Neuroprotective Effects of the Glutathione Precursor *N*-Acetylcysteine against Rotenone-Induced Neurodegeneration

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ABSTRACT | In this study, the neuroprotective potential of the glutathione precursor *N*-acetylcysteine in the rotenone-induced Parkinson's disease (PD) was investigated. Rats were administered rotenone (1.5 mg/kg/day) once every other day for 2 weeks by subcutaneous injection. Starting from the first day of rotenone treatment, rats received the vehicle control or *N*-acetylcysteine (NAC) at doses of 10 and 30 mg/kg orally given at time of rotenone injection. Rats were evaluated for brain malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide content, and paraoxonase-1 (PON-1) activity in the striatum, cerebral cortex, and the rest of the brain tissue. The level of the anti-apoptotic Bcl-2 was also determined in the striatum. In addition, histopathological examination and the expression of cycloxygenase-2 (COX-2) in the striatum and cerebral cortex were performed. Rotenone treatment caused a significant increase in MDA and nitric acid content in the striatum, cerebral cortex, and the rest of the brain tissue. It also significantly decreased brain GSH content and PON-1 activity in these regions and decreased striatal Bcl-2 level compared to control values. Rotenone treatment caused neuronal necrosis, apoptosis, and vacuolization, and increased the expression of COX-2 in both the striatum and cerebral cortex. NAC given at doses of 10 and 30 mg/kg to rotenone-treated rats caused a dose-dependent significant decrease in MDA levels in the cortex and the rest of the brain tissue and at the dose of 30 mg/kg significantly decreased the striatal MDA level. It also significantly decreased the nitric oxide level, increased GSH content and PON-1 activity in the striatum, cerebral cortex, and the rest of the brain when given at doses of 10 and 30 mg/kg. Additionally, there was a significant increase in the striatal Bcl-2 level by NAC at 30 mg/kg. NAC decreased neuronal necrosis and apoptosis as well as COX-2 immunostaining in both the striatum and cerebral cortex in a dose-dependent manner. These findings suggest a potential benefit for NAC in alleviating brain oxidative stress, neuroinflammation, and neurodegeneration in the rotenone model of PD in rats. NAC could thus be a useful adjunct in the treatment of patients with PD.

KEYWORDS | *N*-Acetylcysteine; Bcl-2; Cyclooxygenase; Glutathione; Malondialdehyde; Neurodegeneration; Neuroprotection; Nitric oxide; Paraoxonase; Parkinson's disease; Rotenone

ABBREVIATIONS | COX-2, cycloxygenase-2; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; GSSG, oxidized glutathione; H&E, hematoxylin and eosin; IL, interleukin; IL-1β, interleukin-1β; MDA,

malondialdehyde; NAC, *N*-acetylcysteine; iNOS, inducible nitric oxide synthase; PD, Parkinson's disease; PON-1, paraoxonase-1; ROS, reactive oxygen species; sc, subcutaneous injection; SNpc, substantia nigra pars compacta; TNF-α, tumor necrosis factor-α

CONTENTS

1. INTRODUCTION

Parkinson's disease (PD) is a hypokinetic neurodegenerative disorder that affects ~1% of the elderly population over the age of 65 years [1]. In PD, the pigmented dopamine-containing neurons of the substantia nigra pars compacta (SNpc) of the midbrain basal ganglia undergo preferential and slowly progressive degeneration [2]. The basal ganglia receives information from the cerebral cortex and the intralaminar nuclei of the thalamus and its outflow to the thalamus, which projects to cortex and brain stem nuclei, controls voluntary motor movements [3]. The loss of midbrain dopamine neurons results in the characteristic motor features of PD, namely, hypokinesia or akinesia, rigidity, postural instability, and tremor [3, 4]. Besides, non-motor features, such as anxiety, depression, cognitive changes, autonomic dysfunction, and psychotic symptoms also occur in the course of the disease and are attributable to dopaminergic as well as non-dopaminergic neuronal loss [5, 6].

PD is essentially a sporadic disorder with only 5% of diagnosed cases being of known genetic causes [7, 8]. PD also developed after intravenous use of a meperidine analog in humans and this finding suggested that the disease might be triggered by exposure to an environmental toxin [9, 10]. Later, a number of epidemiological studies indicated an association between the increase in the risk for developing PD and the use of pesticides and fungicides (e.g., rotenone, paraquat and maneb) and insecticides (e.g., chlorpyr-

ifos products) [11, 12]. When injected into rodents, rotenone, a pesticide of plant origin, was able to reproduce the pathological and biochemical changes seen in idiopathic PD, such as nigrostriatal degeneration, dopamine depletion, formation of Lewy body-like inclusions, and hypokinesia [13–15].

The cause of the selective death of SNpc neurons is not yet fully established but accumulating evidence points to several pathogenetic mechanisms such as increased free radicals and consequent oxidative damage to cells as well as neuroinflammation [16–19]. Oxidative stress occurs in the brain of patients with PD because of increased generation of reactive oxygen species (ROS) and decreased antioxidants in the cells [20]. Thus, increased levels of lipid peroxides and products of oxidatively damaged proteins and deoxyribonucleic acid have been encountered in the brain of patients with PD [21–23]. There were also decreased levels of reduced glutathione (GSH) and increased oxidized glutathione (GSSG) in the substantia nigra [24]. Moreover, intravenously given GSH was associated with symptomatic improvement of PD [25, 26]. The occurrence of neuroinflammatory events in PD has been suggested by the findings of raised levels of proinflammatory cytokines, such as interleukin (IL)-1β, IL-2, and IL-6 in the striatum and tumor necrosis factor-α (TNF-α), IL-1β, and interferon-γ and by the presence of activated microglial cells in the substantia nigra of patients with PD [27].

The treatment of PD is only symptomatic and based on replacing the biochemical deficit by administering the dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa) along with a peripheral decarboxylase inhibitor (L-dopa/carbidopa). Other agents aiming at increasing the dopaminergic neurotransmission (i.e., dopamine receptor agonists) or increasing dopamine availability by decreasing its degradation (e.g., catechol-*O*-methyltransferase inhibitors or monoamine oxidase inhibitors) are also used. However, there is no available treatment to prevent the continued cell death in PD [28, 29].

In this study, the glutathione precursor NAC was evaluated for a potential neuroprotective effect in the rotenone model of PD in rats. NAC has been shown to increase peripheral and brain GSH and to provide symptomatic improvement in patients with brain disorders such as attention deficit hyperactivity disorder [30], cannabis use disorder, depression, and schizophrenia [31] as well as in PD subjects [32, 33].

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats, weighing 180–200 g, were used. Rats were group-housed under temperature- and light-controlled conditions and allowed standard laboratory rodent chow and water ad libitum. The experiments were conducted in accordance with the ethical guidelines for care, use, and handling of laboratory animals by the Ethics Committee of the National Research Centre (Cairo, Egypt) and followed the recommendations 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 was purchased from Sigma-Aldrich (St Louis, MO, USA). Rotenone was dissolved in dimethyl sulfoxide (DMSO). NAC was purchased from SEDICO Pharmaceutical (Cairo, Egypt) and dissolved in normal saline. Other chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich.

2.3. Study Design

Rats were randomly divided into four groups, with six rats in each group. The following groups were studied: group 1, vehicle control (DMSO, via subcutaneous injection [sc]); group 2, rotenone 1.5 mg/kg, sc, given once every other day; group 3, rotenone 1.5 mg/kg, sc, given once every other day plus NAC 10 mg/kg, given orally, once a day, at the time of rotenone injection; group 4, rotenone 1.5 mg/kg, sc, given once every other day plus NAC 30 mg/kg, given orally, once a day, at the time of rotenone injection. Treatments were continued for 2 weeks. At the end of the experiment, rats were euthanized for tissue collection; their brains were then quickly removed out on an ice-cold plate, washed with ice-cold phosphate-buffered saline (pH 7.4), and dissected into cortex, striatum, and the rest of the brain tissue, weighed, and stored at −80°C until biochemical studies. The tissues were homogenized in 0.1 M phosphate-buffered saline (pH 7.4) to give a final concentration of 10% weight/volume (w/v) for the biochemical assays.

2.4. Biochemical Assays

2.4.1. Determination of Lipid Peroxidation

Lipid peroxidation was measured in brain homogenates by determining malondialdehyde (MDA) according to Ruiz-Larrea et al. [34]. 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. Results are expressed as nmol/g wet tissue.

2.4.2. Determination of GSH

GSH was determined in brain homogenates according to Ellman et al. [35]. The Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reacts with the free thiol group of GSH to form 2-nitro-5-mercaptobenzoic acid. The chromophore has yellow color and is determined spectrophotometrically at 412 nm. Results are expressed as µmol/g wet tissue.

2.4.3. Determination of Nitric Oxide

Nitric oxide was determined using the 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 spectrophotometrically at 540 nm [36]. Results are expressed as µmol/g wet tissue.

2.4.4. Determination of Paraoxonase-1 (PON-1) Activity

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 [37, 38].

2.4.5. Quantification of Bcl-2

The level of Bcl-2 in the striatum was determined by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Glory Science, Del Rio, TX, USA) according to the manufacturer's instructions. The level of Bcl-2 in a sample was determined by interpolation from a standard curve. The detection range of the kit is 0.5–10 ng/ml.

2.5. Histopathological Studies

The brains were rapidly dissected, fixed in 4% neutral-buffered formalin, processed routinely for paraffin embedding. Paraffin sections (5 µm thick) were prepared and stained with hematoxylin and eosin (H&E) for the histopathological examination. Sections were examined using a light microscope.

2.6. Immunohistochemical Examination of Cyclooxygenase-2 (COX-2)

Immunohistochemistry for COX-2 was performed using formalin-fixed, paraffin-embedded tissue sections (5 µm thick), mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4°C) with primary anti-COX-2 antibody (polyclonal goat anti-mouse) diluted 1:200 in PBS plus bovine serum albumin (PBS-BSA). The slides were then incubated with biotinylated goat anti-IgG, diluted 1:200 in PBS-BSA. After washing, the slides were incubated with avidin-biotin-horseradish peroxidase conjugate for 30 min, COX-2 was visualized with the chromogen 3,3-diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS-BSA 5%. None of the negative controls showed COX-2 immunoreactivity. Slides were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped.

2.7. Quantitative Evaluation of COX-2

Each immunolabeled section was observed at 400×magnification. The optical density measurements of COX-2 positive cells were recorded in the cortex and striatum in 10 fields in slides of each animal from each group. These measurements were av-

eraged to obtain one mean per region for each animal. Counting and morphometric analysis were carried out on Leica Quin 500 Image Analyzer (Leica, Cambridge, UK) in the Pathology Department, National Research Centre (Cairo, Egypt).

2.8. Statistical Analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used for data analysis and post-hoc individual comparisons were performed with Duncan's multiple range test. Statistical significance was considered at $p < 0.05$. The Statistical Package for Social Sciences software (version 16.0; SPSS, Chicago, IL, USA) was used.

3. RESULTS

3.1. Biochemical Results

3.1.1. MDA

Rotenone given alone elicited significant increments ($p < 0.05$) in brain MDA levels in the striatum (30.8%), cerebral cortex (43.9%), and the rest of brain tissue (54.7%) as compared with the vehicle only group. NAC given to rotenone-treated rats at doses of 10 and 30 mg/kg resulted in significantly decreased MDA levels in the cerebral cortex and striatum by 19.8–23.8% and 14.8–28.0%, respectively, and at the dose of 30 mg/kg, NAC significantly decreased the striatal MDA level by 20.4% relative to the rotenone only group (**Table 1**).

3.1.2. Nitric Oxide

In rotenone-treated animals, significantly increased nitric oxide levels were observed in the striatum (55.8%), cerebral cortex (50.2%), and the rest of the brain tissue (67.3%). NAC treatment at doses of 10 and 30 mg/kg resulted in significant decline in nitric oxide levels in these regions by 18.7–30.9%, 19.6–32.0%, and 19.4–26.4% compared with the rotenone only group (**Table 1**).

3.1.3. GSH

There was a significant ($p < 0.05$) decrease in brain GSH content in rotenone-treated rats in the striatum

(36.1%), cerebral cortex (41.0%), and the rest of the brain tissue (34.9%) compared to the vehicle only group. NAC at doses of 10 and 30 mg/kg significantly increased GSH content in the striatum (51.2–95.5%), cerebral cortex (34.4–132.2%), and in the rest of the brain tissue (32.6–64.3%) compared with the rotenone only group (**Table 1**).

3.1.4. PON-1

Rotenone significantly decreased PON-1 activity in the striatum (42.1%), cerebral cortex (37.4%), and the rest of the brain tissue (34.3%) compared to the vehicle only group. NAC administered at doses of 10 and 30 mg/kg during rotenone treatment, resulted in significant increases in PON-1 activity in the striatum (19.4–58.1%), cerebral cortex (27.4–46.8%), and in the rest of the brain tissue (30.3–58.2%) compared with the rotenone only group (**Table 2**).

3.1.5. Bcl-2

Rotenone treatment decreased striatal Bcl-2 levels by 51% as compared to the vehicle treated group. Striatal Bcl-2 showed a significant increase by 24.5% in NAC (30 mg/kg) plus rotenone-treated rats compared to the rotenone only group (**Table 3**).

3.2. Histopathological Results

The H&E-stained sections from the striatum and cerebral cortex of vehicle-treated rats showed normal cellularity with healthy neurons and prominent nuclei (**Figures 1A** and **2A**). Rats treated with only rotenone showed neuropathological changes in the form of necrosis, pyknotic and apoptotic neurons, shrinkage of nuclei with widened pericellular spaces, vacuolization, and scanty acidophilic cytoplasm. Focal gliosis and slight congestion blood vessels were also seen (**Figures 1B** and **2B**). Examination of the striatum and cerebral cortex in rotenone-treated rats given NAC at 10 and 30 mg/kg showed dose-dependent attenuation of the histopathological alterations (**Figure 1C** and **1D** and **Figure 2C** and **2D**).

3.3. Immunohistochemical Detection of COX-2 Expression

Sections from the striatum and cerebral cortex of vehicle-treated rats showed negative immunostaining

TABLE 1. MDA, nitric oxide, and GSH in different brain regions in rats given rotenone alone or in combination with NAC

	Vehicle	Rotenone	Rotenone + NAC 10	Rotenone + NAC 30
MDA				
Striatum	18.68 ± 1.10	$24.45 \pm 1.03^*$	$21.98 \pm 0.77\ (-10.1\%)$	$19.45 \pm 0.85^{*+}\ (-20.4\%)$
Cortex	19.65 ± 1.24	$28.27 \pm 1.72^*$	$22.66 \pm 1.3^+\ (-19.8\%)$	$21.54 \pm 0.95^+\ (-23.8\%)$
Rest of the brain	$22.10 \pm 0.61^*$	$39.21 \pm 2.00^*$	$29.15 \pm 1.32^*\ (-14.8\%)$	$24.61 \pm 1.37^+\ (-28.0\%)$
Nitric oxide				
Striatum	19.64 ± 0.98	$30.61 \pm 1.25^*$	$24.61 \pm 1.62^+\ (-19.6\%)$	$20.80 \pm 1.54^+\ (-32.0\%)$
Cortex	21.67 ± 1.05	$32.55 \pm 1.31^*$	$26.48 \pm 2.72^+\ (-18.7\%)$	$22.48 \pm 1.35^+\ (-30.9\%)$
Rest of the brain	$23.74 \pm 1.23^*$	$39.72 \pm 2.58^*$	$32.00 \pm 1.75^+(-19.4\%)$	$29.23 \pm 2.27^+\ (-26.4\%)$
GSH				
Striatum	3.82 ± 0.09	$2.44 \pm 0.08^*$	$3.69 \pm 0.09^+\ (51.2\%)$	$4.77 \pm 0.12^+\ (95.5\%)$
Cortex	3.85 ± 0.18	$2.27 \pm 0.11^*$	$3.05 \pm 0.16^+\ (34.4\%)$	$5.27 \pm 0.24^+\ (132.2\%)$
Rest of the brain	$4.24 \pm 0.09^*$	$2.76 \pm 0.06^*$	$3.66 \pm 0.07^+\ (32.6\%)$	$4.66 \pm 0.16^+\ (68.8\%)$

Note: The percent changes from the rotenone control group are shown in parentheses. The unit for nitric oxide and GSH is μmol/g tissue; for MDA is nmol/g tissue; for NAC is mg/kg. *, $p < 0.05$ vs. vehicle; +, $p < 0.05$ vs. rotenone control.

TABLE 2. PON-1 activity in different brain regions of rats given rotenone alone or in combination with NAC

	Vehicle	Rotenone	Rotenone + NAC 10	Rotenone + NAC 30
Striatum	$13.10 \pm 0.51^*$	$7.59 \pm 0.34^*$	$9.06 \pm 0.28^{*+}\ (19.4\%)$	$12.00 \pm 0.82^+\ (58.1\%)$
Cortex	$13.43 \pm 0.71^*$	$8.40 \pm 0.54^{*+}$	$10.70 \pm 0.87^+\ (27.4\%)$	$12.33 \pm 0.42^+\ (46.8\%)$
Rest of the brain	$14.72 \pm 0.59^*$	$9.67 \pm 0.47^*$	$12.60 \pm 0.69^*\ (30.3\%)$	$15.30 \pm 1.2^+\ (58.2\%)$

Note: The percent changes from the rotenone control group are shown in parentheses. The unit for PON-1 activity is kU/l; for NAC is mg/kg. *, $p < 0.05$ vs. vehicle; +, $p < 0.05$ vs. rotenone control.

TABLE 3. Bcl-2 level in cerebral cortex of rats given rotenone alone or in combination with NAC

Vehicle	Rotenone	Rotenone + NAC 10 mg/kg	Rotenone + NAC 30 mg/kg
2.84 ± 0.17	$1.39 \pm 0.01^*$	$1.54 \pm 0.09^*\ (10.8\%)$	$1.73 \pm 0.03^{*+}\ (24.5\%)$

Note: The percent changes from the rotenone control group are shown in parentheses. The unit for Bcl-2 is ng/ml. *, $p < 0.05$ vs. vehicle; +, $p < 0.05$ vs. rotenone control.

of COX-2 (**Figures 3A** and **4A**). As shown in Figures 3B and 4B, rotenone induced intense COX-2 expression in neuronal cells. The number of COX-2-positive neurons was reduced in rats treated with NAC (10 and 30 mg/kg) compared to the rotenone only group (**Figure 3C** and **3D** and **Figure 4C** and **4D**). The decrease in COX-2 expression by NAC was dose-dependent.

3.4. Quantitative COX-2 Results

Quantitative morphometric analysis of COX-2 immunoreactivity in the cortex and striatum indicated a significant increase of immunoreactivity in the rotenone only group as compared with the vehicle control group. In rotenone-treated rats, COX-2 immunoreactivity was significantly reduced by co-
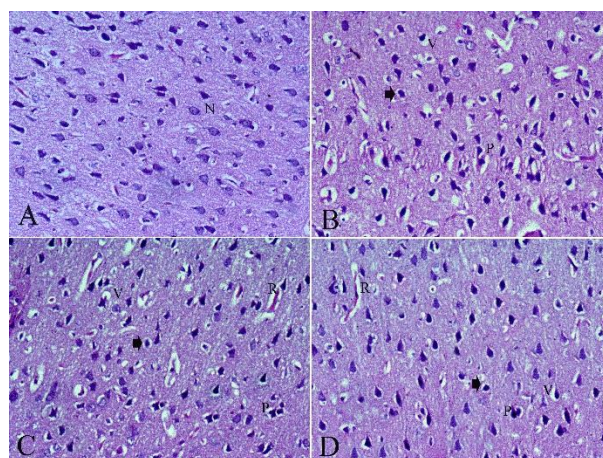
FIGURE 1. Representative photomicrographs of sections of rat striatum stained with H&E. (A) Vehicle control group showing normal neuronal cells with prominent nuclei (N). (B) Rotenone only group showing neuronal degenerative changes, necrosis, vacuolization (V), and scanty acidophilic cytoplasm with pyknotic (P) and apoptotic nuclei (arrowhead). (C) Rotenone and NAC (10 mg/kg) group showing moderate degenerative changes with vacuolization (V), pyknotic (P) and apoptotic nuclei (arrowhead), and red neuron (R). (D) Rotenone and NAC (30 mg/kg) group showing less degenerative changes with few vacuolization (V), pyknotic (P) and apoptotic nuclei (arrowhead), and red neuron (R) (×400).
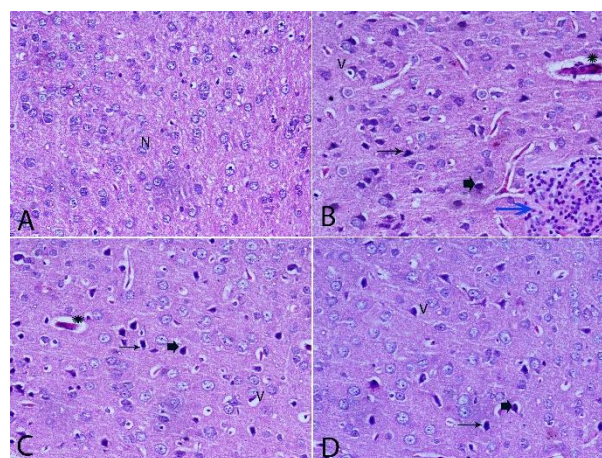

FIGURE 2. Representative photomicrographs of sections of rat cerebral cortex stained with H&E. (A) Vehicle control group showing normal neuronal cells with prominent nuclei (N). (B) Rotenone only group showing necrosis, vacuolization (V), and scanty acidophilic cytoplasm with pyknotic (P) and apoptotic nuclei (arrowhead). Focal gliosis (blue arrow) and slight congestion blood vessels (star) were also seen. (C) Rotenone and NAC (10 mg/kg) group showing less degenerative changes with vacuolization (V) and pyknotic (P) and apoptotic nuclei (arrowhead). Slight congestion blood vessels were also seen (star). (D) Rotenone and NAC (30 mg/kg) group showing nearly normal tissue structure with good cellular morphology and few vacuolizations (V) and pyknotic (P) and apoptotic nuclei (arrowhead) (×400).

administration of NAC in a dose-depended manner (**Table 4**).

4. DISCUSSION

In this study, we have shown that oxidative stress developed in the brain of rats following rotenone injection. Significant elevation in the brain levels of the lipid peroxidation end product MDA was detected. Concomitantly, the level of the antioxidant molecule and free radical scavenger GSH [39] fell, suggesting its consumption due to increased free radical generation. These results confirm our previously published data indicating the ability of rotenone to cause increased brain lipid peroxidation and to decrease the level of GSH and catalase activity in the

brain of rats and mice [40, 41]. Other studies in vitro have shown that rotenone resulted in intracellular accumulation of ROS and protein carbonylation, decreased the level of GSH, and induced apoptotic cell death [42–44]. In vivo, rotenone increased ROS generation in the forebrain and midbrain of mice [45]. Rotenone inhibits mitochondrial complex I (NADH-ubiquinone oxidoreductase) resulting in mitochondrial deficits [46] and the enhanced generation of superoxide [42, 47] or the formation of hydrogen peroxide [48]. The superoxide released can then reduce cytochrome c or transition metals or is converted to hydrogen peroxide by the enzyme superoxide dismutase. Moreover, superoxide reacts with nitric oxide to form peroxynitrite, a strong oxidant [49].
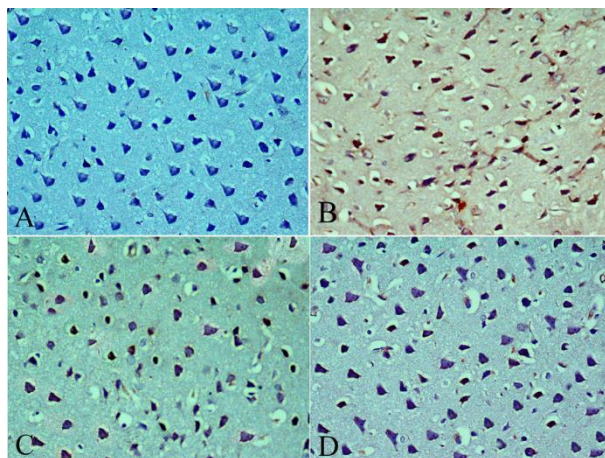
FIGURE 3. Representative photomicrographs of sections of rat striatum stained immunohistochemically with COX-2 antibody. (A) Vehicle control group showing negative immunostaining of COX-2. (B) Rotenone only group showing neurons with intense brown immunostaining of COX-2. (C) Rotenone and NAC (10 mg/kg) group showing moderate immunostaining of COX-2. (D) Rotenone and NAC (30 mg/kg) group showing mild immunostaining of COX-2 (×400).

FIGURE 4. Representative photomicrographs of sections of rat cerebral cortex stained immuno-histochemically with COX-2 antibody. (A) Vehicle control group showing negative immunostaining of COX-2. (B) Rotenone only group showing neurons with intense brown immunostaining of COX-2. (C) Rotenone and NAC (10 mg/kg) group showing moderate immunostaining of COX-2. (D) Rotenone and NAC (30 mg/kg) group showing mild immunostaining of COX-2 (×400).

Rotenone induces the activation of microglia cells [14] and these cells in turn increase the release of superoxide via NADPH oxidase [50] or hypochlorous acid via the myeloperoxidase enzyme [51]. Oxidative stress is likely to be an important mechanism underlying the neurotoxic effects of rotenone because the damage could be alleviated by antioxidants [45, 52]. In vitro, rotenone induces apoptosis which could be inhibited by antioxidants such as GSH, NAC, ascorbate [42], and α-tocopherol [14].

Our results also showed increased brain nitric oxide levels in rotenone-treated animals which is in accordance with previous studies [41, 53, 54]. Rotenone increases nitric oxide release and the expression of the inducible nitric oxide synthase (iNOS) in the striatum of mice and rats [41, 55, 56]. Nitric oxide is a free radical and reacts with oxygen to yield nitrogen oxides such as nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3). It also reacts with superoxide to produce peroxynitrite. These species are capable of inducing oxidation and nitration of tyrosine 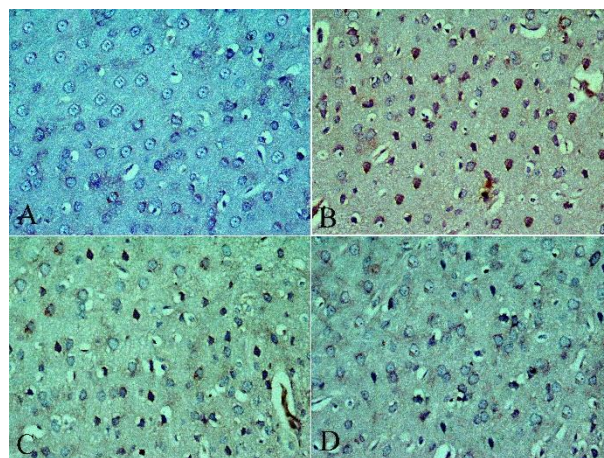residues in proteins, and nitrosylation of thiols. The result is mitochondrial impairment, energy failure, and ultimately neuronal death [57, 58]. Rotenone caused the nitration of tyrosine residues of intracellular proteins and dopaminergic cell apoptosis in vitro which could be decreased by nitric oxide synthase inhibitor or NAC [59, 60]. Rotenone toxicity could also be attenuated by specific inhibitors of iNOS or neuronal nitric oxide synthase [50, 61], thereby implicating an increase in nitric oxide in the rotenone neurotoxicity.

Rotenone inhibits the enzyme PON-1 in the brain of rodents [41, 53, 54]. The significance of this finding derives from the evidence that links variation in the enzyme catalytic efficiency with an increased risk for developing PD [62, 63]. PON-1 acts to hydrolyze the active metabolites of some organophosphate insecticides such as diazoxan, dichlorvos, and chlorpyrifos [64], and exposure to these compounds is thought to contribute to an increase in PD in rural areas [62, 65]. It follows that the decrease in enzyme activity would therefore expose the cells to increasing concentrations of insecticides in agricultural

TABLE 4. Optical density measurements of COX-2 positive cells in cortex and striatum of rats treated with rotenone alone or in combination with NAC

	Vehicle	Rotenone	Rotenone + NAC 10 mg/kg	Rotenone + NAC 30 mg/kg
Striatum	0.081 ± 0.111	$0.85 \pm 0.045^*$	$0.427 \pm 0.011^* (-45.0\%)$	$0.253 \pm 0.011^{*+} (-69.6\%)$
Cortex	0.074 ± 0.027	$0.635 \pm 0.131^*$	$0.343 \pm 0.016^* (-46.0\%)$	$0.209 \pm 0.021^{*+} (-67.1\%)$

Note: The percent changes from the rotenone control group are shown in parentheses. *, $p < 0.05$ vs. vehicle; +, $p < 0.05$ vs. rotenone control.

workers. Furthermore, antioxidative and anti-inflammatory roles have been ascribed to PON-1 [38, 66] and it is thus expected that decreased activity of PON-1 would render the cells vulnerable to free radicals and inflammatory events. PON-1 could be inactivated by oxidative stress [67] which could explain the decline in its activity in rats treated with rotenone [41, 53, 54] or malathion [68].

Caspases are a group of cysteine proteases that mediate the initiation and execution of cellular apoptosis, a morphologically distinct form of cell death [69] and an important mechanism of nigral cell death in PD [70, 71]. In PD, caspase-3 and Bax immunoreactivity are increased in melanized neurons of substantia nigra [72]. Rotenone induces prominent expression of caspase-3 in the substantia nigra, striatum, and cerebral cortex of rats [41, 53, 54]. In vitro, rotenone induced caspase-3-dependent apoptosis in human dopaminergic SH-SY5Y cells, human promyelocytic leukemia cell line, and rat ventral mesencephalic dopaminergic neurons [42, 59, 73, 74]. Caspase-3 is an effector caspase essential for apoptotic chromatin condensation, fragmentation of DNA, and cell dismantling [69]. The process of apoptosis is regulated by the Bcl-2 family proteins which include both pro- or anti-apoptotic proteins. The anti-apoptotic protein Bcl-2 acts to maintain the integrity of the outer mitochondrial membrane, preventing the release of mitochondrial cytochrome c into the cytosol and the subsequent activation of caspase proteins and the execution of apoptosis [75, 76]. In addition, Bcl-2 prevents apoptosis initiated by oxidants such as hydrogen peroxide [77, 78], while the expression of Bcl-2 itself is decreased by oxidative stress. In this study, we showed that the level of Bcl-2 was decreased in the striatum of rotenone-treated rats which is in accordance with our previously published data [53]. Rotenone has also been shown to decrease the expression of Bcl-2 in human dopaminergic cells in vitro [79, 80]. The above observations, therefore,

suggest apoptosis as the likely mechanism of rotenone-induced cell death. Inhibiting the apoptotic cascade in the substantia nigra could be an efficient therapeutic modality to lessen or slow the process of nigral cell death in PD. In this study, NAC increased the Bcl-2 level in the striatum of rotenone-treated rats, suggesting that the agent may prevent apoptosis by increasing Bcl-2.

There is also evidence suggesting the presence of an inflammatory milieu contributing to cell death in the PD brain [16]. Raised levels of proinflammatory cytokines were detected in the striatum and substantia nigra [27]. Glia cells are also activated in the PD brain. These cells contribute to neurodegeneration through the release ROS and inflammatory mediators [81]. In the present study, prominent immunostaining of the enzyme COX-2 was shown in neurons in the striatum and cerebral cortex of rotenone-treated rats. COX enzymes catalyze the conversion of arachidonic acid into prostaglandins. The enzyme COX-2 is not constitutively expressed but is induced in response to inflammatory signals and is the isoform responsible for the production of prostaglandins during inflammatory events [82]. Induction of COX-2 expression in SNpc dopaminergic neurons has been shown in the PD brain post-mortem, suggesting a role for the enzyme in the neurodegenerative process [83]. Rotenone has also been shown to increase the striatal expression of TNF-α and the level of monocyte chemoattractant protein-1 (MCP-1) and IL-1β in the rat brain [41, 53]. These data imply a role for inflammatory mediators and cytokines in the rotenone neurotoxicity. In the present study, NAC was able to decrease COX-2 immunostaining in the striatum and cerebral cortex. NAC might thus protect neurons by interfering with the COX-2 inflammatory cascade.

In conclusion, NAC given to rotenone-treated rats was able to alleviate the increases in brain lipid peroxidation and nitric oxide as well as to increase GSH and PON-1 activity. NAC increased the levels

of the anti-apoptotic factor Bcl-2, decreased COX-2 immunostaining, and afforded neuroprotection against the rotenone-induced neurotoxicity. Based on these findings, NAC might prove a useful adjunct in the treatment of PD. By replenishing the depleted GSH stores in the cells, the agent is likely to tilt the balance in the parkinsonian brain in favor of the antioxidant side, thereby decreasing the available free radicals and protecting cellular macromolecules.

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