Nitric Oxide Synthase Inhibition Protects Against Rotenone-Induced Neurodegeneration *in vivo*

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Abstract: Neuropathologic and motor features of Parkinson's disease can be induced in rodents by the pesticide rotenone. In this study, we aimed to determine whether nitric oxide synthase inhibition by N^G-nitro-L-arginine methyl ester (L-NAME) could prevent neurodegeneration and biochemical changes induced by rotenone in rat brain. For this purpose, rats received subcutaneous injections of rotenone (1.5 mg/kg) every other day for two consecutive weeks and were treated at the same time with L-NAME at doses of 10 or 20 mg/kg, intraperitoneally. Oxidative stress indictors, malondialdehyde, reduced glutathione, and nitric oxide as well as paraoxonase-1 (PON-1) activity in brain, and the concentration of the antiapoptotic protein B cell/lymphoma-2 (Bcl2) in striatum were determined. Motor strength and coordination was assessed using wire hanging and stair tests. Neuronal degeneration was evaluated using hematoxylin & eosin-stained brain sections. Results: L-NAME reduced, in a dose-dependent manner the increase in lipid peroxidation (malondialdehyde) and nitric oxide and restored reduced glutathione levels in brain of rotenone-treated rats. Additionally, L-NAME prevented the inhibition of paraoxonase-1 activity and the decrease in Bcl2 concentration induced by the pesticide. It also prevented motor impairments in a dose-dependent manner. Furthermore, L-NAME treatment ameliorated the neurodegenerative effects of rotenone, as shown by the decrease in degenerated neurons in striatum and cerebral cortex. The results of the present study suggest that rotenone mediates its neurotoxic effects, at least in part by nitric oxide-dependent mechanisms.

Keywords: Nitric oxide synthase, Rotenone, Neuroprotection, Apoptosis, Oxidative stress, Reactive oxygen species.

INTRODUCTION

Parkinson's disease (PD) is a chronic and progressive neurodegenerative motor disorder that affects about 1% of individuals above 65 y of age [1, 2]. In PD, dopamine containing neurons of the substania nigra pars compacta (SNc) in midbrain undergo preferential and progressive death resulting in marked depletion in dopamine in striatum [3]. The consequent disruption of basal ganglia motor circuits and loss of its control over movements initiated in the cerebral cortex result in the emergence of characteristic symptoms of slowness in movements or bradykinesia, muscular rigidity, postural abnormalities and resting tremor [4]. The disease in major part is sporadic (idiopathic PD), and this accounts for about 95% of cases [5]. While the exact cause of the selective death of SNc neurons is not yet defined, convincing evidence points to a role of environmental toxins in initiating Parkinson's disease. Their action can be enhanced by genetic susceptibility in several sites of the genome [1, 6]. In this context, there are several studies, which suggest an association between exposure to pesticides and the increase in the risk for developing PD [7, 8]. Moreover, animal research have shown that rotenone, a naturally occurring pesticide [9] causes motor, and neuropathologic changes like those of idiopathic PD including nigrostriatal dopaminergic cell death, and alpha-synuclein like inclusions [10, 11].

There is evidence that nitric oxide may play a role in the pathogenesis of dopaminergic cell death in PD [12, 13]. The gaseous free radical nitric oxide is produced from L-arginine by two constitutive nitric oxide synthase (NOS) enzymes; neuronal NOS (nNOS), and endothelial NOS (eNOS). The third is an inducible isoform (iNOS) whose expression is stimulated by inflammatory stimuli *e.g.*, bacterial endotoxin and pro-inflammatory cytokines [14]. Low concentrations of nitric oxide produced by the constitutive NOS isoforms are important in intracellular signaling and play a role in neurotransmission, synaptic plasticity and in maintenance of vascular smooth muscle tone. In contrast, iNOS is the source of excessive release of nitric oxide by microglia and astrocytes during pathological conditions such as brain inflammation, infection, and trauma. Such high and long-lasting concentrations of nitric oxide are toxic to neurons [15, 16]. The neurotoxic actions of nitric oxide are ascribed to the formation of peroxynitrite ($ONOO^-$) by the reaction of nitric oxide with superoxide ($O_2^{\bullet-}$) and other reactive oxides of nitrogen such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), resulting in oxidation

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E-ISSN: 2309-6179/22

or nitration of tyrosine residues in proteins, and nitrosylation of thiols in proteins and reduced glutathione [16, 17]. Animal studies have shown that rotenone increased brain nitric oxide and the expression of iNOS in the substantia nigra and striatum, which suggests a role for nitric oxide derived from iNOS in the neurodegeneration caused by this pesticide [18, 19].

The aim of this study was therefore to investigate the effects of NOS inhibition by N^G-nitro-L-arginine methyl ester (L-NAME) on the development of oxidative stress, motor impairment and neuronal injury in brain of rats with rotenone-induced PD.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180-190 g were used in the study. Rats were obtained from the Animal House Colony of the National Research Centre, kept under temperature- and light-controlled conditions (20–22 °C and 12 h/12 h light/dark cycle) and given free access to standard laboratory rodent chow and tap water. Animal procedures followed the guidelines of the institute ethics committee for the use of animals in experimental studies and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Drugs and Chemicals

Rotenone and L-NAME were purchased from Sigma-Aldrich (St Louis, MO, USA). Rotenone was freshly prepared in 100% dimethyl sulfoxide (DMSO). All the used chemicals and reagents in the present study were of analytical grade and obtained from Sigma-Aldrich.

Experimental Design

Rats were randomly assigned to equal treatment groups (6 animals each).

Group 1 received the vehicle (DMSO) three times a week and served as negative control.

Groups 2 was treated with rotenone at 1.5 mg/kg, subcutaneously every other day for two weeks, treated at the same time with the vehicle and served as positive control.

Groups 3 & 4: these two groups were administered rotenone (1.5 mg/kg), subcutaneously every other day for two weeks and received L-NAME (10 or 20 mg/kg), once a day by intraperitoneal (i.p.) injection.

At the end of the study, rats were euthanized by cervical dislocation and each brain was quickly removed, washed with ice-cold phosphate-buffered saline (PBS, pH 7.4), placed on ice-cold plate, dissected, weighed, and stored at −80°C for the biochemical studies. Tissues were homogenized in 0.1 M phosphate-buffered saline at pH 7.4 to give a final concentration of 10 % w/v. Homogenization was performed using a homogenizer (ULTRA-TURAX, IKA T10 basic, Germany) at speed 5000 rpm for 30 seconds. The histopathological study was carried out on separate groups of rats (n = 4 per group).

Biochemical Assays

Lipid Peroxidation Assay

Lipid peroxidation was measured in brain homogenates by determining malondialdehyde (MDA) according to Nair and Turne [20]. In this assay 2-thiobarbituric acid reacts with MDA at 25°C to yield a red colored complex with a peak absorbance at 532 nm.

Reduced Glutathione Assay

Reduced glutathione was determined in brain homogenates according to Ellman [21]. Ellman´s reagent (DTNB; 5, 5'-dithiobis (2-nitrobenzoic acid)) reacts with the free thiol group of GSH to form 2-nitro-s-mercaptobenzoic acid. The chromophore has yellow color and is determined with spectrophotometer at 412 nm.

Nitric Oxide Assay

Nitric oxide was determined using Griess reagent. Nitrate is converted to nitrite with by the enzyme nitrate reductase. Nitrite then reacts with the Griess reagent to form a purple azo compound, and its absorbance is measured at 540 nm with spectrophotometer [22].

Paraoxonase-1 Assay

The arylesterase activity of PON-1 enzyme was determined by the use of phenyl acetate as a substrate. In this assay, arylesterase hydrolyzes phenyl acetate forming phenol and the rate of hydrolysis is measured by monitoring the increase in the absorbance at 270 nm at 25°C with the use of a spectrophotometer. One unit of arylesterase activity is equivalent to 1 µmol of phenol formed per min. Enzyme activity expressed as kU/l is calculated based on the molar extinction coefficient of 1,310 M^{-1} cm^{-1} for phenol at 270 nm, pH 8.0 and 25°C [23].

Quantification of Bcl-2

The level of Bcl-2 in the striatum was quantified using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit purchased from Glory Science, Del Rio, TX, USA, according to the instructions provided by the manufacturer. The level of Bcl-2 in a sample was determined by interpolation from a standard curve.

Behavioral Testing

Stair Test

This test was used to test the rats' skilled reaching. Rats were made to ascend a stair, placed at an angle of 55° above the bench, and the time it took to climb the stair is recorded for the vehicle- and rotenone-treated rats using a stop watch [24].

Wire Hanging Test

This test is used for the measurement of neuromuscular strength where rats were allowed to hang by their forelimbs from a steel rod above the bench. The latency to fall was counted for three trials with a cutoff time of 180 s [25].

Histopathological Studies

Brain samples were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin using standard procedures. Sections of 5 µm thickness were stained with hematoxylin and eosin (Hx&E) for the histopathological study using light microscope (Olympus Cx 41 with DP12 Olympous digital camera; Olympous optical Co. Ltd,Tokyo. Japan).

Statistical Analysis

Results are expressed as mean ± SE. Data were statistically analyzed using one way analysis of variance (ANOVA) followed Tukey's multiple comparisons test. GraphPad Prism 6 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA) was used. Differences were considered statistically significant at a probability value of less than 0.05.

RESULTS

Biochemical Results

Lipid Peroxidation

Repeated injections of rotenone resulted in a marked and significant increase in brain lipid peroxidation as indicated by the increased brain MDA by 42.5% compared with the vehicle-treated animals (23.22 ± 1.21 vs. 16.29 ± 1.45 nmol/g. tissue). There was no significant change in MDA level in the 10 mg/kg L-NAME group (20.82 ± 0.93 nmol/g. tissue) compared with the rotenone control group (23.22 ± 1.21 nmol/g. tissue). In the 20 mg/kg L-NAME group, MDA decreased significantly to 17.10 ± 0.3 nmol/g. tissue (Figure **1**).

Nitric Oxide

In rotenone control rats, the level of nitric oxide increased significantly by 68.5% compared with their vehicle-treated counterparts (30.72 ± 1.26 vs. 18.23 ± 0.50 µmol/g. tissue). In contrast, treatment with L-NAME at 10 or 20 mg/kg significantly decreased the elevated nitric oxide levels by 22.2% and 43%, respectively, compared with the rotenone control group. values are 30.72 ± 1.26 for the rotenone only group, and 23.89 ± 0.76 and 16.27 ± 0.84 µmol/g. tissue for the 10 and 20 mg/kg L-NAME + rotenone groups, respectively (Figure **1**).

Reduced Glutathione

In the rotenone only group, GSH level fell significantly by 45.1% as compared with the vehicle-treated group (1.75 ± 0. 09 vs. 3.19 ± 0.16 µmol/g. tissue). L-NAME treatment prevented the rotenone-induced GSH depletion, increasing brain GSH levels significantly by 58.9% and 71.4%, respectively, compared with the rotenone control group (2.78 ± 0.11 and 3.0 ± 0.13 us. 1.75 ± 0. 09 µmol/g. tissue in rotenone-treated rats without L-NAME) (Figure **1**).

Paraoxonase-1

There was a significant decrease in PON-1 activity in the rotenone only group by 48.3% (6.53 ± 0.35 kU/l) compared with the vehicle control group (11.2 ± 0.37 kU/l). In rotenone + L-NAME groups, PON-1 activity increased significantly to 7.5 ± 0.29 and 9.1 ± 0.39 kU/l (14.9% and 39.4% increase vs. rotenone only group) (Figure **2**).

Bcl-2

Compared with the vehicle control group, a significantly decreased Bcl-2 level in the striatum by 26%was observed in the rotenone only-treated rats (1.85 ± 0.04 vs. 2.5 ± 0.09 ng/ml). There was no significant change in Bcl-2 concentration in the 10 mg/kg L-NAME group compared with the rotenone control group (2.07 ± 0.02 vs. 1.85 ± 0.04 ng/ml). In the 20 mg/kg L-NAME group, however, striatal Bcl-2

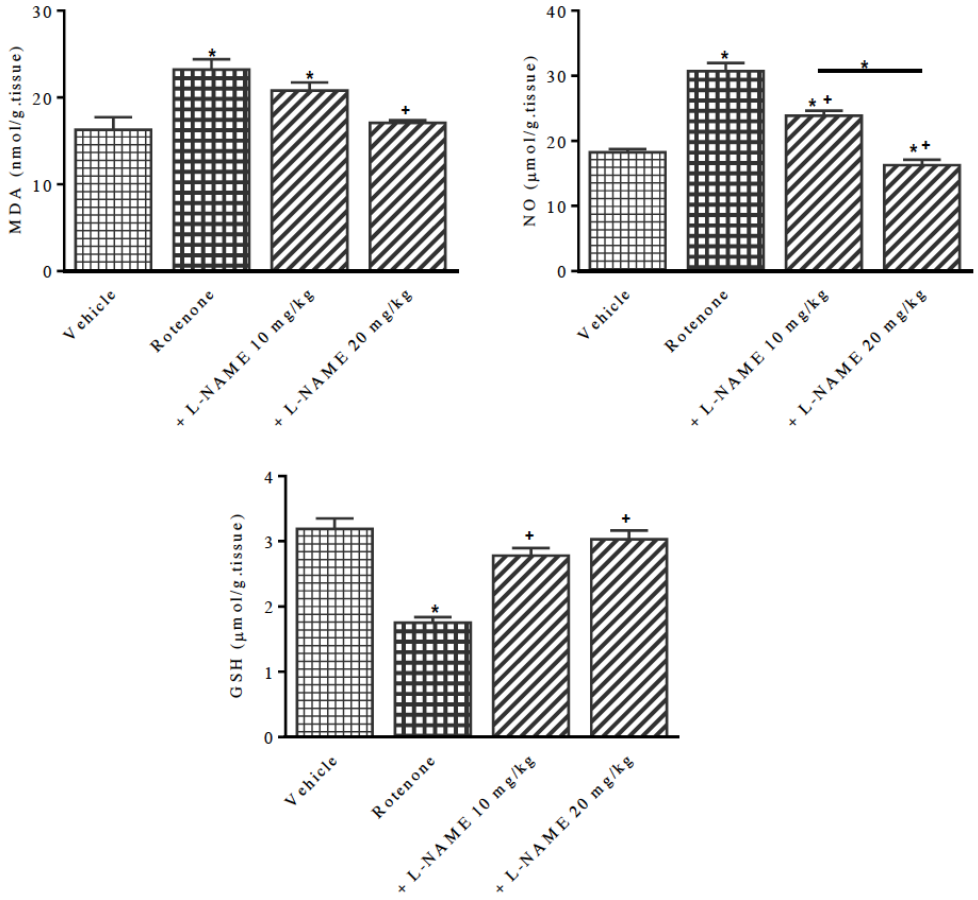

Figure 1: Effects of NOS inhibition by L-NAME on brain levels of malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) in rats treated with rotenone. Values represent means ± SEM. *: P<0.05 vs. vehicle and between different groups as indicated in the graph. +: P<0.05 vs. rotenone control.

increased significantly by 20.5% (2.23 ± 0.08 vs. 1.85 ± 0.04 ng/ml) compared with the rotenone only group (Figure **2**).

Behavioral Results

Wire Hanging Test

In the rotenone only group, rats showed significant and marked decrease in the time they spent

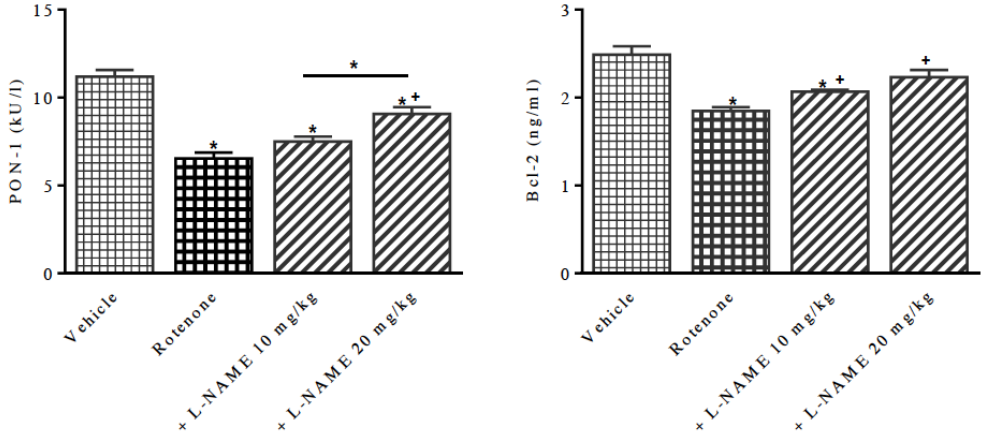

Figure 2: Effects of NOS inhibition by L-NAME on brain paraoxonase-1 (PON-1) activity and striatal Bcl-2 levels in rats treated with rotenone. Values represent means ± SEM. *: P<0.05 vs. vehicle and between different groups as indicated in the graph. +: P<0.05 vs. rotenone control.

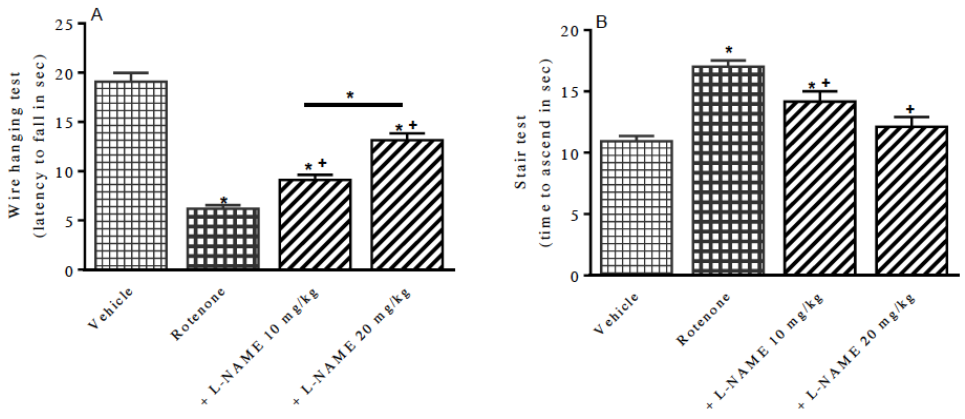

Figure 3: Effects of NOS inhibition by L-NAME on motor strength in rats treated with rotenone. Values represent means ± SEM. *: P<0.05 vs. vehicle and between different groups as indicated in the graph. +: P<0.05 vs. rotenone control.

suspended from the steel rod by 67.5% compared to the vehicle group (6.21 ± 0.35 vs. 19.10 ± 0.88 sec). In the L-NAME + rotenone groups, however, the latency to fall increased significantly by 46.7% and 111.8% compared with the rotenone control value (9.11 ± 0.53 and 13.15 ± 0.69 vs. 6.21 ± 0.35 sec)(Figure **3A**).

Stair Test

Rotenone led to significant increase in the time rat spent to ascend the stair by rotenone by 55.4 % (17.0 ± 0.51 vs. 10.94 ± 0.44 sec). In the L-NAME + rotenone groups, however, the time decreased significantly by 16.6% and 28.6% compared with the rotenone control value (14.17 ± 0.84 and 12.13 ± 0.77 vs. 17.0 ± 0.51sec)(Figure **3B**).

Histopathological Results

Striatum

The striatum from the vehicle control group showed neurons with the surrounding supporting cells with normal nuclei exhibiting dispersed chromatin, basophilic cytoplasm and prominent nucleoli (Figure **4A**). Rotenone caused neurodegenerative

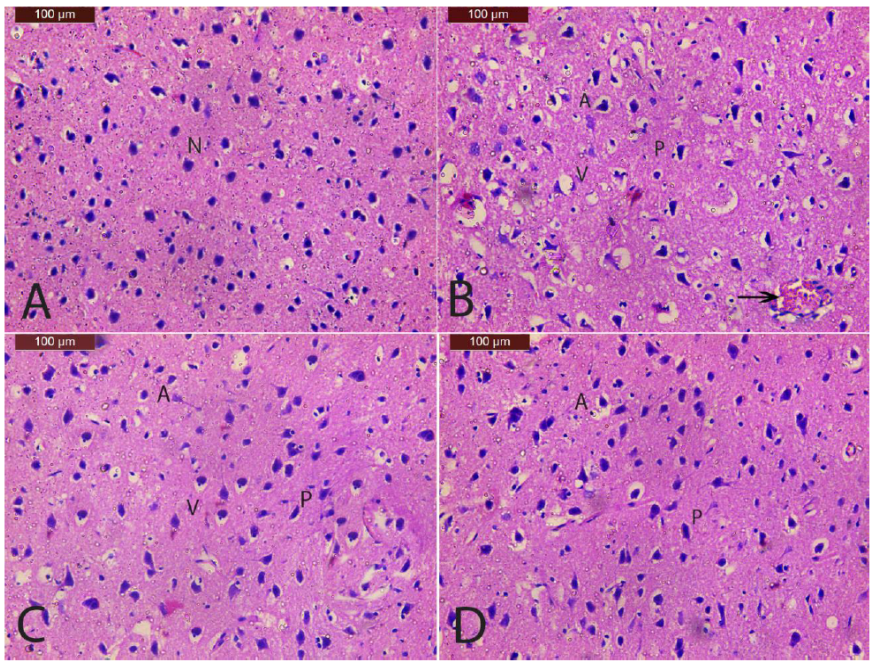

Figure: 4. Representative microphotographs from Hx & E stained sections of the striatum after treatment with: (**A**) Vehicle: showing normal neurons (N). (**B**) Rotenone: showing neurodegenerative changes with perinuclear cytoplasmic vacuoles in neurons (V), congested blood vessels (arrow), pyknotic (P), and apoptotic nuclei (A). (**C**) Rotenone + L-NAME 10 mg/kg: showing less degenerative changes with perivascular vacuolation (V), pyknosis of some neurons (P), and apoptotic cells (A). (**D**) Rotenone + L-NAME 20 mg/kg: showing nearly normal neuronal cells and very few pyknotic nuclei (P) and apoptotic cells (A).

changes with vacuolation of neuropil, congested blood vessels, pyknotic, and apoptotic nuclei (Figure **4B**). In the group treated with rotenone and L-NAME (10 mg/kg) there were less degenerative changes observed with perivascular vacuolation, pyknosis of some neurons, and apoptotic cells (Figure **4C**). However, treatment with L-NAME at 20 mg/kg resulted in nearly normal neuronal cells and few vacuolation, pyknotic and apoptotic nuclei (Figure **4D**)

Cerebral Cortex

Sections of the cerebral cortex from the vehicle-treated group showed normal neurons arranged in neat rows with abundant cytoplasm and round basophilic nuclei (Figure **5A**). Rotenone-treated rats exhibited disorganization, eosinophilic staining, necrotic cells, dilated blood vessels and congested capillaries with red blood cells. Perinuclear cytoplasmic vacuoles in neurons and degenerative changes, pyknotic nuclei, and apoptotic cells were also observed (Figure **5B**). In the group treated with rotenone and L-NAME at 10 mg/kg there was moderate improvement and less histopathological changes except for pyknosis of some neurons, apoptotic nuclei, and congested blood vessels (Figure **5C**). Sections from the group treated with rotenone and L-NAME at 20 mg/kg showed almost normal neuronal cells of cortex. However, few histopathological changes such as very few pyknoticnuclei and apoptotic cells were still seen (Figure **5D**).

DISCUSSION

In this study, we demonstrated that, in the model of rotenone-induced PD in the rat, the concurrent systemic administration of the NOS inhibitor L-NAME conferred significant neuroprotection. It was found that rotenone caused significant increase in brain lipid peroxidation, and nitric oxide, depletion of reduced glutathione, inhibition of PON-1 activity, and decreased striatal Bcl-2. This was accompanied by motor impairment and neuronal atrophy in the striatum and cerebral cortex. The administration of L-NAME decreased brain lipid peroxidation, nitric oxide, restored reduced glutathione, PON-1 activity and Bcl-2 level. L-NAME afforded protection against the histopathological changes induced by rotenone in brain.

Because of its lipophilic properties, rotenone readily crosses the blood–brain barrier and accumulates in the mitochondria. Rotenone is a selective inhibitor of mitochondrial complex I, also known as nicotinamide adenine dinucleotide- ubiquinone oxidoreductase, the first complex of the mitochondrial respiratory chain that transfers electrons to ubiquinone (coenzyme Q) [26,

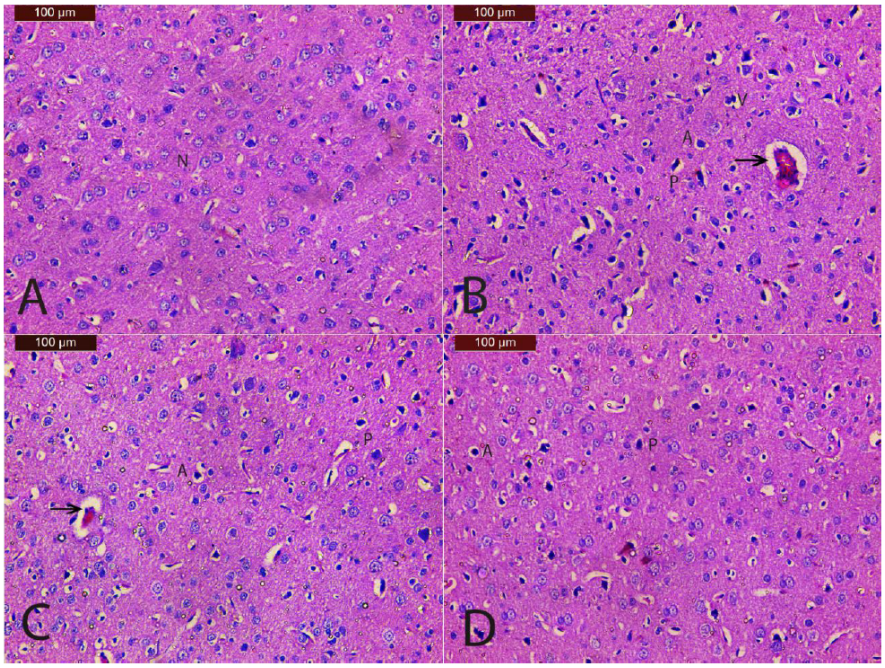

Figure 5: Representative microphotographs from Hx & E stained cerebral cortex sections after treatment with: (**A**) Vehicle: showing normal neurons (N). (**B**) Rotenone: showing disorganization, eosinophilic staining, necrotic cells, perinuclear cytoplasmic vacuoles in neurons (V), pyknotic nuclei (P), and apoptotic cells (A), and dilated blood vessels and congested capillaries with red blood cells (arrow). (**C**) Rotenone + L-NAME 10 mg/kg: showing moderate improvement and less histopathological changes except for pyknosis of some neurons (P), apoptotic nuclei (A) and congested blood vessels (arrow). (**D**) Rotenone + L-NAME 20 mg/kg: showing almost normal neuronal cells, very few pyknotic (P) and apoptotic nuclei (A).

27]. Under physiological conditions, complexes I and III of the mitochondrial respiratory chain are major sites for ROS production. Superoxide radical is generated as a result of electron escape from the electron transport chain, which dismutates to H_2O_2. The reaction of H_2O_2 with the reduced forms of the transition metal ions Fe^{2+}, Cu^{+} gives rise to the most toxic species, the hydroxyl radical ($HO^{\cdot}$)[28, 29]. Rotenone which inhibits mitochondrial complex I activity, induces the accumulation of electrons and generation of $O_2^{\cdot-}$ at complex I, resulting in oxidative stress, mitochondrial damage, and initiation of cell apoptotic pathway [28,30,31]. Rotenone induces dopaminergic cell apoptosis which can be inhibited by antioxidants glutathione, *N*-acetylcysteine, and vitamin C [30]. Moreover, nitric oxide produced by NOS can react with $O_2^{\cdot-}$ to form $ONOO^{-}$ or with O_2 to form NO_2 and NO_3. These species are capable of oxidation, nitration, and nitrosylation reactions [16, 28]. Rotenone may thus evoke neuronal damage by virtue of its ability to increase intracellular reactive nitrogen species. *In vitro*, rotenone increased nitration of the tyrosine residues on intracellular proteins and induced apoptosis of human dopaminergic SH-SY5Y cells, which could be inhibited by L-NAME or *N*-acetylcysteine [32].

In this study, we found that rotenone increased nitric oxide levels and lipid peroxidation in the rat brain. Others have shown increased the level of nitric oxide in striatum and frontal cortex by rotenone [33, 34]. We in addition, have demonstrated increased both iNOS and caspase-3 immunoreactivities in the striatum and SN, with loss of pigmented neurons, and markedly decreased tyrosine hydroxylase-immunoreactivity in rotenone-treated animas, which suggests a role for iNOS-derived NO in neuronal cell death caused by the toxicant [18, 19]. In support of this notion, other reports have shown that NOS inhibitors are protective in models of toxicant-induced nigrostriatal cell death. In mice, iNOS gene deletion or nNOS inhibition was found to confer resistance to the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [35, 36]. In a model of PD induced by i.p. injection of rotenone in rats, the neuronal NOS inhibitor 7-nitroindazole (7-NI) significantly attenuated the increased NOS expression (NADPH-d staining) in the striatum and SN and striatal 3-nitrotyrosine suggestive of nitrosative stress. 7-NI also prevented the loss of tyrosine hydroxylase-positive neurons in the SNc [37]. In another study, rotenone was infused into the right SNc followed by increased iNOS expression and NOS activity in the ipsilateral SN. Either 7-NI or L-NAME (20 mg/kg, i.p.) administered for four days every 12 h attenuated striatal dopamine depletion and the loss of nigrostriatal tyrosine hydroxylase -immunoreactivity induced by rotenone [38]. The above studies therefore, suggest a role for nNOS in mediating neuronal injury caused by rotenone. L-NAME, however, is not selective for the different isoforms of NOS and thus inhibition of both nNOS and iNOS are involved in its protective effect.

Results from this study also demonstrate that PON-1 activity is significantly reduced in brain of rotenone-treated animals, which is in agreement with our previous findings [39, 40] Paraoxonase-1 has an esterase and lactonase activities and catalyzes the hydrolysis many organophosphates, the nerve agents sarin and soman, lipid peroxides and many other xenobiotics [41]. Recent interest in PON-1 focuses on its role in Parkinson's disease, since variation in the catalytic efficiency of the enzyme alters the susceptibility to organophosphate neurotoxicity [42] and consequently the risk of PD in exposed subjects [43]. Moreover, a decrease in enzyme activity was found in neurological disorders, which suggests a neuroprotective function for PON-1 via its antioxidant and anti-inflammatory actions [44]. Paraoxonase-1 is susceptible to oxidative inactivation [45, 46] which may explain the decrease in enzyme activity after rotenone injection and that in turn would render neuronal cells vulnerable to oxidative stress. On the other hand, the increase in PON-1 activity following L-NAME treatment could be the result of lower levels of oxidative/nitrosative stress and hence is indicative of neuroprotection.

The mitochondrial apoptotic pathway is regulated by BcL-2 family, which includes both pro- and anti-apoptotic members [47]. The anti-apoptotic protein Bcl-2 maintains the integrity of the outer mitochondrial membrane, prevents the release of mitochondrial cytochrome c into the cytosol to activate the caspase cascade [48, 49]. The presence of apoptosis has been shown in idiopathic PD as indicated by increased caspase-3 and Bax immunoreactivites in melanized neurons of substantia nigra [50]. Bcl-2 proteins may cause vulnerability of the nigrostriatal system in the adult [51]. In this study, we found that the concentration of Bcl-2 protein in the striatum decreased after rotenone administration which is in agreement with previous observations [52]. Rotenone was shown to decrease the expression of Bcl-2 in human dopaminergic cells *in vitro* [53] In SH-SY5Y cells *in vitro*, rotenone increased expression of Bax, cleaved caspase-3 and decreased expression of Bcl-2 [54]. It has been suggested that Bcl-2 may allow cells to adapt

to high level of oxidative stress [55]. Bcl-2 has an antioxidant action in that it prevents apoptosis initiated by H_2O_2, O_2·- [56, 57] and this may involve increased glutathione concentrations [57] and peroxynitrite-induced cell death [58]. It may also may facilitate DNA repair after oxidative stress [58]. In the present work, the concentrations of Bcl-2 in the striatum of rotenone-treated rats, increased by L-NAME. This may suggest an anti-apoptotic action for the NOS inhibitor or may be due to reducing oxidative stress by L-NAME and hence preventing the oxidative inactivation of Bcl-2.

CONCLUSIONS

In conclusion, our results show that NOS inhibition by L-NAME prevents dopaminergic neuronal degeneration caused by rotenone and suggest that increased nitric oxide production may contribute at least in part to the neurotoxicity.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENT

This work was not supported by research grants.

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Received on 17-05-2022 Accepted on 09-06-2022 Published on 06-07-2022

DOI: https://doi.org/10.12974/2309-6179.2022.07.02