Original Article

The Effect of Nigella Sativa Oil on Frontal Cerebral Cortex and Hippocampus of Rotenone Induced Model of Parkinson's Disease in the Adult Male Albino Rat: Histological and Immunohistochemical Study

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ABSTRACT

Introduction: Parkinson's disease (PD) is the second most common neurological ailment globally, following Alzheimer's disease. Cognitive decline and dementia are one of the most incapacitating symptoms affecting patients. Nigella sativa oil (NSO) has neuroprotective, antioxidant and anti-inflammatory properties.

Aim of the Work: Our study intended to investigate the neurodegenerative alterations that occur in the frontal cortex and hippocampus of a parkinsonian rat model, and to examine the alleviative effect of NSO. This was determined using behavioral, biochemical, histological, immunohistochemical, and morphometric studies.

Materials and Methods: Sixty adult male albino rats were organized into six groups; control, NSO (3 mg/kg b.w/day orally for 30 days), rotenone treated (1.5 mg/kg b.w/day subcutaneous for the last 10 days), pre-treated with NSO for 20 days followed by combined rotenone and NSO for the last 10 days, recovery (1.5 mg/kg b.w/day subcutaneous for the first 10 days) and post-treated with NSO + rotenone. Behavioral tests; open field test, forced swim test and Y maze test were performed. After scarification of the rats, studies involving biochemistry, histology and immunohistochemistry were conducted on the brain. (b.w mean body weight of the rat).

Results: The frontal cortex and CA1 region of the hippocampus showed severe degenerative changes especially in the recovery group. This was manifested clinically by impairment in the spatial working memory. This was accompanied by increased Bax protein, GFAP, TNF-α, iNOS immuno-expression with a decrease in Ki67 immuno-expression. Administration of NSO either as a protective agent or as a therapeutic agent greatly improved the results.

Conclusion: The administration of NSO may protect the risky individuals from catching the disease or at least delay the onset of its occurrence. Also, its administration after establishment of the disease may decrease the rate of the disease progression and delay the development of the cognitive impairment symptoms.

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INTRODUCTION

Parkinson's disease (PD) is the world's second most common neurological ailment, after Alzheimer's disease. Over 6.2 million individuals globally are now affected, and it is anticipated to increase to more than 12 million by 2040[1].

It is already affecting 12% of the population over 65 years[2]. It is a complex neurological condition characterized by stiffness, resting tremor, bradykinesia, and postural instability. This is caused by the degradation of substantia nigra dopaminergic neurons, which leads to the loss of striatal dopamine[3]. Most people acquire non-motor symptoms such as depression, anxiety, sleep difficulties, autonomic dysfunction, cognitive decline, and dementia as a result of affection for other brain areas such as the cerebral cortex and numerous brainstem regions[4].

There are several mechanisms that explain the pathogenesis of Parkinson's disease, including dysfunctional protein clearance system, α-synuclein misfolding and aggregation, mitochondrial dysfunction, oxidative stress, ischemic factors, neuro-inflammatory reactions that may lead to premature neuronal death[5].

One of the risk factors that was documented to initiate PD is the exposure to environmental toxins such as the insecticide[6]. Rotenone is a widely used organic insecticide of natural plant origin that has been shown to cause mitochondrial dysfunction through damage to the mitochondrial complex I electron transport chain and hence reactive oxygen species (ROS) accumulation. All of these lead to loss in the dopaminergic neurons in the nigrostriatal pathway[7].

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Nigella sativa has a rich religious and historical background. It is the black seed that the prophet Mohammed {peace be upon him (PBUH)} described it as having healing properties, the Holy Bible also identified it as the curative black cumin[8].

Nigella sativa oil (NSO) contains many active components; thymoquinone, dithymoquinone and thymo-hydroquinone, carvacrol, and p-cymene[9]. These phytochemicals exhibit anti-inflammatory, antioxidant, anti-carcinogenic, antidiabetic, neuroprotective, hepatoprotective and nephroprotective properties[10].

The goal of the present research was to investigate the neurodegenerative alterations that occur in the frontal cerebral cortex and hippocampus of a parkinsonian rat model, as well as to analyze the alleviative effect of NSO. This was studied using behavioral, biochemical, histological, immunohistochemical and morphometric investigations.

MATERIALS AND METHODS

Chemicals

Rotenone was brought from Sigma-Aldrich (St Louis, MO, USA) (cat#83-79-4) in a powder form, vial of 1 gram. It was dissolved in 100% dimethyl sulfoxide (DMSO). The rotenone solution was prepared till obtaining the concentration of (0.5 mg rotenone / ml DMSO)[11]. Nigella sativa oil (NSO) was purchased from El-Baraka company for natural oils Hurghada, Egypt. DMSO was brought from Sigma-Aldrich (St Louis, MO, USA) (cat#67-68-5) in a liquid form.

Animals

Sixty adult male albino rats with an average weight of 200–250 grams each were used in this study. They were maintained in cages with a 12/12-hours artificial light/dark cycle at ambient temperature (25±2ºC).

Experimental design

The animals were divided into the following six groups:

Group I (Control group): This group composed of 20 animals and was subdivided randomly into two subgroups.

a. Subgroup Ia (negative control): 8 animals were kept without any treatment all through the experiment (40 days).

b. Subgroup Ib (sham control): This group composed of twelve animals, six of them were injected with DMSO (1 ml/kg b w equals 0.2 ml / rat) s.c.[12] for 10 days from the first day till the 10th day of the experiment then they were left without any treatment for the next 30 days. The other 6 animals were left without any treatment for the first 30th days of the experiment then they were injected with DMSO (1 ml/kg b w equals 0.2 ml / rat) s.c. for 10 days from the 31st day to the 40th day of the experiment.

Group II (Nigella sativa oil (NSO) group): This group composed of eight animals left without any treatment for the first 10 days of the experiment, then they were given NSO (3 ml/kg b w equals 0.6 ml / rat) orally by a gastric tube[13] for 30 days from the 11th day- the 40th day of the experiment.

Group III (Rotenone treated group): This group composed of eight animals left without any treatment for the first 30th days of the experiment then they were injected with rotenone solution in a dose of 1.5 mg/kg b w equals 0.3 mg / rat) s.c. once daily for 10 days[14] starting from the 31st day to the 40th day of the experiment.

Group IV (pre-treated with NSO + rotenone): This group composed of eight animals left without any treatment for the first 10 days of the experiment then they were exposed to NSO in the same dose as NSO group orally by a gastric tube from the 11th day to the 30th day of the experiment followed by combined rotenone injection in the same dose as rotenone treated group s.c. once daily and NSO from the 31st day to the 40th day of the experiment[15].

Group V (Recovery group): This group composed of eight animals that were injected with rotenone solution in a dose of 1.5 mg/kg b w subcutaneous once daily for the first 10 days[14] then, animals were left without any treatment for the subsequent 30 days till scarification.

Group VI (post-treated with NSO + rotenone): This group composed of eight animals exposed to rotenone injection in the same dose as rotenone treated group once daily for 10 days starting from the 1st day of the experiment followed by NSO in the same dose as NSO group orally by a gastric tube from 11th day to the 40th day of the experiment[15].

Clinical behavioral tests

These tests were conducted at the end of the experiment.

Open field test (OFT)

It was performed to assess the motor behavior of the animals. Open field apparatus was a square made of wood 100 × 100 cm. Its walls were about of 30 cm height. The animals were positioned in the middle of the field and given three minutes to investigate while a video camera recorded. The entire locomotor activity of each animal was scored, and this was represented as the total number of lines crossed plus the number of rearing[16].

Forced swim test (FST)

It was done to evaluate the animals' depressive-like behavior; every animal was placed within a 25 cm diameter water cylinder containing 50 cm of water. The immobility score was recorded which is the duration of immobility in a 6-min session. The first 2 minutes was deleted from the calculation. This is due to the fact that most animals are very active at the beginning of the test[17]. Immobility was assigned when the animals showed no more movement than what was necessary to maintain its head above the

water. An increase in the animal's immobility period was a sign that it was acting in a depressed manner[18].

The Y-maze test

Y-maze was used to measure spatial working memory. It was made of wood, with three arms of equal length that converged at a right angle to one another. After being positioned in the center of the maze, each animal was given eight minutes to explore all three arms. The formula used to compute the percentage of spontaneous alternation was (actual alternations/maximum alternations) × 100. The total number of arms entered minus two was the maximum alternation number[19].

Following the behavioral tests, the animals were beheaded, sacrificed by cervical dislocation, given diethyl ether inhalation anesthesia, and had their brains removed and processed for biochemical, histological, and immunohistochemical analysis[20].

Biochemical analysis

The striatum of each left cerebral hemisphere was dissected then homogenized in phosphate buffered saline (PH 7.2- 7.4), and centrifugated at 4oC for 20 minutes at 2000-3000 rpm. In preparation for the dopamine level study, the supernatant was extracted and stored at -80 degrees[21].

Histological examination

Each right cerebrum was cut into two parts coronally, anterior for frontal cortex examination and posterior for hippocampus examination. Each part was prepared for examination with hematoxylin & eosin stain[22].

Immunohistochemical examination

Paraffin embedded tissue were deparaffinized and examined immunohistochemically by the following antibodies; Rabbit polyclonal anti- Bax protein marker 1:1000, rabbit polyclonal anti- glial fibrillary acidic protein (GFAP) 1:300, Rabbit polyclonal anti-Inducible nitric oxide Synthase (iNOS) 1:500, mouse monocolonal anti- TNF- alpha 1:1000 and rabbit monoclonal anti- Ki-67 1:500.

Morphometric and Statistical Analyses

Five fields from each animal in the different groups were obtained and the following were measured by the image analyzer software:

- Percentage of the degenerated cells in the frontal cortex and the hippocampus.
- Area percentage of Bax protein, GFAP, TNF-alpha & iNOS immunoreaction and the percentage of ki-67 +ve cells.

On an IBM compatible computer, SPSS (statistical package for social science) version 23.0 was used to analyze the measured data. These data were analyzed using a one-way ANOVA test followed by a least significant difference (LSD) post hoc test. A *P-value* of less than 0.05 was deemed statistically significant.

RESULTS

There was no significant difference as regarding the assessed parameters between the negative control and the sham control so; they were pooled in a single group (control). Also, no significant difference was recorded as regarding the all measured domains between the NSO group and the control one.

Clinical behavioral tests results

Open field test

The total locomotor activity score was significantly diminished in the group that received rotenone (37.50±3.25) in comparison with the control one (59.25±.3.01) *P*<.001. When comparing the total score of locomotor activity between the pretreated group (47.75±3.28) and the rotenone-treated group (37.50±3.25), a significant increase was seen (*P*<.001). The recovery group's total locomotor activity score was significantly lower (18.75±3.28) than that of the control group (59.25±.3.11) and the rotenone-treated group (37.50±3.25), both with a *P-value* of less than .001. When comparing between the post-treated group (32.00±4.9) and the recovery group (18.75±3.28) total locomotor activity score, a significant upregulation was found *P*<0.001. The post-treated group showed a significant decrease in the total locomotor activity (32.00±4.9) compared to the pretreated group (47.75±3.28) *P*<0.001 (Figure 1).

Forced swim test

When compared to the control group (0.25±0.078), the rotenone treated group exhibited a significant elevation in the immobility duration (2.32±0.27) *P*<.001. When compared to the rotenone-treated group (2.32±0.27), the pretreated group's immobility duration (1.33±0.29) was significantly downregulated *P*<.001. while the recovery group showed a significant upregulation in the immobility duration (3.18±0.24) compared with the control group (0.25±0.078) and to the rotenone treated group (2.32±0.27) *P*<.001. The post-treated group demonstrated a significant decrease in the immobility duration (2.30±0.23) compared with the recovery group (3.18±0.24) *P*<0.001. The post-treated group showed a significant increase in the immobility duration (2.30±0.23) compared to the pretreated group (1.33±0.29) *P*<0.001 (Figure 1).

Y maze test

No significant difference exhibited in the percentage of alternation between the control (78.37±.4.62), rotenone treated group (77.25±2.25) and pretreated group (77.62±3.33) *P*>0.05. While the recovery group showed a significant decline in the percentage of alternation (30.62±3.11) compared with the control (78.37±.4.62) and to the rotenone treated one (53.62±3.85) *P*<0.001. The post-treated group showed a significant increase in the percentage of alternation (46.12±2.94) compared with the recovery group (30.62±3.11) *P*<0.001. The post-treated

group showed a significant decrease in the percentage of alternation (46.12±2.94) compared to the pretreated one (77.62±3.33) *P*<0.001 (Figure 1).

Biochemical results

Striatal dopamine level

When compared to the control group (0.81±.014), the rotenone-treated group's striatal dopamine level was significantly downregulated (0.40±.009) *P*<.001. When compared to the rotenone-treated group (.40±.009), the pretreated group's striatal dopamine level was significantly upregulated (0.69±.009) *P*<.001. The striatal dopamine level in the recovery group was significantly lower (0.19±0.01) than in the control group (0.81±.014) and the rotenone-treated group (0.40±.009), with a *P-value* of less than 0.001. The striatal dopamine level was significantly higher in the post-treated group (0.31±.001) than in the recovery group (0.19±0.01), *P*<0.001. The post-treated group showed a significant decrease in the striatal dopamine level (0.31±.001) compared to the pretreated group (0.69±.009) *P*<.001 (Figure 1).

Histopathological results of the frontal cortex

Coronal slices of the frontal cortex of the control group stained with hematoxylin and eosin revealed cortical neurons along with neuroglial cells appeared embedded in an eosinophilic background (neuropil). The most obvious types of cortical neurons were the pyramidal cells with a large vesicular open face nucleus, prominent nucleolus, basophilic cytoplasm and multiple processes. Granular cells appeared with a rounded open face nucleus and prominent nucleolus. Many types of glial cells were scattered among the cortical neurons. Astrocytes appeared with an oval vesicular nucleus. The oligodendroglia appeared with small dark nucleus with a perinuclear halo while microglia with a rod-shaped nucleus. Sections of the rotenone treated group showed degenerated neurons. The degeneration appeared either in the form of pyknotic hyperchromatic nuclei with a perinuclear halo or ghost like cells. The pretreated group showed that the cortical neurons were greatly protected against the damaging effect of rotenone with appearance of normal neurons and neuroglia except few degenerated neurons. The recovery group showed that the degeneration was more extensive in the form of pyknotic shrunken hyperchromatic nuclei or ghost like cells which was encountered here greatly. There was also congested blood vessels. As compared with the recovery group, the administration of nigella sativa oil after the rotenone significantly decreased the percentage of the degenerated neurons. Congested blood vessels also appeared (Figure 2).

Statistically, the percentage of the degenerated cells was significantly higher in the rotenone-treated group (34±2.72) than in the control one (1.87±.83) *P*<.001. When compared to the rotenone-treated group (34±2.72), the pretreated group's percentage of the degenerated cells was significantly downregulated (20.37±1.59) *P*<.001. The percentage of the degenerated cells was significantly higher in the recovery group (61.37±1.40) than in the control group (1.87±.83) and the rotenone-treated group (34±2.72), both *P*<.001. When comparing the percentage of the degenerated cells between the post-treated group (30.37±1.68) and the recovery group (61.37±1.40), a significant upregulation was observed in the post-treated one *P*<.001. The post-treated group showed a significant increase in the percentage of the degenerated cells (30.37±1.68) compared to the pretreated group (20.37±1.59) *P*<.001 (Figure 2).

Histopathological results of the hippocampus

Hematoxylin and eosin stained coronal slices of the CA1 area of the hippocampus proper from the control group showed its three layers; molecular, pyramidal and polymorphic layers. The pyramidal layer consisted of multiple regularly arranged rows of pyramidal neurons. Each neuron obtained large vesicular open face nucleus with a prominent nucleolus and basophilic cytoplasm. The nerve fibers were the main constituents of the molecular layer while the polymorphic layer showed different types of glial cells dispersed in an eisinophilic neuropil. Astrocytes appeared with an oval vesicular nucleus. The oligodendroglia appeared with small dark nucleus with a perinuclear halo. The rotenone treated group showed distorted pyramidal cell layer with an apparent decrease in the pyramidal cell number. Some cells appeard normal while others appeard with pyknotic hyperchromatic nuclei. There were many vacuolations appeard between the cells. Distorted dilated blood vessels also appeard. The pretreated group showed that the neurons were protected against the damaging effect of the rotenone with an appearance of normal neurons and neuroglia except few degenerated ones and vacuolated neuropil. The recovery group showed a complete distortion of the pyramidal layer with a degeneration of almost all the cells. The neuroglial cells in the polymorphic or molecular layers also showed signs of degeneration. The degeneration also here took the form of pyknotic hyperchromatic nuclei with a perinuclear halo. The blood vessels were dilated. Nerve fibers in molecular layer were not uniform compared with the control group. Many vacuolations were found in the neuropil.

The post-treated group showed a picture more or less better than that encountered in the recovery group. Some cells were protected from the extensive damaging effect of rotenone. The blood vessels here were also dilated with appearance of microglia cells and the neuropil still showing vacuolations (Figure 3).

Statistically, the rotenone treated group (28.79±1.08) exhibited a significant upregulation in the percentage of the degenerated cells compared with the control group (1.66±.44) *P*<.001. The pretreated group (8.96±1.12) exhibited a significant downregulation in the percentage of the degenerated cells compared with the rotenone treated group (28.79±1.08) *P*<.001. The recovery group (98.48±.83) showed a significant upregulation in the

percentage of the degenerated cells compared with the control group (1.66±.44) and to the rotenone treated one (28.79±1.08) *P*<.001. The post-treated group (64.37±2.6) showed a significant downregulation in the percentage of the degenerated cells compared with the recovery group (98.48±.83) *P*<.001. The post-treated group showed a significant increase in the percentage of the degenerated cells (64.37±2.6) compared to the pretreated group (8.96±1.12) *P*<.001 (Figure 3).

Immunohistochemical results of the frontal cortex

Regarding the Bax protein immunoreaction, the rotenone-treated group exhibited a significant rise in the area % of Bax protein positive immunoreaction (14.07±0.7) in comparison to the control group (0.12±.017) *P*<.001. When comparing the pretreated group (4.6±0.47) to the rotenone-treated group (14.07±0.7), there was a significant downregulation *P*<.001. The recovery group's area percentage of Bax protein positive immunoreaction was significantly higher than that of the control group (0.12±0.017) and the rotenone-treated group (14.07±0.7) *P*<.001. When compared to the recovery group (23.8±1.2), the post-treated group's area percentage of Bax protein positive immunoreaction was significantly downregulated (11.7±1.03) *P*<.001. The post-treated group showed a significant increase in the area % of Bax protein positive immunoreaction (11.7±1.03) compared to the pretreated group (4.6±0.47) *P*<.001 (Figure 4).

Regarding GFAP immunoreaction, the rotenone-treated group showed a markedly higher area % of GFAP positive immunoreaction (6.5±0.57) than the control group (2.1±0.86) *P*<0.001. When comparing the pretreated group (4.07±0.45) to the rotenone-treated group (6.5±0.57), a significant downregulation was observed (*P*<.001). The area percentage of GFAP positive immunoreaction in the recovery group was significantly elevated (11.6±1.3) than in the control group (2.1±0.86) and the rotenone-treated group (6.5±0.57) *P*<.001. When compared to the recovery group (11. 6±1.3), the post-treated group's area percentage of GFAP positive immunoreaction (7.4±0.70) was significantly downregulated *P*<.001. The post-treated group showed a significant increase in the area percentage of GFAP positive immunoreaction (7.4±0.70) compared to the pretreated group (4.07±0.45) *P*<.001 (Figure 4).

Regarding iNOS immunoreaction, the rotenone-treated group showed a substantial elevation in the area percentage of iNOS positive immunoreaction (8.23±0.76) as compared to the control group (0.12±0.008) *P*<.001. When compared to the rotenone-treated group (8.23±0.76), the pretreated group's area percentage of iNOS positive immunoreaction was significantly downregulated (3.58±0.48) *P*<.001. When compared to the control group (0.12±0.008) and the rotenone-treated group (8.23±0.76), the recovery group's area % of iNOS positive immunoreaction (14.31±1.03) was significantly upregulated *P*<.001. When comparing the area % of iNOS positive immunoreaction between the post-treated group (4.68±0.37) and the recovery group (14.31±1.03), the post-treated group showed a substantial downregulation *P*<.001. The post-treated group showed a significant increase in the area % of iNOS positive immunoreaction (4.68±0.37) compared to the pretreated group (3.58±0.48) *P*<.001 (Figure 5).

Regarding TNF-α immunoreaction, the rotenone treated group exhibited a significant upregulation in the area percentage of TNF-α immunoreaction (3.7±0.32) in comparison with the control group (0.22±0.018) *P*<.001. When compared with the rotenone-treated group (3.7±0.32), the pretreated group's area percentage of TNF-α immunoreaction was significantly downregulated (1.66±.21) *P*<.001. When comparing the recovery group's area % of TNF-α immunoreaction (8.9±0.45) to that of the control group (0.22±0.018) and the rotenone-treated group (3.7±0.32), a substantial upregulation was observed (*P*<.001). When compared to the recovery group (8.9±0.45) *P*<.001, the post-treated group (4.7±0.36) showed a substantial downregulation in the area percentage of TNF-α immunoreaction. The post-treated group showed a significant increase in the the area percentage of TNF-α immunoreaction (4.7±0.36) compared to the pretreated group (1.66±.21) *P*<.001 (Figure 5).

As regard Ki67 immunoreactivity, the group that received rotenone demonstrated a significantly lower percentage of Ki67 positive cells (33±1.8) in comparison with the control group (94.62±3.1) *P*<.001. When compared to the rotenone-treated group (33±1.8%), the pretreated group's percentage of Ki67 positive cells (80.62±2.3) was significantly higher (*P*<.001). The percentage of Ki67 positive cells in the recovery group (2.2±.54) was significantly lower than that in the control group (94.62±3.1) and the rotenone-treated group (33±1.8). The percentage of Ki67 positive cells was significantly elevated in the post-treated group (38.75±1.28) than in the recovery group (2.2±.54), *P*<.001. The post-treated group showed a significant decrease in the percentage of Ki67 positive cells (38.75±1.28) compared to the pretreated group (80.62±2.3) *P*<.001 (Figure 5).

Immunohistochemical results of the hippocamps

As regard Bax protein immunoreaction, the group that received rotenone demonstrated a significant rise in the area percentage of positive immunoreaction (2.8±0.15) when compared to the control group (0.18±0.014) with a *P-value* of less than 0.001. When comparing the pretreated group's area percentage of Bax protein positive immunoreaction (0.83±0.09) to the rotenone-treated group's (2.8±0.15), a substantial downregulation was observed (*P*<0.001). When compared to the control group (0.18±0.014) and the rotenone-treated group (2.8±0.15), the recovery group's area % of Bax protein positive immunoreaction (5.12±0.22) was significantly upregulated *P*<0.001. When comparing the area % of Bax protein positive immunoreaction between the post-treated group (3.26±0.27) and the recovery group (5.12±0.22), a significant rise was observed in the post-treated one *P*<0.001. The post-treated group showed a

significant increase in the area % of Bax protein positive immunoreaction (3.26±0.27) compared to the pretreated group (0.83±0.09) *P*<.001 (Figure 6).

Regarding GFAP immunoreactivity, the group treated with rotenone exhibited a noteworthy increase in the area percentage of GFAP positive immunoreactivity (6.53±0.30) in contrast to the control group (2.01±0.12), with a *P-value* of less than 0.001. When comparing the area % of GFAP positive immunoreaction between the pretreated group (4.58±0.32) and the rotenone-treated group (6.53±0.30), a substantial downregulation was observed (*P*<.001). The area percentage of GFAP positive immunoreaction (16.98±1.33) in the recovery group was significantly higher than that in the control group (2.01±0.12) and the rotenone-treated group (6.53±0.30) *P*<.001. When comparing the area % of GFAP positive immunoreaction between the post-treated group (7.86±0.44) and the recovery group (16.98±1.33), the post-treated group demonstrated a substantial downregulation *P*<.001. The post-treated group showed a significant increase in the area percentage of GFAP positive immunoreaction (7.86±0.44) compared to the pretreated group (4.58±0.32) *P*<.001 (Figure 6).

Regarding iNOS immunoreaction, the group treated with rotenone exhibited a noteworthy increase in the area percentage of iNOS positive immunoreaction (2.66±0.27) in contrast to the control group (0.16±0.04), with a *P-value* of less than .001. When compared with the rotenone-treated group (2.66±0.27), the pretreated group's area % of iNOS positive immunoreaction (1.48±0.19) was significantly downregulated, with a *P-value* of less than 0.001. When compared to the control group (0.16±0.04) and the rotenone-treated group (2.66±0.27), the recovery group's area % of iNOS positive immunoreaction (8.32±0.89) was significantly upregulated *P*<.001. In comparison to the recovery group (8.32±0.89), the post-treated group's area percentage of iNOS positive immunoreaction was significantly downregulated (3.16±0.29) *P*<.001. The post-treated group showed a significant increase in the area percentage of iNOS positive immunoreaction (3.16±0.29) compared to the pretreated group (1.48±0.19) *P*<.001 (Figure 7).

As regard TNF-α immunoreaction, the group that received rotenone treatment had a significantly higher area percentage of TNF-α immunoreaction (5.82±0.54) than the control one (0.08±0.01) *P*<.001. When compared to the rotenone-treated group (5.82±0.54), the pretreated group's area percentage of TNF-α immunoreaction was significantly downregulated (3.56±0.37) *P*<.001. The area percentage of TNF-α immunoreaction in the recovery group was significantly higher (12.73±1.72) than in the control group (0.08±0.01) and the rotenone-treated group (5.82±0.54), with a *P-value* of less than 0.001. When compared to the recovery group (12.73±1.72), the post-treated group's area percentage of TNF-α immunoreaction (5.65±0.45) was significantly downregulated *P*<.001. The post-treated group showed a significant increase in the the area percentage of TNF-α immunoreaction (5.65±0.45) compared to the pretreated group (3.56±0.37) *P*<.001 (Figure 7).

In the context of Ki67 immunoreactivity, the group treated with rotenone demonstrated a noteworthy decrease in the percentage of Ki67 positive cells (48.14±2.08) in contrast to the control group (93.12±2.27), with a *P-value* of less than .001. When compared to the rotenone-treated group (48.14±2.08), the pretreated group's percentage of Ki67 positive cells was significantly higher (80.71±2.9) *P*<.001. The percentage of Ki67 positive cells in the recovery group was significantly lower (0.17±0.02) than in the control group (93.12±2.27) and the rotenone-treated group (48.14±2.08), both with a *P-value* less than .001. The percentage of Ki67 positive cells was significantly higher in the post-treated group (67.22±3.5) than in the recovery group (0.17±0.02) *P*<.001. The post-treated group showed a significant decrease in the percentage of Ki67 positive cells (67.22±3.5) compared to the pretreated group (80.71±2.9) P<.001 (Figure 7).

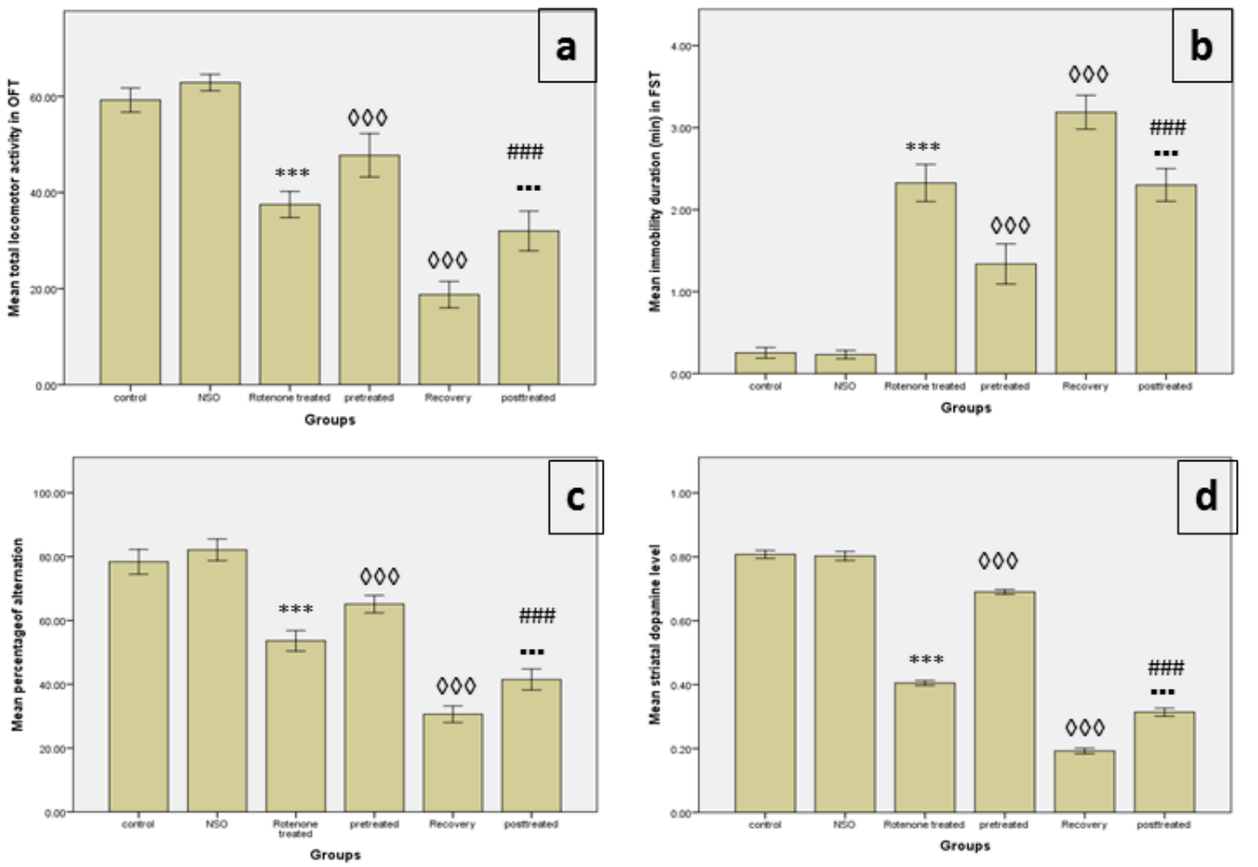

Fig. 1: (a, b, c, d) Mean total locomotor activity in OFT, mean immobility duration in FST, mean percentage of alternation in Y maze test and mean striatal dopamine level. ***$p < 0.001$ compared to the control group, ◊◊◊$p < 0.001$ compared to the rotenone treated group, ▪▪▪$p < 0.001$ compared to the recovery group. ###$P < 0.001$ compared to the pretreated group.

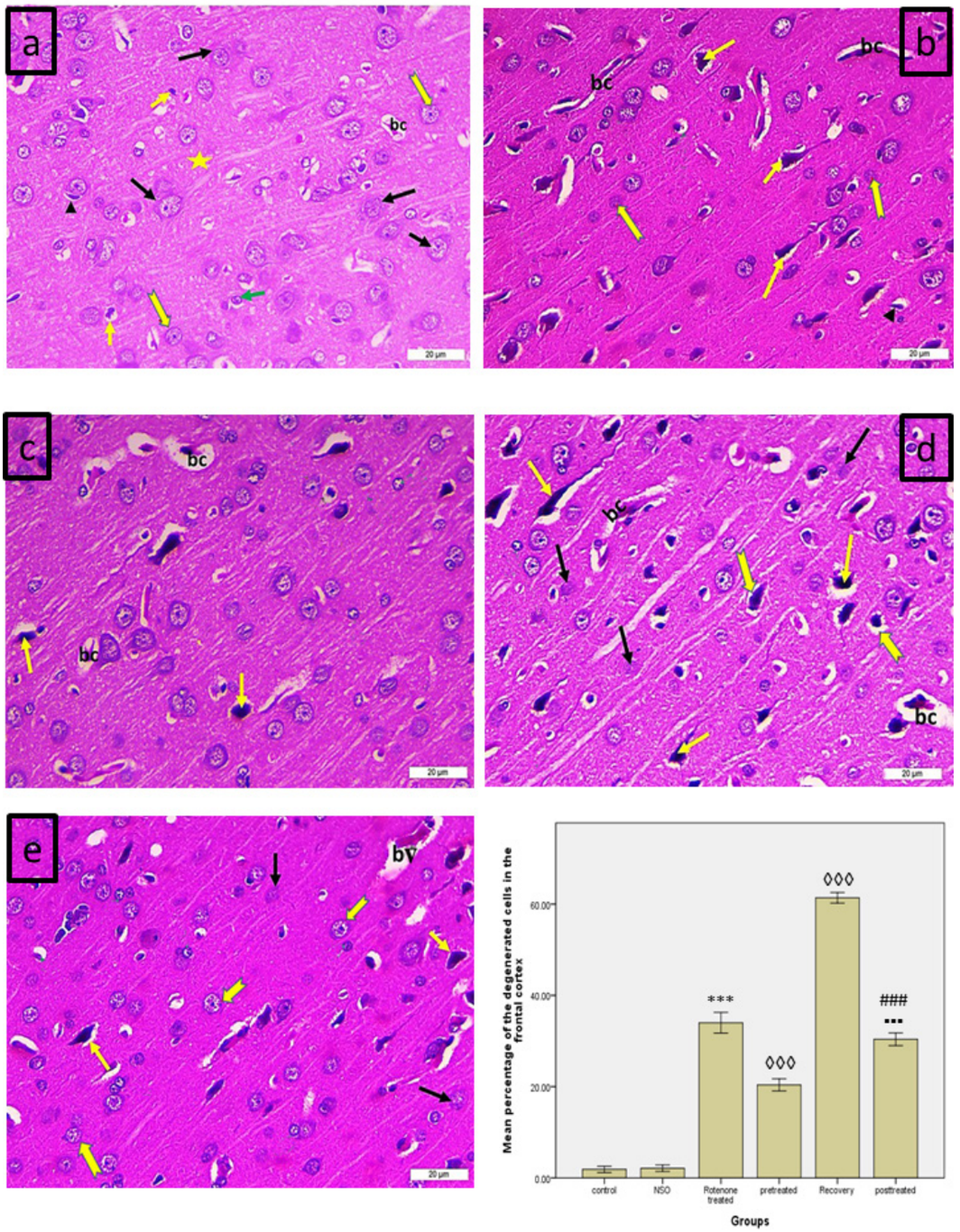

Fig. 2: H&E staining of coronal sections in the rat frontal cortex of the different groups. (A) The control group showing pyramidal (black arrow), granular (notched yellow arrow) and different types of glial cells; astrocyte (green arrow), oligodendroglia (yellow arrow) and microglia (arrow head) distributed within an eosinophilic neuropil (star). Blood capillaries also appear (bc). (b) The rotenone treated group showing many degenerated cells with hyperchromatic pyknotic nuclei surrounded by a perinuclear halo (yellow arrow). Dilated blood capillaries also appear (bc) with many ghost cells (notched yellow arrow). (c) The pretreated group showing few degenerated cells (yellow arrow) with dilated blood capillaries can be seen (bc). (d) The recovery group showing multiple degenerated cells with hyperchromatic pyknotic nuclei (yellow arrow) surrounded with a perinuclear halo (notched yellow arrow). Multiple ghost like cells appear (black arrow). Dilated congested blood capillaries can be seen (bc). (e) The post-treated group showing apparent decrease in the percentage of the degenerated cells (yellow arrow) compared to the recovery group. Ghost cell (black arrow) also appear among surrounded normal neurons (notched yellow arrow). Congested blood vessel also appears (bv). (Scale bar = 20 µm). ***$p < 0.001$ compared to the control group, ◊◊◊$p < 0.001$ compared to the rotenone treated group and ▪▪▪$p < 0.001$ compared to the recovery group. . ###$P < 0.001$ compared to the pretreated group.

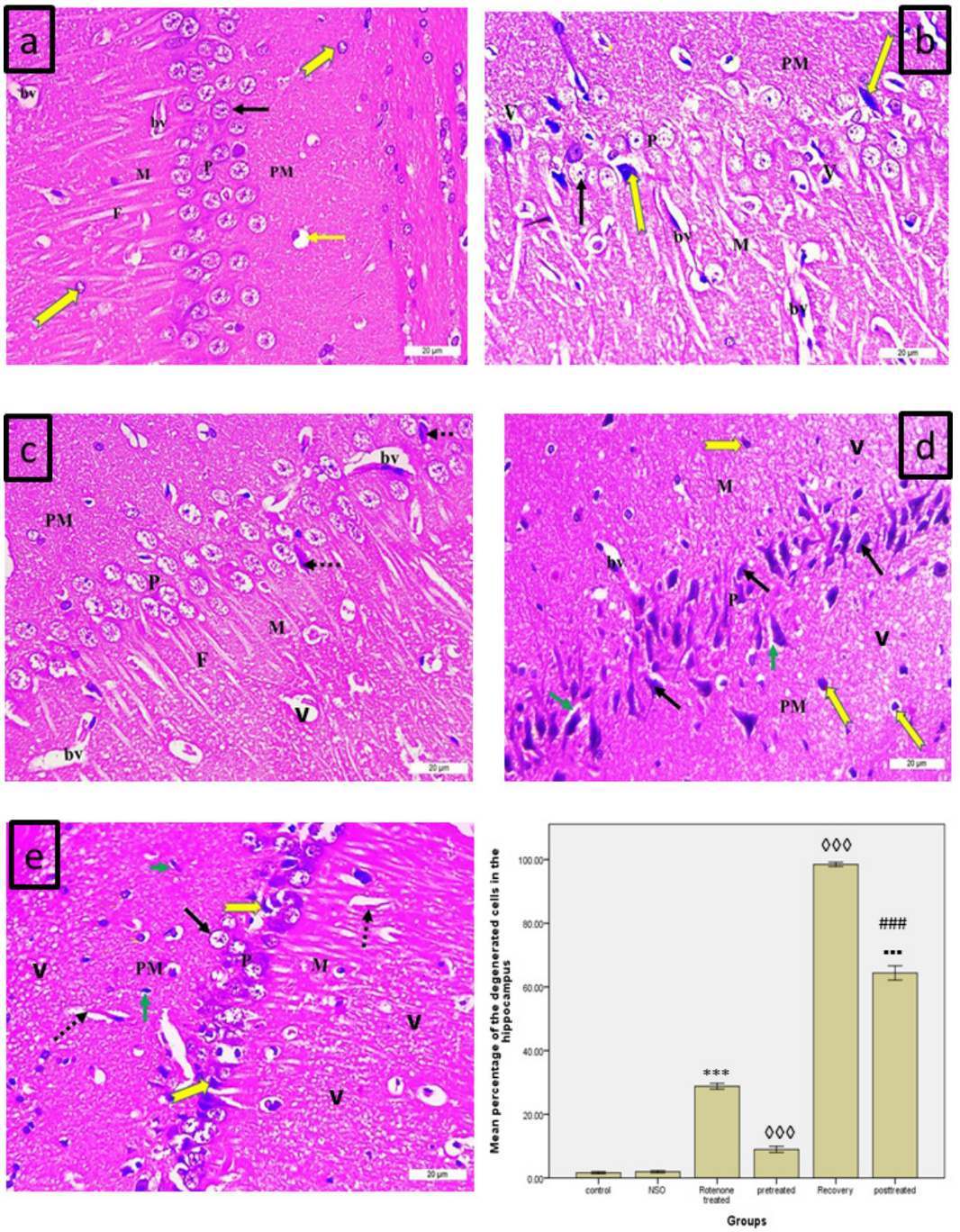

Fig. 3: H&E staining of a coronal section of the brain at CA1 region of the rat hippocampus of the different groups showing the molecular (M), pyramidal (P) and polymorphic (PM) layers. (a) The control group showing multiple regular rows of pyramidal cells (black arrow) with different neuroglial cells scattered in the eosinophilic neuropil of the polymorphic and molecular layers; astrocytes (notched yellow arrow) and oligodendroglia (yellow arrow). Blood vessel (bv) and multiple nerve fibers (F) appear in the molecular layer. (b) The rotenone treated group showing distorted pyramidal cell layer (P) with an apparent decrease in pyramidal cell number. Some cells are normal (black arrow), others appear with pyknotic nuclei (notched yellow arrows). Notice the vacuolations that appear between the cells (V). Distorted dilated blood vessels also appear (bv). (c) The pretreated group showing dilated blood vessel (bv) and few abnormal pyknotic nuclei (dashed arrow). Notice the vacuolation in the neuropil (v). (d) The recovery group showing almost all pyramidal cells in pyramidal layer are degenerated with pyknotic hyperchromatic nuclei (black arrows) and a perinuclear halo (green arrows). The neuroglial cells in the polymorphic (PM) or molecular (M) layers also show pyknosis with hyperchromasia (notched yellow arrows). A dilated blood vessel appears in the molecular layer (bv). Nerve fibers in molecular (M) layer are not uniform compared to control group. Notice the vacuolations in the neuropil (v). (e) The post-treated group showing disorganization of the pyramidal layer (P) with presence of few normal cells (black arrow). Others appear degenerated with pyknotic hyperchromatic nuclei surrounded with a perinuclear halo (notched yellow arrow). Microglia also appear with an elongated nucleus (green arrow). Dilated blood vessel (dashed arrow)) also appear. Notice the vacuolations in the neuropil (v). (Scale bar = 20 µm). ***$p < 0.001$ compared to the control group, ◊◊◊$p < 0.001$compared to the rotenone treated group and ▪▪▪$p < 0.001$ compared to the recovery group. ###$P < 0.001$ compared to the pretreated group.

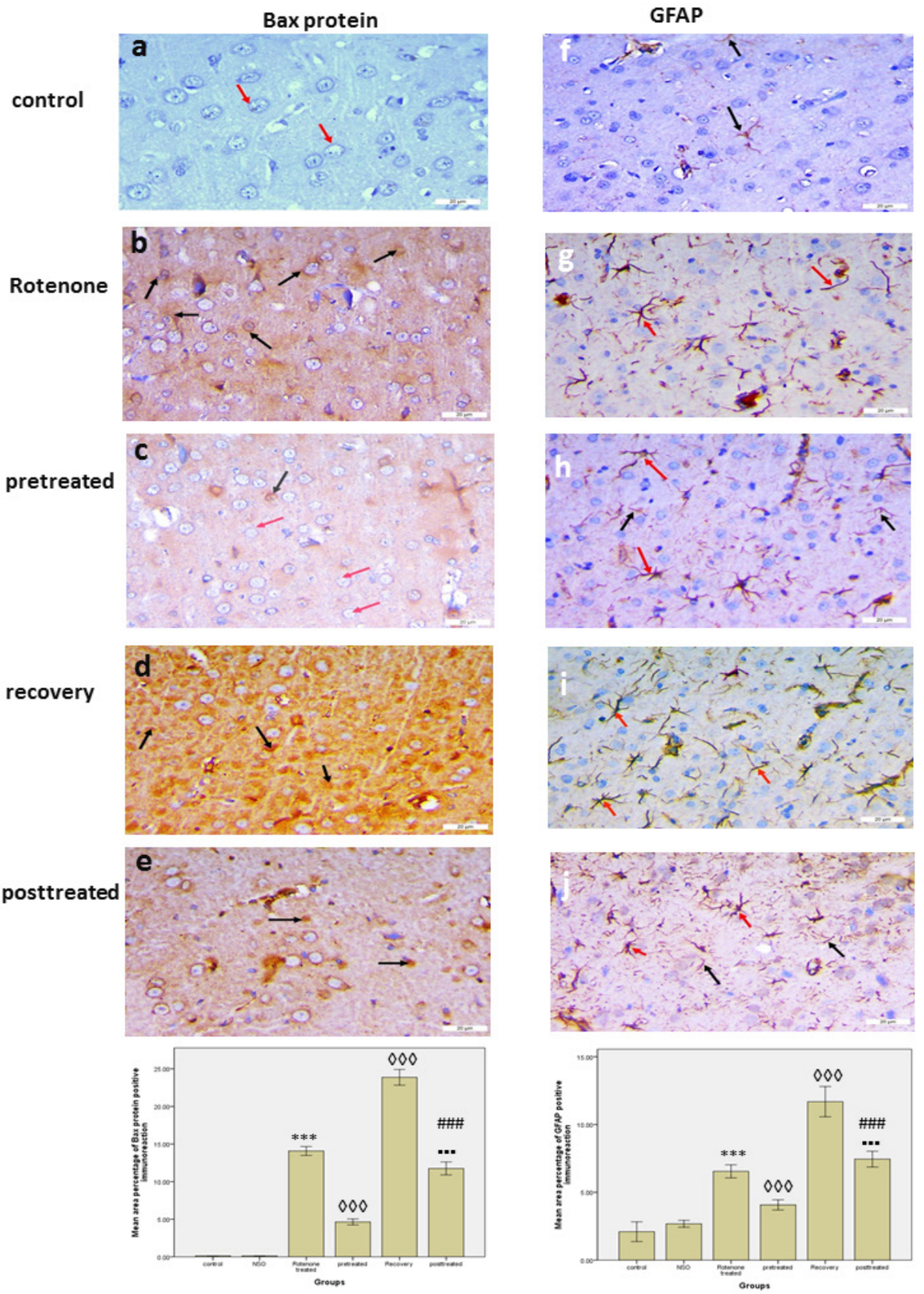

Fig. 4: Representative immunostaining of the rat frontal cortex of the different groups showing a significant increase in the immunoreactivity to Bax protein (a-e) and GFAP (f-g) in the rotenone treated and recovery groups. Administration of NSO in the pretreated and post-treated groups significantly attenuated the reaction compared to the rotenone treated and recovery groups respectively. (Scale bar = 20 µm). ***p < 0.001 compared to the control group, ◊◊◊p < 0.001compared to the rotenone treated group and ▪▪▪p < 0.001 compared to the recovery group. ###P < 0.001 compared to the pretreated group.

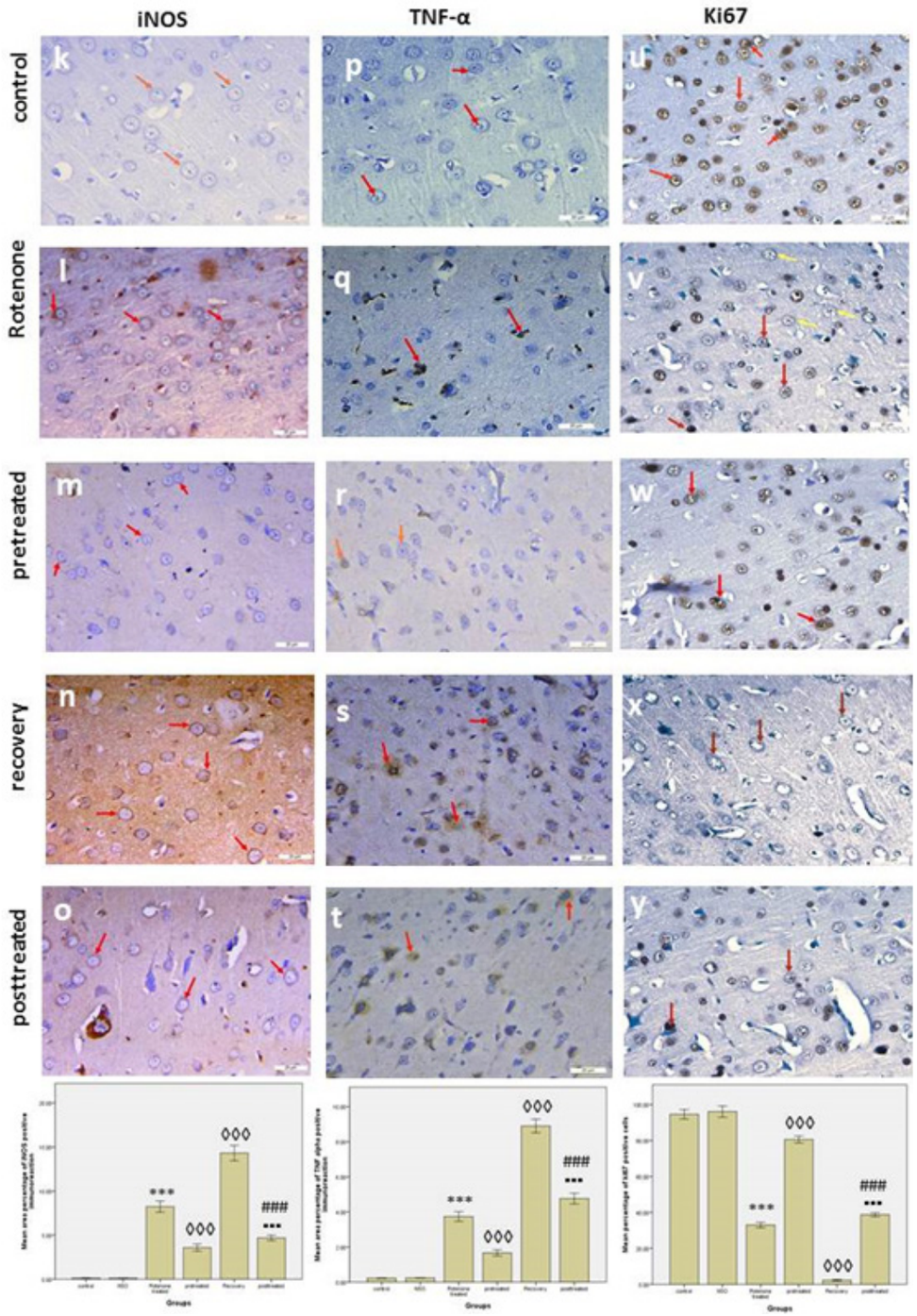

Fig. 5: Representative immunostaining of the rat frontal cortex of the different groups showing a significant increase in the immunoreactivity to iNOS (k-o) and TNF-α (p-t) in the rotenone treated and recovery groups. Administration of NSO in the pretreated and post-treated groups significantly attenuated the reaction compared to the rotenone treated and recovery groups respectively. Ki67 (u-y) immunostaining showed significant decrease in the rotenone treated group and recovery group which was significantly increased in the pretreated group compared to the rotenone and post-treated groups compared to the recovery. ***p < 0.001 compared to the control group, ◊◊◊p < 0.001compared to the rotenone treated group and ▪▪▪p < 0.001 compared to the recovery group. ###P < 0.001 compared to the pretreated group.

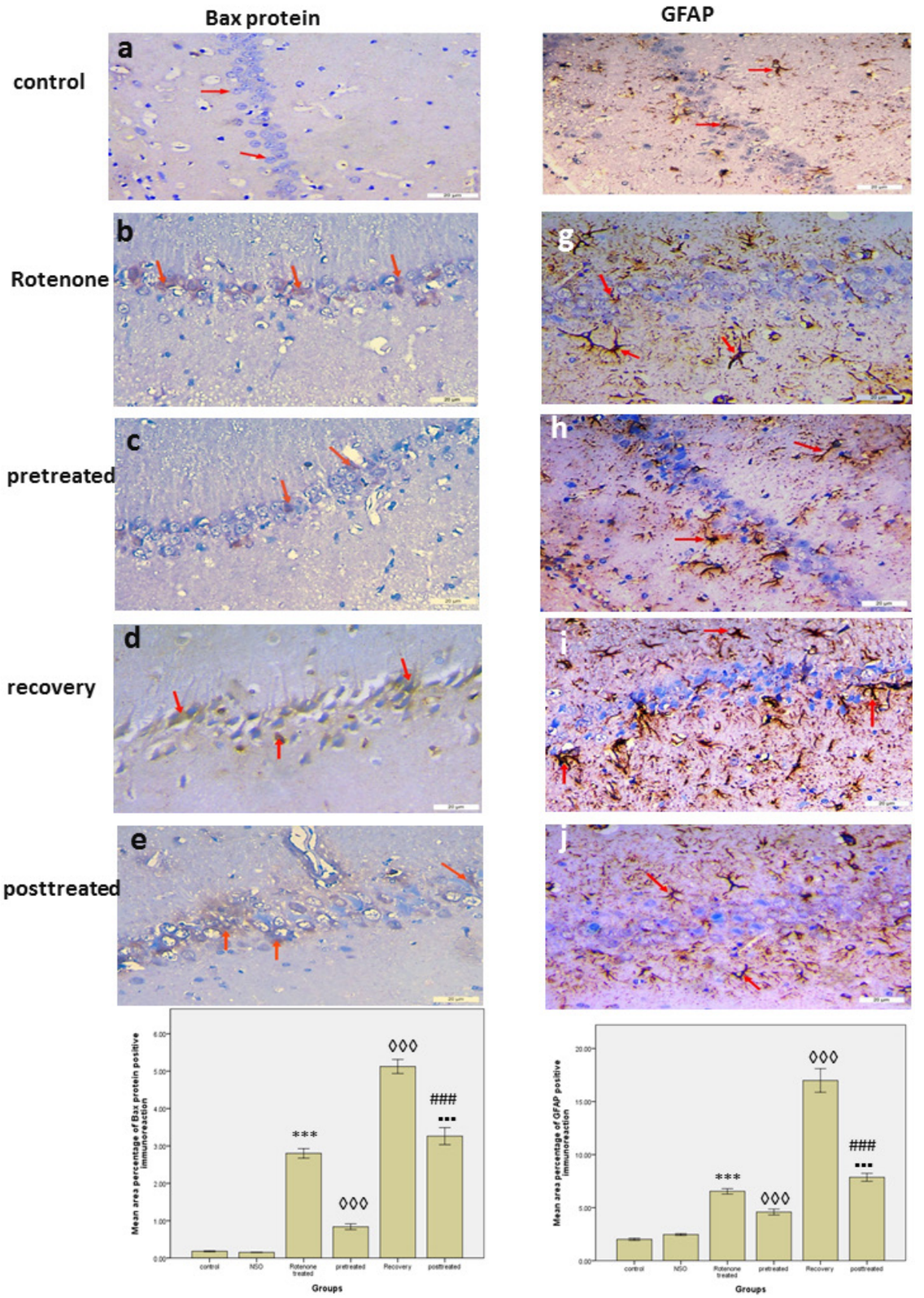

Fig. 6: Representative immunostaining of the CA1 region of the rat hippocampus of the different groups showing a significant increase in the immunoreactivity to Bax protein (a-e) and GFAP (f-j) in the rotenone treated and recovery groups. Administration of NSO in the pretreated and post-treated groups significantly attenuated the reaction compared to the rotenone treated and recovery groups respectively. ***$p < 0.001$ compared to the control group, ◊◊◊$p < 0.001$compared to the rotenone treated group and ▪▪▪$p < 0.001$ compared to the recovery group. ###$P < 0.001$ compared to the pretreated group.

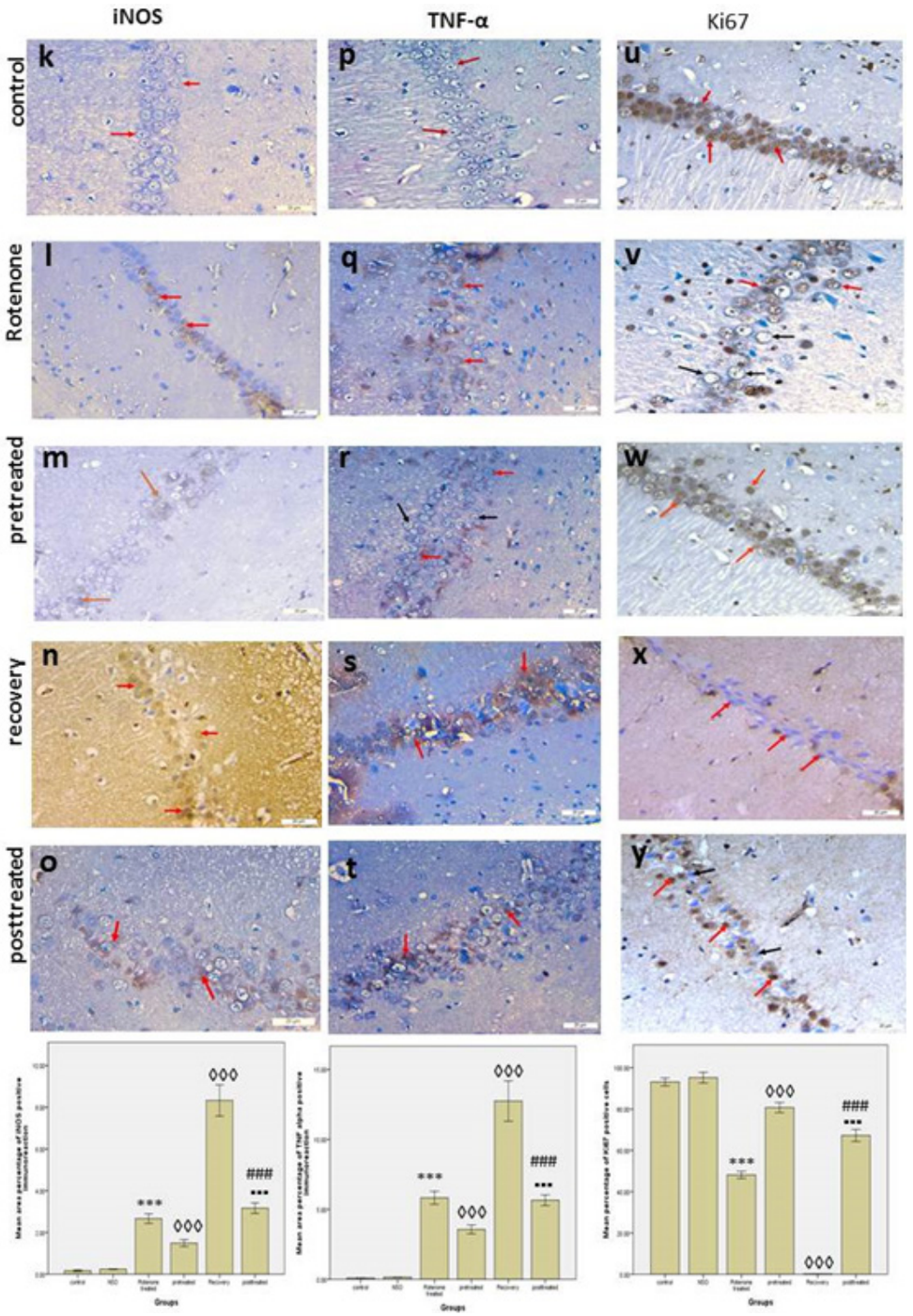

Fig. 7: Representative immunostaining of the CA1 region of the rat hippocampus of the different groups showing a significant increase in the immunoreactivity to iNOS (k-o) and TNF-α (p-t) in the rotenone treated and recovery groups. Administration of NSO in the pretreated and post-treated groups significantly attenuated the reaction compared to the rotenone treated and recovery groups respectively. Ki67 (u-y) immunostaining showed significant decrease in the rotenone treated group and recovery group which was significantly increased in the pretreated group compared to the rotenone and post-treated groups compared to the recovery. ***p < 0.001 compared to the control group, ◊◊◊p < 0.001compared to the rotenone treated group and ▪▪▪p < 0.001 compared to the recovery group. ###P < 0.001 compared to the pretreated group.

DISCUSSION

The symptoms of Parkinson's disease (PD), motor and non-motor, are commonly well recognized. Motor symptoms are due to changes that occur in the nigrostriatal system, while the non-motor ones are due to affection of other brain regions[1].

Much attention has been given to the changes that occur in the nigrostriatal system. Here we paid attention to the changes that occur in the prefrontal cortex and the hippocampus since they are the reason for the cognitive impairment encountered in this disease[23].

The rotenone induced model was chosen in this study as it is implicated in many pathogenic pathways that mediate dopaminergic cell death including nigral iron accumulation that lead to ferroptosis with subsequent oxidative stress and neuroinflammation. Rotenone also causes α-synuclein phosphorylation and aggregation, LB pathology and proteasomal dysfunction[38].

In the present study, OFT and FST were performed to ensure the disease induction[16]. Regarding the OFT, it was shown that the recovery and rotenone-treated groups had significantly lower total locomotor activity when compared to the control one. This was in accordance with Monir *et al*.[17] and Ali *et al*.[24] who declared that the OFT's locomotor activity had decreased in the rotenone induced models of PD. This may be due to degeneration of the midbrain dopaminergic neurons with subsequent hypodopaminergic status that affect basal ganglia circuits implicated in selection and planning of movement as postulated by Masini, Kiehn[25]. This coincides with Venkateshgobi *et al*.[26] who reported that decreased exploration and square crossing in rotenone- lesioned animals might be linked to the dopaminergic neuron loss.

This clinical observation was confirmed biochemically by a significant downregulation in the striatal dopamine in the rotenone treated and the recovery group.

Also, PD is characterized clinically by a group of non-motor symptoms like depression Fontoura *et al*.[27]. So, FST was performed to examine the depressive like behavior of the animals. In our study, the immobility period was significantly longer in the recovery and rotenone-treated groups than in the control group. This may be as the result of a decline in the dopamine level which is greatly implicated in the pathophysiology of the depression. These results also were reported by McDowell, Chesselet[28] and Arida *et al*.[16]. Arida *et al*.[16] explained this by depletion of the dopamine due to affection of the dopaminergic connection to the mPFC and hence affecting the function of the cortex.

In the recovery group, OFT and FST were more affected than in the rotenone treated one. This may be as the result of the disease's progressive nature. This was in line with Lard *et al*.[29] who clarified that when rats were given rotenone for 14 days, their motor behavior significantly decreased beginning on the seventh day of the trial and continued to drop until the 28th. The experiment's 28th day had the least amount of motor activity.

To assess the spatial working memory, one of the cognitive functions of the brain, which require intact hippocampus, prefrontal cortex and the neural connection between them, the Y maze test was conducted. The control, rotenone treated and pretreated groups showed non-significant difference as regard spontaneous alternation percentage. This may be due to the pathological changes that occur in rotenone treated group were not more severe enough to be manifested clinically.

When compared to the rotenone-treated group, the recovery group's percentage of alternations significantly decreased. This may be due to the severe degenerative changes that encountered here manifest itself by a positive test. This conclusion coincides with Weir[30] who stated that the pathology in the brain accumulates for many years or decades before the dementia and other cognitive symptoms manifest itself. Also, Halliday *et al*.[31] Postulated that the pathologic alterations in PD occur gradually over years with a period of clinically asymptomatic cellular dysfunction.

As far as we know, this is the first research to investigate the spatial working memory through Y maze test in a recovery group of rotenone model of PD.

This was supported by the results of the histopathology that showed a significant impairment in all observed data in the recovery group in comparison with the rotenone treated group.

As regard the histopathological results, hematoxylin and eosin-stained sections of the frontal cortex and CA1 area of the hippocampus revealed a picture of degeneration. These findings were concordant with Gaballa *et al*.[32] and Fikry *et al*.[11] who declared that the rotenone`s degenerative changes were not restricted only to the substania nigra, but also affected other brain areas, including the striatum, cerebral cortex, cerebellum and hippocampus. This picture of degeneration may be probably apoptotic changes. This was confirmed in our research by over-expression of Bax protein. Abdel-Salam *et al*.[33] reported the same result. Also, dilated congested blood vessels were also encountered here in the histopathological results which may be probably a sign of inflammation. These finding can be owed to the nature of the rotenone in causing a state of inflammation and oxidation in the tissue as mentioned by Khadrawy *et al*.[34] and Azmy *et al*.[35] who declared that the pathological changes caused by the rotenone may be the result of rotenone induced neuroinflammation, mitochondrial dysfunction, energy deficiency and oxidative stress.

As regard the neuroinflammatory changes, there was a significant upregulation of TNF- α immunoreaction in the rotenone treated and the recovery group in the present study. This was in line with Sherer *et al*.[36], Sharma *et al*.[37] and Radad *et al*.[38] who found that in rat brain tissues, rotenone enhanced the release of pro-inflammatory mediators such as TNF-α and IL-6.

As regard the oxidative changes, there was an upregulation of iNOS in the frontal cortex and hippocampus of the rotenone treated and recovery groups with a consecutive increase in the level of nitric oxide. Superoxide and nitric oxide can combine directly to form peroxynitrite and nitrogen dioxide[39], which are potent oxidizers that react with DNA, proteins, and lipids and destroy them[40]. This was consistent with Abdel-Salam *et al*.[41] who clarified that in mice and rats, rotenone itself causes the substantia nigra and striatum to express iNOS. Moreover, Gao *et al*.[42] stated that iNOS specific inhibitor protects the rat from the rotenone induced neurotoxicity.

In our research, there was a significant upregulation in the GFAP immunoreaction of the tissue, marker for astrogliosis, in the rotenone treated group and the recovery group. Astrocytes release GFAP and neurotoxic compounds in response to any insult to the nervous system. GFAP is therefore thought to be an indicator of astrogliosis[43]. This may probably be a reactive astrogliosis or secondary to the oxidative stress. ROS causes activation of astrocytes and microglia with a subsequent rise in the produced proinflammatory mediators, which triggers the apoptotic pathways and causes neurodegeneration as mentioned by Sharma *et al*.[37].

This was matching with the findings of Fikry *et al*.[11] who reported a rise in the astrocytic activity and an increased GFAP expression in the brain tissue after rotenone treatment. They explained this to be a compensatory mechanism to the neurodegeneration. On the other side, Abdel-Salam *et al*.[44] and Arida *et al*.[16] who found a substantial drop in the GFAP immunoexpression in the frontal cortex of the group receiving rotenone as opposed to the control group. Arida *et al*.[16] attributed this to the astrocytes' deterioration due to an accumulation of α synuclein.

Adult neurogenesis is the process by which neural stem cells found in the cerebellum, cerebral cortex, substantia nigra, subventricular zone (SVZ), and subgranular zone (SGZ) of the hippocampus give rise to new neurons[45]. Learning, tissue healing, memory consolidation, and mood modulation all depend on the adult neurogenesis[46].

Ki67 is an efficient immunohistochemical marker for detection of adult neurogenesis as mentioned by Allen *et al*.[47]. Upon using Ki67 staining, this study showed a significant downregulation of Ki67 immunoexpression of the tissue in the rotenone treated and recovery group in comparison to the control one. This finding was consistent with the finding of Wang *et al*.[48] who reported that the expression of ki67 was decreased by 58% in the SVZ of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Also, Scopa *et al*.[49] demonstrated that the patients with PD show an impaired neurogenesis.

The mechanism of defective neurogenesis in PD still unknown but many previous studies explained that either by deficiency of dopamine as SVZ receives dopaminergic afferents from the substantia nigra, alpha synuclein accumulation in SVZ or SGZ or by mitochondrial dysfunction[50,51,52].

Nigella sativa and its isolated component thymoquinone have antioxidant, anti-inflammatory and neuroprotective effects[9].

Nigella sativa (500 mg pill twice daily for nine weeks) was shown by Bin Sayeed *et al*.[53] to enhance memory, cognition, and attention in healthy volunteers. Cascella *et al*.[54] confirmed the NSO effects on learning and memory in adult male rat. So, in the present research, the promising effect of NSO in preventing and /or reversing the pathological changes that occur in frontal cortex and hippocampus was studied.

This is the first work according to our knowledge to investigate the outcome of NSO on the frontal cortical and hippocampal pathological changes in a PD rat model.

Upon using the NSO as a protective agent before and during the rotenone administration, it significantly protected the neurons from the damaging effects of this toxin. Moreover, its administration after the rotenone has already exerting its deleterious effects on neurons; it also succeeded in damping its toxic effects on the tissues on the long run but, its administration as a protective agent exceeds its usage as a therapeutic agent in PD.

As regard the biochemical results, when comparing the pretreated group to the rotenone-treated one and the post-treated group to the recovery group, the administration of NSO markedly raised the striatal dopamine levels. This was evident clinically by improvement in the results of the behavioural tests; OFT and FST.

The histopathological results revealed its ability to prevent the degenerative alterations occurred as a result of rotenone in both the frontal cortex or CA1 region of the hippocampus to a great extent in pretreated group when contrasted with the rotenone treated one. While, in the post-treated group, it dampened the effect of the rotenone with decline in the neurodegenerative changes in the studied tissues compared with the recovery one. This was obvious by a noteworthy decline in the percentage of the degenerated cells which was confirmed by a decrease in Bax protein immunoreaction.

This protective ability of the NSO can be explained by its richness with several bioactive molecules as thymoquinone which was well known by its antioxidant, anti-inflammatory and multiorgan safeguarding effects. This was confirmed in this work by a significant downregulation of TNF-α and iNOS immunoexpression either in the frontal cortex or the hippocampus.

These finding cope with the work of Sedaghat *et al*.[55] who declaired that TQ pretreatment for intra-striatal 6 hydroxy dopamine lesioned rats significantly prevented neuronal loss in substantia nigra and lowered level of MDA. They explained this by the ability of thymoquinone to attenuate lipid peroxidation in tissue. Also, Aminu *et*

al.[56] reported that NSO was important in preventing the increase in the ROS and NO production in the hippocampus of dichlorvos exposed rodent.

Bargi *et al*.[57] demonstrated that Rats with lipopolysaccharide-induced memory deficits showed improvement with TQ.

Also, administration of NSO caused a significant downregulation of GFAP immunoreaction either in the frontal cortex or in CA1 region of the hippocampus. This may be probably due to decreased percentage of degenerated cells with subsequent decrease in reactive gliosis or due to decreased oxidative stress in tissue which cause astrocytes activation. This goes in line with the study of Nor-Eldin and Elsayed.[58] who found that, in comparison to the group exposed to the x-rays, nigella sativa dramatically reduced the number of GFAP positive cells in the cerebral cortex.

As regard the results of Ki67 immunohistochemical reaction, NSO administration caused a significant upregulation of Ki67 immunoexpression in both the frontal cortex and CA1 area of the hippocampus. This also can be explained by the antioxidant and neuroprotective effects of NSO. Its administration protected brain stem cells from rotenone-induced oxidative damage and neuroinflammatory alterations. This is consistent with Imam *et al*.[59], who said that rats treated with chlorpyrifos showed a drop in Ki67 expression in the SGZ, indicating a decrease in the proliferation of adult-born neurons. Otherwise, the rats treated with NSO and chlorpyrifos demonstrated an increase in Ki67 in the SGZ.

CONCLUSION

In conclusion, this work revealed that the frontal cortex and the CA1 area of the hippocampus were greatly affected pathologically in PD especially after a period of the establishment and diagnosis of the disease which translated clinically by an impairment in the results of Y maze test. So, affection of these two regions in PD share to a great extent to the cognitive impairment encountered in PD patients. Administration of NSO may protect the risky individual from catching the disease or at least delay the onset of its occurrence as it protected the neurons from degeneration. Also, its administration after establishment of the disease may decrease the rate of the disease progress and postpone the onset of symptoms related to cognitive impairment.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تأثير زيت حبة البركة علي القشرة المخية الجبهية وقرن آمون في نموذج الشلل الرعاش المستحث بالروتينون في ذكر الجرذ الأبيض البالغ

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المقدمة: يعد الشلل الرعاش ثاني أكثر الاضطرابات العصبية انتشارًا في جميع أنحاء العالم بعد مرض الزهايمر كما ان التدهور المعرفي والخرف الذي يصاحبه يعد من أكثر الأعراض المنهكة التي تؤثر على مرضى الشلل الرعاش. يحتوي زيت حبة البركة على خصائص مضادة للأكسدة ومضادة للالتهابات وواقية للأعصاب.

الهدف: دراسة التغيرات التي تحدث في القشرة الدماغية الأمامية وقرن آمون في نموذج الشلل الرعاش المستحث تجريبيا في الفئران وكذلك دراسة دور زيت حبة البركة علي هذه التغيرات. واستند هذا على التحليلات السلوكية والكيميائية الحيوية والنسيجية والكيميائية المناعية والقياسات الكمية.

مواد و طرق البحث: تم استخدام ستون من ذكور الفئران البيضاء البالغة وقد تم تقسيمهم إلى ست مجموعات: المجموعه الضابطة، مجموعة زيت حبة البركة ٣ مجم / كجم / يوم عن طريق الفم لمدة ٣٠ يومًا، المجموعة المعالجة بالروتينون (١,٥ مجم / كجم وزن الجسم / يوم تحت الجلد خلال الأيام العشرة الأخيرة)، المجموعة المعالجة مسبقًا بـ زيت حبة البركة لمدة ٢٠ يومًا يليها اضافة روتينون مع زيت حبة البركة لآخر ١٠ أيام من مدة التجربة، مجموعة الاسترداد: (١,٥ مجم/كجم/يوم تحت الجلد للأيام العشرة الأولى) والمجموعة المعالجة بـالروتينون ثم زيت حبة البركة. تم إجراء الاختبارات السلوكية بما في ذلك اختبار المجال المفتوح، واختبار السباحة القسرية، واختبار المتاهة Y بالإضافة إلى التحليلات الكيميائية الحيوية. بعد ذلك تم تخدير الحيوانات ثم تم قطع رأسها واستئصال أدمغتها ، تم إخضاع الدماغ للدراسات النسيجية والكيميائية المناعية.

النتائج: أظهرت القشرة الدماغية الأمامية ومنطقة CA١ في قرن آمون تغيرات هستولوجية شديدة في مرض الشلل الرعاش خاصة في مجموعة التعافي. وقد تجلى ذلك سريريًا من خلال ضعف الذاكرة العاملة المكانية. كان ذلك مصحوبًا بزيادة التعبير المناعي لبروتين Bax وGFAP وTNF-α وiNOS مع انخفاض في التعبير المناعي Ki٦٧.

الاستنتاج: قد يؤدي تناول زيت حبة البركة إلى حماية الشخص الأكثر عرضة للاصابة بمرض الشلل الرعاش من الاصابة به أو على الأقل تأخير ظهوره.. كما أن تناوله بعد ظهور المرض قد يقلل من معدل تطور المرض ويؤخر أيضًا تطور أعراض الضعف الإدراكي.