Cacoa prevents degeneration of dopamine neurons and motor impairment caused by rotenone

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Abstract: - We investigated the effects of cacoa powder on oxidative stress and neurodegeneration in rotenone-induced Parkinson's disease in mice. Rotenone was given subcutaneously at 1.5 mg/kg, every other day for two weeks and mice were treated at the same time with the cacoa at 1 and 2 g/kg, or L-dopa at 25 mg/kg, orally once a day. The control group received the vehicle (DMSO). Oxidative stress biomarkers; malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO) were measured in brain tissue. Behavioral testing for motor performance and histologic study of the brain were also done. Results indicated that rotenone led to significant elevations in brain MDA and NO and this was accompanied by a marked GSH depletion. Mice exhibited impaired grip strength, motor balance and coordination in the wire hanging, wood walking, and stair tests. The histological study revealed marked decrease in number and size of pigmented cells in substantia nigra and an increase in the number of deeply stained neurons and karyorrhexis in the cerebral cortex and hippocampus after rotenone injection. Treatment with cacoa reduced brain MDA, NO and increased GSH levels and alleviated the motor deficits induced by rotenone. Cacoa given at 2 g/kg increased the size of pigmented cells in the substantia nigra, decreased the number of deeply stained neurons in hippocampus, while most of cortical neurons appeared normal. These results indicate that cacoa provides protection against rotenone-induced neurotoxicity and this involves an antioxidant action.

Key-Words: - cacoa; neuroprotection; rotenone; antioxidants; oxidative stress; reactive oxygen species

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1 Introduction

Parkinson's disease (PD) is neurodegenerative motor disorder characterized by bradykinesia, tremor, rigidity, and postural abnormalities. In addition to these, neuropsychiatric symptoms such as depression, apathy, anxiety, cognitive impairment, psychosis, and autonomic and gastrointestinal dysfunction may occur in the course of the disease with considerable impact on the quality of living in these patients [1]. Parkinson's disease is an age-related, occurring in 1% of individuals above 65 y of age with increasing prevalence in more advanced age [2]. In these patients, the cause is not known (idiopathic PD) but minority of cases (~5%) are of genetic origin [3]. Neuropathologically, PD is characterized by loss of substantia nigra dopaminegic neurons which project to the striatum, resulting in profound defect in striatal dopamine content and emergence of the motor symptoms of the disease [4]. Moreover, there

is also affection of other neurotransmitter systems e.g., cholinergic, noradrenergic, and serotonergic both in the brain and periphery [5] which suggests that PD is a multisystem disorder.

The cause underling the preferential death of dopaminergic neurons in PD is not yet explained although increasingly available evidence suggests a role for environmental toxins besides a genetic susceptibility [6],[7]. In particular, the involvement of insecticides and pesticides like rotenone has been emphasized [8]. The death of pigmented substantia nigra neurons appears to be largely driven by free radical-mediated mechanisms [9],[10]. Oxidative stress whereby the cell's antioxidants are overwhelmed by increasingly generated reactive oxygen species (ROS) leading to oxidative damage of cellular biomolecules like membrane lipids, enzyme proteins and DNA has been shown in the brain of PD patients [11],[12]. While the brain tissue with its high metabolic demands, oxygen consumption and rich content of polyunsaturated fatty acids is susceptible to oxidative stress [13], several other factors in PD add to this vulnerability. These include increased iron content, dopamine oxidation [14], [15], glutathione deficiency [16], and mitochondrial dysfunction [17].

In the present time, the treatment of PD is based on the dopamine precursor l-dopa which results in marked improvement of the motor disabilities by correcting the biochemical deficit in PD [18[. After several years, the ability of l-dopa to alleviate motor symptoms decreases and there is the problem of L-dopa side effects like dyskinesia [19]. Clearly, this illustrates the immense need for finding out new therapeutics with the aim to improve disease manifestations at the best to stop the progression of the disease.

For these reasons, much hope has been placed on natural products and natuiceuticals to treat neurodegenerative disorders including PD [20]. Cacoa with its rich content of antioxidant flavonoids, and caffeine [21],[22] may prove of usefulness. Caffeine in particular has been shown to improve symptoms in PD patients, possibly *via* adenosine A_{2A} receptors antagonism [23],[24]. In this study, we aimed to investigate the potential protective effect of cacoa on oxidative stress, brain neurodegeneration, and motor dysfunction induced by rotenone in mice. Rotenone is a naturally occurring pesticide which has been linked to an increase in the risk for developing idiopathic PD [8],[25]. Rotenone causes nigrostriatal damage in experimental animals [26] and thus is widely used as an experimental model of PD to explore pathogenetic mechanisms involved in dopaminergic cell death and likely therapeutic interventions.

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 was obtained from Sigma-Aldrich (St Louis, MO, USA). Rotenone was freshly prepared in 100% dimethyl sulfoxide. Commercially available cacoa powder (100% cacao) was used in the study (Hershey's cocoa). Cacoa powder was freshly prepared every day in hot water, left to cool at room temperature, and then given orally to mice at a dose of 1 or 2g/kg. 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 divided into different treatment groups (6 animals in each of group). Group 1 received the vehicle (DEMSO) three times a week and served as control -ve. 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; control +ve), L-dopa at 25 mg/kg orally once a day (group 3) or cacoa at 1 or 2 g/kg, orally once a day (groups 4 & 5). Behavioral testing was done 24 hrs after last injection of rotenone. Mice were euthanized by cervical dislocation at the end of the procedure and the brain of each muse was immediately dissected out on ice-cold plate, washed with ice-cold phosphate-buffered saline (PBS, pH 7.4), 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. Representative brain samples were used for histologic assessment of tissue injury.

2.4 Biochemical studies

2.4.1. Lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation was measured in tissue homogenates by determining malondialdehyde (MDA) according to Nair and Turne [27]. 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. [28]. 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 [29]. 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 [30].

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 [31].

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 [32].

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 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

Repeated injections of rotenone led to a significant increase in brain MDA content by 68.8% from vehicle control value of 17.89 ± 1.04 to 30.2 ± 1.0 nmol/g. tissue. Administration of l-dopa resulted in significant decrease in MDA by 23.9% compared to the rotenone control (22.98 ± 1.17 *vs.* 30.2 ± 1.0 nmol/g. tissue). The level of MDA showed significant decrements by 29.1% and 38.4% after treatment with cacoa at 1 and 2 g/kg, respectively (21.4 ± 0.48 and 18.6 ± 0.66 *vs.* 30.2 ± 1.0 nmol/g. tissue) (Fig. 1).

3.1.3 Nitric oxide

The level of nitric oxide was increased by 121.1% after rotenone administration from vehicle control value of 21.6 ± 0.58 to 47.76 ± 0.75µmol/g. tissue. Nitric oxide decreased by 29.0% after l-dopa and by 24.4% and 27.8% by cacoa administration compared to the rotenone only group (38.12 ± 0.76, 33.26 ± 1.42, 29.63 ± 0.65 *vs.* 47.76 ± 0.75µmol/g. tissue) (Fig. 1).

3.1.2 Reduced glutathione

In the rotenone-treated animals, brain GSH showed significant decrease by 50.9% compared to vehicle control value (1.54 ± 0.14 *vs*. 3.14 ± 0.13 µmol/g. tissue). The GSH level increased by 75.3%, 81.2% and 83.1% following administration of l-dopa and cacoa at 1 or 2 g/kg, respectively 2.39 ± 0.09, 2.79 ± 0.12, 2.87 ± 0.06 *vs.* 3.14 ± 0.13 µmol/g. tissue) (Fig. 1).

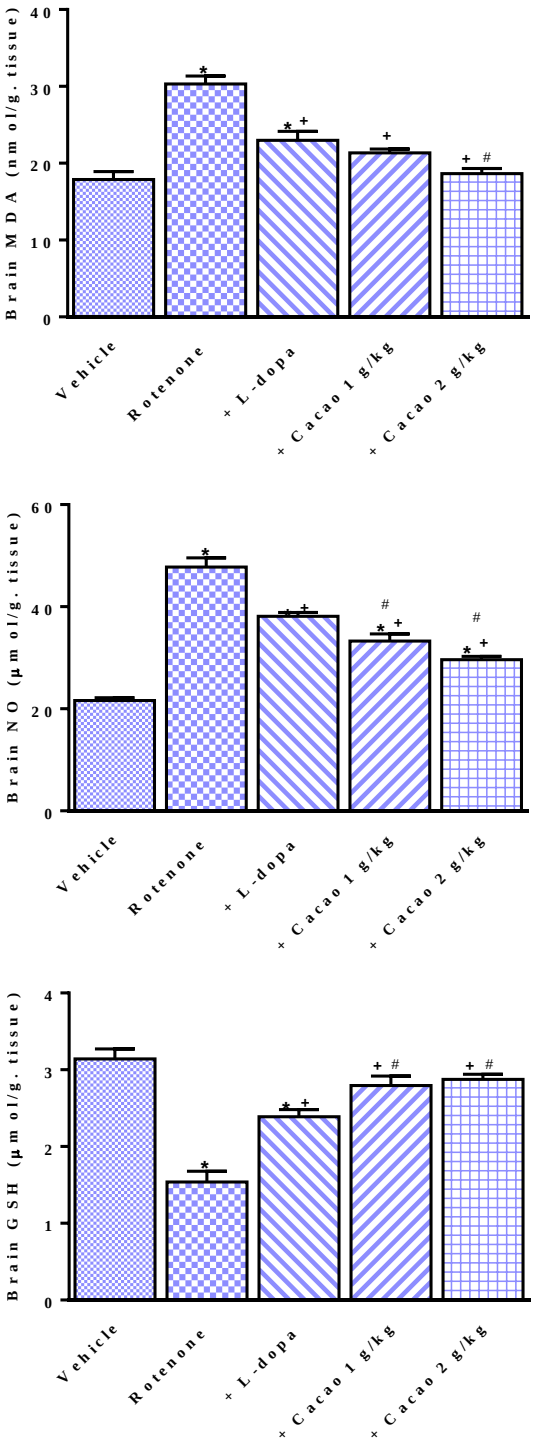

Fig. 1 Oxidative stress biomarkers in brain of mice treated with rotenone alone or in combination with cacoa. *: $p<0.05$ *vs.* corresponding vehicle-treated group. +: $p < 0.05$ *vs.* rotenone control group.

2.4 Behavioral studies

2.4.1. Stair test

Rotenone-treated mice spent significantly more time in ascending the stair by 55.1% compared to their vehicle controls (18.01 ± 0.69 *vs.* 11.61 ± 0.35 sec). The ascending time were significantly decreased by 53.4% after L-dopa and by 28.7% and 57.1% after cacoa administration as compared with rotenone control (8.38 ± 0.32, 12.85 ± 0.51, 7.73 ± 0.36 *vs.* 18.01 ± 0.69 sec).

2.4.1. Wire hanging test

Rotenone exposed mice showed significantly shorter hanging time by 68.4% compared to control mice (4.57 ± 0.24 *vs.* 14.45 ± 0.41 sec). Co-administration of L-dopa and cacoa significantly increased the latency to fall by 142.9%, 118.4% and 163.4% as compared with rotenone control value (11.10 ± 0.31, 9.98 ± 0.31, 12.04 ± 0.71 *vs.* 4.57 ± 0.24 sec).

2.4.1. Wood walking test

Animals treated with rotenone mice were significantly slower to traverse a wooden stick as compared with vehicle control group (18.26 ± 0.64 *vs.* 8.48 ± 0.46 sec). Co-administration L-dopa and cacoa was associated with significantly shorter time to traverse the stick by 64.8%, 50.7% and 59.0%, respectively as compared with the rotenone control (6.72 ± 0.41, 9.0 ± 0.66, 7.49 ± 0.46 *vs.* 18.26 ± 0.64 sec).

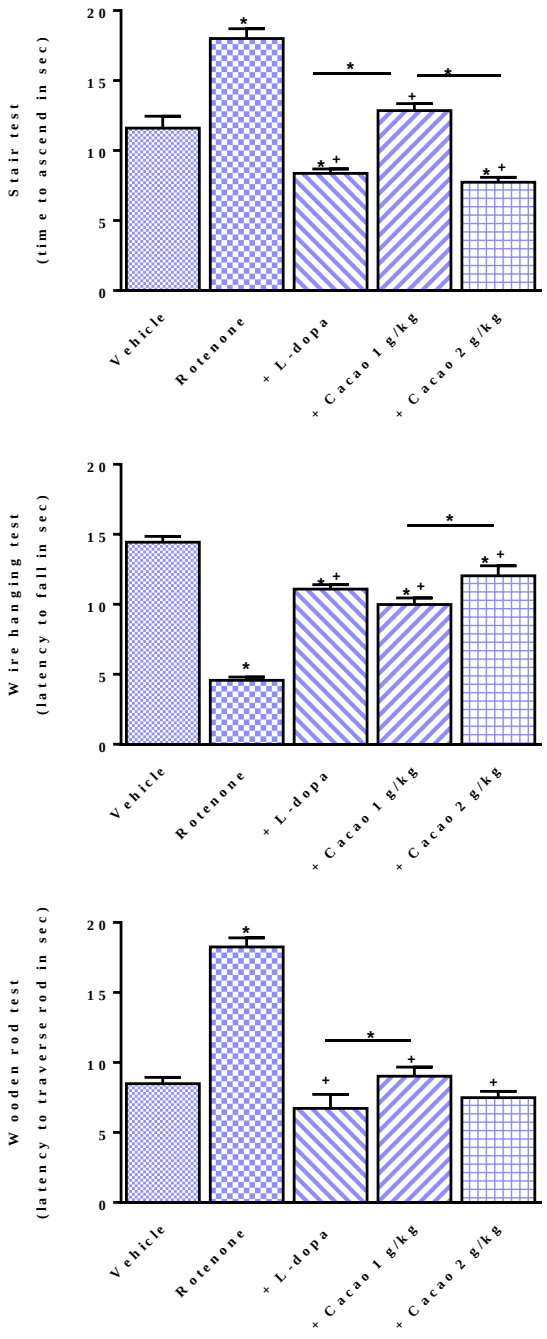

Fig. 2 Changes in behavioral parameters (neuromuscular strength, motor coordination and balance) in mice treated with rotenone alone or co-administered with cacoa. *: $p<0.05$ *vs.* corresponding vehicle-treated group and between different groups as indicated in the graphs. +: $p < 0.05$ vs. rotenone control group.

2.4 Histopathological studies

Rotenone led to marked neurodegenerative changes in different brain regions. There was a marked decrease in the number and size of pigmented dopaminergic cells in the substantia nigra. Rotenone also resulted in an increase in the number of deeply stained neurons and karyorrhexis in the cerebral cortex and hippocampus brain regions. These pathological changes were ameliorated by the co-administration of cacoa in a dose-dependent manner (Figs. 3-5).

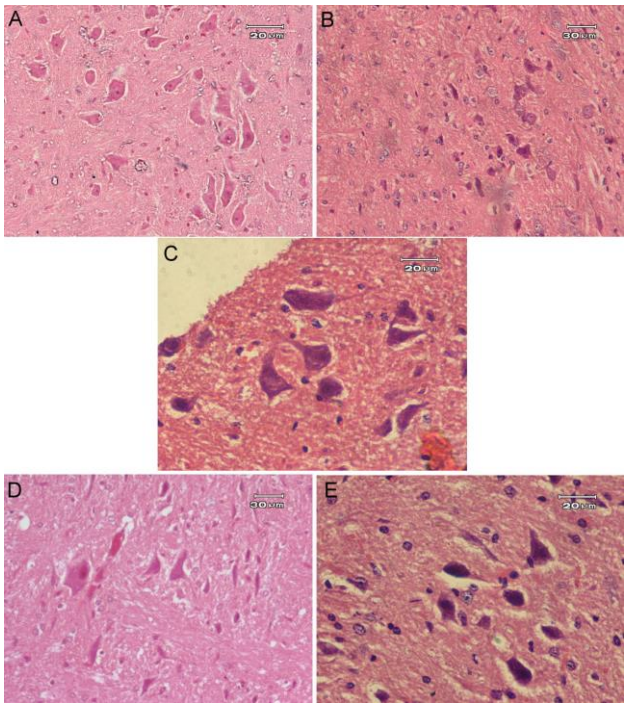

Fig. 3 Representative photomicrographs of Hx & E stained sections of the substantia nigra area after treatment with: **(A)** Vehicle the normal structure of the pigmented neurons in this area. **(B)** Rotenone: a marked decrease in size and number of pigmented cells. **(C)** Rotenone + L-dopa: shows marked amelioration of pigmented cells size, but they are still less than normal in number. **(D)** Rotenone + cacoa 1 g/kg shows a decrease of pigmented cells. **(E)** Rotenone + cacoa 2 g/kg shows slight increase in pigmented cells' size, while their number is still below normal.

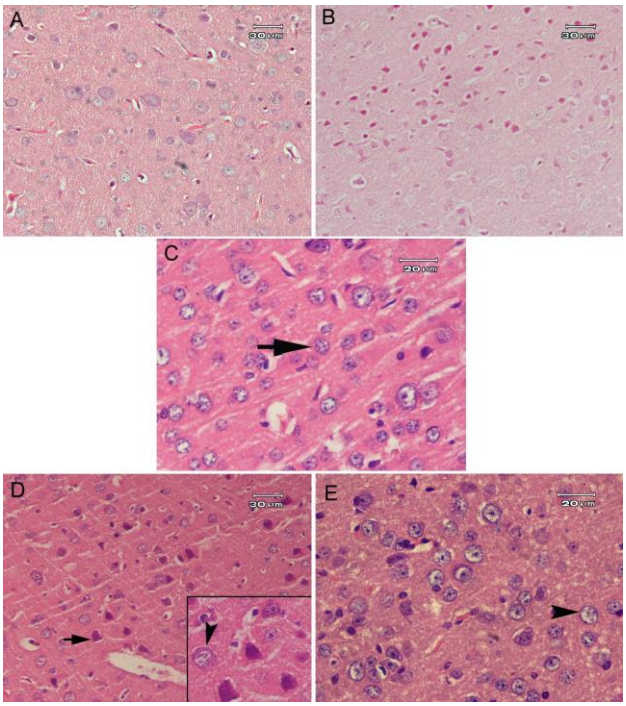

Fig 4 Representative photomicrographs of Hx & E stained sections of the cerebral cortex tissue from mice treated with: **(A)** Vehicle: shows normal appearance of neurons. **(B)** Rotenone: shows many deeply stained neurons among normal cells. **(C)** Rotenone + L-dopa: shows most of neurons appear normal, although some neurons show karyorrhexsis (arrow). **(D)** Rotenone + Cacoa 1g/kg shows slight decrease of both deeply stained (arrows) and karyorrhectic neurons (arrowhead). **(E)** Rotenone Cacoa 2g/kg shows most neurons appear normal.

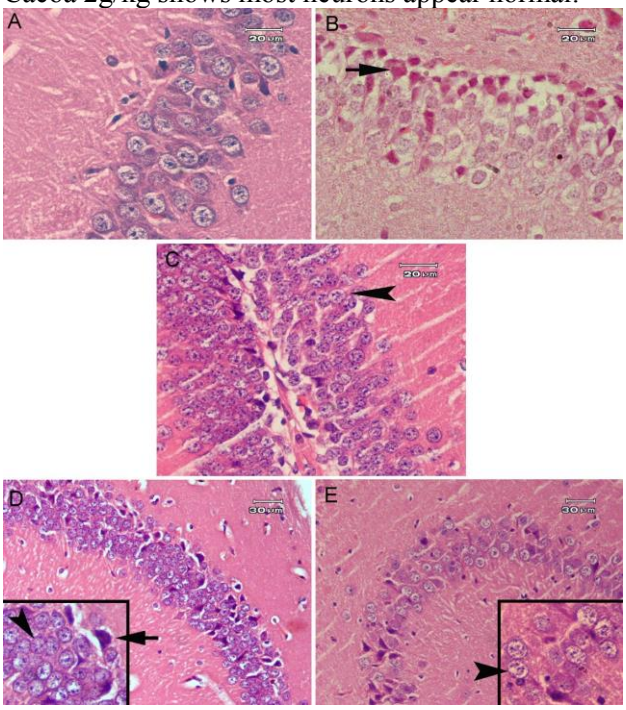

Fig. 5 Representative photomicrographs of Hx & E stained sections of the hippocampus area from mice treated with: **(A)** Vehicle: shows the normal structure of this tissue. **(B)** Rotenone many deeply stained neurons (arrow). No reduction of the granular cell layer thickness is observed. **(C)** Rotenone + L-dopa: shows decrease of deeply stained neurons. Neurons with karyorrhexis are also noticed (arrowhead). **(D)** Rotenone + cacoa 1 g/kg shows slight decrease of deeply stained (arrows) and also karyorrhectic neurons (arrowhead). **(E)** Rotenone + cacoa 2 g/kg shows marked decrease of deeply stained neurons, while cells showing karyorrhexis are more pronounced (arrowhead).

4 Discussion

In this study, cacoa was evaluated for a potential therapeutic effect in the rotenone model of PD in mice. Results of this study indicate that rotenone caused increased brain oxidative stress, a decrease in neuromotor strength, motor balance and neurodegenerative changes in the striatum, cerebral cortex and hippocampus brain regions which were attenuated by cacoa. The pesticide rotenone is an inhibitor of mitochondrial complex I or NADH-quinone oxireductase, which causes nigrostriatal neurodegeneration and thus is commonly used to delineate the pathogenetic mechanisms underlying dopaminergic cell death that occur in Parkinson's disease and find possible therapeutic interventions [26]. In this study, rotenone treatment led to significant increase in brain lipid peroxidation and nitric oxide. In addition, there was markedly decreased level of the antioxidant and radical scavenger GSH. These data indicate an increase in ROS and consequent attack on membrane lipids in brain of rotenone-treated animals and are consistent with other studies indicating high production of ROS with rotenone exposure [33],[34],[35]. Rotenone induces neuronal cell death by virtue of its ability to increase ROS generation. Rotenone which inhibits mitochondrial complex I, increases the generation of superoxide and hydrogen peroxide at complex I and complex III, resulting in oxidative stress, mitochondrial damage, decreased energy production, eventually leading to neuronal cell damage and death [36],[37],[38]. Rotenone thus disrupt the redox balance in the cell in favor of oxidative stress together with depletion of depletion of reduced glutathione and decreased activity of the antioxidant enzyme superoxide dismutase [39]. Oxidative stress is an important contributor to rotenone-induced dopaminergic cell damage and decline in neuromotor function which can be prevented by antioxidants such as glutathione, and vitamin E [36],[40]. In addition, rotenone increases

the formation of nitric oxide and has been shown to increase inducible nitric oxide synthase immunoreactivities in the striatum and substantia nigra of rodents. Excessive generation of nitric oxide has been linked to neurodegeneration through the formation of reactive nitrogen species such as peroxynitrite or nitrogen oxides causing oxidation, nitration, and nitrosylation reactions [41],[42]. Nitration of protein tyrosine residues by rotenone may contribute to apoptosis and death of dopaminergic cells by the toxicant, which can be attenuated by nitric oxide synthase inhibitor or an antioxidant [43].

In this study, co-administration of cacoa to rotenone-treated mice caused a significant decrease in brain lipid peroxidation, nitric oxide whilst increasing GSH levels, indicative of a decrease in ROS. Combating oxidative stress represents an important target in Parkinson's disease with the aim to prevent or slow the process of dopaminergic cell death and hence the significance of the present findings. In Parkinson's disease, bradykinesia and rigidity are disabling motor symptoms, for which dopamine-based drugs are used [44]. Over the time, because of the progressive nature of the disease, more dopaminergic cells are being lost, and hence these agents decline in their effectiveness to control symptoms. There is in addition, the problem of emerging motor complications such as fluctuations and dyskinesia, after several years of continued l-dopa therapy [19]. In this study, we tested motor function in rotenone-treated mice. We assessed balance with the wooden beam test, sensorimotor function using stair test and grip strength with the wire hanging test and showed impaired performance in all motor tests compared with the vehicle controls. We found that cacoa improved balance, motor coordination and grip strength which is indicative of a decreased dopaminergic neurodegeneration. Results of the histological studies support this notion. While rotenone only-treated mice exhibited marked decrease in size and number of pigmented cells in the substantia nigra, those treated with rotenone and cacoa 2 g/kg showed slight increase in pigmented cells' size. Our study showed in addition the presence of neurodegenerative changes in the cerebral cortex and hippocampus after rotenone treatment which is consistent with previous observations [45]. In these brain regions, cacoa 2g/kg exerted clear protective effect, markedly decreasing the number of deeply stained degenerated neurons.

Cacoa is rich in procyanidins, catechins, and epicatechins [21],[22]. Studies have shown that the presence of these flavonoids in high amounts in diet may modulate the risk for developing Parkinson's disease [20] possibly via their antioxidant properties. Cocoa flavonoids were shown to improve endothelial function [46], cause peripheral and cerebral vasodilatation [47],[48], increase brain oxygenation and cognitive performance [49]. Cocoa is also a rich source of caffeine (1,3,7-trimethylxanthine) [21]. Caffeine and coffee intake is associated with a significantly lower incidence [50],[51] and lower rate of progression of Parkinson's disease [24]. Caffeine was reported to improve motor scores, tremor and daytime sleepiness in Parkinson's disease [23]. Caffeine is an antagonist at adenosine A_1 and A_{2A} receptors [52]. A_{2A} receptors are densely expressed on striatopallidal neurons and their blockade by drugs like istradefylline reduces the decrease in L-dopa effectiveness or "off episodes" [53]. Cocoa also contains high amounts of theobromine, another methylxanthine, a phosphodiesterase inhibitor which increases intracellular cyclic adenosine monophosphate (cAMP) and an antagonist at adenosine receptors [54] with neuroprotective properties [55]. In mice, a diet supplemented with 0.05% theobromine, increases brain brain-derived neurotrophic factor levels and motor learning [56].

5 Conclusion

In conclusion, the results of the present study indicate that in an experimentally-induced Parkinson's disease model, the administration of cacoa exerted antioxidant effect and markedly reduced neuronal damage and motor impairment. Cacoa which is rich in flavonoids antioxidants besides its content of caffeine and theobromine will be a useful additive natriceutical for the prevention of dopaminergic cell death in Parkinson's disease.

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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 wrote and prepared the manuscript. Marwa El-Shamarka, Omar Abdel-Salam, and Nermeen Shaffie approved the final version of the manuscript.

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Conflict of Interest

The authors state that there are no conflicts of interest