Capsaicin protects against nigrostriatal neurodegeneration induced by rotenone

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Abstract: - Capsaicin, the principal pungent ingredient of hot pepper exerts neuroprotective effects. In this study, the effect of capsaicin on rotenone-induced Parkinson's disease in mice was investigated. Mice were given subcutaneous rotenone injections (1.5 mg/kg, every other day) and at the same time treated with the vehicle, L-dopa (25 mg/kg) or capsaicin at doses of 0.5 or 1.0 mg/kg orally once a day for two weeks. Biochemical indices of oxidative stress, malondialdehyde, reduced glutathione and nitric oxide were determined in brain tissue and histopathological study of the brain was done. Behavioral tests included stair, wire hanging and wood walking tests. Results showed that rotenone treatment led to significant increases in brain malondialdehyde and nitric oxide contents parallel with marked depletion of reduced glutathione. Rotenone induced degeneration of pigmented neurons in substantia nigra and of cerebral cortex and hippocampus neurons. Rotenone impaired neuromuscular strength, motor balance and coordination. Treatment with capsaicin significantly ameliorated the neuronal degeneration caused by rotenone and improved motor function. Capsaicin alleviated the increase in lipid peroxidation (malondialdehyde) and nitric oxide and prevented the depletion of reduced glutathione in brain of rotenone-treated animals. These data indicate that capsaicin protects against rotenone-induced neuronal damage and this involves decreased level of oxidative stress. Capsaicin therefore might prevent cell death in the brain of Parkinson's disease patients.

Key-Words: - capsaicin; neuroprotection; substantia nigra; oxidative stress; rotenone

1 Introduction

Parkinson's disease (PD) is a common neurological movement disorder affecting about 1% of individuals above 65 years of age [1]. Classically, the disease manifests as bradykinesia or difficulty in initiation and slowness of voluntary movements, muscle rigidity, resting tremor and postural instability [2]. These symptoms result from a progressive death of dopaminergic cells in midbrain substantia nigra pars compacta (SNc) and consequent dopamine depletion in SNc and striatum [3]. It is this loss of dopamine which leads to inability of basal ganglia motor circuits to exert control over motor action *via* its connections to the thalamus (which in turn projects to motor cortex, cortical supplementary motor area and other premotor cortical areas) or other brainstem nuclei [4]. In addition to motor symptoms, cognitive decline, apathy, depression and autonomic dysfunction occur during the course of illness [2].

The disease occurs in a predominantly sporadic form (idiopathic PD) with only about 3-5% of cases being of genetic origin [5],[6]. The exact cause of the preferential dopaminergic cell death in PD is not yet clear, but interaction between environmental factors (probably toxins) and genetic susceptibility is a widely accepted hypothesis [7]. In support of this notion, a number of epidemiological studies have suggested a link between exposure to pesticides and an increased risk for developing PD [7],[8].

Increased level of oxidative stress is a key event that underlies cell death in PD [9],[10]. Reactive oxygen species (ROS) are produced within the cell as byproducts of normal cellular metabolism, the most important source being the mitochondrial respiratory chain where leakage of electrons onto molecular oxygen results in the formation of superoxide $(O_2^{\bullet-})$. Other sources are the autoxidation or metabolism of monoamine neurotransmitters by the enzyme monoamine oxidase, activated phagocytes that release superoxide, hydrogen peroxide and hypochlorous acid, activated lipoxygenase and cyclooxygenase and nitric oxide synthases [11]. In face of ROS, the cell is equipped by a number of antioxidant defenses that includes the enzymes superoxide dismutase and glutathione peroxidase, reduced glutathione (GSH), ascorbate, and α-tocopherol [12]. Oxidative stress develops when the cell's antioxidants are overwhelmed by the increased production of ROS, resulting in oxidative damage to membrane lipids, nucleic acids and proteins [11]. The abundance of highly polyunsaturated fatty acids coupled with the paucity of antioxidants makes the brain particularly susceptible to oxidative stress [13]. Moreover, mitochondrial complex I damage in the brain of PD patients can lead to increased production of superoxide [14].

Currently, there are no treatments which can cure or halt the progression of PD. Dopamine replacement therapy with the dopamine precursor levodopa (l-dopa) is accepted as the most effective in alleviating symptoms [15]. However, several years after initiation of L-dopa, complications such as motor fluctuations and dyskinesias arise [16]. This highlights the need of finding novel therapies for PD.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent principle of hot chilli pepper of the plant genus Capsaicin is a selective sensory excitotoxin that in low doses excites while in high doses desensitizes a subset of mammalian primary afferents with unmyelinated C fibers and thinly myelinated Aδ fibres that express the transient receptor potential vanilloid-1 (TRPV1). Capsaicin is a selective agonist at the TRPV1 or capsaicin receptor [17]. TRPV1 channel is also expressed in the cerebral cortex, hippocampus and substantia nigra in neuronal cell bodies and dendrites, microglia, astrocytes and pericytes [18].

In light of the above, the present study aimed to examine the effect of capsaicin administration on oxidative stress and the neurodegeneration in brain of rotenone-treated mice. Rotenone is a botanical insecticide and pesticide derived from the roots of tropical plants of the Leguminosae family. It is used in home gardens for insects, for lice and ticks on pets, and to kill fish populations [19]. Rotenone has been shown to result in the neuropathologic and motor changes similar to that of PD when injected into rodents [20],[21],[22]. Rotenone is thus widely used to investigate the pathogenetic mechanisms underlying nigrostriatal degeneration and possible therapeutic targets.

2 Materials and Methods

2.1 Animals
For the experiment, male Swiss albino mice weighing between 22 and 25 g were employed. The mice were allowed unlimited access to standard lab food and water. The Institute Ethics Committee's and the U.S. National Institutes of Health's Guide for Care and Use of Laboratory Animals' requirements (Publication No. 85-23, revised 1996) were adhered to in the animal studies.

2.2 Drugs and Chemicals
Rotenone and capsaicin were purchased from Sigma-Aldrich (St Louis, MO, USA). Rotenone was freshly prepared in 100% dimethyl sulfoxide. Stock solutions of capsaicin (4 mg/ml) contained 10% ethanol, 10% Tween 80, and 80% saline solution. Capsaicin was diluted in saline obtain the necessary doses. Levodopa/carbidopa (Sinemet, Merck Co.) was used and dissolved in saline. All the used chemicals and reagents in the present study were of analytical grade and obtained from Sigma-Aldrich.

2.3 Experimental Groups
Mice were randomly assigned to different treatment groups (6 animals each). Group 1 received the vehicle (DEMSO) three times a week and served as negative control. Groups 2, 3, 4 & 5 were given rotenone at 1.5 mg/kg, subcutaneously every other day for two weeks and treated at the same time with

the vehicle (group 2; positive control), L-dopa/carbidopa (sinemet) at 25 mg/kg orally once a day (group 3) or capsaicin at 0.5 or 1 mg/kg, orally once a day (groups 4 & 5).

Treatments were continued for two weeks. Behavioral tests were done 24 hrs after last injection of rotenone. After that, mice 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 study. 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).

2.4 Biochemical studies
2.4.1. Lipid peroxidation
Malondialdehyde (MDA), a product of lipid peroxidation [23] was measured in tissue homogenates by determining malondialdehyde (MDA) according to Nair and Turne [24]. 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 that can then be measured using a spectrophotometer at 532 nm (UV-VI8 Recording Spectrophotometer, Shimadzu Corporation, Australia).

2.4.3. Nitric oxide
Nitric oxide was determined in tissue homogenates by using Griess reagent, according to the method of Archer et al. [25]. Nitrate is converted to nitrite via nitrate reductase. Griess reagent then act to convert nitrite to a deep purple azo compound, the absorbance of which can be measured with a spectrophotometer at 540 nm.

2.4.3. Reduced glutathione
Reduced glutathione was determined in brain homogenates according to Ellman procedure [26]. In this assay, 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.

2.5. Behavioral studies
2.5.1. Stair test
In order to assess skilled reaching, each mouse were placed at the bottom of a stair (30 cm in length) placed at an angle of 55° above the bench. The latency to climb the stair is recorded for each mouse using a stopwatch [27].

2.5.2. Wood walking test
To assess motor coordination, each mouse was made to walk over a wooden stick (~1 m in length, 1 cm in width). The time each mouse spent to reach the end was recorded [28].

2.5.3. Wire hanging test
To examine motor strength, mice were made to hang by their forelimbs from a steel rod (25 cm long, 0.2 cm in diameter), 0.25 m above the bench. The time each mouse could hang suspended from the rod was recorded for three trials with a cut-off time of 180 s [29].

2.6 Histopathological studies
Brain sections were fixed in freshly prepared 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Paraffin sections 5 µm thick were prepared and stained with hematoxylin and eosin (Hx & E) for histopathological examination. Images were examined and photographed under a digital camera (Microscope Digital Camera DP70, Tokyo, Japan), and processed using Adobe Photoshop version 8.0 (San Jose, CA, USA).

2.7 Statistical analysis
The study's data were shown as mean ± SEM. Duncan's multiple range test was used in conjunction with a one-way ANOVA to determine statistical significance. The program used was Graphpad Prism version 6 (GraphPad Prism Software Inc., San Diego, CA, USA). A probability value was considered statistically significant if it was less than 0.05.

3 Results
3.1 Biochemical parameters
3.1.1 Lipid peroxidation
The level of MDA, an indicator of lipid peroxidation, was significantly higher in the rotenone-treated group by 84.7% compared with the vehicle control (31.19 ± 0.8 *vs.* 16.89 ± 0.71 nmol/g.tissue). MDA levels were significantly decreased by 38.7% and 44.3% in the rotenone + capsaicin groups, respectively, compared with those of the rotenone control group. It also decreased by 29.5% in the rotenone + l-dopa group compared with the rotenone control. Values were 21.98 ± 0.62 nmol/g.tissue in the rotenone + l-dopa group and 19.12 ± 1.24 and 17.36 ± 1.11 nmol/g.tissue in the rotenone + capsaicin groups, respectively (Fig. 1).

3.1.3 Nitric oxide

In the group treated with only rotenone, nitric oxide level was significantly increased by 110% compared with the vehicle control value (49.93 ± 1.47 *vs.* 23.77 ± 1.57 μmol/g. tissue). The level of nitric oxide fell by 23.7% and 30.1% in the rotenone + capsaicin (0.5 and 1 mg/kg)-treated groups, respectively, compared with the rotenone only group (38.10 ± 2.5, 34.54 ± 1.68 *vs.* 49.93 ± 1.47 μmol/g. tissue). There was also significant decrease in the level of nitric oxide by 27.7% in mice treated with rotenone + l-dopa (36.10 ± 1.77 *vs.* 49.93 ± 1.47 μmol/g. tissue) (Fig. 1).

3.1.2 Reduced glutathione

Mice treated with only rotenone had significantly lower GSH levels by 48.1% compared with the vehicle control (1.61 ± 0.05 *vs.* 3.1 ± 0.20 μmol/g. tissue). Meanwhile, GSH levels increased by 124.8% and 155.9% by capsaicin administration compared to the rotenone only group (3.62 ± 0.13, 4.12 ± 0.2 *vs.* 1.61 ± 0.05 μmol/g. tissue). In rotenone + l-dopa group, GSH levels were increased by 60.2% compared with that of rotenone only group (2.58 ± 0.10 *vs.* 1.61 ± 0.05 μmol/g. tissue). (Fig. 1).
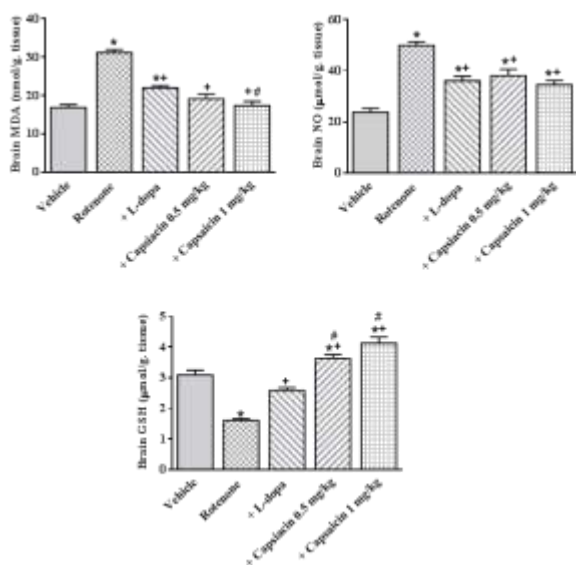

Fig. 1 Effects of treatment with L-dopa, sucrose or sucrose/metformin on malondialdehyde (MDA) and nitric oxide in brains of mice given s.c. rotenone injections. *: p<0.05 *vs.* the vehicle group and between the different treated groups as shown in the graph. +: p<0.05 *vs.* the rotenone control group. #: p<0.05 *vs.* the L-dopa group.

2.4 Behavioral studies

2.4.1. Stair test

In the rotenone group, the time spent in ascending the stair was significantly increased by 58.4% compared to the vehicle group (19.44 ± 0.68 *vs.* 12.27 ± 0.56 sec). In the groups treated with rotenone and capsaicin (0.5 and 1 mg/kg) or rotenone + L-dopa, the time taken by mice to ascend the stair was significantly shorter by 47%, 64.1% and 57.8%, compared to the rotenone only group (10.30 ± 0.95, 6.98 ± 0.74, 8.21 ± 0.57 *vs.* 19.44 ± 0.68 sec).

2.4.1. Wire hanging test

Mice receiving rotenone showed decreased ability to hang suspended from the steel rod by 65.7% compared to their vehicle controls (5.18 ± 0.49 *vs.* 15.10 ± 1.25 sec). When treated with capsaicin, the latency to fall increased by 59.6% and 126.8% as compared with rotenone only group (8.27 ± 0.15, 11.75 ± 0.77 *vs.* 5.18 ± 0.49 sec). Mice treated with rotenone + L-dopa exhibited 108.9% increase in heir latency to fall (10.82 ± 0.46 vs. 5.18 ± 0.49 sec).

2.4.1. Wood walking test

Compared with the vehicle control group, rotenone-treated mice were significantly slower to traverse a wooden stick by 114.2% (17.46 ± 0.66 *vs.* 8.15 ± 0.39 sec). In the groups treated with capsaicin, the latency to traverse the stick was significantly decreased by 72.1% and 78.1% compared with the rotenone only group (4.88 ± 0.51, 3.82 ± 0.22 *vs.* 17.46 ± 0.66 sec). Meanwhile, mice given rotenone + L-dopa showed 58.6% decrease in latency (7.22 ± 0.48 *vs.* 17.46 ± 0.66 sec).
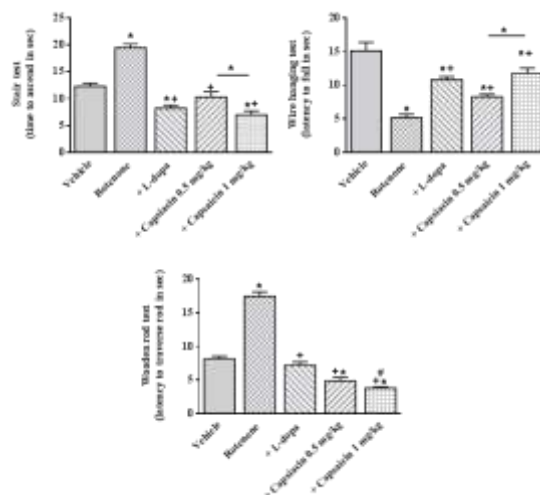

Fig. 2 Effect of capsaicin and L-dopa on motor strength and coordination in mice treated with rotenone. Values represent means ± SEM. *: P<0.05 *vs.* vehicle and between different groups as indicated in the graph. +: P<0.05 *vs.* rotenone control. #: P<0.05 *vs.* L-dopa-treated group.

2.4 Histopathological studies

Following rotenone injections, neurodegenerative changes could be observed in the substantia nigra, cerebral cortex and hippocampus brain regions. The pigmented cells in the substantia nigra were markedly reduced in size and number.   In the cerebral cortex and hippocampus, many deeply stained neurons with shrunken cytoplasm and damaged nuclei could be seen. The pathological changes caused by rotenone were attenuated by capsaicin and L-dopa (Figs. 3,4 & 5).
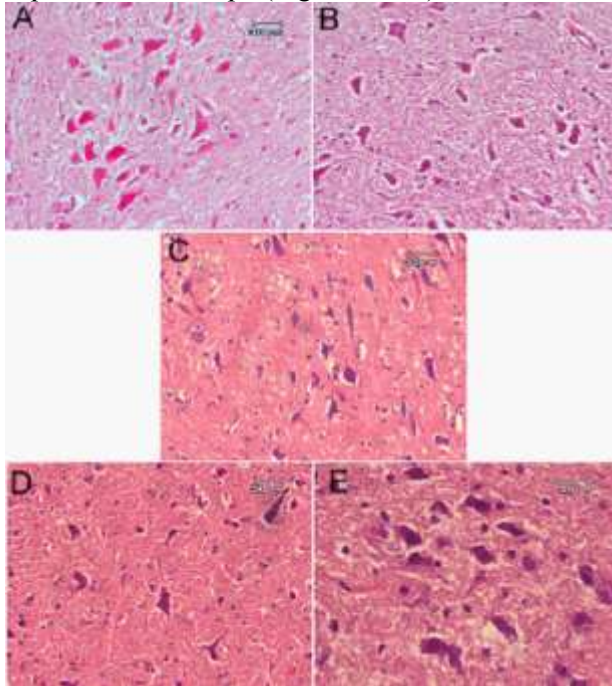

Fig. 3 Representative photomicrographs of Hx & E stained sections of the substantia nigra area after treatment with: **(A)** Vehicle showing the normal structure of the pigmented neurons in this area. **(B)** Rotenone showing decrease in size and number of pigmented cells. **(C)** Rotenone + L-dopa showing increase of pigmented neurons in number, but many of them are still smaller in size than normal. **(D)** Rotenone + capsaicin 0.5 mg/kg showing marked decrease of size and number of pigmented neurons. **(E)** Rotenone + capsaicin 1 mg/kg showing noticeable increase in number and size of pigmented cells.
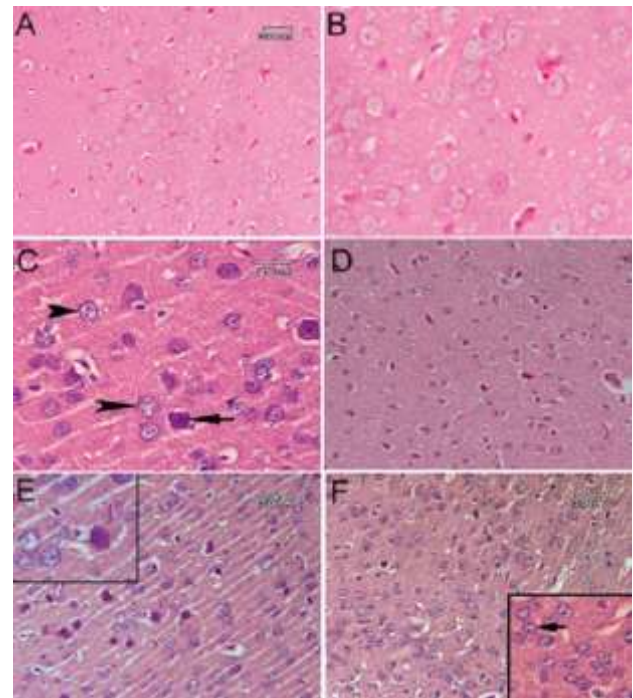

Fig 4 Representative photomicrographs of Hx & E stained sections of the cerebral cortex tissue from mice treated with: **(A)** Vehicle: showing the normal structure of cerebral cortex tissue. **(B)** A higher magnification of the previous section shows the same result. **(C)** Rotenone showing deeply stained neurons (arrow) and karyorrhexia in others (arrowhead). **(D)** Rotenone + L-dopa showing shows marked decrease of deeply stained neurons, only a few deeply stained cells are noticed among normal neurons. **(E)** Rotenone + capsaicin 0.5 mg/kg showing a slight decrease of darkly stained and karyorrhectic neurons. (F) Rotenone capsaicin 1 mg/kg showing marked decrease of darkly stained nuclei, although neurons with karyorrhectic nuclei are still noticed (arrow).
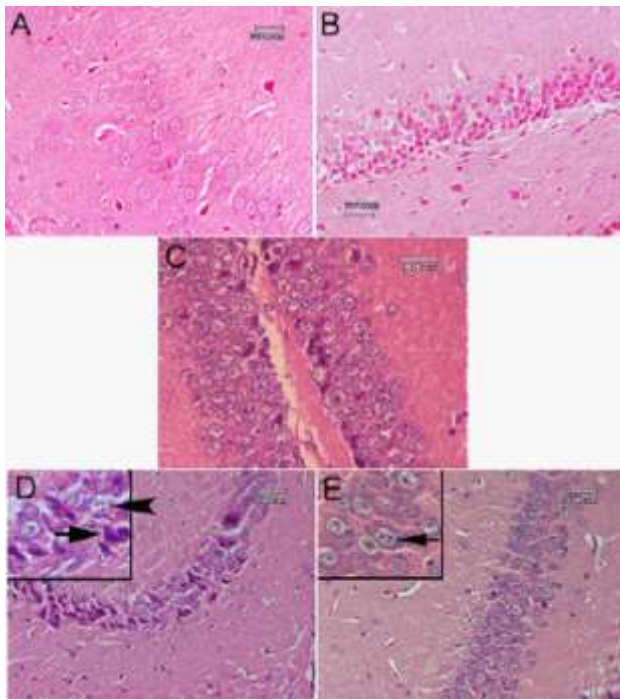
Fig. 5 Representative photomicrographs of Hx & E stained sections of the hippocampus area from mice treated with: **(A)** Vehicle: showing the normal structure of this tissue. **(B)** Rotenone showing many deeply stained neurons (arrow). A slight decrease of the granular cell layer thickness is observed. **(C)** Rotenone + L-dopa showing decrease of deeply stained neurons. **(D)** Rotenone + capsaicin 0.5 mg/kg showing many darkly stained (arrowhead) and karyorrhectic (arrow) neurons. A noticeable decrease of the granular cell layer thickness is seen. **(E)** Rotenone + capsaicin 1 mg/kg showing no darkly stained neurons are observed, although neurons with karyorrhectic nuclei are still noticed (arrow).

4 Discussion

This study demonstrates a neuroprotective effect for theTPRV1 agonist capsaicin on neuronal cell degeneration in brain after systemic rotenone administration in mice. The number of degenerated neurons in substantia nigra, cerebral cortex and hippocampus after rotenone was reduced by capsaicin. This was accompanied by a decrease in oxidative stress and improved motor strength and coordination.

The neuroprotective potential of capsaicin reported in the present study is supported by previous findings. Capsaicin (0.01–0.6 mg/kg, s.c.) protected against global cerebral ischemia in Mongolian gerbils [30]. Capsaicin (0.5 mg/kg) was also reported to prevent dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced PD in mice [31]. Capsaicin (0.15 or 1.5 mg/kg, i.p.) showed antioxidant effects increasing brain reduced glutathione content and decreasing serum nitric oxide levels after systemic administration of the endotoxin lipopolysaccharide (100 μg/kg, i.p.) in rats [32] and when given at 1 mg/kg, s.c., it decreased plasma nitric oxide, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) during endotoxaemia in rats [33].

Rotenone is a known mitochondrial complex I inhibitor which can lead to oxidative stress and results in dopaminergic cell death. Because of its lipophilic properties, rotenone readily permeates the blood–brain barrier and cell membranes and accumulates in mitochondria, where it inhibits oxidative phosphorylation by blocking the activity of complex I of the electron transport chain [34]. Inhibition of complex I results in the increased production of reactive oxygen species leading to the development of oxidative stress, depletion in cellular ATP and consequent neuronal death [35],[36]. Rotenone was found to activate microglia cells causing the increased production of superoxide and hypochlorous acid [37],[38].

The development of oxidative stress in the brain following rotenone injection is evidenced by the increased brain lipid peroxidation, depletion of reduced glutathione. The latter is an essential antioxidant capable of scavenging ROS in both cytosol and mitochondria [39]. Rotenone was also found to decrease total antioxidant capacity and the activity of the antioxidant enzymes superoxide dismutase [40]. Neuronal death caused by rotenone could be prevented by administering antioxidants such as glutathione, *N*-acetylcysteine, and α-tocopherol, thereby, lending further support to the important contribution of oxidative stress in rotenone neurotoxicity [35],[41].

Our results also indicated a significant in the level of brain nitric oxide following rotenone treatment which is consistent with previous studies [42],[43]. Nitric oxide is synthesized from L-arginine by the action of nitric oxide synthase (NOS). The constitutive endothelial (eNOS) and neuronal (nNOS) isoforms produce small amounts of nitric oxide for short period. In contrast, high concentrations result from inducible NOS (iNOS) in resident brain immune cells, microglia and astrocytes following their activation by ROS, cytokines, and bacterial lipopolysaccharide [44]. High levels of nitric oxide can be damaging to neurons. This is largely thought to be caused by the strong oxidant peroxynitrite generated from the

reaction of nitric oxide and superoxide. The result is oxidation of membrane lipids, proteins, and DNA, nitrosylation of thiol residues in proteins or glutathione, and nitrotyrosination of proteins [45],[46]. Studies indicated increased iNOS expression in substantia nigra and striatum in rotenone-treated animals [47]. Rotenone neurotoxicity is reduced by iNOS or nNOS inhibitors, thereby, suggesting that both isoenzymes contribute to the production of the nitric oxide that mediates the rotenone-induced dopaminergic neuron apoptosis and degeneration [37],[48],[49].

The presence of bradykinetic motor function is the most important feature of human PD. Therefore, in the present study, the effect of rotenone treatment on motor functions was evaluated using stair, wire hanging and wood walking tests. We also found that administration of rotenone induced motor dysfunction which can be alleviated by treating animals with capsaicin or l-dopa. Previous studies using the present regimen of rotenone have shown depletion of striatal dopamine and tyrosine hydroxylase [50]. It is likely, therefore, that the improvement of motor function by capsaicin or l-dopa reflects interference with the rotenone-induced degeneration of nigrostriatal neurons and the consequent depletion of striatal dopamine levels. This conclusion is supported by the histpathological study which showed noticeable increase in the number and size of dopaminergic pigmented cells in the substantia nigra.

5 Conclusion

In summary, our findings indicate that capsaicin was capable of preventing neuronal loss and motor dysfunction in experimental model of PD induced by rotenone in mice, possibly *via* decreased oxidative stress. By interfering with oxidative-mediated neuronal injury and death, capsaicin may also prove beneficial in Parkinson's disease.

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Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

Marwa El-Shamarka and Omar Abdel-Salam designed the study and conducted the experiments. Nermeen Shaffie performed the histological studies and its interpretation. Omar Abdel-Salam prepared the manuscript. Marwa El-Shamarka, Omar Abdel-Salam, and Nermeen Shaffie approved the final version of the manuscript.

Conflict of Interest

The authors state that there are no conflicts of interest