Original Article

Beneficial effects of L-arginine on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neuronal degeneration in substantia nigra of Balb/c mice

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Abstract

Background: L-arginine has been recently investigated and proposed to reduce neurological damage after various experimental models of neuronal cellular damage. In this study, we aim to evaluate the beneficial effects of L-arginine administration on the numerical density of dark neurons (DNs) in the substantia nigra pars compacta (SNc) of Balb/c mice subjected to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration.

Materials and Methods: Male Balb/c mice were randomly divided into 4 groups ($n = 7$ each): MPTP only; saline only (control); MPTP + L-arginine; and L-arginine only. The animals were infused intranasally with a single intranasal administration of the proneurotoxin MPTP (1 mg/nostril). L-arginine (300 mg/kg) was administrated intraperitoneally once daily for 1-week starting from 3 days after MPTP administration. Cavalieri principle method was used to estimate the numerical density of DNs in the SNc of different studied groups.

Results: Twenty days following MPTP administration, the number of DNs was significantly increased when compared to sham-control and L-arginine-control groups ($P < 0.05$). Nevertheless, our results showed that L-arginine administration significantly decreased the numerical density of DNs in SNc of mice.

Conclusion: This investigation provides new insights in experimental models of Parkinson's disease, indicating that L-arginine represents a potential treatment agent for dopaminergic neuron degeneration in SNc observed in Parkinson's disease patients.

Key Words: 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine, Balb/c mice, L-arginine, substantia nigra

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INTRODUCTION

The substantia nigra (SN) - a crescent-shaped nucleus - lies in the midbrain immediately dorsal to the cerebral peduncles. This nucleus is an important motor center that is thought to be the lesion site in Parkinson's disease (PD).[1,2] PD is recognized as the second most common neurodegenerative disorder

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affecting 1% of the population worldwide after the age of 65 years.[2,3] Movement impairments including tremor, bradykinesia, akinesia, rigidity, and postural instability are among the most obvious symptoms in the course of the disease.[1] This disorder is caused by degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) that project to the striatum. The loss of DA neurons is responsible for the major symptoms of the disease.[1] Although the most cases of PD are idiopathic, the etiology of DA neuronal demise is elusive. Dopamine degeneration process in PD involves oxidative stress, mitochondrial dysfunction, apoptotic processes, and microglial activation.[1,2,4]

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a systemic neurotoxin, which causes a specific loss of DA neurons in the nigrostriatal system, and recapitulates the permanent symptoms of PD.[5-9] Therefore, in a series of earlier experimental investigations, intranasal (i.n.) administration of MPTP has become the most commonly used animal model of PD.[10-16] In addition, the some of them demonstrated that low concentrations of MPTP can enter the brain via the olfactory mucosa and alter DA function in a range of brain structures.[11,13-16] Because of safety considerations, the i.n. administration of MPTP which is not constrained by such factors may be more effective in getting higher levels of MPTP into the brain and to induce alterations in central nervous system structure and function.[11,13-16]

When MPTP is injected into an animal, the chemical penetrates the brain through the blood-brain barrier and is metabolized to 1-methyl-4-phenyl1-2,3-dihydropyridium (MPP+) by monoamine oxidase-B enzyme in glia. MPP+ has a high affinity for the dopamine transporter (DAT) on DA cells and is taken up into the cell.[17] MPP+ is then released from the glia and enters neurons via the DAT on DA cells.[18] MPP+ is then accumulated in the mitochondria and creates further neuronal damage through the activation of reactive microglia and subsequent generation of free radicals.[7,19] By 7-day postadministration of MPTP, a significant loss of DA neurons in the SNc is evident, along with a significant reduction of DA production in the terminal field within the striatum.[20] Thus, MPTP administration to animals induces a DA neuron loss that mirrors the loss seen in end-stage PD.

L-arginine is a semi-essential amino acid that involved in two major metabolic pathways. One of them is the nitric oxide synthase (NOS) pathway where L-arginine is converted to nitric oxide (NO) and L-citruline. The other pathway is the arginase pathway.[21-24] L-arginine and its analogues are also potent NOS inhibitors.[21-26] Growing evidence from studies in human PD brain, in addition to genetic and toxicological models, indicates that neurons of SN express the considerable amount of inducible nitric oxide synthase (iNOS). On the other hand, experimental and clinical findings show a close relationship between iNOS induction and neurodegeneraion suggesting the inhibition of iNOS can have a therapeutic effect in PD.[26-30] From etiological view point, it is clearly showed that L-arginine have a regulatory effect on arterial pressure and improve blood flow after brain trauma resulting in neural death reduction.[31-33]

In previous experimental studies, dark neurons (DNs) production has been reported in the brain of animals exposed to various pathological conditions.[34,35] DNs are the final product of a series of physico-chemical reactions initiated from extracellular milieu and propagate into the neuron.[36-40] Morphologically DNs are characterized by at least six features namely: Hyperbasophilia, argyrophilia, disappearance of antigenicity, ultrastructural compaction, volume reduction, and increased electron density.[34] This kind of degenerating neurons have been reported in Huntington disease, epilepsy, spreading depression, and also in aging process.[35,41]

Since L-arginine and its product, NO, exert such a range of critical roles in regulating physiological functions of the brain, we hypothesize that L-arginine can have a therapeutic effect on the MPTP-induced neurodegeneration in the SNc of mice. So, this study was designed to evaluate the beneficial effects of L-arginine on the numerical density of DNs in the SNc of Balb/c mice subjected intranasally to MPTP administration.

MATERIALS AND METHODS

Animals and study design

Healthy adult male Bulb/c mice (20–30 g body weight, 6–8-week-old) were purchased from the Experimental Animal Facility of Birjand University of Medical Sciences (Birjand, Iran). The animals were housed in polypropylene cages (four per cage) under controlled temperature and light conditions (22 ± 3°C, 40–70% relative humidity, 12-h light phase with daylight).[42] They were fed with standard pellet diet (Javaneh co., Iran) and water *ad libitum*. All procedures involving animals were conducted in accordance with the guide for the care and use of Laboratory Animals of the Birjand University of Medical Sciences, Iran. All efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were

randomly assigned to four equal groups (n = 7 each):

- Control Group: Mice were administrated intranasally with the same dose of vehicle (saline 0.9%)
- MPTP Group: Mice were administrated intranasally with the single dose of MPTP (Sigma–Aldrich, St. Louis, MO, USA; dissolved in saline 0.9%) at the dose of 1 mg/nostril[14,43]
- L-arginine Group: Mice only received intraperitoneally L-arginine (Sigma–Aldrich, St. Louis, MO, USA; 300 mg/kg dissolved in saline 0.9%) once daily for 1-week
- L-arginine - MPTP Group: Mice received intraperitoneally L-arginine (Sigma–Aldrich, St. Louis, MO, USA; 300 mg/kg dissolved in saline 0.9%) once daily for 1-week starting from 3 days after MPTP administration.

MPTP (1 mg\nostril) was administered by i.n. route according to the procedure previously described[11,13,14] and modified in our laboratory. Briefly, mice were lightly anesthetized with xylazine/ketamine (10–75 mg/kg body weight, intraperitoneal injection) and a 7 mm piece of PE-10 tubing was inserted through the nostrils. The tubing was connected to a calibrated peristaltic pump set at a flow rate of 12.5 IU/min. The MPTP was dissolved in saline at a concentration of 20 mg/ml, after which it was infused at 1-min intervals for 4 min. The control solution consisted of saline. Animals were given a 1 min interval to regain normal respiratory function and then this procedure was repeated with infusions administered through the contralateral nostrils.

Tissue preparation and toluidine blue staining

Twenty days after the MPTP administration, mice were anesthetized with chloral hydrate (100 mg/kg). The mice were subjected to thoracotomy and perfusion with ice-cold 0.9% sodium chloride 50 ml and then with 4% paraformaldehyde 100 ml in 0.01 M phosphate buffered saline (PBS) through the left ventricle. After fixation, the brains were removed immediately and postfixed overnight at room temperature in the following fixative: 10% formaldehyde in 0.01 M PBS.

Following fixation, samples were dehydrated using an ascending ethanol series, cleared in xylene, and infiltrated with paraffin. They were then embedded in paraffin and sectioned through the SN coronally at 5 μm thickness using rotary microtome (Leica, Germany). All of the sections containing substintia Nigra[44] were mounted on slides. Sections were stained with 1% toluidine blue in 1% sodium borate for 1 min at 60°C.

Quantification of dark degenerating neurons

DNs in SNc were counted by an investigator blinded to the protocol treatment, using the optical dissector technique described in detail by Gundersen *et al.*[45] The optical dissector technique eliminates bias in counting as a result of cell size and shape. Briefly, DNs were counted as they came into focus while scanning through the section.

For each section, 4–6 unbiased counting frames were sampled in a systematically random fashion inside the area of SNc. The preparations were examined under a light microscope using a × 60 objective lens (UPlanFI, Japan), and images were transferred to computer using a high-resolution camera (B × 51, Japan). The number of DNs was counted using a 10,000 μm^2 counting frame. The mean numbers of neurons per unit area in SNc were calculated using the formula as follows:

$$N_A = \frac{\Sigma \overline{Q}}{a/f \bullet \Sigma P}$$

In this formula "$\Sigma \overline{Q}$" is the summation of counted DNs appeared in sections, "a/f" is the area associated with each frame (10,000 μm^2), "∑P" is the sum of frames associated points hitting the reference space.

Statistical analysis

The numbers of 8–10 sections from each animal were averaged, and the data from seven animals of each group were presented as a mean ± standard error of the mean. Results were analyzed using one-way ANOVA, followed by Tukey's *post-hoc* test for multiple comparisons between different groups studied. The level of statistical significance was set at $P < 0.05$. SPSS for Windows (Version 22; SPSS Inc., Chicago, IL, USA) was used to perform the total statistical analysis.

RESULTS

To explore the beneficial effects of L-arginine against MPTP-induced neuronal degeneration, toluidine blue staining was used to examine the numerical density of DNs in the SNc of Balb/c mice. Normal cells showed round and pale stained nuclei with a distinct nucleolus. The shrunken cells after MPTP administration with the morphological features of proapoptosis such as nuclear shrinkage and condensed chromatin were counted as DNs. To determine the numerical density of DNs in the SNc of Balb/c mice, we traced the boundaries for SNc as in Figure 1. The Numerical density of DNs was stereologically counted in SNc of mice in different studied groups.

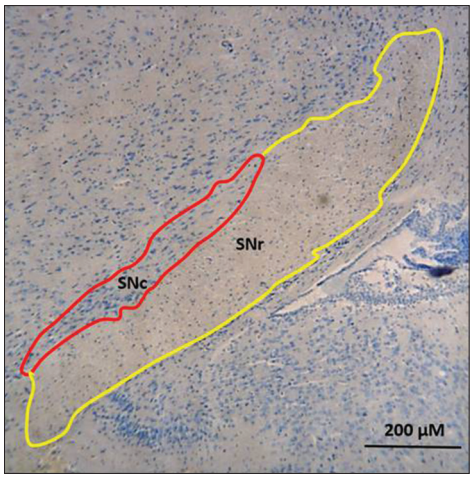

Figure 1: Photomicrograph of coronal section from the substantia nigra in Balb/c mice substantia nigra pars compacta (red); substantia nigra pars reticulata (yellow) illustrating methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dark neurons production in substantia nigra pars compacta subregion where used for the stereological study. Regional boundaries were determined by cross-referencing with the atlases of Paxinos and Watson (2006)

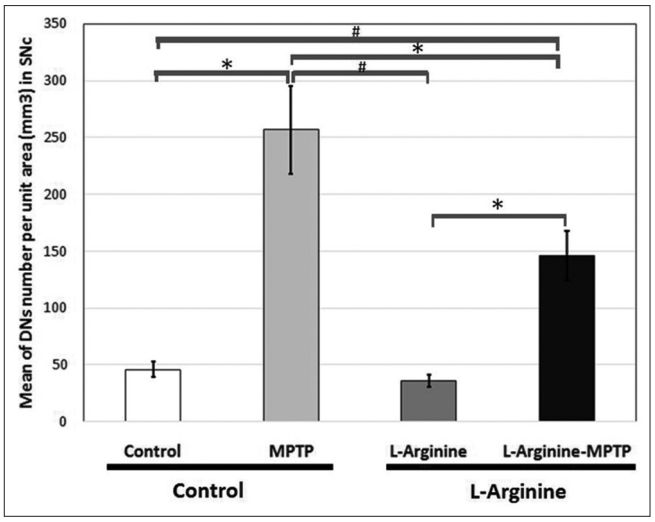

Figure 2: *P < 0.05; significant difference. #P < 0.01; significant difference. Graph 1: Mean of dark neuron numbers per unit area (mm^3) in the substantia nigra pars compacta subdivision of Balb/c mice and its comparison in the different studied groups. The data show that the mean number of dark neurons per unit area in methyl-4-phenyl-1,2,3,6-tetrahydropyridine group significantly increased in substantia nigra pars compacta comparing to L-arginine and control Balb/c mice. Evaluation of therapeutic effects on dark neuron production in substantia nigra pars compacta subregion revealed a significant reduction in the mean number of dark neurons in L-arginine - methyl-4-phenyl-1,2,3,6-tetrahydropyridine group. Data present in mean ± standard error of the mean

In control and L-arginine groups, there were a few numbers of DNs in SNc of the Balb/c mice [Figures 2 and 3a, c]. Whiles following 20 days after MPTP administration induced severe DNs production. Our results revealed a marked increase in the number of DNs in SNc of the Balb/c mice in MPTP group when compared with both control and L-arginine groups ($P < 0.05$ and $P < 0.01$, respectively) [Figure 3a, b, d and Graph 1]. Additionally, the number of DNs in the L-arginine-MPTP group also increased significantly when compared with both control and L-arginine groups ($P < 0.05$ and $P < 0.01$, respectively) [Figures 2 and 3a, c, d].

Nevertheless, injection of L-arginine (300 mg/kg; i.p.) once daily for 7 days starting from 3 days after MPTP administration significantly decreased the numerical density of dark degenerating neurons in SNc subregion of substintia nigra of the Balb/c mice ($P < 0.05$); [Figure 3a, d, and Graph 1]. We found a statistical decrease in the number of DNs in the SNc in the L-arginine-MPTP group Balb/c mice comparing to the MPTP group ($P < 0.05$) [Figures 2 and 3a, d].

DISCUSSION

To our knowledge, this is the first report investigating the neuroprotective effects of L-arginine in the animal model of PD. The results of this study show a novel beneficial effect of L-arginine against MPTP-induced neurodegeneration in SN of Balb/c mice. The SN is an important motor center that is thought to be the lesion site in PD.[1-3] Therefore, therapeutic or preventive strategies that stop or even slow the progress of PD are expected to have a major impact on the prevention or treatment of this kind of neurodegenerative disorders.[46] A recent series of studies demonstrated that a single i.n. infusion of MPTP in rodents produces diverse signs of PD such as impairments in the motor, cognitive, and emotional functions.[11,14,15] On the other hand, there is a little experience about the pharmacology of L-arginine administration in the doses given in experimental studies, especially in neurodegenerative diseases.[21,47] Nevertheless, there is sufficient evidence suggesting that the 300-mg/kg dose of L-arginine in the rodents provides the best results.[47] Recent studies on laboratory animals revealed that the administration of L-arginine has potential therapeutic importance, including anticonvulsant, anxiolytic, and antidepressant-like actions.[48-50]

In this study, we showed that the repeated treatment with l-arginine during 7 consecutive days after MPTP administration was able to decrease significantly the numerical density of DNs in SNc of Balb/c mice administrated intranasally with MPTP. These data corroborate the therapeutic potential of L-arginine in PD, since it attenuated the DA cell loss in the SNc of Balb/c mice infused intranasally with MPTP.

The therapeutic effects of L-arginine may result from different mechanisms including inhibition of

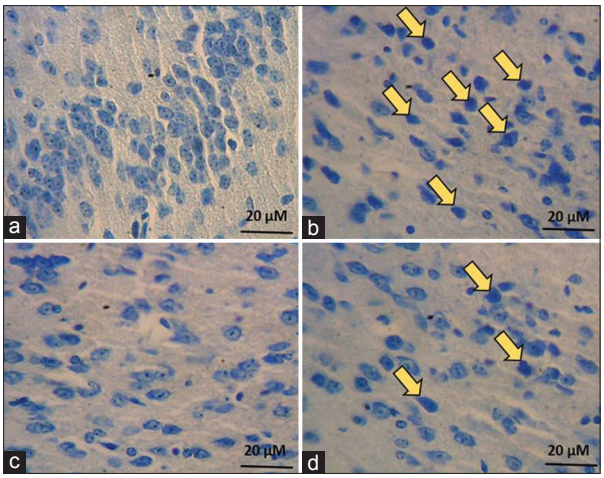

Figure 3: Photomicrographs showing distribution of dark neurons in substintia nigra pars compacta subdivisions of Balb/c mice in the control, (a) methyl-4-phenyl-1,2,3,6-tetrahydropyridine, (b) L-arginie, (c) and L-arginine - methyl-4-phenyl-1,2,3,6-tetrahydropyridine (d) groups. Dark neurons pointed with arrows. As shown the distribution of dark neurons in substantia nigra pars compacta subregion was strikingly increased in methyl-4-phenyl-1,2,3,6-tetrahydropyridine and L-arginine -methyl-4-phenyl-1,2,3,6-tetrahydropyridine group animals, compared to control and L-arginine Balb/c mice. Toluidine blue staining

NOS,[51] oxygen radical scavenging,[52] blocking of N-methyl-D-aspartate (NMDA) receptors,[48-50] and protection against mitochondrial membrane potential collapse.[53] However, the sequence of events leading to the protective effects of L-arginine against cell damage has not been fully elucidated.[52,53] In addition, the therapeutic effects of L-arginine administration could occur from its effects on the vasculature.[54]

Some of beneficial effects L-arginine are also presumed to occur via production of NO, as L-arginine is the precursor of NO in the reaction mediated by the enzyme NOS. NO is produced by many different tissues and has numerous physiological and pathological effects.[21,55,56] In the brain, NO plays a role as a neurotransmitter.[57] Experimental studies have well documented the synthesis of NO in the brain, and its role in a variety of neuronal functions including learning and memory processes, cortical arousal, and blood vessel dilatation and immune response.[57] NO is also a potent vasodilator and inhibits the platelet aggregation and leukocyte adhesion and may improve blood flow by preventing microvascular plugging by platelets and leukocytes.[58]

The current hypothesis about the mechanisms by which neurons come into apoptotic or necrotic process of degeneration has led to belief that the use of drugs modulating the function of glutamate NMDA receptors may have beneficial effects in PD cases.[59] There is increasing evidence of the beneficial effects of L-arginine blockades NMDA receptors, against different insults of the CNS.[48-50] Previous studies have also demonstrated that MPTP decreases glutamate uptake by astrocytes in cell culture.[60] Therefore, one possible mechanism by which L-arginine may exert beneficial effects against MPTP neurotoxicity may be due to the modulation of glutamate reuptake into neural cells.[60]

In earlier investigations, administration of L-arginine has also been shown to increase cerebral blood flow and reduce neurological damage after experimental traumatic brain injury.[31,47,56,61]

On the other hand, agmatine, formed by the decarboxylation of L-arginine, has been shown to be neuroprotective in experimental brain trauma and ischemia models.[43] Recently, agmatine has been proposed as a novel neuromodulator that plays protective roles in several models of neuronal cellular damage.[43,62] A study by Matheus *et al.*[43] demonstrated that treatment with agmatine increased the survival rate of old mice infused with a single i.n. administration of MPTP, improving the general neurological status of the surviving animals. The researchers claimed that agmatine represents a novel potential therapeutic tool for the management of cognitive and motor symptoms of PD.[43]

Of high importance, the administration of L-arginine demonstrated its protective properties as previously described in several models of neuronal damage.[61,63] These results corroborate recent findings on L-arginine neuroprotection in cellular models of neurodegenerative diseases.[21]

CONCLUSIONS

Taken together, these findings reinforce i.n. MPTP administration as a valuable rodent model for testing novel palliative compounds for PD. More importantly, this study provides the first preclinical data indicating that repeated systemic treatment with L-arginine prevents DA cell loss in the SNc of mice submitted to an experimental model of PD. These results provide new insights in experimental models of PD, indicating that L-arginine may represent a new neuroprotective agent from DA neuron degeneration observed in PD patients.

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Conflicts of interest

There are no conflicts of interest.

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