Grape Seed Extract Exerts an Anti-Apoptotic Effect and Attenuates the Decrease in Striatal Tyrosine Hydroxylase in Rotenone-Treated Mice

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ABSTRACT | The potential neuroprotective effect of grape seed extract (GSE) was evaluated in the rotenone-induced Parkinson's disease in mice. Rotenone was administered at the dose of 1.5 mg/kg subcutaneously (sc) three times per week for 2 weeks alone or in combination with GSE at doses of 13.5 and 27 mg/kg, sc, daily. The control group received the vehicle. The brain levels of the lipid peroxidation product malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (nitrite), and paraoxonase-1 (PON-1) were determined. Histopathology, caspase-9 immunohistochemistry in different brain regions, and tyrosine hydroxylase immunoreactivity (TH-ir) in the substantia nigra were performed. Behavioral testing included rearing activity, locomotor activity, and stair test paradigms. Results indicated significantly increased lipid peroxidation and nitric oxide contents along with a significant decrease in GSH level and marked inhibition of PON-1 activity in the striatum and in the rest of the brain tissue in rotenone-treated mice. Rotenone caused significant decreases in rearing and locomotor activities and impaired motor strength. Treatment with GSE at 27 mg/kg resulted in decreased MDA and nitric oxide by 22.8%/17.9% and 38.5%/45.5%, respectively, in the striatum and the rest of the brain. GSH was increased by 20.8% and 26%, while PON-1 activity increased by 204% and 142.9% after GSE treatment in the striatum and in the rest of the brain tissue, respectively, compared with the corresponding rotenone control values. GSE given at 27 mg/kg almost completely corrected the decrease in motor activity and motor strength caused by rotenone. Neuronal degeneration and the increase in caspase-9 expression caused by rotenone in different brain regions as well as the loss of substantia nigra TH-ir were markedly reduced by GSE. These data indicate that GSE was effective in improving brain oxidative stress and in preventing the behavioral deficits and neurodegeneration induced by rotenone in the mouse brain. It is suggested that GSE might be useful as an adjunctive treatment in patients with Parkinson's disease.

KEYWORDS | Grape seed extract; Lipid peroxidation; Oxidative stress; Parkinson's disease; Rotenone

ABBREVIATIONS | DA-ergic, dopaminergic; DMSO, dimethyl sulfoxide; GSE, grape seed extract; GSH, reduced glutathione; H&E, hematoxylin and eosin; MDA, malondialdehyde; PBS, phosphate-buffered saline; PD, Parkinson's disease; PON-1, paraoxonase-1; SNpc, substantia pars compacta; TH, tyrosine hydroxylase; TH-ir, tyrosine hydroxylase immunoreactivity

CONTENTS

1. INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder that affects approximately 1% of the population above the age of 65 years [1]. The loss of the dopaminergic pigmented neurons of the substantia pars compacta (SNpc) of the midbrain results in disordered motor activity with slowness in movement initiation, muscular rigidity, postural instability, and a resting tremor of the hands [2, 3]. Non-motor manifestations, including neuropsychiatric symptoms, depression, autonomic disturbances, and paresthesia, also occur in the disease process and account for significant morbidity [4, 5]. Parkinson's disease is idio-

pathic in the majority of cases and the exact cause for the selective dopaminergic (DA-ergic) cell loss is not yet known. It is widely believed that the disease process is triggered by an environmental toxin in those subjects who are genetically susceptible [1, 6]. In this context, there are increasingly available data implicating organophosphorus insecticides in the development of PD in the exposed population [7, 8]. The pathogenetic processes most likely implicated in DA-ergic cell death are oxidative stress and neuroinflammation [9, 10]. Oxidative stress arises when there is excessive formation of reactive oxygen metabolites in amounts that exceed the capacity of antioxidants in the cell. The result is oxidative damage to the cell membrane, mitochondria, and DNA, leading to cellular perturbation [11, 12]. In this context, studies indicated increased lipid peroxidation [13], oxidative DNA damage [14], and protein carbonyls [15] indicative of increased generation of reactive oxygen metabolites in the brain of PD patients [13–15].

Currently, the treatment of PD is still based on alleviating the midbrain DA-ergic deficit with the use of the dopamine precursor L-3,4-dihydroxyphenylalanine (levodopa or L-dopa). Other agents, such as dopamine receptor agonists, irreversible selective monoamine oxidase-B inhibitors, or catechol-O-methyltransferase inhibitors, are used in mild cases or as an add-on therapy to L-dopa [16–18]. Over time and owing to the continued loss of midbrain DA-ergic neurons, these agents become less effective in alleviating symptoms together with emergence of the troublesome L-dopa motor complications, necessitating dose reduction [16]. Moreover, none of the available anti-parkinsonian drugs can halt or lessen the neurodegeneration, thereby, necessitating finding newer agents for controlling the disease process [19].

In search for novel remedies that might be able to interfere with the pathogenetic mechanisms of DA-ergic cell death in PD, botanicals present such a rich source for drugs [20]. Grape seed polyphenolic extracts obtained from *Vitis vinifera* seeds are rich in the polyphenolic compounds proanthocyanidins, (+)-catechin, (-)-epicatechin, (-)-epicatechin-*O*-gallate [21, 22]. GSEs exert antioxidant [23, 24] and antiapoptotic [23, 25] actions. In vitro, GSE protected neonatal mouse hippocampal neurons against glutamate excitotoxicity with the effect being attributable to polyphenols and/or procyanidin oligomers [26]. In vivo, GSEs were shown to be protective in rodent models of ischemia-reperfusion brain injury [23], chronic cerebral hypoperfusion injury [27], and malathion-induced neuronal degeneration [28].

In this study, the potential protective effect of a standardized GSE rich in proanthocyanidins was investigated in an experimental model of PD induced in mice by the administration of the pesticide rotenone. The latter has been widely used to model human PD in rats [28, 29] and mice [30–35].

2. MATERIALS AND METHODS

2.1. Animals

Swiss albino mice (male), weighing 25–30 g of body weight, were obtained from the Animal House of the National Research Centre, Cairo. Mice were group-housed under temperature- and light-controlled conditions and provided with standard laboratory food and water ad libitum. Animal studies were performed in accordance with the Ethics Committee of the National Research Centre (Cairo, Egypt), following 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 (Sigma-Aldrich, St Louis, MO, USA) was used and dissolved in dimethyl sulfoxide (DMSO). Grape seed extract containing ~95% standardized proanthocyanidins was obtained from Arab Company for Pharmaceuticals and Medicinal Plants (MEPACO, Egypt) and dissolved in isotonic (0.9 % NaCl) saline solution immediately before use. Other chemicals and reagents were of analytical grade and obtained from Sigma-Aldrich.

In human studies, the dose of GSE used was in the range of 300–600 mg per day [36, 37]. The doses of GSE in this study were based on 150 mg and 300 mg, respectively, after conversion applicable to mice using Paget and Barnes conversion tables [38].

2.3. Study Design

Mice were randomly divided into four groups, with six mice in each group. The following groups were studied: Group 1. Vehicle (DMSO); Group 2. Rotenone 1.5 mg/kg, sc, once every other day; Group 3.

Rotenone 1.5 mg/kg, sc, once every other day + GSE 13.5 mg/kg, sc, daily; Group 4. Rotenone 1.5 mg/kg, sc, once every other day + GSE 27 mg/kg, sc, daily.

GSE was given at the time of rotenone injection. Treatments were continued for 2 weeks. Behavioral testing was done 24 h after the last rotenone injection. Mice were then euthanized by decapitation for tissue collection; their brains were quickly removed and placed on an ice-cold plate, washed with ice-cold phosphate-buffered saline (PBS), dissected into striatum and the rest of the brain tissue, weighed, and stored at −80°C until further biochemical studies. The tissues were homogenized in PBS to give a final concentration of 10% weight/volume (w/v) for the biochemical assays.

2.4. Biochemical Analyses

2.4.1. Determination of Lipid Peroxidation

Lipid peroxidation products in brain homogenates were assayed by measuring the level of malondialdehyde (MDA) using the method of Ruiz-Larrea et al. [39]. In this assay, the thiobarbituric acid-reactive substances derived from lipid peroxidation 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 brain homogenates using the method of Ellman et al. [40]. The procedure is based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-5-mercaptobenzoic acid, which is intense yellow in color and determined spectrophotometrically at 412 nm.

2.4.3. Determination of Nitric Oxide

Nitric oxide was determined indirectly using the Griess reagent. Nitrate is converted to nitrite via nitrate reductase. The Griess reagent then acts to convert nitrite to a deep purple azo compound and the absorbance was read at 540 nm using a spectrophotometer [41].

2.4.4. Determination of PON-1 Activity

Paraoxonase-1 (PON-1) arylesterase activity in brain homogenates was determined with the use of phenyl acetate as a substrate. PON-1 catalyzes the cleavage of phenyl acetate with the formation of phenol. The rate of phenol formation is measured using a spectrophotometer by monitoring the increase in absorbance at 270 nm and 25°C. One unit of arylesterase activity is defined as 1 μmol of phenol formed per min. Enzyme activity is calculated based on the extinction coefficient of phenol of $1,310\ M^{-1}\ cm^{-1}$ at 270 nm, pH 8.0, and 25°C. The enzyme activity is expressed in kilo international units/liter (kU/L) [42].

2.5. Behavioral Testing

2.5.1. Cylinder Test

The cylinder test is used to assess the spontaneous forelimb use. Mice are placed in a transparent Plexiglas cylinder and the number of spontaneous rears made during 5 min in the cylinder was measured for each animal [43].

2.5.2. Horizontal Bar Test

To evaluate the motor strength, mice were made to hang by their forelimbs from a steel rod (25 cm long, 0.2 cm in diameter), 0.25 m above the bench. The time each mouse could hang suspended from the rod was recorded for three trials with a cut-off time of 180 s [44].

2.5.3. Wood Walking Test

To assess the motor coordination, mice were made to walk over a wooden stick (∼1 m in length, 1 cm in width) and the time each mouse spent to reach the end was recorded [45].

2.5.4. Stair Test

In order to assess skilled reaching, mice were placed at the bottom of a stair (30 cm in length) placed at an angle of 55° above the bench, and the latency to climb the stair was recorded for each mouse [46].

2.6. Histopathological Examination

Brain specimens were fixed in freshly prepared 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Paraffin sections (5 μm thick) were prepared and stained with hematoxylin and eo-
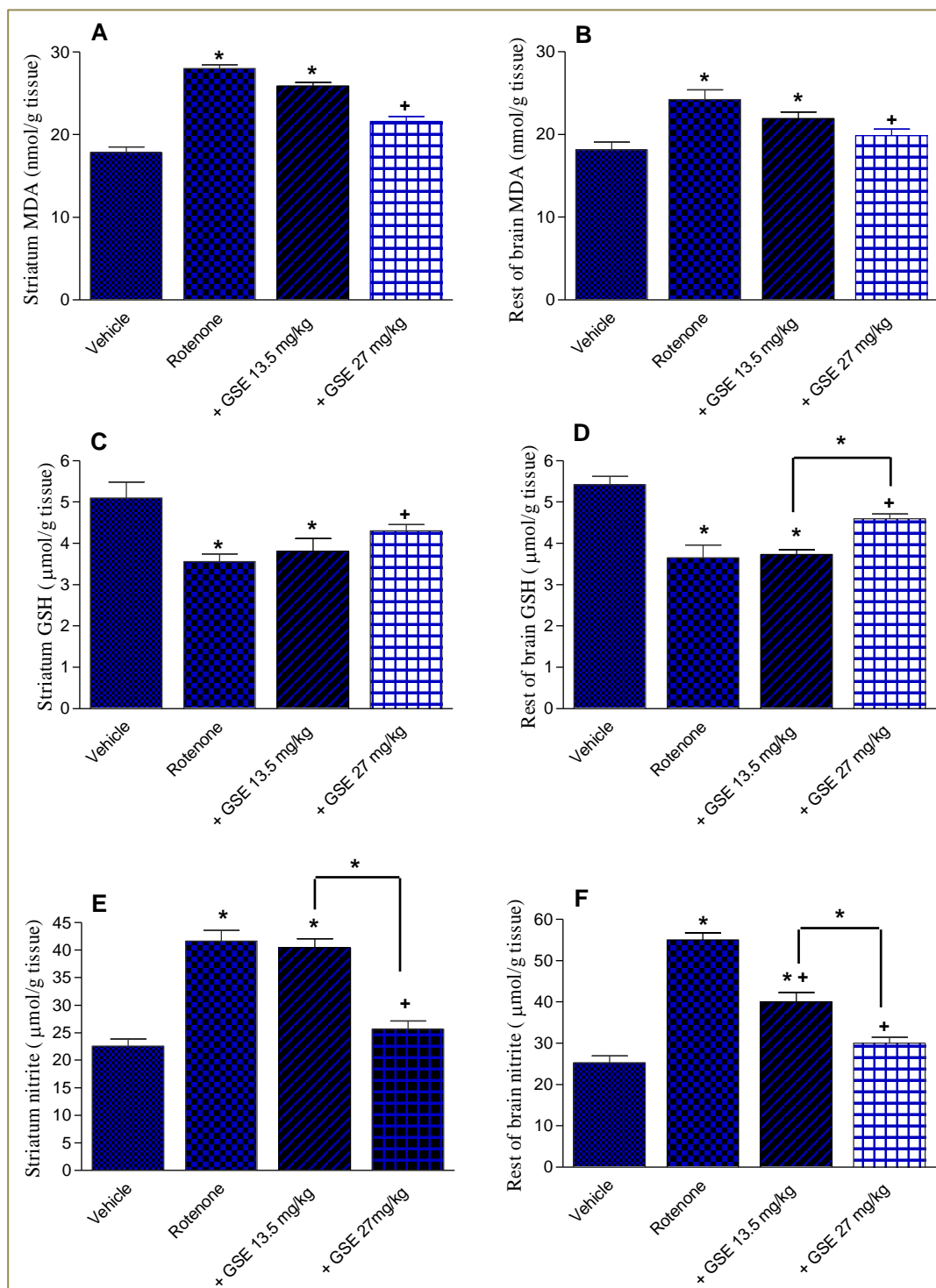
FIGURE 1. Effect of grape seed extract (GSE) on malondialdehyde (MDA), reduced glutathione (GSH), and nitrite in the brains of rotenone-treated mice. *, $p < 0.05$ vs. corresponding vehicle-treated group and between different groups as indicated on the graphs; +, $p < 0.05$ vs. rotenone control group.

FIGURE 2. Effect of grape seed extract (GSE) on paraoxonase-1 activity (PON-1) in the brains of rotenone-treated mice. *, $p < 0.05$ vs. corresponding vehicle-treated group and between different groups as indicated on the graphs; +, $p < 0.05$ vs. rotenone control group.
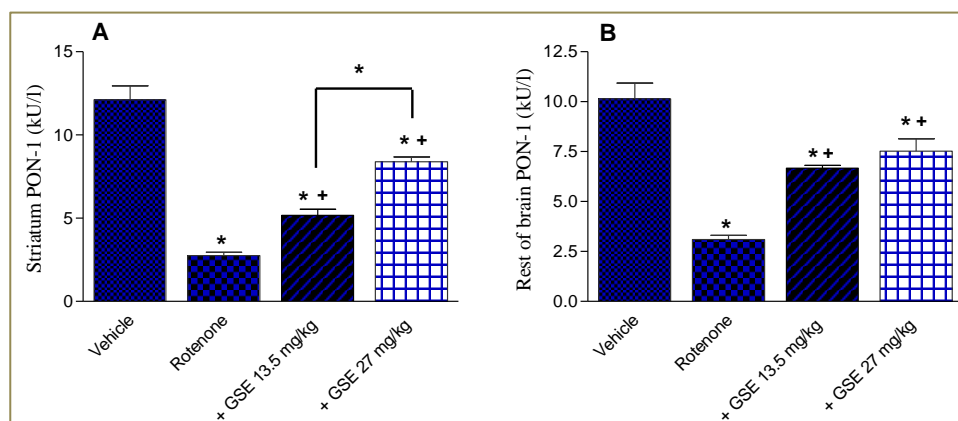
sin (H&E) for the histopathological examination. Sections were examined using a light microscope (Nikon, Japan).

2.7. Immunohistochemical Examination of Caspase-9

Brain sections were deparaffinized in xylene and rehydrated in graded alcohol. The tissues were pretreated with 10 mM citrate buffer, pH 6.0, in microwave oven at 500 W for 10 min for antigenic retrieval. The slides were washed with PBS for 5 min. Sections were incubated overnight at 4°C in a humidified chamber with mouse monoclonal antibody to caspase-9 antibody diluted 1:50. The sections were rinsed again with PBS and then incubated with a biotinylated goat anti rabbit and mouse antibody for 10 min. The sections were rinsed again with PBS. Finally, sections were incubated with Streptavidin peroxidase. To visualize the reaction, slides were incubated for 10 min with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich). The slides were counterstained with hematoxylin then dehydrated and mounted. Primary antibodies were omitted and replaced by PBS for negative controls.

2.8. Immunohistochemistry for Tyrosine Hydroxylase

Sections were immunostained for tyrosine hydroxylase (TH) (Abcam, Cambridge, UK) at room tem-perature with a biotinylated peroxidase-based kit. Deparaffinized sections were rinsed in PBS (2.5 min) and then placed in 0.3 % H_2O_2 for 30 min to reduce endogenous peroxidase activity. The tissue was then rinsed in PBS (2.5 min) and blocked in a solution of PBS containing 3% horse serum and 0.1% Triton X-100 for 60 min. Without rinsing, the tissue sections were incubated in the blocking solution that also contained a 1:20,000 dilution of monoclonal mouse TH antibody for 60 min. Tissues were rinsed (2.5 min) with PBS and incubated with the blocking solution that included a 1:300 dilution of biotinylated anti-mouse antibody made in horse. After PBS rinses (2.5 min), slices were incubated with avidin–peroxidase reagent diluted as instructed by the manufacturer. Sections were transferred to a 50 mM Tris-buffered 0.9% saline solution, rinsed, then treated with DAB and a final rinse in PBS (2 min), and then counterstained with H&E. Sections were mounted on slides, allowed to air-dry for 24 h, then dehydrated with a series of ethanol rinses, and cleared with xylene. Sections were cover slipped after at least 24 h drying in air.

2.9. Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA; post-hoc individual comparisons were made using Duncan's multiple range test. A p value of less than 0.05 was considered to be statistically significant.
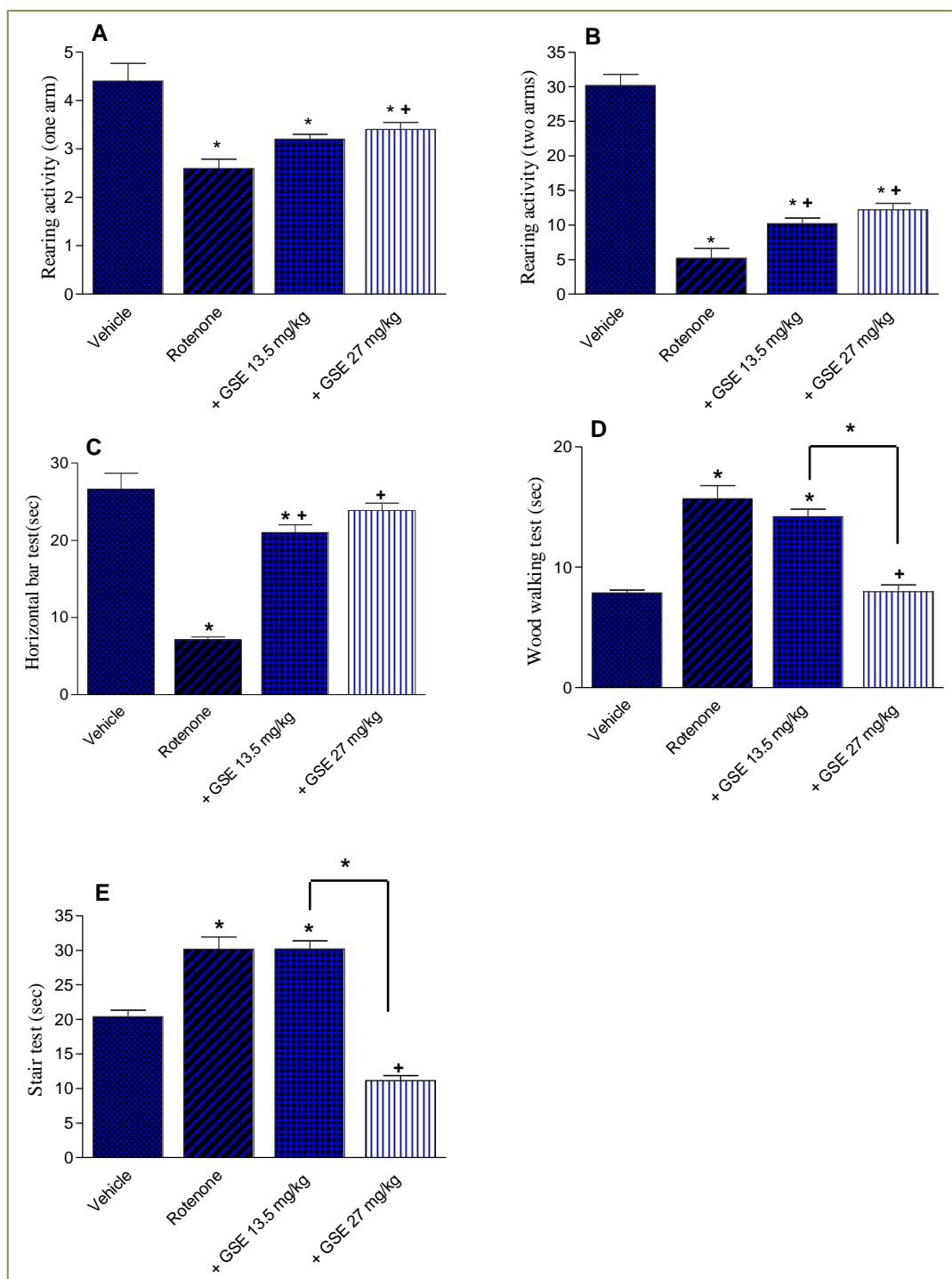
FIGURE 3. Changes in behavioral parameters (rearing activity, locomotor activity, and stair test paradigms) in mice treated with rotenone alone or combined with grape seed extract (GSE). *, $p < 0.05$ vs. corresponding vehicle-treated group and between different groups as indicated in the graphs; +, $p < 0.05$ vs. rotenone control group.

3. RESULTS

3.1. Biochemical Results

3.1.1. Lipid Peroxidation

Rotenone caused significantly higher MDA concentrations in the striatum and in the rest of the brain tissue than those of the vehicle-treated group. MDA increased by 56.9% (28.0 ± 0.46 vs. 17.84 ± 0.67 nmol/g.tissue) and 33.4% (24.2 ± 1.2 vs. 18.14 ± 96 nmol/g.tissue) in the striatum, and in the rest of the brain, respectively. Grape seed extract given at 13.5 mg/kg had no significant effect on brain MDA of rotenone-treated mice. The higher dose of GSE (27 mg/kg), however, decreased MDA by 22.8% and 17.9% in the striatum and in the rest of the brain of tissue, respectively, compared with the corresponding rotenone control values (21.62 ± 0.59 vs. 28.0 ± 0.46 and 19.86 ± 0.76 vs. 24.2 ± 1.2 nmol/g.tissue) (**Figure 1**).

3.1.2. GSH

Compared with vehicle-treated mice, the rotenone only group exhibited 30.2% and 32.7% decrements

FIGURE 4. Representative photomicrographs of H&E-stained sections of substantia nigra of mice. (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400. N, normal SNpc neurons; V, vacuolated neurocytes; P, shrunken pyknotic nuclei.

in GSH in the striatum, and in the rest of the brain, respectively (3.56 ± 0.18 vs. 5.1 ± 0.39 and 3.65 ± 0.31 vs. 5.42 ± 0.20 µmol/g.tissue). Only at the high dose of 27 mg/kg did GSE increase GSH level in these two brain regions by 20.8% and 26%, respectively (4.3 ± 0.15 vs. 3.56 ± 0.18 and 4.6 ± 0.11 vs. 3.65 ± 0.31 µmol/g.tissue) (**Figure 1**).

3.1.3. Nitric Oxide

Mice treated with rotenone alone exhibited 84.6% and 117.4% increments in nitric oxide content in the striatum and in the rest of the brain compared with the vehicle control values (41.61 ± 1.9 vs. 22.54 ± 1.3 and 55 ± 1.71 vs. 25.3 ± 1.6 µmol/g.tissue). Nitric oxide in the striatum decreased by 38.5% after treatment with 27 mg/kg GSE (25.6 ± 1.48 vs. 41.61 ± 1.9 µmol/g.tissue). It was reduced by 27.3% and 45.5% in the rest of the brain tissue after treatment with 13.5 and 27 mg/kg GSE, respectively (40.0 ± 2.3 and 30.0 ± 1.42 vs. 55 ± 1.71 µmol/g.tissue) (**Figure 1**).

3.1.4. PON-1

Rotenone caused significant decreases in PON-1 activity by 77.2% and 69.4% in the striatum and in the rest of the brain tissue, respectively, compared with the vehicle control values (2.76 ± 0.18 vs. 12.1 ± 0.85 and 3.1 ± 0.21 vs. 10.14 ± 0.79 kU/l). Treatment with GSE at 13.5 and 27 mg/kg resulted in increased PON-1 activity by 87.3%/204% and 115.2%/142.9%, respectively, in these brain regions compared with the rotenone control group (**Figure 2**).

3.2. Behavioral Testing

3.2.1. Cylinder Test

The exploratory behavior (rearing activity) of mice was significantly decreased by rotenone. The number of spontaneous rears using one arm decreased by 41% while that using both arms decreased by 82.8% of the corresponding control values. Rearing activity was improved, but not corrected by GSE treatment. Grape seed extract given at 13.5 and 27 mg/kg resulted in a dose-dependent increase in the rearing activity by 23.1–30.8% and 86.1–134.6%, respectively, compared with the corresponding control values (**Figure 3**).
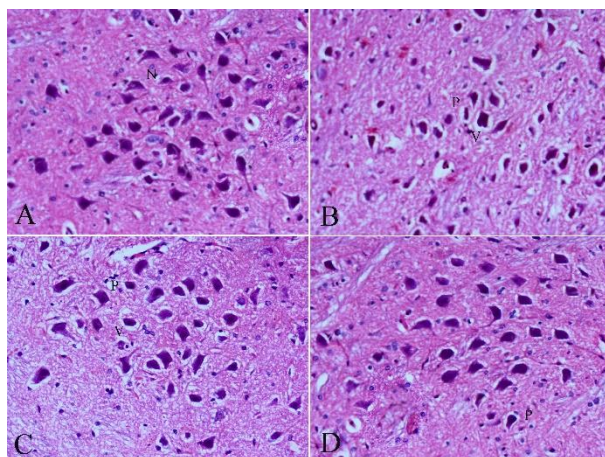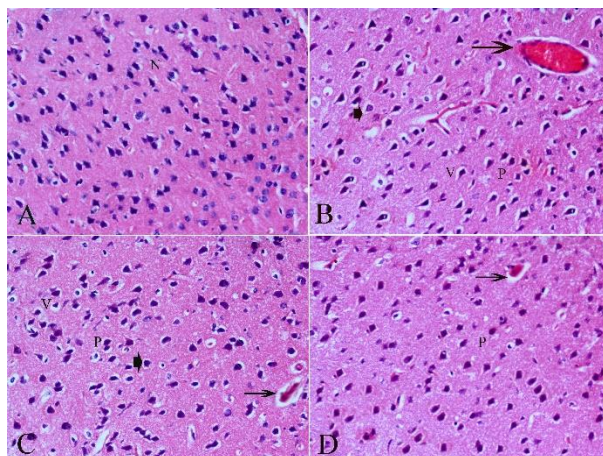
FIGURE 5. Representative photomicrographs of H&E-stained sections of mice striatum. (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400. N, normal neurons; V, vacuolated neurocytes; P, shrunken pyknotic nuclei; arrowhead, apoptotic nuclei; thin arrow, congestion in blood vessels.
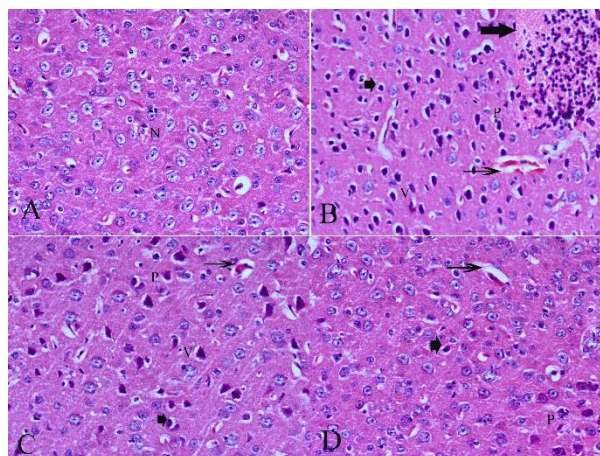

FIGURE 6. Representative photomicrographs of H&E-stained sections of mice cerebral cortex. (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400. N, normal neurons; V, vacuolated neurocytes; P, shrunken pyknotic nuclei; arrowhead, apoptotic nuclei; thin arrow, congestion in the blood vessels; thick arrow, focal gliosis.

3.2.2. Horizontal Bar Test

Rotenone treatment caused a significant decrease in the time taken by mice to hang suspended from a steel rod by 71% (7.12 ± 0.39 vs. 26.6 ± 2.1 sec). GSE given at 13.5 and 27 mg/kg resulted in a dose-dependent increase in the ability of mice to hang suspended from the rod by 194.5% and 235.1%, respectively (21.0 ± 1.0 and 23.9 ± 0.94 vs. 7.12 ± 0.39 sec) (**Figure 3**).

3.2.3. Wood Walking Test

The time spent by mice to traverse a wooden stick was significantly increased by rotenone (a 102.8% increase: 15.96 ± 1.1 vs. 7.87 ± 0.25 sec). GSE given at 27 mg/kg almost reversed this effect of rotenone (**Figure 3**).

3.2.4. Stair Test

Mice treated with only rotenone exhibited a 48% increase in the time taken to ascend the stair (30.14 ± 1.8 vs. 20.36 ± 0.97 sec). Treatment with GSE at 27 mg/kg reversed this effect of rotenone (**Figure 3**).

3.3. Histopathological Results

3.3.1. Substantia Nigra

Mice treated with the vehicle control showed normal SNpc neurons with obvious nuclei (**Figure 4A**). Rotenone treatment caused marked SNpc degeneration; neurons appeared with low number per field and with indistinct neuronal boundaries (**Figure 4B**). The above histopathological changes were ameliorated by GSE in a dose-dependent manner (**Figure 4C** and **4D**).

3.3.2. Striatum

The striatum from the vehicle-treated group showed the normal structure of the tissue (**Figure 5A**). Rotenone caused degenerated and vacuolated neurocytes with dystrophic changes in the form of shrunken, pyknotic, and hyperchromatic nuclei. Congestion in the blood vessels of the meninges was noted (**Figure 5B**). These changes were prevented by GSE (**Figure 5C** and **5D**).
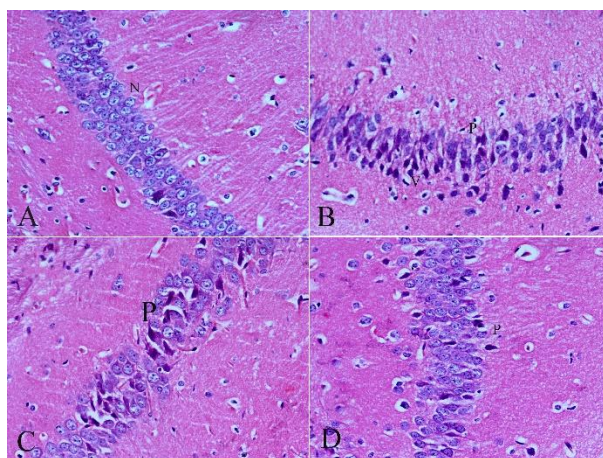
FIGURE 7. Representative photomicrographs of the hippocampus stained by H&E. (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400. N, normal neurons; V, vacuolated neurocytes; P, shrunken pyknotic nuclei.

3.3.3. Cerebral Cortex

Figure 6A represents section of the cerebral cortex from the vehicle-treated mice with normal structure and neuronal cells with prominent nuclei. Rotenone caused neuronal degeneration and vacuolation with shrunken, pyknotic, and hyperchromatic nuclei. Congestion was observed in the blood vessels of the meninges. Focal gliosis was also noticed (**Figure 6B**). Mice treated with GSE showed amelioration of the rotenone-induced neurodegeneration (**Figure 6C** and **6D**).

3.3.4. Hippocampus

The hippocampus from the vehicle-treated group showed the normal neuronal morphology of pyramidal cell (**Figure 7A**). In rotenone only-treated mice, the region of the hippocampus showed decreased thickness of the pyramidal layer and severe damage in the form of pyknotic shrunken, apoptotic neurocytes, vacuolated neurons with hyperchromatic nuclei, and dilated hemorrhagic blood vessels (**Figure 7B**). These histopathological alterations were prevented dose-dependently by GSE (**Figure 7C** and **7D**).

3.4. Immunohistochemical Results

3.4.1. Caspase-9 Immunoreactivity

Mice treated with the vehicle showed negative immunostaining reaction for caspase-9 in the striatum, cortex, and hippocampus (**Figures 8A**, **9A**, and **10A**). Rotenone caused a strong positive nuclear reaction of caspase-9 in these brain areas (**Figures 8B**, **9B**, and **10B**). In contrast, a small number of neurocytes showed a positive immune reaction in mice treated with rotenone and GSE (**Figures 8C**, **8D**, **9C**, **9D**, **10C**, and **10D**).

3.4.2. TH Immunoreactivity

Immunohistochemistry of TH in the substantia nigra revealed intense reaction in vehicle-treated mice as brown color (**Figure 11A**). In the rotenone-treated group, TH immunostaining showed obvious loss of reaction, reflecting neurodegeneration in this region (**Figure 11B**). Treatment GSE resulted in increased expression of TH-immunostained neurons in a dose-dependent manner (**Figure 11C** and **11D**).

4. DISCUSSION

The pesticide rotenone is widely used to induce experimental PD in laboratory animals. In rats, intravenous [47], subcutaneous [29, 48], intraperitoneal [49], and intrastriatal [50, 51] routes have been employed. In these studies, rotenone was reported to induce nigrostriatal cell loss, decrease striatal dopamine content, TH-ir, and to result in PD-like motor signs such as hypokinesia and rigidity [29, 48, 49]. There were also α-synuclein-like deposits in the remaining SNpc neurons [29, 49]. Other studies have shown that rotenone given to mice via subcutaneous or intragastric routes was capable of inducing nigrostriatal degeneration [31–35, 52]. The mouse model of rotenone administration was used in this study. Rotenone was subcutaneously given at the dose of 1.5 mg/kg/day, once a day, for two consecutive weeks (a total of 6 injections). This dosing regimen has been shown to induce nigrostriatal degeneration (shrunken, distorted neurons, pericellular haloes, and inflammation in the striatum), neuronal apoptosis, loss of pigmented neurons in the SN, decreased striatal dopamine, serotonin, as well as a decrease in TH-ir in the striatum of
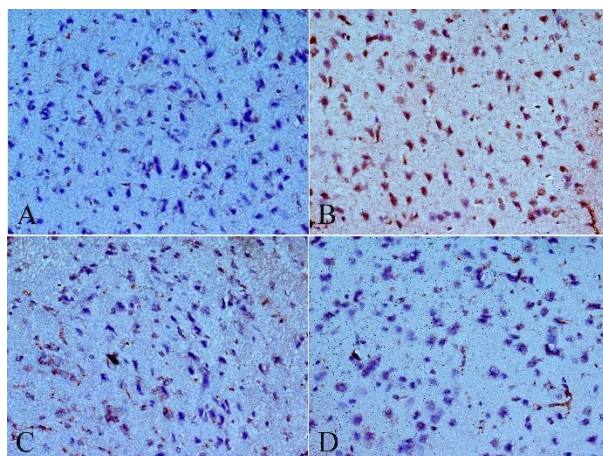
FIGURE 8. Representative photomicrographs of caspase-9 immunoreactivity in the striatum (identified by brown color). (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400.
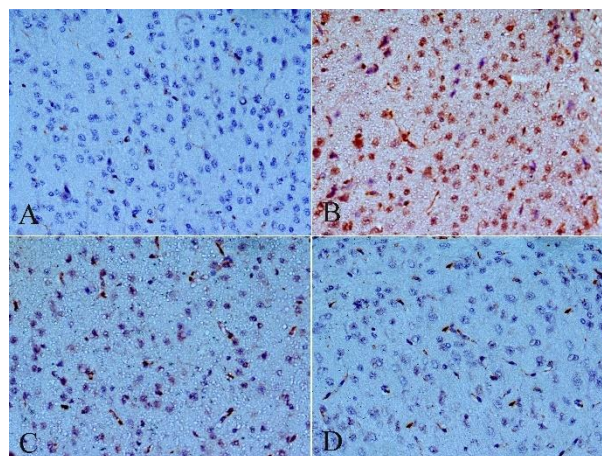

FIGURE 9. Representative photomicrographs of caspase-9 immunoreactivity in the cerebral cortex (identified by brown color). (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400.

treated mice. Rotenone also caused a significant impairment in the motor function of mice [33–35]. Much more important is that this dosing regimen did not cause protracted illness and thus allowed for careful assessment of the mouse's motor behavior. The results of the present study are in accordance with these effects of rotenone. There were nigrostriatal damage, loss of substantia nigra TH-ir, neuronal degeneration, focal gliosis in the cerebral cortex, and an increase in apoptotic marker caspase-9 in different brain regions. Rotenone impaired motor performance and grip strength as indicated by several behavioral tests.

The results of this study indicate that rotenone caused an increase in lipid peroxidation and nitric oxide and a decrease in the antioxidant molecule GSH in the striatum and in the rest of the brain tissue. Rotenone causes neuronal cell death via increased oxidative stress [53], a major pathogenetic mechanism contributing to DA-ergic neurodegeneration in human PD [9, 54]. Studies thus showed the increased formation of reactive oxygen metabolites [55], increased lipid peroxidation products [33–35], and protein carbonyls [56] in the rodent brain after rotenone treatment. Rotenone activates microglia that release reactive oxygen metabolites via myeloperoxidase enzyme [57]. Cellular antioxidants such as GSH, su-

peroxide dismutase activity, catalase activity, and total antioxidant capacity also decreased in the brain tissue of rotenone-treated animals [33–35, 51, 58]. The findings of the present study are also indicative of increased reactive oxygen metabolites and the occurrence of oxidative damage as evidenced by the increase in brain MDA content and the decline in GSH.

Rotenone also caused a significant increase in brain nitric oxide, a finding that is in agreement with previously published observations [33–35]. We have also shown that rotenone resulted in increased nitric oxide release and prominent inducible nitric oxide synthase (iNOS) immunostaining in the striatum and SNpc [33–35, 59]. The increase in nitric oxide by rotenone is thus likely to be mediated by the enhanced expression of iNOS. This increased expression of iNOS by microglia and astrocytes results in the generation excessive amounts of nitric oxide for prolonged time and neuronal cell death [60]. However, neurons could be another source of the increase in brain nitric oxide by rotenone. This is because the neuronal NOS inhibitor 7-nitroindazole was able to decrease the level of 3-nitrotyrosine and nigrostriatal damage in rotenone-treated rats [61]. The neurotoxic actions of nitric oxide generated in excess are ascribed to the nitrogen oxides, such as nitrogen diox-
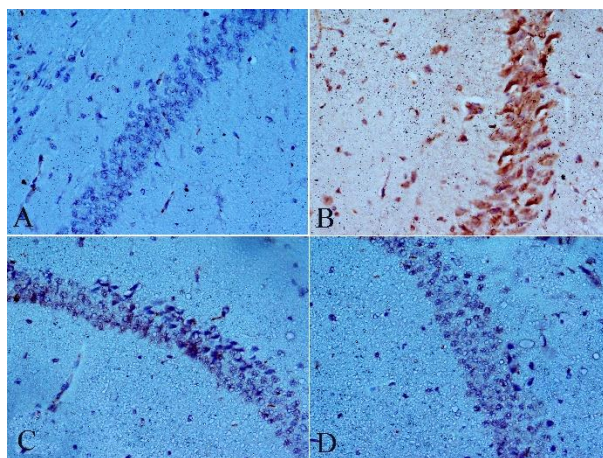
FIGURE 10. Representative photomicrographs of caspase-9 immunoreactivity in the hippocampus (identified by brown color). (A) Vehicle. (B) Rotenone. (C) Rotenone + GSE 13.5 mg/kg. (D) Rotenone + GSE 27 mg/kg. Magnification scale, ×400.

FIGURE 11. Representative photomicrographs of tyrosine hydroxylase (TH) immunoreactivity (identified by brown color). (A) Vehicle: intense immunostaining for TH. (B) Rotenone: obvious loss of TH immunostained neurons. (C) Rotenone + GSE 13.5 mg/kg: mild increase in TH immunostaining. (D) Rotenone + GSE 27 mg/kg: moderate increase in TH immunostaining (TH, hematoxylin counterstain, ×1,000).

ide (NO_2), and dinitrogen trioxide (N_2O_3) formed by the reaction of nitric oxide and oxygen and peroxynitrite ($ONOO^-$) formed by the reaction of nitric oxide and superoxide ($O_2^{\cdot-}$). These species are capable of causing oxidation and nitration of tyrosine residues in proteins, and nitrosylation of thiols in proteins or GSH, inhibition of mitochondrial respiration, cellular energy failure, and neuronal death [60, 62, 63].

Our results also demonstrate marked inhibition of PON-1 activity in the brain of rotenone-intoxicated mice, a finding which is in agreement with our previous studies [33, 34]. The enzyme PON-1 hydrolyzes the active metabolites, i.e., oxons of a number of organophosphate insecticides including diazoxan, dichlorvos, and chlorpyrifos oxon [64]. The catalytic efficiency of the enzyme appeared to determine the susceptibility to organophosphates, while the purified PON-1 itself was able to protect against toxicity induced by paraoxon or chlorpyrifos oxon in rodents [65–67]. There is an increasingly recognized role for PON-1 in a number of central nervous system diseases, including dementia [68], and autism [69]. Studies also implicated exposure to organophosphate insecticides in the risk for developing PD [7, 8]. Moreover, the PON-1 status of the individual, i.e., the slow metabolizer variants, may affect the susceptibility to organophosphate neurotoxicity in humans
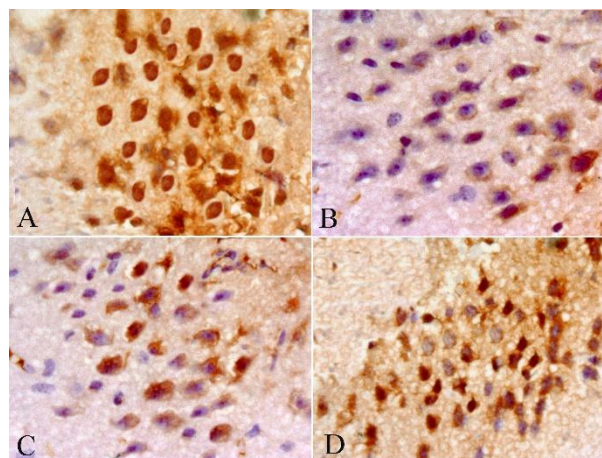
[7, 70]. Thus, on the one hand, PON-1 hydrolyzes the active metabolites of some insecticides, and in this way its tissue and plasma level might determine the individual's susceptibility to the neurotoxic effects of organophosphates being exposed to [7, 65, 70]. On the other hand, the enzyme possesses antioxidant and anti-inflammatory effects [71, 72] and hence a decrease in activity renders the cell vulnerable to oxidants. These data therefore suggest a neuroprotective role for PON-1. Rotenone might inhibit PON-1 activity directly or via the increased oxidative/nitrosative stress. It was also noted that the restoration of the enzyme activity coincided with neuroprotection by different agents, possibly due to a decrease in oxidative stress [48, 58, 59].

Caspases are cysteine proteases that function in the initiation and execution of apoptosis or "programmed cell death". The initiator caspases-1, -2, -4, -5, -8, -9, -10, -11, and -12 respond to proapoptotic signals, and downstream ''executioner'' or "effector" caspases-3, -6, and -7 [73, 74]. Hartmann et al. [75] reported an increase in the percentage of activated caspase-3-positive neurons in SNpc of PD patients

compared with controls. In addition, activated caspase-8 and caspase-9 were detectable in postmortem PD brain tissue [76]. Evidence has also been provided for the occurrence of apoptosis in experimental models of PD. Thus, activated caspase-3 and capase-9 immunoreactivity was detected in dopaminergic neurons in culture after 6-hydroxydopamine [77]. Increased caspase-3 expression in the rat and mouse brain was also reported after treatment with 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) [78, 79] or rotenone [33, 80]. Moreover, the level of the anti-apoptotic protein Bcl-2 was also significantly altered in the striatum of rotenone-intoxicated rats [48, 59]. The present study shows increased level of caspase-9 protein in the striatum, cerebral cortex, and hippocampus of rotenone-treated mice. Caspase-9 is an important initiator caspase, and its inhibition was found to prevent the activation of both caspase-3 and caspase-8 and to inhibit cleavage of the proapoptotic protein Bid in the SN of MPTP-treated mice [76].

The treatment of PD is still a challenging issue to the physician and there is no doubt that there is a need to find new therapeutic agents to improve therapeutic outcome or decrease neuronal loss in the course of the disease. In this study, the effect of a standardized GSE rich in proanthocyanidins that possesses antioxidant [23, 24] and antiapoptotic properties [23, 25], was therefore evaluated for a potential neuroprotective effect against the rotenone neurotoxicity. Our findings suggest that GSE could be of value in this respect. The extract was demonstrated to exert an antioxidant effect decreasing brain lipid peroxidation. It also increased brain GSH and PON-1 activity, reflecting an improved redox status of the cell with consequent neuroprotection. GSE resulted in marked inhibition of the raised nitric oxide level in the brain of rotenone-treated mice, which could underlie at least partly the neuroprotection observed. Besides nigrostriatal cell loss, rotenone caused neurodegeneration in other brain regions like the cerebral cortex and hippocampus, which is in agreement with previous studies. The rotenone-induced neurodegeneration was prevented by GSE which also improved TH-ir and exerted an antiapoptotic effect. The improvement in biochemical and pathological changes caused by rotenone in the mouse brain was reflected in functional improvement. Mice given GSE showed not only better exploratory activity, but also recovery of motor strength as measured by wire handing and stair tests. These re-sults clearly suggest that GSE could be of benefit as an adjunctive treatment in subjects with PD.

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