

Neuroprotection by Montelukast against Rotenone-Induced Rat Brain Damage

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ABSTRACT | Montelukast is a cysteinyl-leukotriene receptor antagonist used in asthma prophylaxis. In this study, the effect of montelukast (10 or 20 mg/kg) on neuronal damage and oxidative stress induced in the rat brain by rotenone was examined. Rats were treated with rotenone subcutaneously at 1.5 mg/kg every other day alone or along with montelukast. The control group received the vehicle dimethyl sulfoxide (DMSO). The results showed that compared with the vehicle-treated group, rotenone resulted in increased brain lipid peroxidation by 84.5% as assessed by malondialdehyde (MDA) content. Nitric oxide increased by 77.4% while reduced glutathione (GSH) and total antioxidant capacity (TAC) decreased by 37.7% and 68.6%, respectively. In addition, the activities of superoxide dismutase (SOD), paraoxonase-1 (PON-1), and butyrylcholinesterase (BChE) significantly decreased by 34.6%, 68%, and 75.2%, respectively, after rotenone injection. Rotenone caused neurodegenerative changes in the cerebral cortex and substantia nigra. The administration of montelukast along with rotenone decreased MDA by 34.1–53.6%, nitric oxide by 51.6–64.7%, increased GSH content by 20.7–65.8%, and increased TAC by 109.6–156.2%. SOD activity increased by 50–62.5%, PON-1 activity by 161.1–203.7%, and BChE activity by 135.3–274.3% compared with respective rotenone control values. The rotenone-induced neuronal damage was ameliorated dose-dependently by montelukast. These results indicate that montelukast exerts a neuroprotective effect in the rotenone model of neurotoxicity. The neuroprotective action of montelukast is likely to involve an inhibitory effect on oxidative stress and nitric oxide. It is suggested that montelukast could be of value in the adjunctive treatment of Parkinson's disease.

KEYWORDS | Cysteinyl-leukotriene receptor; Montelukast; Neurotoxicity; Oxidative stress; Rotenone

ABBREVIATIONS | BChE, butyrylcholinesterase; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; MDA, malondialdehyde; PON-1, paraoxonase-1; SOD, superoxide dismutase; TAC, total antioxidant capacity

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1. INTRODUCTION

Parkinson's disease is a neurodegenerative disorder in which there is substantial loss of the dopamine producing neurons of the substantia nigra pars compacta of the midbrain basal ganglia [1]. The function of the latter is to control and smoothen motor movements initiated in the cortex [2]. The midbrain dopamine deficiency results in disordered motor activity which manifests as slow or absent movements, increased muscular rigidity, and a resting tremor [1, 3]. In advanced disease, there is also involvement of serotonergic, cholinergic, GABAergic, and glutamatergic neurons with the emergence of non-motor symptoms such as depression, apathy, anxiety, cognitive impairment, and autonomic dysfunction [4, 5]. Parkinson's disease is sporadic in ~95% of cases, occurring in the older adults over the age of 65 years with increasing incidence as the age advances [6, 7]. The cause of Parkinson's disease is not completely understood but is probably the result of exposure of an environmental toxin (s) combined with genetic susceptibility [7, 8]. The pathogenic process most commonly implicated in the dopaminergic nigral cell death is oxidative stress [9]. The latter term is used to describe as state in which the redox balance within the cell is shifted in favor of pro-oxidants either because an increase in reactive oxygen metabolites or deficient cellular antioxidant machinery. This results

in disordered redox signaling and control and consequent damage to cellular macromolecules, which constitutes potential threat to the cell [10, 11]. In Parkinson's disease, a biochemical evidence of oxidative damage to cell membrane lipids [12], proteins [13], nucleic acids [14], and reduced glutathione (GSH) [15] have been demonstrated in the substantia nigra. The occurrence of neuroinflammation in the brain of Parkinson's disease subjects is another mechanism underlying cell death in which there is microglia activation and increased levels of pro-inflammatory cytokines such as interleukin-1 β and interleukin-6 [16, 17].

Drug therapy in Parkinson's disease is largely directed towards maintaining midbrain dopaminergic activity by correcting the biochemical deficit. This might be achieved through the administration of L-3,4-dihydroxyphenylalanine or L-dopa, the precursor of dopamine, the use of dopaminergic receptor agonists, or by decreasing the metabolism of L-dopa via the use of catechol-*O*-methyltransferase inhibitors. By time, these drugs become less efficient in controlling the motor symptoms and complications such as motor fluctuations, and dyskinesia often develops after prolonged levodopa therapy [3, 18, 19]. There is no drug available to prevent the continued nigral cell loss in Parkinson's disease [20]. This indicates the immense need for the identification of novel therapeutic agents. Montelukast is a cysteinyl leuko-

trienes receptor antagonist in use in patients with bronchial asthma to reduce airway inflammation [21]. Montelukast displays anti-inflammatory and antioxidant effects and has been shown to exert neuroprotective action in rodent model of traumatic brain damage [22] and to inhibit microglia activation by rotenone in vitro [23].

The aim of the present study was to investigate the neuroprotective potential of montelukast in a rat model of nigrostriatal damage caused by rotenone in vivo. Rotenone is a pesticide known to cause Parkinson's disease-like syndrome in rodents [24, 25]. This toxin induces nigrostriatal cell damage via inhibiting mitochondrial complex I activity [25] and increasing brain reactive oxygen metabolites and nitric oxide [26, 27].

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague–Dawley rats weighing 160–180 g, obtained from the Animal House of the National Research Centre (Cairo, Egypt) were used in the study. Rats were group-housed under temperature- and light-controlled conditions. Standard laboratory rodent chow and water were given ad libitum. The study was done in accordance with the institutional Ethics Committee and the regulations of the United States National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and Chemicals

Rotenone (Sigma-Aldrich, St. Louis, MO, USA) and montelukast (European Egyptian Pharm. Ind., Alexandria, Egypt) were used in the study. Rotenone was dissolved in dimethyl sulfoxide (DMSO). Montelukast was dissolved in isotonic saline (0.9% NaCl) immediately before use. Other chemicals and reagents were purchased from Sigma-Aldrich.

2.3. Study Design

Rats were randomly divided into four equal groups with six rats in each group. Group 1 was treated with the vehicle (DMSO) given subcutaneously (sc). Group 2 was treated with rotenone (1.5 mg/kg, sc)

every other day for two weeks. Groups 3 and 4 were treated with sc rotenone (1.5 mg/kg) along with montelukast at doses of 10 and 20 mg/kg. At the end of the study, rats were euthanized by decapitation for tissue collection. The brains were quickly removed out on an ice-cold plate, washed with ice-cold phosphate-buffered saline (pH 7.4), weighed, and stored at -80°C until the biochemical assays. The brain tissues were homogenized with 0.1 mL phosphate buffer saline at pH 7.4 to give a final concentration of 10%.

2.4. Biochemical Studies

2.4.1. Determination of Lipid Peroxidation

Lipid peroxidation products in the brain homogenates was assayed by measuring the level of malondialdehyde (MDA) using the method of Ruiz-Larrea et al. [28]. In this assay, the lipid peroxidation-derived substances react with thiobarbituric acid to produce a red colored complex having a peak absorbance at 532 nm.

2.4.2. Determination of GSH

GSH was determined in the brain homogenates using the method of Ellman et al. [29]. The procedure is based on the reduction of the Ellman's reagent by $-SH$ groups of GSH to form 2-nitro-5-mercapto-benzoic acid, which is intense yellow in color and determined spectrophotometrically at 412 nm.

2.4.3. Determination of Nitric Oxide

Nitric oxide was determined using the Griess reagent, according to the method of Moshage et al. [30]. Nitrite, a stable end-product of nitric oxide radical, is commonly used as an indicator for the production of nitric oxide.

2.4.4. Determination of Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined using a commercially available kit (Biodiagnostic, Egypt). In this assay, the SOD enzyme inhibits the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye, which is measured spectrophotometrically [31].

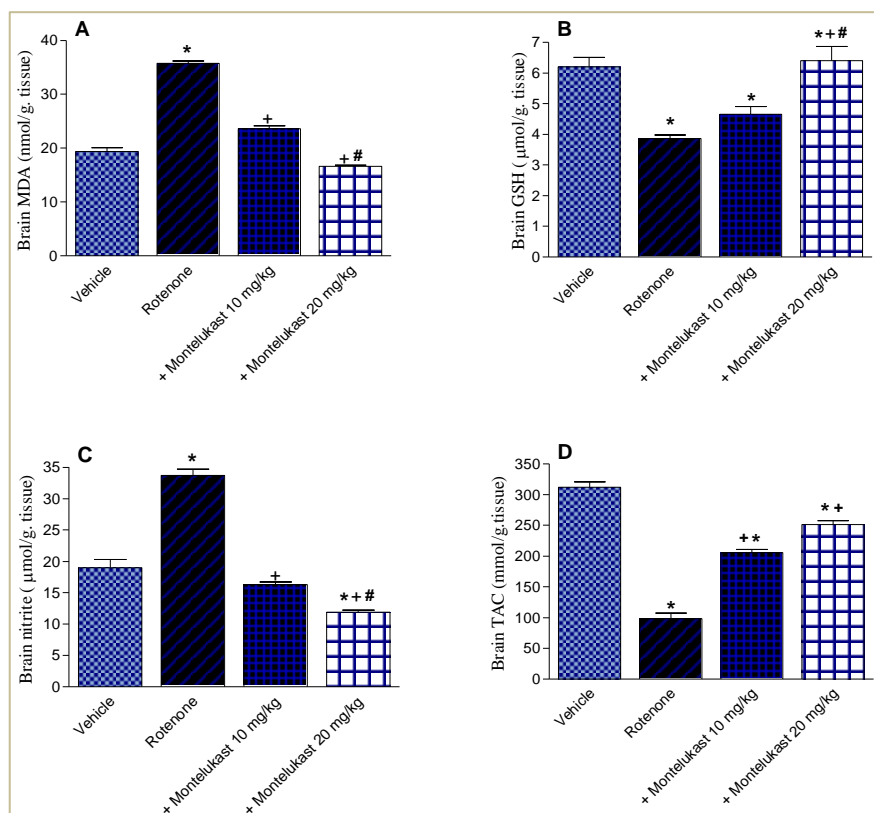


FIGURE 1. Effect of montelukast on the levels of (A) malondialdehyde (MDA), (B) reduced glutathione (GSH), (C) nitric oxide, and (D) total antioxidant capacity (TAC) in the brain tissues of rats treated with rotenone. *, $p < 0.05$ versus vehicle; +, $p < 0.05$ versus rotenone only; #, $p < 0.05$ versus rotenone + 10 mg/kg montelukast.

2.4.5. Determination of Paraoxonase-1 Activity

Arylesterase activity of paraoxonase-1 (PON-1) was measured spectrophotometrically in supernatants using phenyl acetate as a substrate. In this assay, PON1 catalyzes the cleavage of phenyl acetate, resulting in the formation of phenol. The rate of the formation of phenol is measured spectrophotometrically by monitoring the increase in absorbance at 270 nm [32].

2.4.6. Determination of Butyrylcholinesterase Activity

Butyrylcholinesterase (BChE) activity was measured in supernatants using a commercially available kit (Ben Biochemical Enterprise, Milan, Italy). Cholinesterase catalyzes the hydrolysis of butyrylthiocho-

line, forming butyrate and thiocholine. The thiocholine then reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) forming a colored compound. The increase in absorbance in the unit time at 405 nm is proportional to the activity of the cholinesterase in the sample.

2.5. Histopathological Studies: Hematoxylin and Eosin Staining

Brain samples of all animals were dissected immediately after euthanasia. The specimens were then fixed in 10% neutral-buffered formalin saline for at least 72 h. All the specimens were washed in tap water for 30 min and then dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin wax. Serial sections of 6 μm thick were

cut and stained with hematoxylin and eosin (H&E) for histopathological investigation.

2.6. Statistical Analysis

Data are presented as mean \pm SEM. Statistical significance was determined with one-way analysis of variance and Duncan's multiple range test using SPSS software (SAS Institute Inc., Cary, NC, USA). A probability value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Oxidative Stress

As shown in **Figure 1**, brain MDA levels significantly increased by 84.5% ($p < 0.05$) with respect to control levels after rotenone injection (35.8 ± 0.42 versus 19.4 ± 0.67 nmol/g.tissue). There was also a significant and marked increase in the levels of nitric oxide by 77.4% (33.7 ± 1.0 versus 19.0 ± 1.3 μ mol/g.tissue). In addition, rotenone treatment significantly lowered brain GSH concentration by 37.7% (3.86 ± 0.12 versus 6.2 ± 0.31 μ mol/g.tissue) and TAC by 68.6% (98.1 ± 9.0 versus 312.2 ± 8.4 mmol/g.tissue), respectively, compared to controls. Animals receiving 10 and 20 mg/kg montelukast along with rotenone exhibited significantly decreased brain MDA concentrations by 34.1–53.6% compared to the rotenone only group (23.6 ± 0.58 and 16.6 ± 0.24 versus 35.8 ± 0.42 nmol/g.tissue). A significant decrease ($p < 0.05$) of nitric oxide concentrations by 51.6–64.7% was also observed in the brains of rats given montelukast (16.3 ± 0.41 and 11.9 ± 0.34 versus 33.7 ± 1.0 μ mol/g.tissue). The drug also resulted in significantly higher GSH levels by 20.7–65.8% compared to the rotenone only group (4.66 ± 0.25 and 6.4 ± 0.47 versus 3.86 ± 0.12 μ mol/g.tissue). Furthermore, TAC showed 109.6–156.2% increments by montelukast treatment (205.6 ± 5.2 and 251.3 ± 6.0 versus 98.1 ± 9.0 mmol/g.tissue).

3.2. SOD Activity

A significant decrease in SOD activity by 34.6% was found in the brain tissues of rotenone-treated rats as compared to the vehicle group (192.0 ± 2.4 versus 293.4 ± 6.8 U/g.tissue). In rats treated with rotenone

and montelukast, SOD activity increased by 50–62.5% compared to the rotenone only group (288.1 ± 2.6 and 312.0 ± 5.6 versus 192.0 ± 2.4 U/g.tissue) (**Figure 2A**).

3.3. PON-1 Activity

Rotenone-treated animals exhibited significantly depressed PON-1 activity by 68% compared to the vehicle group (5.4 ± 0.14 versus 16.9 ± 1.3 kU/l). A dose-dependent increase in PON-1 activity (161.1–203.7% of controls) was found in the brain tissues of rats given 10 and 20 mg/kg montelukast (14.1 ± 0.38 and 16.4 ± 1.0 versus 5.4 ± 0.14 kU/l) (**Figure 2B**).

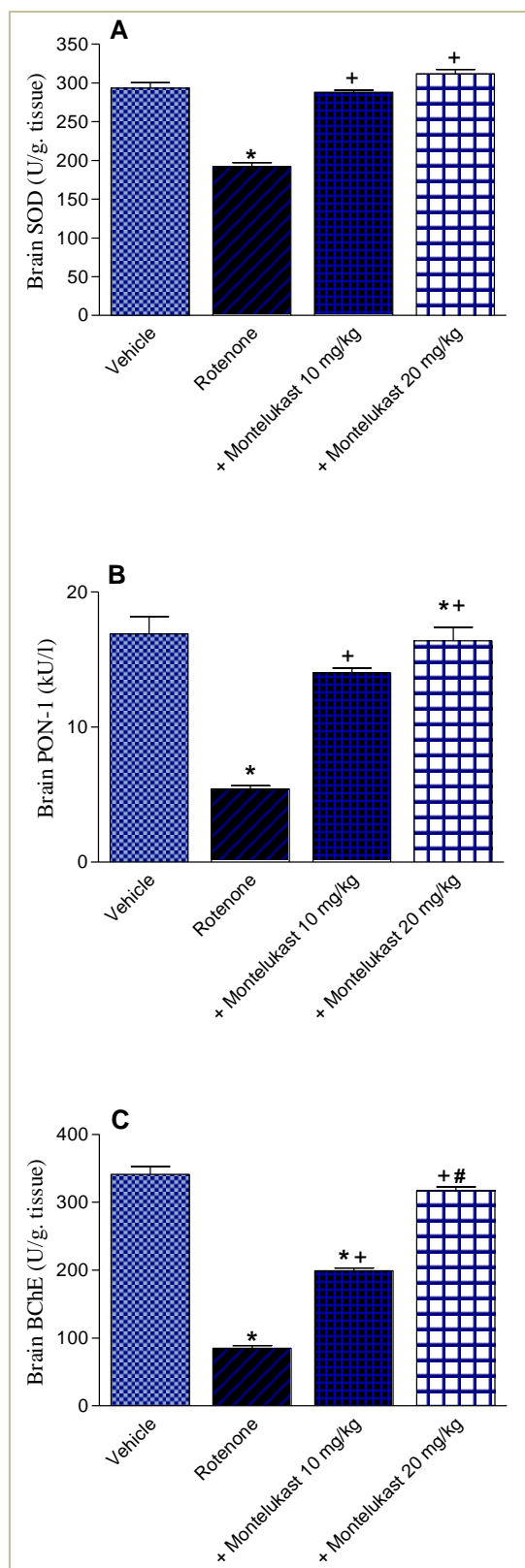
3.4. BChE Activity

Rotenone produced a significant decrease in BChE activity by 75.2% compared to the vehicle control value (84.7 ± 4.1 versus 341.2 ± 11.5 U/g.tissue). In rats treated with both rotenone and montelukast, BChE activity rose by 135.3–274.3% compared to the rotenone only group (199.3 ± 3.7 and 317.0 ± 5.9 versus 84.7 ± 4.1 U/g.tissue) (**Figure 2C**).

3.5. Histopathological Findings

Microscopic examination of the cerebral cortex from vehicle-treated rats showed normal structure (**Figure 3A**). Investigation of the brain sections from rats treated with rotenone only revealed serious damage inflicted by the toxin on the brain tissues. Many neurons in the cerebral cortex showed signs of degradation in the form of acidophilia of cytoplasm and pyknosis of nuclei (**Figure 3B** and **3C**). Treatment with montelukast at 10 mg/kg slightly reduced the damaging effect of rotenone with the number of affected neurons being decreased (**Figure 3D** and **3E**). Results obtained with the high dose of this drug were better, as neurons in the cerebral cortex were close to normal (**Figure 3F** and **3G**).

The substantia nigra of vehicle-treated rats showed normal structure (**Figure 4A**). Rotenone caused flattening of neurons with a decrease in their size (**Figure 4B** and **4C**). Treatment with montelukast at 10 mg/kg reduced the number of affected neurons, and some cells regained their normal size (**Figure 4D** and **4E**). Rats treated with the higher dose of the drug showed morphologically close to normal neurons (**Figure 4F** and **4G**).



4. DISCUSSION

This study shows that the administration of the neurotoxin rotenone induces oxidative stress in the brain tissues of the rats as evidenced by the increase in lipid peroxidation (MDA formation), and in nitric oxide content. This occurred along with depletion in GSH, the brain's most important antioxidant and free radical scavenger [33] and also with decreased TAC. Moreover, the activities of the antioxidant enzyme SOD, PON-1, and BChE were markedly depressed in the brain tissues of rotenone-intoxicated rats. Our results thus agree with previous studies demonstrating the ability of the pesticide to induce the production of reactive oxygen species as well as lipid peroxidation in vitro [34, 35] and in the brain tissues of rodents [26, 36-39] and to decrease the activity of the antioxidant enzymes in the rat brain [40]. Rotenone caused neuronal damage in the striatum, cerebral cortex, and hippocampus which is in agreement with previous studies [36-40]. Under these circumstances, the cysteinyl-leukotriene receptor 1 antagonist montelukast conferred significant neuronal protection accompanied with a dose-dependent decrease in brain MDA and nitric oxide contents. Moreover, the drug restored brain GSH level, TAC, and the activities of SOD, PON-1, and BChE.

The mitochondrial complex I (NADH-ubiquinone oxidoreductase) in the inner membrane is an important source of intracellular reactive oxygen metabolites in the brain tissue [41]. Rotenone induces oxidative stress by inhibiting the activity of complex I, thereby, increasing the formation of superoxide [25, 42, 43] which then can reduce cytochrome c or transition metals. Superoxide could be also converted to hydrogen peroxide (H_2O_2) by the enzyme SOD or react with nitric oxide to form peroxynitrite ($ONOO^-$) which is a strong oxidant [41, 44, 45]. The result is damage to the mitochondria and activation of the apoptotic cell death pathway [25, 42, 46]. Studies indicated that rotenone induces apoptotic cell death

FIGURE 2. Effect of montelukast on the activities of (A) superoxide dismutase (SOD), (B) paraoxonase-1 (PON-1), and (C) butyrylcholinesterase (BChE) in the brain tissues of rats treated with rotenone. *, $p < 0.05$ versus vehicle; +, $p < 0.05$ versus rotenone only; #, $p < 0.05$ versus rotenone + 10 mg/kg montelukast.

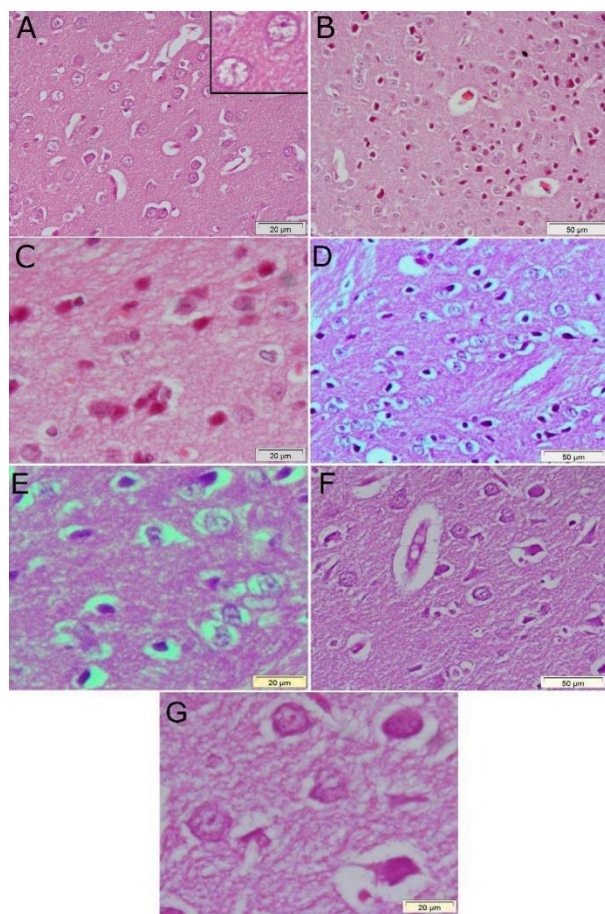


FIGURE 3. Photomicrographs of representative brain sections from the cortex of rats under different treatment conditions. (A) vehicle control cerebral cortex, showing large vesicular nuclei of neurons with their characteristic owl eye appearance; (B and C) rotenone only, showing many cells with signs of degeneration in the form of acidophilic cytoplasm and pyknotic nuclei; (D and E) rotenone and 10 mg/kg montelukast, showing reduced number of affected cells compared with the rotenone only group; (F and G) rotenone and 20 mg/kg montelukast, showing normalization of brain tissue (H&E staining with a magnification scale of 100 and 200).

[42, 47]. Strong cleaved caspase-3 immunoreactivity was observed in the striatum, substantia nigra, and cerebral cortex while the antiapoptotic protein Bcl-2 decreased in the striatum of rotenone-treated rodents

[38, 39, 48]. It has also been shown that cells that are transduced with Ndi1 (nicotinamide-adenine dinucleotide-ubiquinone oxidoreductase) of *Saccharomyces cerevisiae* which replaces complex I were resistant to rotenone neurotoxicity [49]. The increase in brain nitric oxide is also likely to contribute to the rotenone neurotoxicity [27]. The neurotoxin has been demonstrated to increase the expression of the inducible form of nitric oxide synthase (iNOS) in the striatum and substantia nigra [37, 48]. This enzyme is responsible for the excessive and prolonged release of nitric oxide by microglia and astrocytes observed during inflammation and under toxic conditions [50]. Nitric oxide causes oxidative/nitrosative stress by reacting with oxygen to produce other reactive oxides of nitrogen such as ONOO^- , NO_2 , N_2O_3 , or by liberating catalytic iron from ferritin [50, 51]. Excessive nitric oxide can result in inhibition of mitochondrial respiration, and cellular energy depletion [52, 53]. Rotenone also induces a state of neuroinflammation in the brain tissue [47, 54]. This toxicant has been shown to increase the expression of tumor necrosis factor- α (TNF- α) as well as the level of monocyte chemoattractant protein-1 (MCP-1) [47] and interleukin-1 β [54] in the rat brain. Rotenone thus causes the development of oxidative stress and neuroinflammation, two fundamental pathologic processes encountered in the brain of human Parkinson's disease [9, 16, 17].

In this study, rotenone was shown to cause marked inhibition PON-1 activity in the brain which agrees with previous studies [37-40, 48, 54]. This enzyme acts to detoxify organophosphate insecticides [55], and variation in its catalytic efficiency has been shown to alter the susceptibility to organophosphate compounds [56] and thus possibly increases the risk for developing Parkinson's disease in susceptible individuals [57, 58]. An antioxidant and anti-inflammatory role has been described for PON-1 [59, 60], and a decrease in enzyme activity occurs in conditions of oxidative stress [61]. In several studies, restoration of the PON-1 enzyme activity was associated with alleviation of oxidative stress and/or neuronal protection [37-40], suggesting that the enzyme could be a sensitive indicator of the oxidative status in the cell [39].

Our data also show that rotenone can result in significant inhibition of BChE activity in the brain tissues which is in agreement with previous observations [54]. Rotenone was also demonstrated

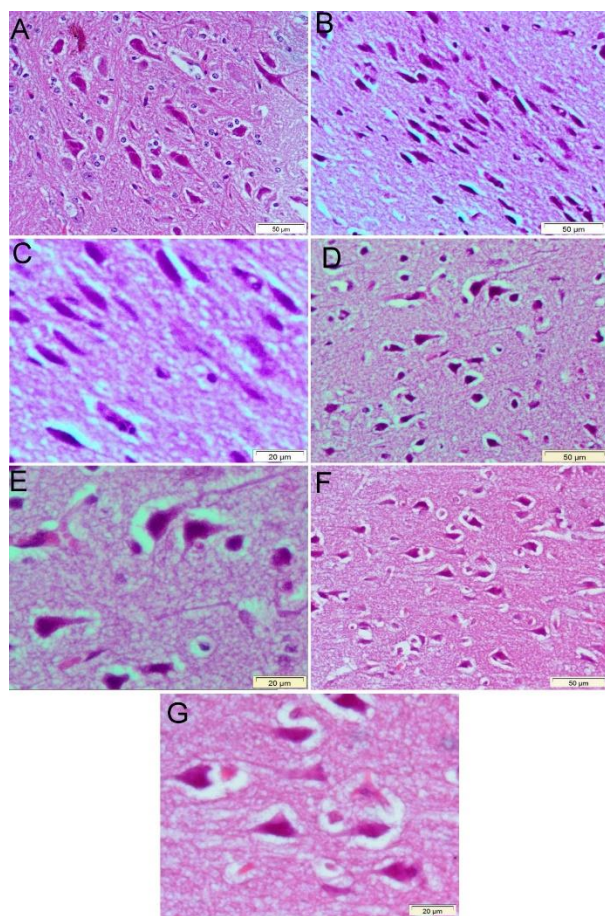


FIGURE 4. Photomicrographs of representative brain sections from the substantia nigra of rats under different treatment conditions. (A) vehicle only, showing large pigmented neurons; (B and C) rotenone only, showing that most of cells are flattened and appear smaller than normal; (D and E) rotenone + 10 mg/kg montelukast, showing improvement of the neurons size; and (F and G) rotenone + 20 mg/kg montelukast, showing normalization of brain tissue (H&E staining with a magnification scale of 100 and 200).

to inhibit acetylcholinesterase (AChE) activity in the cerebral cortex of injected rats [38, 48]. This decrease in the activity of cholinesterases could be due to damage to cholinergic neurons and/or direct enzyme inhibition by the toxicant and is likely to contribute to the development of motor manifestations in response to rotenone administration. In Parkinson's

disease, there is an imbalance between cholinergic and dopaminergic neurotransmission in the basal ganglia [62], and hence, anticholinergic drugs have a place in the management of mild cases especially for the static tremor [63]. Notably, treatment with montelukast was associated with restoration of both PON-1 and BChE activities in rotenone-intoxicated rats; an effect that is likely to be a consequence of its neuroprotective action.

The lipid mediators, leukotrienes, synthesized from arachidonic acid by the action of 5-lipoxygenase are fundamental to the development of inflammation [64]. Montelukast is a cysteinyl leukotrienes receptor antagonist that is used in the management of bronchial asthma reducing the airway inflammatory response [21]. The drug was shown to possess an antioxidant effect and a neuroprotective potential in traumatic brain damage in rats, reducing brain MDA and neutrophil infiltration (myeloperoxidase activity) [22]. In vitro, montelukast prevented the rotenone-induced microglia activation, decreasing the release of cytokines such as interleukin-1 β and TNF- α [23]. This study showed that the systemic administration of montelukast was able to protect against the biochemical changes caused by rotenone in the rat brain. This neuroprotective effect of montelukast was confirmed by histopathological studies. The signs of neuronal degeneration in the cerebral cortex and substantia nigra in the rotenone only group were reduced by montelukast in a dose-dependent manner.

In summary, the present study shows that montelukast is able to afford neuroprotection against the nigrostriatal damage caused by the neurotoxin rotenone in rats. This effect of montelukast is likely to involve attenuation of oxidative stress and the inflammatory response in the brain tissues.

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The authors declare that there are no conflicts of interest.

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