The Impact of Recombinant Human Erythropoietin Treatment on Motor Impairment in Rotenone-Induced Parkinsonism in Rats

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Abstract

Background: Parkinson's disease is a neurodegenerative disease mainly characterized by loss of dopaminergic neurons in the substantia nigra pars compacta and their terminals in the striatum. The development of neuroprotective drugs that slow or delay neurodegeneration became of a considerable interest. In numerous animal models, exogenously administered EPO exhibits neuroprotective effects. *Aim:* The current research investigated the impact of administration of recombinant human EPO (rhEPO) in rotenone parkinsonian rats. *Materials and Methods:* Thirty two adult male albino Sprague-Dawly rats were equally and randomly divided into four groups; group 1 the vehicle-treated group, group 2 rotenone-treated group, group 3 treated with rotenone in addition to intranasal rhEPO and group 4 treated with rotenone in addition to intraperitoneal rhEPO. The motor performance of the rats was evaluated. Malondialdehyde and reduced glutathione were assayed. Blood indices were measured. Histopathology of the substantia nigra was also done. *Results:* Results showed that rotenone-treated rats exhibited significant impairment of motor coordination and marked degeneration of substantia nigra neurons was observed. Both intranasal and intraperitoneal rhEPO treatment improved the motor deficit and significantly increased the number of neurons in the SNpc. intraperitoneal rhEPO significantly increased lipid peroxide and significantly affected blood indices. *Conclusion:* Our findings suggest that, EPO may have neuroprotective effect in PD. Systemic rhEPO neuroprotective effects may be attenuated by its adverse effects such as increase of OS in the vascular system and stimulation of erythropoiesis. Small doses of intranasal EPO may be sufficient to produce neuroprotection without affecting erythropoiesis and further researches are required to address the mechanisms of neuroprotective effects of EPO.

Keywords: *neuroprotection; oxidative stress; neurodegeneration*

Introduction

Parkinson's disease (PD) is a neurodegenerative disease. It is characterized clinically by motor complications (asymmetric onset of bradykinesia, rigidity, resting tremor), and non-motor complications (depression, sleep disturbances, memory deficits). These complications are mainly the outcome of neurodegeneration of the substantia nigra pars compacta (SNpc), which leads to subsequent reduction of dopaminergic input

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to the striatum[1]. Although the pathogenesis of PD is not completely understood, reactive oxygen species (ROS) over-generation, oxidative stress and mitochondria dysfunction are well recognized in the pathogenesis of PD[2,3]. Rotenone induces systemic inhibition of mitochondrial complex I and cause selective nigrostriatal dopaminergic degeneration[4]. The dopaminergic neurodegeneration caused by rotenone is mediated through contribution of reactive oxygen species (ROS), specifically nitric oxide (NO) and hydroxyl radicals (˙OH)[5]. Erythropoietin (EPO) is a cytokine that acts on erythroid progenitor proliferation and differentiation. It has been reported that, EPO might have a direct protective role against a variety of neurotoxic insults, such as free radical injury[6], and exposure to neurotoxic agent[7,8]. EPO has been shown to have beneficial effects against oxidative stress[9], and inflammation[10,11]. In the brain, EPO increases in response to oxidative or nitrosative stress[12]. Protection against oxidative stress by EPO involves preventing excitotoxicity[10,13] and free radical exposure[14,15]. Recombinant human EPO (rhEPO) was proved to have neuroprotective effects in several models of nervous tissue damage[16,17]. A study in Parkinson's disease patients using rhEPO produced in Cuba (ior-EPOCIM) found that, (ior-EPOCIM) was safe and the preliminary positive responses in motor, cognitive and affective domains that were observed could suggest its potential effectiveness as a treatment for PD[18]. An exploratory pilot study investigated the effects of (rhEPO) on motor and non-motor symptoms (NMS) in PD patients. It found that rhEPO had beneficial effects on NMS but not on motor function[19]. Nasal drug delivery has major advantages including direct transport of absorbed drug into systemic circulation and bypassing first-pass effect of liver and gastrointestinal tract so that dose can be minimized, bypassing the blood brain barrier, providing an alternative to invasive methods of drug administration, lower enzymatic activity when compared with gastrointestinal tract and liver, avoidance of gastrointestinal membrane irritation, reduced risk of overdose and infection, self-medication and ease of convenience that increases patients' compliance. Recently, several studies confirmed the delivery of EPO to the brain via the nasal route by avoiding the blood brain barrier, and reported that this pathway could be safer and 10 times faster than the intravenous route[20]. Taken together, these results offered an interesting perspective to our research, which aimed to investigate the neuroprotective effects of rhEPO on rotenone-induced Parkinsonism in rat.

Materials and Methods

This study was performed at the Physiology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

Animals
Thirty-two adult male albino Sprague-Dawly rats (body weight 120 -180 gm) purchased from center for experimental animals, Faculty of Veterinarian Medicine, Zagazig University were used in the study. All rats were left to acclimatize for one week prior to the experiment and were housed in plastic cages maintained at controlled room temperature (22-24 C) with 12-hour diurnal (day and night change) with free access to standard pellet animal diet and tap water. The animals were equally and randomly divided into four groups; Group 1: (the vehicle-treated group), group 2: (rotenone-induced parkinsonism without intervention group), group 3: (rotenone-induced parkinsonism with intranasal recombinant human erythropoietin treatment group), and group 4: (rotenone-induced parkinsonism with systemic

(intraperitoneal) recombinant human erythropoietin treatment group).

Induction of experimental parkinsonism:
Rotenone (Sigma-Aldrich, MO, USA) was dissolved in 1:1 (v/v) dimethylsulfoxide (DMSO, Sigma-Aldrich, MO, USA) and polyethylene glycol (PEG-300; Sigma-Aldrich, MO, US). Rats in group 2, 3 and 4 were injected with rotenone (1.5 mg/kg subcutaneously) every 48 hours ± 2 hours. Total number of injections was six injections. Group 1 received six subcutaneous injections of the vehicle (1:1, DMSO/PEG-300) with the same schedule as other groups [21].

Treatment with recombinant human erythropoietin (rhEPO):
In group 3, 2.4 IU/rat of rhEPO (EPICO, Egypt) were administered intranasal [22]. While, in group 4, rhEPO (EPICO, Egypt) was injected intraperitoneally (5000 IU/kg) 30 minutes before each rotenone injection.

Functional assessment:
Twenty four hours after the last injection of rotenone (day 12), rats were screened for motor impairments using the open-field test and rota-rod test.

A- Open field test:
Assessment was done in the open field apparatus which consists of a rectangular box arena with the measurements 115 X 115 X 44 cm. The arena floor is painted with dark lines that form a 5 X 5 cm rectangular unit's pattern [23]. Rats were introduced individually into the center of the open field arena and behavioral parameters were observed for 5 minutes, the observer recorded ambulation frequency (the number of squares crossed), number of stops and the vertical movement (rearing frequency). Furthermore, activity index (the number of squares passed in a locomoting interval =total number of squares divided by the total number of stops) was calculated for each rat[21].

B- Rota-rod test:
To assess balance and motor coordination, each rat was placed on a rotating rod (10 cm long and 4 cm in diameter). The rod rotation speed was 20 rpm. Each rat was left on the rotating rod and the time spent from putting the animal on the shaft of the rotarod, till it falls to the ground (latency) was recorded. The maximal time that was allowed for each rat was 5 minutes [24].

Measurement of blood indices:
After functional assessment, rats were anesthetized and one milliliter of blood was withdrawn from retro-orbital venous plexus from each rat into an EDETA-containing eppendorfs and the following tests were performed: hemoglobin concentration, hematocrit, total erythrocyte count, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration. Blood testing was carried out using the hematology autoanalyzer.

Processing of the brains
Anesthetized rats were killed by decapitation. Brains were quickly dissected and washed with ice-cold saline. Two brains from each group were fixed using 4% paraformaldehyde and then embedded with paraffin. 4 μm thicknesses sections were prepared from each paraffin block for histopathological staining with hematoxylin & eosin (H & E) and cresyl violet for Nissl-staining. The remaining brains in each group were homogenized in Tris buffer (10 mM Tris HCl, 1 mM EDTA, 0.32 M sucrose, pH=7.8) as 10% (w/v) using a Teflon homogenizer (Glas Col homogenizer system, Vernon hills, USA). The homogenate was sonicated and centrifuged at 20,000×g for 10 min, then, supernatant was kept at –80 °C until determination of the biochemical

markers of oxidative stress (malondi-aldehyde and glutathione peroxidase)[21].

Determination of lipid peroxides (Malondi-aldehyde) in brain tissues
Tissue malondialdehyde (MDA) were estimated according to the Spectrophotometric method[25] using lipid peroxides kits (Biodiagnostics, Egypt).

Determination of total glutathione peroxidase (GPx) activity in brain tissues
GPx activity in brain tissues were estimated according to the spectrophotometric method using glutathione peroxidase kits (Biodiagnostics, Egypt).

Statistical analysis
All the data was expressed as mean ± standard error of mean (SEM) and analyzed using Statistical Package for Social Sciences (SPSS) program version 20. All the comparisons among groups were carried out using one way Analysis of Variance (ANOVA) followed by Bonferroni post hoc test to test the significance difference among group means. Data were considered statistically significant with P ≤ 0.05.

Results

Functional assessment results:
rhEPO treatment (intranasally and intraperitoneally) partially ameliorated motor impairment in rotenone-induced parkinsonism.

A- Open field results:
Rotenone injections in group 2 significantly decreased rearing (vertical movement) frequency (p= 0.03). Rotenone injections in group 2 decreased the ambulation frequency, number of stops and activity index but this change was not significant (p > 0.05). rhEPO treatment partially ameliorated this change in groups 3 and 4 with no significant change between group 1 and groups 3 and 4 (table 1). There was no significant difference between group 3 and group 4 (table 1).

Table 1: Open field test results in the study groups

Open field test	Group 1 (vehicle-treated)	Group 2 (rotenone-treated)	Group 3 (rotenone + intranasal EPO)	Group 4 (rotenone + i.p EPO)	P value
Ambulation frequency	43 ± 4.6	26 ± 5	27 ± 5	43 ± 12.5	0.3
Number of stops	7 ± 1.5	5.6 ± 1	4.5 ± 1	4.5 ± 0.6	0.4
Activity index	7 ± 0.6	4.7 ± 0.6	6 ± 1.4	9.8 ± 3	0.2
Rearing frequency	5 ± 1.1	*1 ± 0.4	2 ± 0.3	2 ± 0.6	0.025

Data are presented as Mean ± SEM. * Significant decrease in rearing frequency in group 2 (rotenone only) vs. group 1 (vehicle-treated) (p= 0.03).

B- Rota-rod test results:
Rotenone injections decreased the latency time in rats in group 2 and this change was significantly different from the latency time in normal rats in group 1, while rhEPO treatment in groups 3 and 4 partially improved this effect and there was no signify cant difference between latency time in group 1 and groups 3 and 4 (figure 1). There was no significant difference between group 3 and group 4 (figure 1).

Oxidative stress parameters results:
A- Lipid peroxide:
Rotenone injections in group 2 increased lipid peroxide level (marker of lipid peroxidation) in brain tissue but this increase was not significantly different from lipid perox-

ide mean level in group 1. In group 4, lipid peroxide mean level significantly increased than lipid peroxide mean level in group 1 so intraperitoneal rhEPO injections in group 4 caused more increase in lipid peroxide mean level . In group 3, intranasal rhEPO treatment didn't significantly affect the oxidative stress parameters (Table 2).
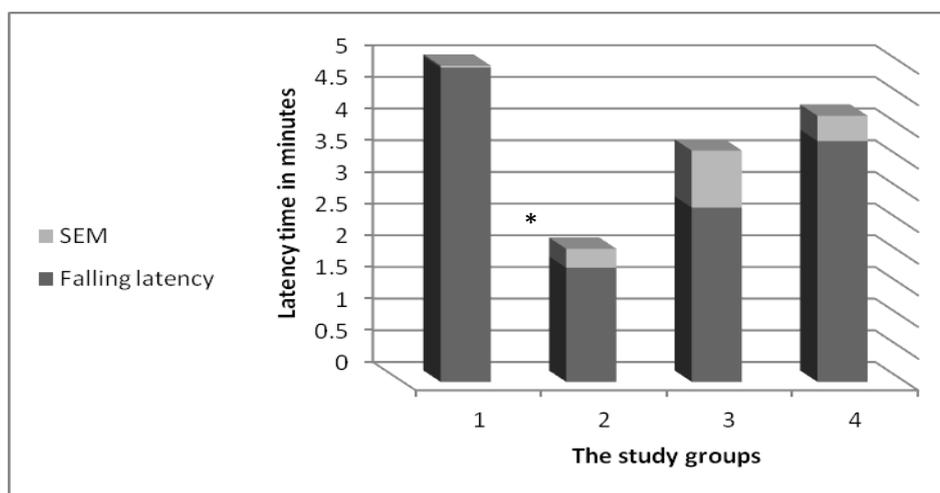
Figure 1: Rota-rod test results in the study groups.
*Significant decrease in latency time in group 2 (rotenone only) vs. group 1 (vehicle-treated) (*p*= 0.007)**.**

B- Glutathione peroxidase:
There was a trend of decreasing the level of glutathione peroxidase levels (antioxidant activity) in groups 2, 3 and 4 but this difference was statistically not significant (*p*= 0.07) (Table 2). Taken together these results suggested that rotenone alone in group 2 increased the oxidative stress marker and decrease the antioxidant marker but both effects failed to reach a significant level. Intranasal rhEPO had no significant change concerning the oxidative markers in group 3. Systemic rhEPO significantly increased the oxidative stress marker and decreased the antioxidant marker but insignificantly in group 4

Table 2: Comparison of oxidative stress parameters in the study groups

Oxidative stress Parameters	Group 1 (vehicle-treated)	Group 2 (rotenone-treated)	Group 3 (rotenone + intranasal EPO)	Group 4 (rotenone + i.p EPO)	*p* value
Lipid peroxide (nmol/gram of tissue)	651 ± 51	785 ± 52	730 ± 71	*a855 ± 18	*0.03
Glutathione Peroxidase (U/gram of tissue)	32 ± 5	26 ± 11	19 ± 14.5	5 ± 2.8	0.07

Data are presented as Mean ± SEM, *=significant at *p*<0.05 level *a =Significant increase in lipid peroxide concentrations in group 4 (rotenone + i.p. rhEPO) *vs.* group 1 (vehicle-treated).
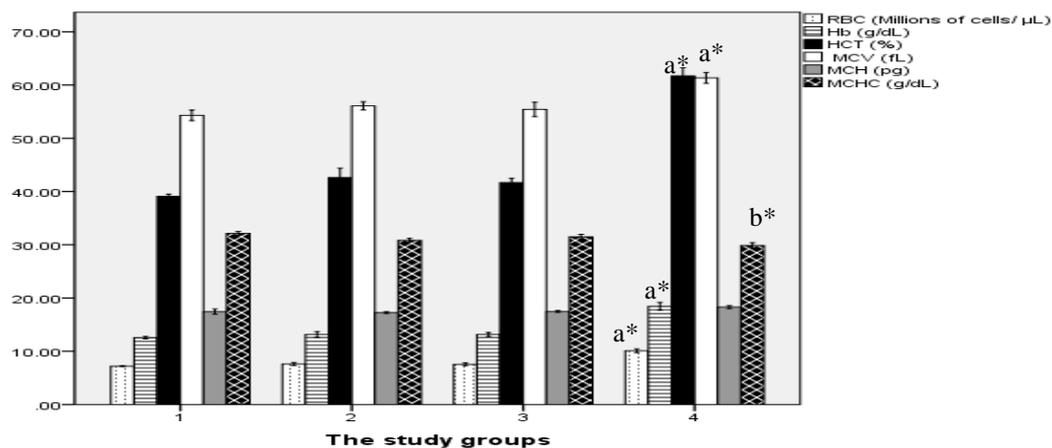
Figure 2: Comparison of means of blood indices in the study groups
a*=significant increase in RBC, Hb, HCT and MCV in group 4 (rotenone + i.p rhEPO) vs. groups 1, 2, and 3; b*=significant decrease in MCHC in group 4 (rotenone + i.p. rhEPO) vs. group 1 (vehicle-treated).

Blood indices results:

Intraperitoneal rhEPO injections of 5000 IU/kg significantly increased RBC, Hb, HCT and MCV, significantly decreased MCHC and did not affect MCH. Intranasal rhEPO administration of 2.4 IU/rat had no significant effect on all blood indices (figure 2).

Histopathological results

Rotenone injections significantly decreased the number of neurons in basal ganglia and rhEPO injections protected basal ganglia neurons against rotenone effect. The number of basal ganglia neurons was significantly decreased in group 2 *vs.* groups 1, 3 and 4 (p=0.007, p=0.048 and p=0.011 respectively) (figure 3). Histological examination showed that vehicle-treated rats had normal neurons in substantia nigra. Rotenone injections caused marked neuronal degeneration in substantia nigra. Intranasal and systemic rhEPO treatment significantly ameliorated this effect in rats in groups 3 and 4 as compared to group 2 (Figure 4). Nissl bodies staining by cresyl violet stain confirmed the previous results and revealed that vehicle treated rats had a high number of Nissl stained neurons in the substantia nigra, whereas, rotenone treated rats showed a significant marked decrease in the number of Nissl stained neu-

rons. Marked improvement was observed with rhEPO administration in groups 3 and 4 (Figure 5).

Discussion

In this study, rotenone treated animals exhibited significant impairment of motor coordination in the rota-rod test and exhibited motor deficits in the open-field test. However, in the open-field test, the decrease in the rearing frequency was significant. While the decrease in the ambulation frequency, number of stops and activity index did not reach statistical significance. Also, the histopathological analysis in this study showed that, rotenone injection in rats caused marked degeneration of the substantia nigra pars compacta (SNpc) neurons. PD is a neurodegenerative disease mainly characterized by loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Rotenone models for PD appear to mimic most clinical features of idiopathic PD and recapitulate the slow and progressive loss of dopaminergic (DA) neurons[26]. Consistently, Morais et al. 2012[27] have shown that, short term intraperitoneal rotenone administration caused an important locomotor impairment. Moreover, Fleming et al. 2004[28] re-

ported that rearing was the only test consistently affected by rotenone. Therefore, in our study, the motor impairment came on line with the marked degeneration of the neurons in the SNpc. In the present study, the animals that received only rotenone showed non- significant increase in lipid peroxide and non-significant decrease in glutathione peroxidase. It is well known that, the neurotoxin rotenone directly inhibits mitochondrial complex I [29]. Mitochondrial complex I inhibition resulted in increased oxidative stress (OS) [29, 30] which leads to neuronal cell death.
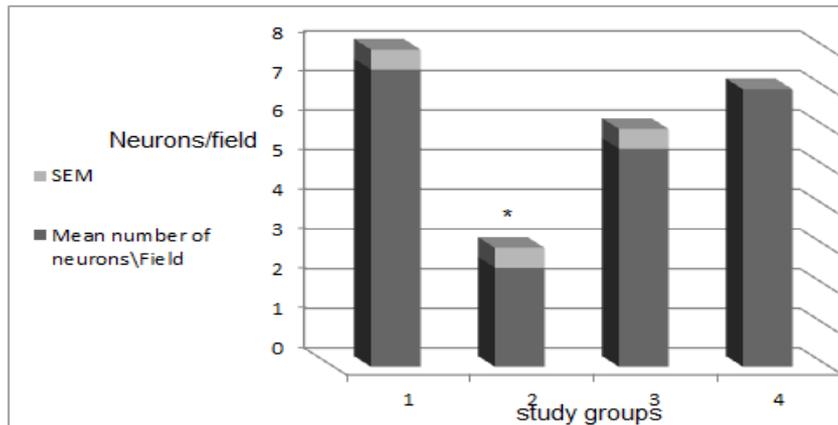
Figure 3: Comparison of the number of neurons in substantia nigra pars compacta in the study groups *significant decrease in the number of neurons in group 2 (rotenone treated) vs. groups 1, 3 and 4 (vehicle-treated, rotenone + intranasal EPO and rotenone + i.p EPO respectively)

However, later studies showed that rotenone effects can be mediated independently of complex I inhibition[31, 32]. This neurotoxin affects not only the mitochondria function but also microtubule (MT) stability, Ca2+ homeostasis, OS, DNA damage response (DDR), proteasome function, inflammatory response and apoptosis[30,31, 33-43] investigated the toxic effect of rotenone on mouse neuroblastoma cells. They indicated that, intracellular calcium rather than oxidative stress is a major factor for rotenone-induced apoptosis in neuronal cells. In the current study, both intranasal and intraperitoneal rhEPO treatment of the rotenone animals improved the motor deficit but failed to restore the control level and this was confirmed in the histopathological analyses where both intranasal and intraperitoneal rhEPO treatment increased the number of neurons in the SNpc in comparison to rotenone group. In agreement with our results; Erythropoietin (EPO) has been shown to be neuroprotective in experimental Parkinson's disease (PD) following direct intra-cerebral injection[44]. Similarly, Zhoua et al.[45] demonstrated that, brain penetrating form of EPO is neuroprotective in PD following IV administration. Also, it was found that, in traumatic brain injury intraperitoneally administered EPO improved mitochondrial function and exerted neuroprotective effect[46,47]. EPO has been proven to be neuroprotective against mechanisms involved in neuronal death. It has anti-inflammatory [48-50], anti-excitotoxic[51], anti-oxidant[52], and anti-apoptotic[53] effects on neurons and oligodendrocytes[54]. It also promotes neurogenesis[55,56], and angiogenesis[57], which are essential for injury repair and normal neurodevelopment.

Group 1

Group 2
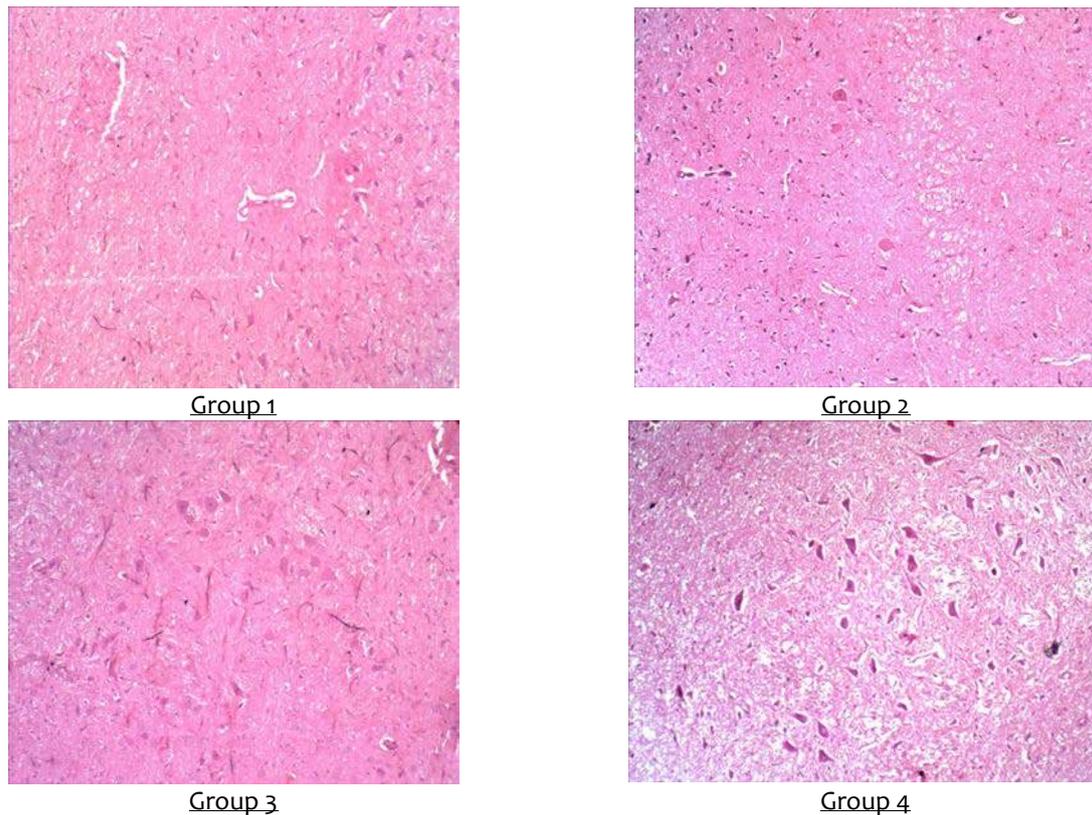
Group 3

Group 4

Figure 4: Photomicrographs of sections in the substantia nigra of the study groups showing normal nigral neurons (group 1), marked neuronal degeneration with indistinct neuronal boundaries and invisible nuclei in (group 2), and increased number of intact nigral neurons in (groups 3, 4), H&E x10.

In this study, it was noticeable that, intraperitoneal rhEPO injection of 5000 u/kg into rotenone-treated rats was relatively more efficient than intranasal rhEPO delivery of 2.4μ. This finding could be attributed to the low dose of the intranasal rhEPO. Interestingly it was found that, EPO neuroprotection is dose dependent and EPO treatment was found to be ineffective at low dose and at multiple high doses[58]. In this study, treatment of rotenone animals with intraperitoneal rhEPO significantly increased lipid peroxide and caused non-significant decrease in glutathione peroxidase as compared to controls. This finding could be attributed to the fact that, pharmacologic doses of EPO may show side effects arising from the vascular system such as elevated arterial blood pressure[59,60]. In animal models of uremia, it was found that, oxidative stress (OS) contributes to the development of hypertension and to the progression of renal injuries. EPO administration further increases OS, which might partly account for the accentuation of hypertension and renal injury[61]. Similarly, it was suggested that in human endothelial cell lines, EPO increased the extracellular and intracellular OS which decreased the activity of dimethylarginine dimethylaminohydrolase (DDAH)[62]. DDAH is the enzyme which metabolize asymmetric dimethylarginine (ADMA). ADMA is an endogenous inhibitor of nitric oxide synthase (NOS). In addition, both allantoin, a marker of oxygen free radical generation, and reactive oxygen species increased significantly after EPO treatment compared with control.

A	B

Group 1

Group 2
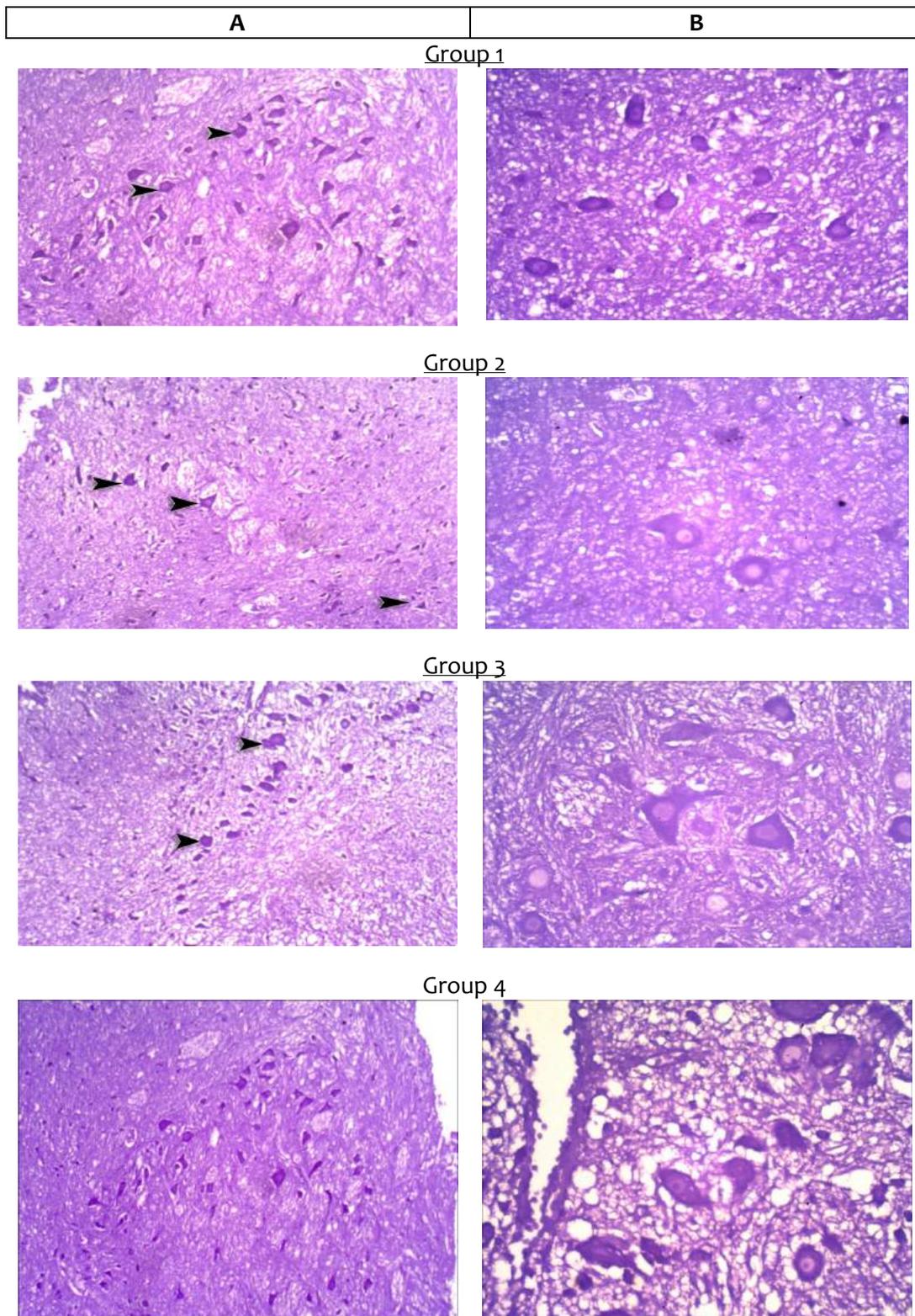
Group 3

Group 4

Figure 5: Photomicrographs of sections in the substantia nigra of the study groups showing normal Nissl stained nigral neurons in group 1, marked decrease in Nissl stained nigral neurons in group 2 and increased number of Nissl stained nigral neurons in groups 3 and 4 vs. group 2, (head of arrow: Nissl stained neurons. Cresyl violet Nissl stain (A) ×10 and (B) × 40).

Accordingly, in this study, lipid peroxide, a marker of OS, may be increased by rhEPO treatment. Also in the current study, intraperitoneal rhEPO injections significantly increased RBC, Hb, HCT and MCV, significantly decreased MCHC and did not affect MCH. EPO has a known role in erythropoiesis stimulation. It has been shown that, transgenic mice overexpressing systemic EPO developed larger infarcts than wild-type controls, indicating that rhEPO systemic chronic treatment might increase hematocrit and thus deteriorate the after-stroke outcome[63]. Since stimulation of erythropoiesis is unwanted for neuroprotection. Therefore, in our study EPO may be unable to produce a significant improvement in the motor deficit because of its adverse effects in the erythropoiesis and OS.

Conclusion

Our findings suggest that, EPO may have neuroprotective effect in PD. Systemic rhEPO neuroprotective effects may be attenuated by its adverse effects such as increase of OS in the vascular system and stimulation of erythropoiesis. Small doses of intranasal EPO may be sufficient to produce neuroprotection without affecting erythropoiesis and further researches are required to address the mechanisms of neuroprotective effects of EPO.

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