

The Effect of 2,4-Dinitrophenol on Oxidative Stress and Neuronal Damage in Rat Brain Induced by Systemic Rotenone Injection

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ABSTRACT | 2,4-Dinitrophenol (2,4-DNP) is an uncoupler of mitochondrial oxidative phosphorylation which shows promise as a neuroprotectant. In this study, the effect of 2,4-DNP on oxidative stress and neuronal damage in rat brain induced by systemic rotenone injection was investigated. Rats were treated with rotenone 1.5 mg/kg alone or in combination with 2,4-DNP (1 or 3 mg/kg) injected subcutaneously three times per week for 2 weeks. The brain content of the lipid peroxidation product malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (nitrite), paraoxonase 1 (PON1) activity, and nuclear factor kappaB (NF-κB) were determined. Histopathology and caspase-3 immunohistochemistry were also performed. Rotenone produced a significant increase in the levels of MDA, nitric oxide, and NF-κB by 56.3%, 99.3%, and 59.2%, respectively, compared with the vehicle control group. In contrast, there was significant decrease in both GSH and PON1 activity by 30.6% and 29.6%, respectively. Neuronal damage and strong caspase-3 immunoreactivity were observed. Administration of 3 mg/kg 2,4-DNP to rotenone-treated rats resulted in near control levels of MDA, GSH, nitric oxide, and NF-κB. PON1 activity, however, showed further decrease by 2,4-DNP treatment. 2,4-DNP decreased the neuronal damage inflicted by rotenone in the cerebral cortex and hippocampus and ameliorated the loss of pigmented neurons in the substantia nigra. 2,4-DNP also resulted in decreased caspase-3 immunostaining. These data show that 2,4-DNP is effective in decreasing brain oxidative stress, neuronal damage, and apoptosis caused by rotenone. Thus, it should be further explored as a useful adjunct in the treatment of Parkinson's disease.

KEYWORDS | 2,4-Dinitrophenol; Mitochondrial uncoupling; Parkinson's disease; Rotenone

ABBREVIATIONS | 2,4-DNP, 2,4-Dinitrophenol; GSH, reduced glutathione; MDA, malondialdehyde; NF-κB; nuclear factor kappaB; PD, Parkinson's disease; PON1, paraoxonase 1; SNPc, substantia nigra pars compacta; TNF-α, tumor necrosis factor-alpha

CONTENTS

1. Introduction
2. Materials and Methods

- 2.1. Animals
- 2.2. Drugs and Chemicals
- 2.3. Study Design
- 2.4. Biochemical Analyses
 - 2.4.1. Lipid Peroxidation
 - 2.4.2. Reduced Glutathione
 - 2.4.3. Nitric Oxide
 - 2.4.4. Paraoxonase 1 Activity
 - 2.4.5. Nuclear Factor kappaB (NF-κB)
- 2.5. Histopathology
- 2.6. Immunostaining for Caspase-3
- 2.7. Statistical Analysis
- 3. Results
 - 3.1. Oxidative Stress
 - 3.2. PON1 Activity
 - 3.3. NF-κB
 - 3.4. Histopathological Results
- 4. Discussion

1. INTRODUCTION

Parkinson's disease (PD) is characterized by neurodegeneration involving the dopaminergic neurons in the substantia nigra pars compacta (SNPc), which project to the striatum [1]. This results in profound loss of dopamine in the SNPc and striatum with a consequent development of the motor manifestations of the disease namely, bradykinesia or akinesia, muscular rigidity, postural imbalance, and a resting tremor [2]. It is not clear how neurons are lost in SNPc, but there is accumulating evidence to suggest a pivotal role for free radical mechanisms [3, 4]. In PD, there is excessive generation of oxygen- and nitrogen-derived free radicals since there is biochemical evidence of oxidative damage to cell membrane lipids, proteins, and DNA detected in the brain post-mortem [5–7]. One important site for the generation of reactive oxygen metabolites in the cell is the mitochondria [8, 9]. These reactive oxygen species are produced as a consequence of oxidative phosphorylation which occurs in the inner mitochondrial membrane and involves the oxidation of NADH to produce energy. This energy is then used to phosphorylate adenosine diphosphate (ADP) to adenosine triphosphate (ATP). Leakage of electrons from the mitochondrial respiratory chain onto molecular oxygen (O_2) results in the generation of the superoxide anion radical ($O_2^{\cdot-}$) which in turn might reduce cytochrome c, transition metals, or be converted to hy-

drogen peroxide (H_2O_2) by superoxide dismutase. The latter can be reduced to the more reactive hydroxyl radical (HO^{\cdot}). $O_2^{\cdot-}$ might also react with nitric oxide to produce the highly reactive peroxynitrite anion [10, 11]. Complex I (NADH-ubiquinone reductase or NADH dehydrogenase) and complex III (ubiquinol-cytochrome c reductase) of the electron-transport chain are the main sites that generate reactive oxygen metabolites [11]. In brain tissue, complex I is the main source for $O_2^{\cdot-}$ formation under normal and pathological conditions [9].

Mitochondrial dysfunction that occurs in the context of hypoxic-ischemic injury, toxin exposure, or associated with aging, has been implicated in increased production of reactive oxygen metabolites and consequent neuronal injury [12, 13]. Mitochondrial uncouplers, such as 2,4-dinitrophenol (2,4-DNP), interfere with cellular energy metabolism. 2,4-DNP acts as a H^+ carrier (H^+ ionophore) and provides a pathway for the flow of H^+ across the inner mitochondrial membrane, thereby bypassing the ATP synthase. 2,4-DNP thus uncouples the electron transport chain from oxidative phosphorylation diminishing ATP production [14]. It has been suggested that mild uncoupling effectively decreases $O_2^{\cdot-}$ production that occurs from complex I (NADH-ubiquinone reductase) during reverse electron transport [15]. 2,4-DNP has been shown to result in decreased mitochondrial membrane potential, calcium influx, and free radical formation [16]. The agent

showed protective effects in experimental models of spinal cord injury [17] and brain injury [18–20]. In this study, we have examined the ability of 2,4-DNP to prevent neuronal damage in the rotenone model of Parkinson's disease in the rat. Studies in rodents have demonstrated that rotenone, a naturally occurring pesticide, is able to cause pathological brain changes similar to those found in human idiopathic PD [21, 22].

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats (Animal House of the National Research Centre, Cairo, Egypt) weighing 180–200 g were group-housed under temperature- and light-controlled conditions and allowed standard laboratory rodent chow and water ad libitum. Animal procedures followed the recommendations of the Ethics Committee of the National Research Centre (Cairo, Egypt) and 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 and 2,4-DNP were purchased from Sigma-Aldrich (St Louis, MO, USA). Rotenone and 2,4-DNP were dissolved in dimethyl sulfoxide.

2.3. Study Design

Rats were randomly divided into four groups, with six rats in each group. Group 1 received the vehicle (dimethyl sulfoxide); group 2 received a subcutaneous injection of rotenone 1.5 mg/kg; groups 3 and 4 received rotenone 1.5 mg/kg subcutaneously along with 2,4-DNP at 1 and 3 mg/kg (subcutaneous injection), respectively. Drugs were given every other day for 2 weeks. Rats were then euthanized by decapitation for tissue collection; their 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 further 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 Analyses

2.4.1. Lipid Peroxidation

Lipid peroxidation in the brain homogenates was assayed by measuring the level of malondialdehyde (MDA) using the method of Ruiz-Larrea et al. [23]. In this assay, MDA (along with its equivalents) reacts with thiobarbituric acid to produce a red colored complex that exhibits a peak absorbance at 532 nm.

2.4.2. Reduced Glutathione

Reduced glutathione (GSH) was determined in the brain homogenates using the method of Ellman et al. [24]. The procedure is based on the reduction of Ellman's reagent by $-\text{SH}$ groups of GSH to form 2-nitro-5-mercaptobenzoic acid, which is intense yellow in color and determined using a spectrophotometer at 412 nm.

2.4.3. Nitric Oxide

Nitric oxide was determined using the Griess reagent, according to the method of Moshage et al. [25]. Nitrite, a stable end product of nitric oxide, is mostly used as an indicator for the production of nitric oxide.

2.4.4. Paraoxonase 1 Activity

The arylesterase activity of paraoxonase 1 (PON1) was measured spectrophotometrically in the tissue supernatants using phenylacetate as a substrate, according to the method described before [26].

2.4.5. Nuclear Factor κB (NF- κB)

NF- κB was measured in the tissue supernatants using a commercially available human NF- κB ELISA kit (Glory Science, Del Rio, TX, USA) according to the manufacturer's instructions. The kit uses a double antibody sandwich enzyme-linked immunosorbent assay to measure the level of NF- κB .

2.5. Histopathology

Brain tissues were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin using standard procedures. Sections of 5 μm

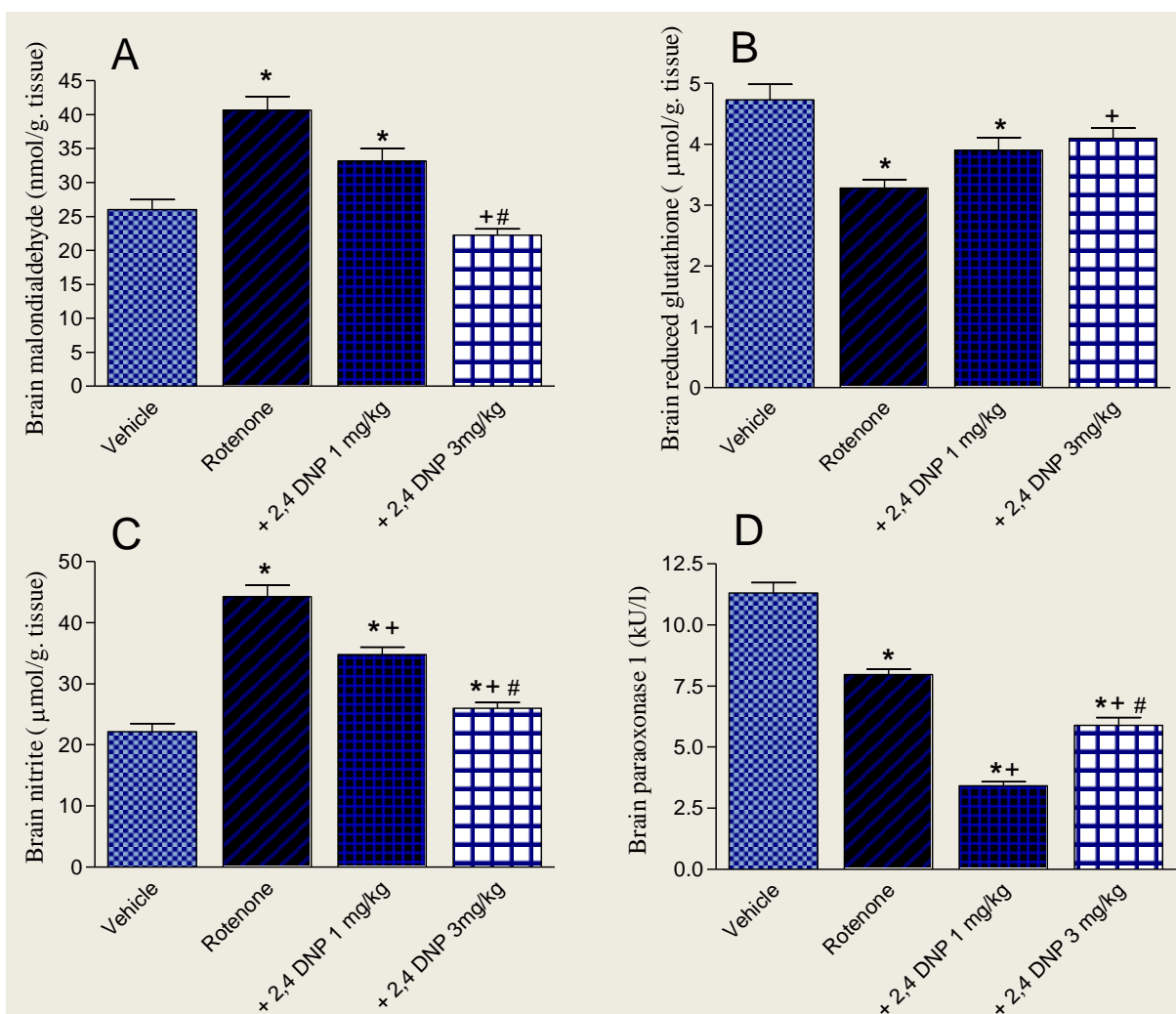


FIGURE 1. Effect of 2,4-DNP on MDA, GSH, nitrite, and PON1 activity in the brain of rotenone-treated rats. *: $p < 0.05$ versus corresponding vehicle-treated group; +: $p < 0.05$ versus rotenone control group; #: $p < 0.05$ versus 1 mg/kg 2,4-DNP.

thickness were stained with hematoxylin and eosin (H&E) for histopathological examination under a light microscope.

2.6. Immunostaining for Caspase-3

Immunostaining of anti-caspase-3 antibody was performed using streptavidin-biotin. Sections of 4 μm thick were deparaffinized and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The specimens were then incubat-

ed with anti-caspase-3 antibody as the primer antibody at a 1: 100 dilution. The specimens were counter stained with H&E. Negative controls were prepared by substituting normal mouse serum for each primary antibody.

2.7. Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was determined using an ANOVA, followed by Duncan's multiple range test using SPSS software

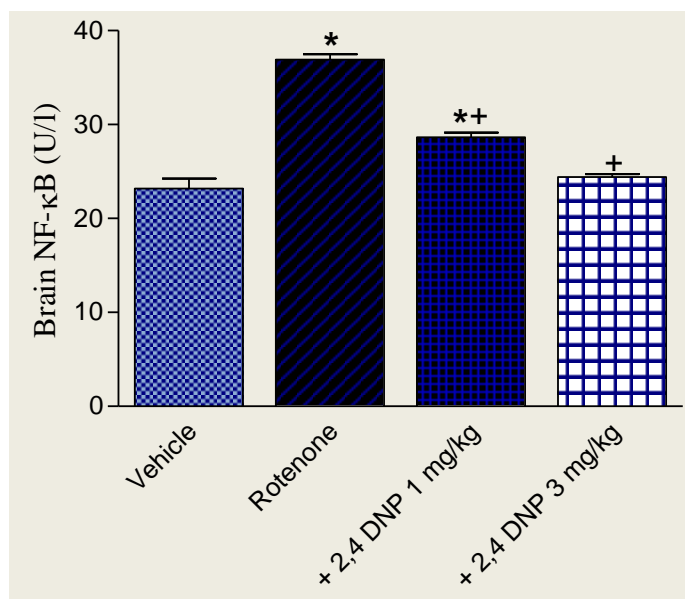


FIGURE 2. Effect of 2,4-DNP on NF-κB in the brain of rotenone-treated rats. *: $p < 0.05$ versus corresponding vehicle-treated group; +: $p < 0.05$ versus rotenone control group.

(SAS Institute Inc., Cary, NC, USA). A probability value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Oxidative Stress

Rats treated with rotenone alone exhibited significantly higher MDA levels in the brain than those of the control group (56.3% increase: 40.64 ± 2.0 versus 26.0 ± 1.5 nmol/g.tissue) (**Figure 1A**). On the other hand, brain GSH content was significantly decreased by 30.6% by rotenone (3.28 ± 0.14 versus 4.73 ± 0.26 μmol/g.tissue) (**Figure 1B**). The concentrations of nitric oxide increased by 99.3% (44.25 ± 1.9 versus 22.2 ± 1.3 μmol/g.tissue) as compared with those of the control group (**Figure 1C**).

In the presence of 2,4-DNP (1 and 3 mg/kg), a dose-dependent decrease in MDA levels (18.5-45.3% of controls) was found compared with the rotenone only group (33.12 ± 1.9 and 22.2 ± 1.0 versus 40.64 ± 2.0 nmol/g.tissue) (**Figure 1A**). In addition, an increment in GSH by 18.9% and 25%, respectively,

was observed in the 2,4-DNP-treated group compared with the rotenone only group (3.9 ± 0.21 and 4.1 ± 0.17 versus 3.28 ± 0.14 μmol/g.tissue) (**Figure 1B**). 2,4-DNP also decreased nitric oxide levels in rotenone-treated rats by 21.3% and 41.2%, respectively (34.83 ± 1.2 and 26.0 ± 0.98 versus 44.25 ± 1.9 μmol/g. tissue) (**Figure 1C**).

3.2. PON1 Activity

Rotenone resulted in significant inhibition of PON 1 activity by 29.6% compared with the control group (7.96 ± 0.23 versus 11.3 ± 0.41 kU/l). 2,4-DNP (1 and 3 mg/kg) elicited a further and significant decrease in this brain enzyme activity by 57% and 26%, respectively (3.42 ± 0.17 and 5.89 ± 0.32 versus 7.96 ± 0.23 kU/l) (**Figure 1D**).

3.3. NF-κB

In rats treated with rotenone, there was significant increase in NF-κB levels in the brain compared to controls (59.2% increase: 36.91 ± 0.58 versus 23.18 ± 1.1 U/l). A significant decrease in NF-κB concentrations by 22.3% and 33.9% was observed in the

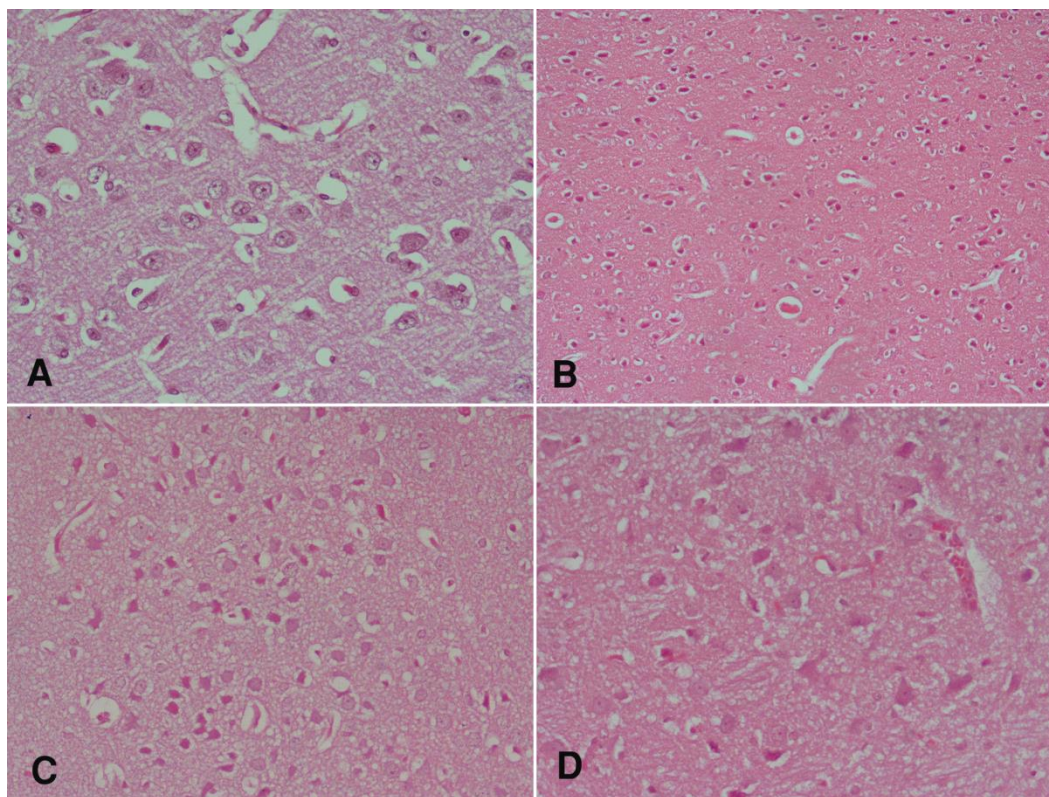


FIGURE 3. Photomicrographs of sections from the cerebral cortex following treatment with different agents. (A) vehicle control: showing the normal structure of this tissue; (B) rotenone: showing many cells with dark cytoplasm; (C) rotenone + 2,4-DNP 1 mg/kg: showing reduction in dark neurons; (D) rotenone + 2,4-DNP 3 mg/kg: showing more amelioration of the damaging effect of rotenone. H&E staining with a magnification scale of $\times 200$ for A, C, and D, and $\times 100$ for B.

brain of the animals receiving 1 and 3 mg/kg 2,4-DNP, respectively (28.66 ± 0.48 and 24.4 ± 0.3 versus 36.91 ± 0.58 U/l) (Figure 2).

3.4. Histopathological Results

From the histopathological investigation it was clear that rotenone caused many neurons in the cerebral cortex to appear darker than normal (both the cytoplasm and the nucleus) (Figure 3B). Treatment with 2,4-DNP ameliorated this damaging effect in a dose-dependent manner. However even with the high dose, a few dark cells were still observed (Figure 3C and 3D). The same results could be seen in other areas of the brain (hippocampus and substantia nigra) (Figures 4 and 5). The results of immunohistochem-

ical staining with anti-caspase-3 antibody confirmed that 2,4-DNP decreased apoptosis caused by rotenone (Figure 6).

4. DISCUSSION

In this study, rotenone resulted in increased brain lipid peroxidation as indicated by the increased level of the lipid peroxidation product MDA. This finding agrees with previous studies suggesting increased intracellular reactive oxygen metabolites by rotenone [27–30]. There was also marked decrease in brain GSH content. This could be due to increased formation of reactive oxygen metabolites, and subsequent consumption of the antioxidant. GSH is the

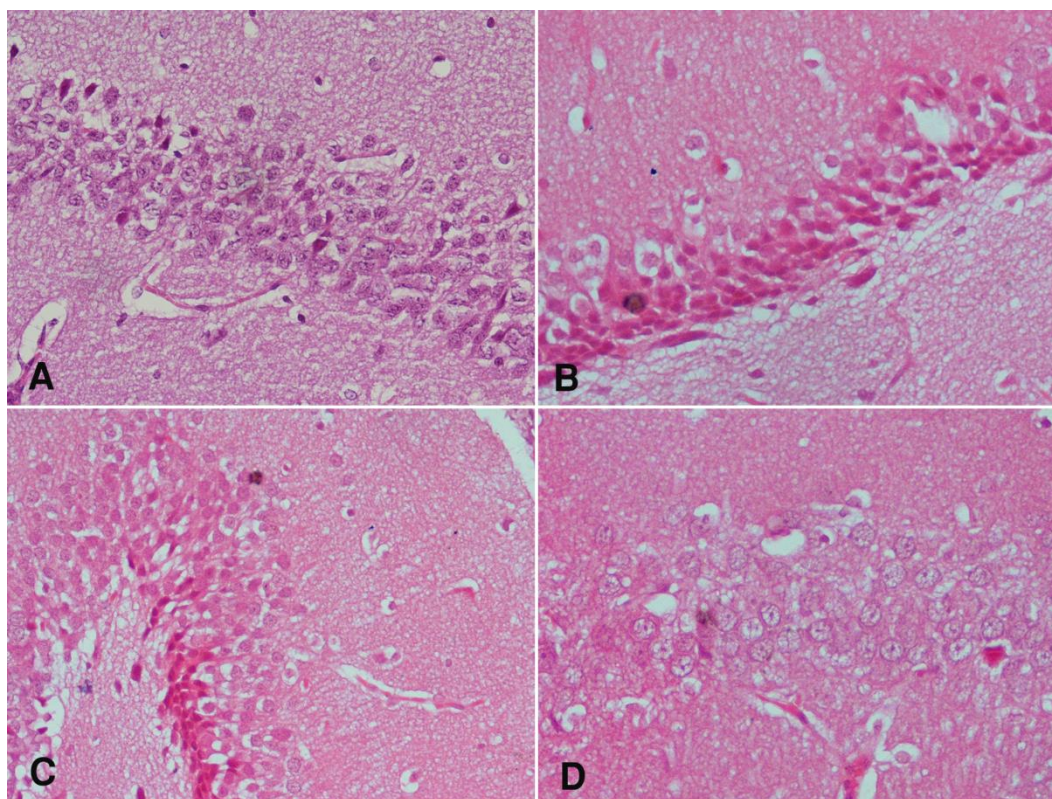


FIGURE 4. Photomicrographs of sections from hippocampus following treatment with different agents. (A) vehicle control: showing the normal structure of this tissue; (B) rotenone: showing decrease in cell layers in which many cells show dark cytoplasm; (C) rotenone + 2,4-DNP 1 mg/kg: showing reduction in dark neurons. (D) rotenone + 2,4-DNP 3 mg/kg: showing disappearance of dark neurons but with disarrangement of layers. H&E staining with a magnification scale of $\times 200$.

most abundant intracellular thiol which has an important role in maintaining the redox balance in the cell. GSH acts as a direct antioxidant scavenging such reactive species as hypochlorous acid and peroxynitrite anion. GSH also functions as a co-factor for enzymes such as glutathione peroxidase [31, 32]. The thiol exists mostly in its reduced form while less than 1% is present in the oxidized or disulfide form. A decrease in GSH content is likely to contribute to the oxidative stress seen in patients with PD and other neurodegenerative diseases [33, 34]. In this context, studies indicated decreased GSH levels in the substantia nigra of PD subjects [35, 36]. This decrease in GSH causes mitochondrial impairment and decreases complex I activity and consequently results in nigrostriatal neurodegeneration [37, 38].

In agreement with previous studies [39–42], rotenone also resulted in markedly increased levels of brain nitric oxide. Other researchers showed increased nitric oxide synthase (NOS) activity and 3-nitrotyrosine in the substantia nigra of rats following rotenone treatment. These effects were decreased by the neuronal NOS inhibitor 7-nitroindazole, which also reduced nigrostriatal damage [43]. Moreover, rotenone caused prominent inducible nitric oxide synthase (iNOS) immunostaining in the striatum and substantia nigra after systemic administration in rodents, suggesting that iNOS mediated the increase in brain nitric oxide levels by rotenone [40]. Increments in nitric oxide levels via iNOS are thus likely to be involved in the mechanism by which rotenone induced brain damage. Increased generation of nitric

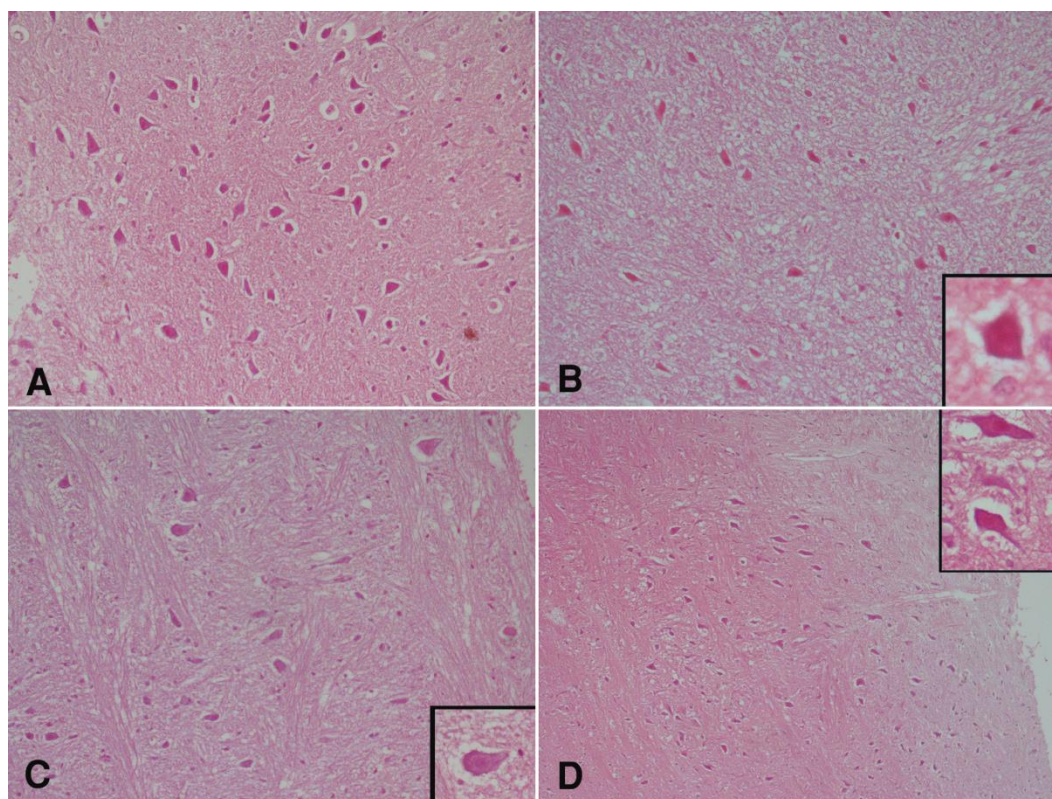


FIGURE 5. Photomicrographs of sections from the substantia nigra after treatment with different agents. (A) vehicle control: showing the normal appearance of the pigmented neurons in this area; (B) rotenone: showing marked decrease in the number of pigmented cells; (C) rotenone + 2,4-DNP 1 mg/kg: showing mild restoration of pigmented neurons; (D) rotenone + 2,4-DNP 3 mg/kg: showing more amelioration of the damaging effect of rotenone in the form of an increased number of normal pigmented neurons. H&E staining with a magnification scale of $\times 200$ for A, B, and C, $\times 100$ for D, and $\times 500$ for the corners).

oxide causes neuronal damage via several pathways. The peroxynitrite anion formed by the reaction of nitric oxide with $O_2^{\cdot -}$ can result in oxidation, nitration, and nitrosation of mitochondrial proteins and the inactivation of several mitochondrial electron transport complexes including complex I [44–46].

The present study showed that brain NF- κ B increased significantly following rotenone injection. The transcription factor NF- κ B is present in the cytoplasm in an inactivated dimeric form by binding to the inhibitory molecule I κ B. Being activated by inflammatory mediators, oxidative stress, tissue injury or infection, NF- κ B translocates to the nucleus and induces the expression of several genes encoding inflammatory mediators, such as cyclooxygenase-2,

proinflammatory cytokines (e.g., interleukin 1 β [IL-1 β], IL-6 and tumor necrosis factor- α [TNF- α]), cellular adhesion molecules, and monocyte chemoattractant protein-1 (MCP-1) [47, 48]. Previously, rotenone has been shown to increase TNF- α and MCP-1 in the rat striatum [41, 42]. NF- κ B is sensitive to oxidative stress being activated by intracellular reactive oxygen metabolites and kept in its inactive form by antioxidant enzymes [49, 50]. Increased NF- κ B activation has been demonstrated in dopaminergic neurons from the brains of patients with PD, which appears to be mediated by oxidative stress-induced apoptosis [51]. Moreover, inhibition of NF- κ B decreased DNA damage induced by oxidative stress and delayed cellular senescence in mice.

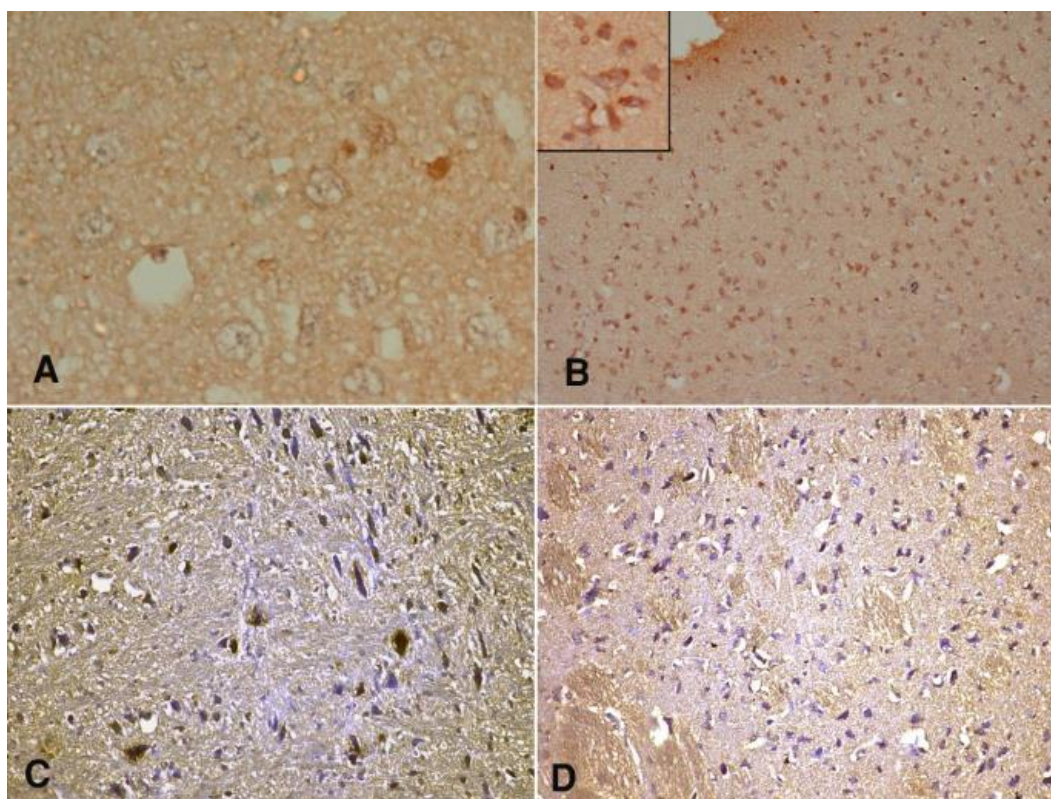


FIGURE 6. Photomicrographs of sections from the cerebral cortex stained immunohistochemically for caspase-3 after treatment with different agents. (A) vehicle control: showing negative reaction to the stain in almost all of the cells; (B) rotenone: showing many cells with positive reaction; (C) rotenone + 2,4-DNP 1 mg/kg: showing that positively stained cells are still observed; (D) rotenone + 2,4-DNP 3 mg/kg: showing reduction in the number of positively stained cells. Immunostaining of anti-caspase-3 with a magnification scale of $\times 200$ for A and the corner in B, and $\times 100$ for B, C, and D.

This suggested a role for NF- κ B in aging or in age-related neurodegenerative diseases [52]. Our data suggest that rotenone induces the activation of NF- κ B which could be explained by the increased oxidative stress. This also suggests a role for NF- κ B in the inflammatory response in the brain of rotenone-treated rats.

We also found decreased activity of PON1 enzyme in the brain of rotenone-treated rats, which is in agreement with our previous studies [41, 42]. PON1 hydrolyzes the active metabolites (oxons) of several organophosphorus insecticides, such as parathion, diazinon, and chlorpyrifos [53]. The enzyme also has anti-inflammatory and antioxidant effects [54] and there is evidence that functional impairment in PON1

activity could be associated with increased susceptibility to PD in subjects exposed to insecticides [55, 56]. PON1 is inactivated by oxidative stress [57] and thus the observed decrease in its activity in the brain of rotenone-treated rats could be due to the increased oxidative stress.

In the present study, we demonstrated that the administration of the mitochondrial uncoupler 2,4-DNP was able to inhibit neurodegeneration induced in the rat brain by rotenone. The neuroprotective action of 2,4-DNP was associated with decreased oxidative stress and NF- κ B in the brain. Histopathological investigations indicated that 2,4-DNP decreased neuronal damage in the striatum, cerebral cortex, and hippocampus. In the striatum, this was reflected in

preserving the pigmented neuromelanin-containing neurons. Caspases are cysteine proteases involved in the initiation and execution of apoptosis or programmed cell death [58, 59]. Our results showed that rotenone caused prominent caspase-3 immunostaining in the cerebral cortex, suggesting enhanced neuronal apoptosis. It was also clear that 2,4-DNP resulted in decreased neuronal caspase-3 immunoreactivity. Neuroprotective effects for 2,4-DNP have also been shown under such conditions as focal ischemia-reperfusion injury in the rat brain [19], quinolinic acid injection into the rat striatum [18], and spinal cord injury in rats [17]. 2,4-DNP displayed neuroprotective action when given at the dose of 5 mg/kg [17, 19, 20]. In this study, the doses employed were 1 and 3 mg/kg. In rats subjected to ischemic/reperfusion brain injury, 2,4-DNP (5 mg/kg) did not cause brain barrier disruption or edema in the intact cerebral hemisphere [60].

Our histopathological studies also indicated that 2,4-DNP at 1 or 3 mg/kg protected against the liver and kidney damage caused by rotenone (unpublished observations). The ability of 2,4-DNP to exert neuroprotective effects has been attributed to a decrease in the mitochondrial membrane potential, which in turn decreases the formation of reactive oxygen metabolites and mitochondrial calcium uptake, improves mitochondrial function, and maintains mitochondrial bioenergetics [17–19]. Moreover, the agent was shown to stimulate neurite outgrowth and neuronal differentiation and exert an anti-amyloidogenic action [61]. Being a complex I inhibitor, rotenone selectively targets the mitochondria [62, 63]. The likely cause of cell death due to rotenone is thought to be the mitochondrial damage caused by the increase in reactive oxygen metabolites [62–65].

It has been suggested that the increase in $O_2^{\cdot-}$ at complex I by rotenone induces further damage to complexes I and II, which shifts $O_2^{\cdot-}$ generation from CoQ10 sites to a more proximal site and thus becomes independent of the functional state of the mitochondria [65]. Rotenone-induced apoptosis and cell death could be rescued with antioxidants like α -tocopherol [28], GSH, N-acetylcysteine, and ascorbate [64]. Thus, it can be suggested that the ability of 2,4-DNP to decrease neuronal damage inflicted by rotenone is mediated via the decreased generation of reactive oxygen metabolites, thereby protecting the mitochondria against oxidative damage.

REFERENCES

1. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* 1992; 55(3):181–184.
2. Fahn S. Description of Parkinson's disease as a clinical syndrome. *Ann N Y Acad Sci* 2003; 991:1–14.
3. Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol* 2003; 53 Suppl 3:S26–36; discussion S36–28. doi: 10.1002/ana.10483.
4. Miller RL, James-Kracke M, Sun GY, Sun AY. Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem Res* 2009; 34(1):55–65. doi: 10.1007/s11064-008-9656-2.
5. Dexter DT, Holley AE, Flitter WD, Slater TF, Wells FR, Daniel SE, et al. Increased levels of lipid hydroperoxides in the parkinsonian substantia nigra: an HPLC and ESR study. *Mov Disord* 1994; 9(1):92–97. doi: 10.1002/mds.870090115.
6. Zhang J, Perry G, Smith MA, Robertson D, Olson SJ, Graham DG, et al. Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *Am J Pathol* 1999; 154(5):1423–1429. doi: 10.1016/S0002-9440(10)65396-5.
7. Alam ZI, Daniel SE, Lees AJ, Marsden DC, Jenner P, Halliwell B. A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease. *J Neurochem* 1997; 69(3):1326–1329.
8. Figueira TR, Barros MH, Camargo AA, Castilho RF, Ferreira JC, Kowaltowski AJ, et al. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. *Antioxid Redox Signal* 2013; 18(16):2029–2074. doi: 10.1089/ars.2012.4729.
9. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003; 552(Pt 2):335–344. doi: 10.1113/jphysiol.2003.049478.
10. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*. 3rd ed. Oxford University Press, Oxford, UK. 1999.
11. Casteilla L, Rigoulet M, Penicaud L. Mitochondrial ROS metabolism: modulation by uncoupling proteins. *IUBMB Life* 2001; 52(3–5):181–188. doi: 10.1080/15216540152845984.

12. Rego AC, Oliveira CR. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem Res* 2003; 28(10):1563–1574.
13. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. *Biochim Biophys Acta* 2006; 1757(5–6):509–517. doi: 10.1016/j.bbabi.2006.04.029.
14. Terada H. Uncouplers of oxidative phosphorylation. *Environ Health Perspect* 1990; 87:213–218.
15. Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, et al. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med* 2004; 37(6):755–767. doi: 10.1016/j.freeradbiomed.2004.05.034.
16. Maragos WF, Korde AS. Mitochondrial uncoupling as a potential therapeutic target in acute central nervous system injury. *J Neurochem* 2004; 91(2):257–262. doi: 10.1111/j.1471-4159.2004.02736.x.
17. Jin Y, McEwen ML, Nottingham SA, Maragos WF, Dragicevic NB, Sullivan PG, et al. The mitochondrial uncoupling agent 2,4-dinitrophenol improves mitochondrial function, attenuates oxidative damage, and increases white matter sparing in the contused spinal cord. *J Neurotrauma* 2004; 21(10):1396–1404. doi: 10.1089/neu.2004.21.1396.
18. Korde AS, Sullivan PG, Maragos WF. The uncoupling agent 2,4-dinitrophenol improves mitochondrial homeostasis following striatal quinolinic acid injections. *J Neurotrauma* 2005; 22(10):1142–1149. doi: 10.1089/neu.2005.22.1142.
19. Korde AS, Pettigrew LC, Craddock SD, Maragos WF. The mitochondrial uncoupler 2,4-dinitrophenol attenuates tissue damage and improves mitochondrial homeostasis following transient focal cerebral ischemia. *J Neurochem* 2005; 94(6):1676–1684. doi: 10.1111/j.1471-4159.2005.03328.x.
20. Pandya JD, Pauly JR, Nukala VN, Sebastian AH, Day KM, Korde AS, et al. Post-Injury Administration of mitochondrial uncouplers increases tissue sparing and improves behavioral outcome following traumatic brain injury in rodents. *J Neurotrauma* 2007; 24(5):798–811. doi: 10.1089/neu.2006.3673.
21. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 2000; 3(12):1301–1306. doi: 10.1038/81834.
22. Sherer TB, Kim JH, Betarbet R, Greenamyre JT. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp Neurol* 2003; 179(1):9–16.
23. Ruiz-Larrea MB, Leal AM, Liza M, Lacort M, de Groot H. Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. *Steroids* 1994; 59(6):383–388.
24. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82(1):70–77.
25. Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* 1995; 41(6 Pt 1):892–896.
26. Higashino K, Takahashi Y, Yamamura Y. Release of phenyl acetate esterase from liver microsomes by carbon tetrachloride. *Clin Chim Acta* 1972; 41:313–320.
27. Liu HQ, Zhu XZ, Weng EQ. Intracellular dopamine oxidation mediates rotenone-induced apoptosis in PC12 cells. *Acta Pharmacol Sin* 2005; 26(1):17–26. doi: 10.1111/j.1745-7254.2005.00003.x.
28. Testa CM, Sherer TB, Greenamyre JT. Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. *Brain Res Mol Brain Res* 2005; 134(1):109–118. doi: 10.1016/j.molbrainres.2004.11.007.
29. Choi WS, Palmiter RD, Xia Z. Loss of mitochondrial complex I activity potentiates dopamine neuron death induced by microtubule dysfunction in a Parkinson's disease model. *J Cell Biol* 2011; 192(5):873–882. doi: 10.1083/jcb.201009132.
30. Parameshwaran K, Irwin MH, Steliou K, Pinkert CA. Protection by an antioxidant of rotenone-induced neuromotor decline, reactive oxygen species generation and cellular stress in mouse brain. *Pharmacol Biochem Behav* 2012; 101(3):487–492. doi: 10.1016/j.pbb.2012.03.007.

- 10.1016/j.pbb.2012.02.011.
31. Dickinson DA, Forman HJ. Glutathione in defense and signaling: lessons from a small thiol. *Ann N Y Acad Sci* 2002; 973:488–504.
32. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother* 2003; 57(3–4):145–155.
33. Bains JS, Shaw CA. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Brain Res Rev* 1997; 25(3):335–358.
34. Mytilineou C, Kramer BC, Yabut JA. Glutathione depletion and oxidative stress. *Parkinsonism Relat Disord* 2002; 8(6):385–387.
35. Sofic E, Lange KW, Jellinger K, Riederer P. Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett* 1992; 142(2):128–130.
36. Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Ann Neurol* 1994; 36(3):348–355. doi: 10.1002/ana.410360305.
37. Merad-Boudia M, Nicole A, Santiard-Baron D, Saille C, Ceballos-Picot I. Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochem Pharmacol* 1998; 56(5):645–655.
38. Chinta SJ, Kumar MJ, Hsu M, Rajagopalan S, Kaur D, Rane A, et al. Inducible alterations of glutathione levels in adult dopaminergic midbrain neurons result in nigrostriatal degeneration. *J Neurosci* 2007; 27(51):13997–14006. doi: 10.1523/JNEUROSCI.3885-07.2007.
39. Bashkatova V, Alam M, Vanin A, Prast H, Schmidt WJ. Chronic administration of rotenone induces Parkinsonian symptoms and increases levels of nitric oxide in rat brain. *BMC Pharmacology* 2008; 8 (Suppl 1):A33.
40. Abdel-Salam OME, Mohammed NA, Youness ER, Khadrawy YA, Omara EA, Sleem AA. Cerebrolysin protects against rotenone-induced oxidative stress and neurodegeneration. *J Neurorestoratol* 2014; 2:47–63.
41. Abdel-Salam OME, Omara EA, Youness ER, Khadrawy YA, Mohammed NA, Sleem AA. Rotenone-induced nigrostriatal toxicity is reduced by methylene blue. *J Neurorestoratol* 2014; 2:65–80.
42. Xiong ZK, Lang J, Xu G, Li HY, Zhang Y, Wang L, et al. Excessive levels of nitric oxide in rat model of Parkinson's disease induced by rotenone. *Exp Ther Med* 2015; 9(2):553–558. doi: 10.3892/etm.2014.2099.
43. He Y, Imam SZ, Dong Z, Jankovic J, Ali SF, Appel SH, et al. Role of nitric oxide in rotenone-induced nigro-striatal injury. *J Neurochem* 2003; 86(6):1338–1345.
44. Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch Biochem Biophys* 1996; 328(1):85–92.
45. Riobo NA, Clementi E, Melani M, Boveris A, Cadenas E, Moncada S, et al. Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *Biochem J* 2001; 359(Pt 1):139–145.
46. Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. *Free Radic Biol Med* 2002; 33(11):1451–1464.
47. Blackwell TS, Christman JW. The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* 1997; 17(1):3–9. doi: 10.1165/ajrcmb.17.1.f132.
48. Haddad JJ. Redox regulation of pro-inflammatory cytokines and IkappaB-alpha/NF-kappaB nuclear translocation and activation. *Biochem Biophys Res Commun* 2002; 296(4):847–856.
49. Schreck R, Albermann K, Baeuerle PA. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* 1992; 17(4):221–237.
50. Kretz-Remy C, Mehlen P, Mirault ME, Arrigo AP. Inhibition of I kappa B-alpha phosphorylation and degradation and subsequent NF-kappa B activation by glutathione peroxidase overexpression. *J Cell Biol* 1996; 133(5):1083–1093.
51. Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M, et al. Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. *Proc*

- Natl Acad Sci USA* 1997; 94(14):7531–7536.
52. Tilstra JS, Robinson AR, Wang J, Gregg SQ, Clauson CL, Reay DP, et al. NF-kappaB inhibition delays DNA damage-induced senescence and aging in mice. *J Clin Invest* 2012; 122(7):2601–2612. doi: 10.1172/JCI45785.
53. La Du BN. Human serum paraoxonase: arylesterase. In: *Pharmacogenetics of Drug Metabolism* (W Kalow). Pergamon Press, New York, NY, USA.1992. pp. 51–91.
54. Ng DS, Chu T, Esposito B, Hui P, Connelly PW, Gross PL. Paraoxonase-1 deficiency in mice predisposes to vascular inflammation, oxidative stress, and thrombogenicity in the absence of hyperlipidemia. *Cardiovasc Pathol* 2008; 17(4):226–232. doi: 10.1016/j.carpath.2007.10.001.
55. Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, et al. Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. *BMC Neurol* 2008; 8:6. doi: 10.1186/1471-2377-8-6.
56. Narayan S, Liew Z, Paul K, Lee PC, Sinsheimer JS, Bronstein JM, et al. Household organophosphorus pesticide use and Parkinson's disease. *Int J Epidemiol* 2013; 42(5):1476–1485. doi: 10.1093/ije/dyt170.
57. Nguyen SD, Sok DE. Preferential inhibition of paraoxonase activity of human paraoxonase 1 by negatively charged lipids. *J Lipid Res* 2004; 45(12):2211–2220. doi: 10.1194/jlr.M400144-JLR200.
58. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; 281(5381):1312–1316.
59. Yuan J, Yankner BA. Apoptosis in the nervous system. *Nature* 2000; 407(6805):802–809. doi: 10.1038/35037739.
60. Ennis SR, Keep RF. Effects of 2,4-dinitrophenol on ischemia-induced blood-brain barrier disruption. *Acta Neurochir Suppl* 2006; 96:295–298.
61. Wasilewska-Sampaio AP, Silveira MS, Holub O, Goecking R, Gomes FC, Neto VM, et al. Neuritogenesis and neuronal differentiation promoted by 2,4-dinitrophenol, a novel anti-amyloidogenic compound. *FASEB J* 2005; 19(12):1627–1636. doi: 10.1096/fj.05-3812com.
62. Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, et al. Mechanism of toxicity in rotenone models of Parkinson's disease. *J Neurosci* 2003; 23(34):10756–10764.
63. Richardson JR, Quan Y, Sherer TB, Greenamyre JT, Miller GW. Paraquat neurotoxicity is distinct from that of MPTP and rotenone. *Toxicol Sci* 2005; 88(1):193–201. doi: 10.1093/toxsci/kfi304.
64. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem* 2003; 278(10):8516–8525. doi: 10.1074/jbc.M210432200.
65. Panov A, Dikalov S, Shalbuyeva N, Taylor G, Sherer T, Greenamyre JT. Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. *J Biol Chem* 2005; 280(51):42026–42035. doi: 10.1074/jbc.M508628200.