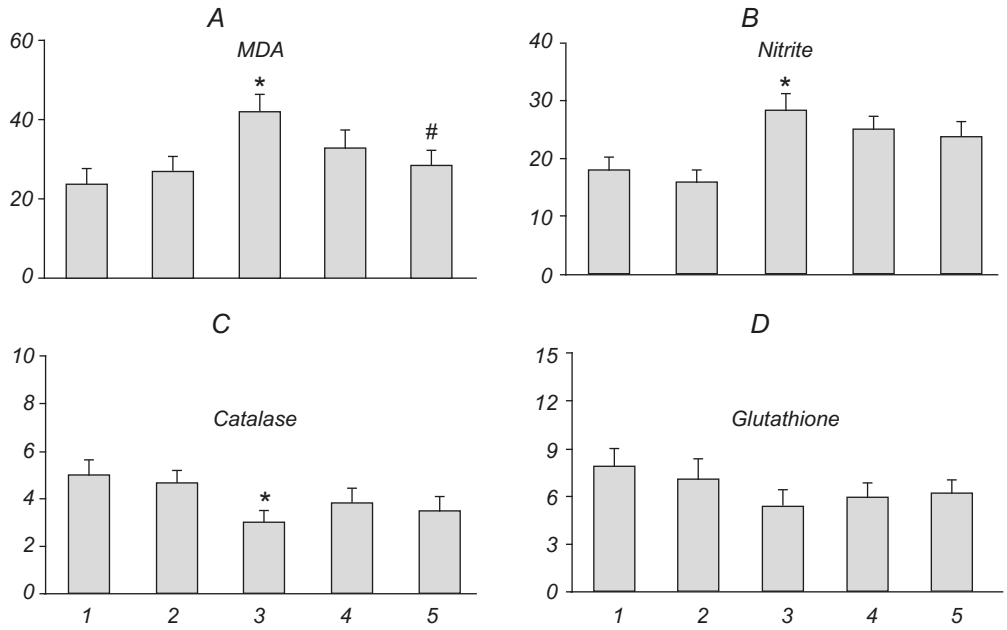


**Fig. 3.** Oxidative stress markers (A-D) in the midbrain of animals of different experimental groups. Other designations are similar to those in Figs. 1 and 2.

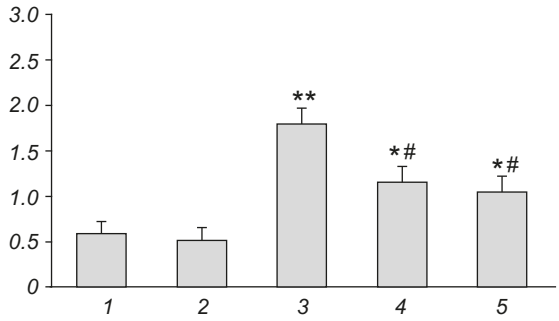


**Fig. 4.** Effect of trigonelline pretreatment on fragmentation in the 6-OHDA-model. Designations are similar to those in Fig. 3.

rats with trigonelline at a dose of 100 mg/kg only significantly lowered the MDA content ($P < 0.05$). Meanwhile, changes in the trigonelline(100)-treated group relatively to lesion animals, regarding other above-mentioned parameters, were moderate but did not reach the significance level. Furthermore, the measurement of chromosomal breakdown of DNA (which is a reliable indicator of apoptosis) showed a significant increase of DNA fragmentation in 6-OHDA-lesioned rats ($P < 0.005$) vs. the sham group. The trigonelline pretreatment at a dose of 100 mg/kg significantly reduced this index in comparison to that in the 6-OHDA group ($P < < 0.05$), as shown in Fig. 4.

## DISCUSSION

In our study, we confirmed that trigonelline pretreatment in the 100 mg/kg daily dose significantly reduced apomorphine-induced rotations in the respective model, increases the viability of SNC neurons, prevents their apoptosis, and restores the MDA level in 6-OHDA-lesioned rats.

Selective degeneration of dopaminergic neurons in patients with PD results from a genetically and/or environmentally-induced process of neurodegeneration (mostly in the SNC) [23]. 6-OHDA, the agent generally used for PD induction in rodent models, is assumed to cause selective degeneration of dopaminergic neurons in this structure [24]. The unilateral damage of the nigrostriatal dopaminergic system, induced by intrastriatal injection of 6-OHDA, is followed by a reduction in the striatal dopamine level and up-regulation of dopaminergic postsynaptic receptors at the same side. These changes produce a prominent functional and motor asymmetry that can be evaluated by direct-acting dopaminergic agonists, apomorphine in particular [25]. These rotations are considered reliable indicators of nigrostriatal dopamine depletion [26]. In our study, significant attenuation of apomorphine-induced rotational behavior was observed in trigonelline(100)-pretreated 6-OHDA-lesioned rats. The observed attenuation of rotational

behavior in the mentioned group could be attributed to a clear neuroprotective effect of trigonelline against SNC neurodegeneration and maintenance of striatal dopamine at a level not accompanied by a marked rotational behavior. In other words, in the presence of trigonelline nigro-striatal neurons within the SNC were noticeably protected against neurodegenerative effects induced by the neurotoxin 6-OHDA. In agreement with our earlier observations, a standardized hydroalcoholic extract of *Trigonella foenum-graecum* seeds, which contains considerable amounts of trigonelline, was capable of significantly lowering the intensity of induced rotations in unilaterally 6-OHDA-lesioned rats and to reverse motor dysfunctions, including enhanced spontaneous motor activity in 4-phenyl-1,2,3,6-tetrahydropyridine-induced lesioned mice. These effects have been attributed to the neuroprotective potential of this extract [15].

In addition, an overproduction of free radicals, especially of reactive oxygen species, is also considerably involved in 6-OHDA-induced neurodegeneration [26]. Oxidative stress is among the most important factors affecting the survival of dopaminergic neurons in PD. Neurons crucially depend on an adequate energy supply produced by mitochondria and are simultaneously faced with high levels of reactive oxygen species, as well as with increased levels of free iron. The latter phenomenon can promote hydroxyl production [27]. Overloading by the free radicals inevitably leads to intensification of the process of cell death. In addition, auto-oxidation of dopamine may produce dopamine quinine [28]. The formation of such species (semiquinones and other free radicals) can especially intensely damage nucleic acids, proteins, and membrane lipid components [29]. Therefore, adequate therapeutic approaches should be aimed at attenuation of oxidative stress. Free radical scavengers may also be helpful in prolonging the survival time of dopaminergic neurons [30]. In this respect, trigonelline has been demonstrated to attenuate neuronal damage and to strongly counteract oxidative stress. The measurement of oxidative stress markers in our study also showed that trigonelline is able to attenuate this stress and to augment the antioxidant defensive system in the midbrain. In this way, this compound can significantly decrease the toxicity of 6-OHDA. This finding is consistent with literature data on the antioxidant ability of this means via regulating the antioxidant defense system, as well as via suppression of free radical generation [11].

The inflammatory process initiated in the brain structures is also an important causative factor for PD [31, 32]. Pro-inflammatory cytokines released from glial cells can considerably stimulate nitric oxide production and exert a deleterious effect on dopaminergic neurons by activating receptors that contain intracytoplasmic death domains involved in the apoptotic pathway [33]. It has been shown that coffee (a food product that also contains high amounts of trigonelline) demonstrates noticeable anti-inflammatory activity [11]. Thus, it is possible that trigonelline may also lower the level of the respective "PD-related" inflammatory mediators within the brain, and this also contributes in some way to neuroprotection in 6-OHDA-induced PD model in rats, as was observed in our study. In addition, apoptosis is another factor that plays a critical role when cells are exposed to neurotoxins, including 6-OHDA [34]. In our study, trigonelline was able to prevent apoptosis, as evidenced by a lower DNA fragmentation. The anti-apoptotic effect of trigonelline has also been reported before [35]. Nevertheless, some aspects of the respective issue need further investigation.

Taken together, our results demonstrated that pre-lesion treatment with trigonelline in the animal model of PD exerts, in a dose-dependent manner, clear neuroprotective and antiapoptotic effects against 6-OHDA toxicity. Hence, the above agent may be included in a set of therapeutic means advantageous for the management of early PD.

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The procedures used for animal experimentation and their care were in accordance with the existing international ethical norms for experimental work mentioned in the NIH guidelines.

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