BENEFICIAL EFFECTS OF ERYTHROPOIETIN ON STRIATAL DOPAMINERGIC NEURONS IN 6-OHDA-TREATED AGED RAT MODEL OF PARKINSON'S DISEASE

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Abstract: Background & objectives: It has been reported that erythropoietin played important role in prevention of striatal dopaminergic neurons loss against 6-hydroxydopamine (6-OHDA) in young rats. As the degeneration of dopaminergic neurons occurred commonly in aged individuals. The present study was undertaken to investigate the beneficial effects of erythropoietin against 6-hydroxydopamine neurotoxicity in aged rat model of Parkinson's disease. Literature is silent about it. **Methods**: Sprague-Dawley rats were pre-treated with erythropoietin and subsequently administered the neurotoxin 6-hydroxydopamine into the aged rat striatum. Various behaviour and immunohisotochemical tests were used to investigate the beneficial effects of erythropoietin. **Results**: Statistically significant difference was found between post-lesion values of all groups (ANOVA, $P<0.001$ in apomorphine-induced rotational behaviour, staircase test (success rate) and disengage time; $P<0.05$ in stepping test, initiation time and postural balance test). Erythropoietin also increased more survival of dopaminergic neurons in the 6-OHDA lesioned striatum. **Interpretation & conclusion**: On the basis of changes in these tests, the present study concludes that erythropoietin enhances the beneficial effects in 6-OHDA treated aged rat. Howsoever less improvement are found in aged rats than that of young rats.

Keywords: Erythropoietin, 6-hydroxydopamine and aged rat.

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Introduction:

Erythropoietin is a 30.4-kDa glycoprotein hormone which represents the major regulator of erythropoiesis or red blood cell production. It is naturally synthesized by fetal liver and adult kidney, and it can stimulate erythropoiesis in the bone marrow in response to hypoxia. For more than a decade, it has been used clinically in treating anaemia resulting from chronic renal failure or from cancer chemotherapy.It prevents effectively adriamycin-induced heart failure in Wistar rats[1]. It is associated with a decreased level of oxidative stress and apoptosis in cardiomyocytes[1]. It improves cardiac contractile function in excitable murine and human left ventricular muscle preparations[2]. It alters the rate of cerebral aneurysm formation and progression in rats[3]. It improves post ischemic injury of rat heart by attenuating nitrosative stress[4]. It reduces infarct size and alleviated left ventricular function in experimental models of Myocardial Infarction[5]. It improves memory function with reducing endothelial dysfunction and amyloid-beta burden in Alzheimer's disease models[6]. It prevents L-DOPA neurotoxicity by activating the phosphoinositide 3-kinase pathway as well as reducing oxidative stress[7]. It prevents the progression of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Epilepsy, Multiple sclerosis and Motor neuron diseases[8]. Erythropoietin and granulocyte-colony stimulating factor efficiently repress expression of the pro-apoptotic protein p53 up-regulated

modulator of apoptosis (PUMA)[9]. It has been reported to protect dopaminergic neurons from neonatal hypoxia-ischemia[10], 6-hydroxydopamine lesioning in vitro[11] and 1 methyl -4phenyl 1,2,3,6-tetrahydroxydopamine in a mouse model of PD[12]. The above mentioned studies focused on the use of young animals to examine the in vivoeffects of erythropoietin. However, since the degeneration of substantia nigra dopaminergic (DA) neurons that occurs in Parkinson's disease is more often than not confined to elderly individuals, it is of interest to determine whether the beneficial effects of erythropoietin against 6-OHDA in young adult rats can be extended to aged animals. Therefore in the present study, beneficial effects of erythropoietin were investigated in aged rat model of PD. Both behavioural and immunohistochemical changes were examined after animals were pretreated with erythropoietin and subsequentally administered the neurotoxin 6-OHDA into the rat striatum. No information is available on beneficial potential of erythropoietin in 6-OHDA induced aged rat model of PD.

Material and Methods:

Animals: Sprague-Dawley young (3-4 months, weight 180±50 gms)[13] and aged (22-24 months, weight 280±50 gms)[14] rats at the beginning of the experiment, were housed at temperature (23±3°C) [15] with a 12-hour light/dark cycle and were provided standard rodent chow and water *ad libitum*. Experiments were performed between 10:00 and 15:00 in compliance with the regulation, policies and principles of Institutional Animal Ethical Committee.

Animal groups: The animals were equally divided into the 4 groups of 10 rats each. Group 1 is the aged sham control group. Aged animals of group 2 received 8 µg 6-OHDA into left striatum of rat. Young animals of group 3 received first erythropoietin (20 IU, intrastriatal dissolved in 0.9% saline) and than 8 µg 6-OHDA was injected into left striatum. Aged animals of group 4 received first erythropoietin (20 IU, intrastriatal dissolved in 0.9% saline) and than 8 µg 6-OHDA was injected into left striatum.

6-hydroxydopamine lesion: Rats were anaesthetised with the help of ketamine-xylazine (50-100mg/kg; 5-10 mg/kg, i.p.). Animal was placed into a David Kopf stereotaxic frame (INCO, Ambala). Lesion was made at the following co-ordinates: anterior-posterior, 0 mm; lateral, 3.5 mm; dorso ventral to the dura, 5.5 mm; from bregma. After scalp incision, burr hole was drilled over the injection sites, and a blunted 26-gauge cannula, connected to a Hamilton syringe, was lowered to the injection site. The 6-OHDA solution was injected over a 4 min period and the needle was left in place for an additional 5 min before retraction[16-17].

Quantitation of rotational behaviour: Rats were tested for apomorphine induced rotations in response to apomorphine (Sigma, 0.05mg / kg s.c.) at the base line (prelesion) and after 5 weeks of 6-OHDA induced lesion (postlesion) in all groups for 30 minutes duration[18] by the help of rota count 8 (Columbus Instruments, USA).

Staircase test: The staircase test was used to assess skilled forelimb test in the rat. The staircase apparatus was filled with 30 chow pellets on each side. In each test session, several measures were evaluated; the number of pellets taken (pellets eaten plus pellets grasped but dropped), the number of pellets eaten (successful reaches), and the success rate (pellets eaten divided by pellets taken) [19].

Stepping test: The stepping test was used to assess forelimb function and the motivational component of akinesia. The rats were held with one hand by the experimenter fixing the both hindlimbs and with the other hand fixing the forelimb that was not to be monitored. The rat was moved across the surface of the wooden plank (0.9 m wooden plank at a consistent speed in 30 sec), in such a way that the rat must bear weight on the

remaining forelimb in both direction first in the forehand direction i.e. movement of the paw toward the torso to compensate for an outward lateral movement of the body, and then in the backhand direction i.e., movement of the paw away from the torso to compensate for the inward medial movement of the body. The number of adjusting steps for both directions and both forelimbs were counted while the rat was moved sideways along the wodden plank. Each test consisted of one tests per day for 3 consecutive days, and the mean of the three subtests were calculated[20].

Initiation time: In this test, the time to actively initiate a forelimb movement was determined in the same test sessions as for the stepping test. During the test, the rat was held as described for the stepping test and placed with its unrestrained forepaw (the one to be monitored) on the bottom of the ramp. The time elapsed before the rat actively initiated movement with the unrestrained forelimb and started to step forward along the ramp (1.1m) toward the home cage were recorded[21].

Disengage test: A blunt wooden probe touched the perioral region beneath the vibrissae of the rat repeatedly at 1s interval when the rat was engaged in eating a piece of milk chocolate. The latency of an orienting response was recorded to the nearest second. The animals were tested once daily on 3 consecutive days. Final results were expressed as the mean of the three days score[22].

Postural Balance Test: In this test sessions, the rats' ability to regain balance was measured in a balance or side-falling test, the rat was held in the same position as described in the stepping test, with the rat standing with one forepaw on the table. Instead of being moved sideways, the rat was titled by the experimenter towards the side of the paw touching the table. This resulted in loss of balance, and the ability of the rat to regain balance by movement of the forelimb during the tilting movement of the forelimb was monitored by a scoring system ranging from 0 to 3. When the rat fell onto the side and there was no detectable muscles reaction in the forelimb, score 0 was given. Score 1 represented a clear forelimb reaction, as seen by muscle concentration, but lack of success in recovering balance etc., the rat still fell on to the side. Score 2 was given when the rat showed an incomplete recovery of balance etc. the rat perform clear forelimb movement, But the placement of the paw was compared with rat seen in controls in that the digits were not be plainly split on the table but partially crossed over one another. Score 3 was given for a normal forelimb placement movement and total recovery of balance, similar to unlesioned control. The test was repeated six time a day on each side for thee consecutive days, giving a maximum score of 18 at each of the three test based. Final results was expressed as the mean of the 3 days[23-24].

Cresyl violet staining: When the behavioural study was completed, rats were sacrificed with an overdose of ketamine and xylazine (200 mg/kg, i.p. and 20 mg/kg, i.p.) and perfused intracardially with heparin saline (0.1% heparin in 0.9% saline; 100 ml/rat) followed by paraformaldehyde (4% in phosphate buffer). Brains were removed and postfixed for 10 hours in 4% paraformaldehyde. Brains were submerged in 30% sucrose until equilibrated. All stains were carried out on a 1 in 6 series of sections. One series of sections from each brain sections were stained using the cresyl violet. Slides were mounted and coverslipped with DPX per mount[25].

Tyrosine hydroxylase immunohistochemical test were carried out on free-floating sections. Sections were treated for 10 minutes in 3% hydrogen peroxide, were held 3 times in 0.1 M PBS, and were incubated in 2% normal goat serum with 0.1% Triton X-100 for 30 minutes prior to overnight incubation at 4°C with primary antibody

diluted in 2% normal goat serum and 0.1% Triton X-100. The primary antibodies utilized were rabbit anti-tyrosine hydroxylase (Sigma). After six washes in 0.1 M PBS (5 minutes each), sections were incubated in 0.1 M PB containing 1% normal goat serum and biotinylated goat anti-rabbit secondary antibody for 60 min at 37ºC. The sections were rinsed three times in PBS and incubated in avidin-biotin-peroxidase complex (ABC-Elite kit) for 50 minutes at room temperature. Following thorough rinsing with PBS, staining was visualized by incubation in 3, 3-Diaminobenzidine solution with nickel enhancement. This immunostaining allowed the determination of the extent of dopaminergic cell degeneration. After immunostaining, floating tissue sections were mounted on glass slides and counterstained before dehydrating, clearing and coverslipping[26-27].

Statistical analysis: Behavioural test results were subjected to express as the mean ± S.E.M. Statistical analysis between pre lesion and post lesion within the group was performed using a paired Student's t-test. Differences between all groups were evaluated using ANOVA. The significance level was established at $p<0.05$[28].

Results:

Rats subjected to erythropoietin and receiving stereotaxic injection of 6-OHDA had significant effect in various behaviour tests (Table 1). There were no statistical significant differences between pre lesion values of group 1(student's t test, $p > 0.1$). Comparative analysis between prelesion value and post lesion value of group 2 was found highly significant (student's t test, $p<0.001$) in apomorphine-induced rotational behavior, stepping test, initiation time, staircase test, disengage time and postural balance test. Statistical significant difference in various behavior tests was observed between pre lesion and post lesion values of group 3 (student's t test, $p<0.001$ in apomorphine-induced rotational behavior, initiation time, disengage time and $p<0.05$ in staircase test, stepping test, postural balance test). Significant difference (student's t test, $p<0.001$) between prelesion and post lesion value of group 4 was found in apomorphine-induced rotational behavior, staircase test (success rate), stepping test, initiation time, disengage time and postural balance test. Statistical evaluation revealed significant difference between all the groups (ANOVA, $p<0.001$) in apomorphine-induced rotational behavior, staircase test (success rate) & disengage time and (ANOVA, $p<0.05$) in stepping test, initiation time & postural balance test (Table 1).

Cresyl violet staining was performed in all the rat groups. The animals of group 1 exhibited normal dopaminergic neurons in striatal brain sections. Cresyl violet stained nissl body of the dopamergic neurons. No neuronal loss was seen in these brain sections. Significant loss of striatal DA neurons was observed in-group 2 animals. Injected 6-OHDA alone resulted in almost complete loss of DA neurons compared to the group1. Partially protected DA neurons were found in Erythropoietin treated animals of group 3 and 4. The number of dopminergic neurons in group 3 and 4 were found significantly more than that of animals of group 2. Howsoever there is slight decrease in dopaminergic neurons in group 4 than that of group 3.

Tyrosine hydroxylase immunohistochemical test were also investigated in all groups of the rats. Microscopic examination of slides showed abundant dark brown stained DA neurons in the brain section of group1. Very less dark brown stained TH-immunoreactive neurons were demonstrated in brain section of group 2. It is due to loss of normal dopaminergic neurons. The brain sections of group 3 and 4 showed abundant dark brown stained neurons. The dark brown color neurons were found significantly more in group 3 and 4 than that of group 2. The aged rats showed less protection in neurons loss

(46 %) than that of young (65 %) rats (Figure1).

In the present study, the beneficial effects of erythropoietin were tested against 6-OHDA neurotoxicity for dopaminergic neurons in aged rats. Although there have been numerous studies on the beneficial effects of erythropoietin in young model of PD. The current investigation was used to focus the beneficial effects of erythropoietin in aged rat model of PD. Various behaviour and immunohistochemical tests were used as an index of striatal dopaminergic function. Intrastriatal administration of erythropoietin

Discussion:

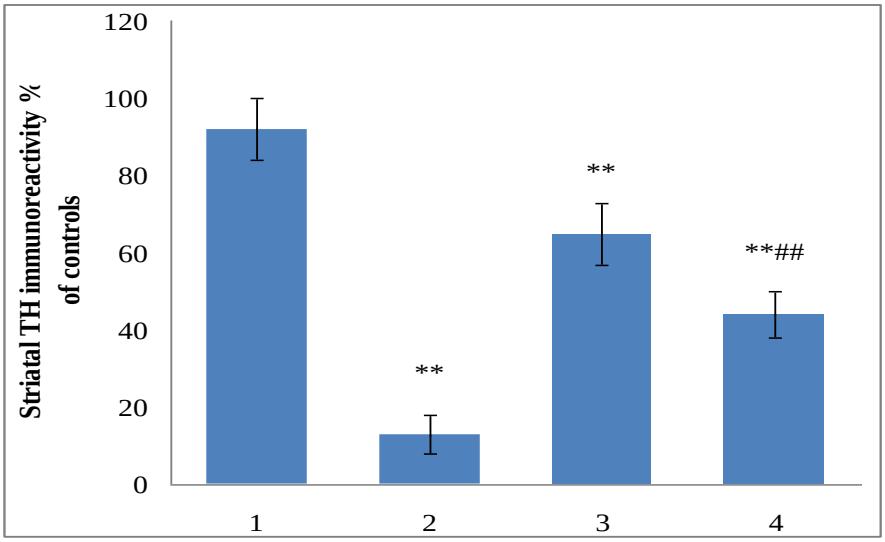
was found to protect dopaminergic neurons of striatum in 6-OHDA induced aged rat model of PD. Less neuroprotection was found in aged rats than that of young rats.

Parkinson disease is one of the most common chronic, progressive neurodegenerative diseases, affecting over 1% of the aged people in Western countries and with a present estimated prevalence in the world of around 4.5 million persons[29]. It is characterized by clinical abnormalities i.e. resting tremor,

Table:1 : Effect of erythropoietin on behavior tests in all groups of rats.

Behavior tests		Group-1		Group-2		Group-3		Group-4	
		Pre-lesion	Post-lesion	Pre-lesion	Post-lesion	Pre-lesion	Post-lesion	Pre-lesion	Post-lesion
Apomorphine-induced rotational test		8 ±1	9 ± 1	9 ± 1	240 ± 12**	8 ± 1	126 ± 11**	10 ± 1	142 ± 6**##
Staircase test	Taken	19± 4	17±2	20 ± 2	14 ± 1**	23 ± 2	26 ± 3 *	27 ± 2	24 ± 3 *#
	Eaten	17± 3	15±1	18 ± 1	5 ± 1**	21 ± 2	20 ± 2 *	24 ± 2	15 ± 2 *#
	Success rate	89 ± 6	88 ± 2	90 ± 2	36± 3**	91 ± 3	77 ± 4*	88 ±3	62 ± 4**##
Stepping test	In forehand direction	31 ± 3	34 ± 1	34 ± 2	7 ± 1**	28 ± 2	22 ± 1*	28 ± 2	15 ± 1**#
	In backhand direction	28 ± 3	32 ± 4	30 ± 3	5 ± 1**	26 ± 2	20 ± 2*	27 ± 1	13 ± 1**#
Initiation time		5 ± 1	6 ± 1	6 ± 1	24 ±2**	5 ± 1	18 ± 2**	4 ± 1	13 ± 1**#
Postural balance test score		16 ± 1	15 ± 1	17± 1	2 ± 1**	16 ± 3	11 ± 2*	16 ± 3	8 ± 1**#
Disengage behaviour		11 ± 3	12 ± 2	10 ± 1	118 ± 14**	10 ± 1	60 ± 7**	9 ± 1	48 ± 6**##

Graph-1: Neuroprotective effect of erythropoietin in young and aged rat.



rigidity, postural instability and bradykinesia[30]. The pathological hallmark of PD is degeneration of dopaminergic neurons in the substantia nigra pars compacta, with subsequent damage of nerve terminals accompanied by deficiency of DA in the striatum. The etiology of dopaminergic neurons loss is not known. However, it was reported that oxidative stress as the probable candidate to mediate in the original unknown cause.6-OHDA-induced PD models also produce similar changes[31].

Our findings are consistent with earlier reports of improved behavioural activity and histological test in PD[11,32-35]. Intrastriatal administration of 20 IU Erythropoietin protects dopaminergic neurons and improves behavioural outcome in a rat model of Parkinson's disease[34]. Intrastriatal injection of 5-20 IU erythropoietin rescue both immortalized dopaminergic cells and primary dopamine neurons from 6-OHDA-induced neurotoxicity[11]. It was also reported that systemic administration of erythropoietin did not protect dopaminergic neurons from 6-OHD neurotoxicity[34]. Therefore in the present study, 20 IU erythropoietin was injected into the rat striatum.

It has suggested that erythropoietin may exerts its beneficial effects through multiple mechanism including antioxidant[36], anti-apoptosis[11], anti-inflammation[37], inhibition of glutamate release, reactive oxygen species formation[38], activation of Akt/protein kinase B via the phosphoinositide 3-kinase pathway[11] and activation of Janus kinase-2 and nuclear factor-kappaB signalling pathway[39].

In the present study, the extent of neuroprotection against 6-OHDA induced dopaminergic neurotoxicity is less in aged hemiparkinsonium animals. It may be due to neurochemical and cellular changes in the nigrostriatal dopaminergic changes during aging. There are decrease in striatal levels of dopamine as well as DA receptors[40]. Neuronal death in the substantia nigra reaches about 50 % by the ninth decade in humans[41]. Reduction in high affinity DA uptake sites[42], DA transporter messenger RNA[43] and TH

messenger RNA[44] also become evident as individual's age.

Conclusions:

The results of present study demonstrated that intrastriatal administration of erythropoietin protects striatal dopaminergic neurons against 6-OHDA neurotoxicity in the aged rat. The effect was accompanied by a significant recovery in behaviour and immunohistochemical tests. Further studies are needed to elucidate the mechanism of erythropoietin.

References:

1. Ammar HI, Saba S, Ammar RI, Elsayed LA, Ghaly WB, Dhingra S. Erythropoietin protects against doxorubicin-induced heart failure. Am J Physiol Heart Circ Physiol. 2011; 301(16):2413-2421.
2. Hefer D, Yi T, Selby DE, Fishbaugher DE, Tremble SM, Begin KJ, et al. Erythropoietin induces positive inotropic and lusitropic effects in murine and human myocardium. J Mol Cell Cardiol. 2012; 52(1):256-263.
3. Xu Y, Tian Y, Wei HJ, Chen J, Dong JF, Zacharek A, et al. Erythropoietin increases circulating endothelial progenitor cells and reduces the formation and progression of cerebral aneurysm in rats. Neuroscience. 2011; 181:292-299.
4. Lu MJ, Chen YS, Huang HS, Ma MC. Erythropoietin alleviates post-ischemic injury of rat hearts by attenuating nitrosative stress. Life Sci. 2012; 90(19-20):776-784.
5. Najjar SS, Rao SV, Melloni C, Raman SV, Povsic TJ, Melton L, et al. Intravenous erythropoietin in patients with ST-segment elevation myocardial infarction: REVEAL: A randomized controlled trial. Jama. 2011; 305(18):1863-1872.
6. Lee ST, Chu K, Park JE, Jung KH, Jeon D, Lim JY, et al. Erythropoietin improves memory function with reducing endothelial dysfunction and amyloid-β burden in Alzheimer's disease models. J Neurochem. 2012; 120(1):115-124.
7. Park KH, Choi NY, Koh SH, Park HH, Kim YS, Kim MJ, et al. L-DOPA neurotoxicity is prevented by neuroprotective effects of erythropoietin. Neurotoxicology. 2011; 32(6):879-887.
8. Chong ZZ, Shang YC, Mu Y, Cui S, Yao Q, Maiese K. Targeting erythropoietin for chronic neurodegenerative diseases. Expert Opin Ther Targets. 2013; 17(6):707-720.
9. Kook YH, Ka M, Um M. Neuroprotective cytokines repress PUMA induction in the 1-methyl-4-phenylpyridinium (MPP(+)) model of Parkinson's disease. Biochem Biophys Res Commun. 2011; 411(2):370-374.
10. Demers EJ, McPherson RJ, Jull SE. Erythropoietin protects dopaminergic neurons and improves neurobehavioural outcomes in juvenile rats after neonatal hypoxia-ischemia. Pediatr Res. 2005; 58:297-301.
11. Signore AP, Weng Z, Hastings T, Van Laar AD, Liang Q, Lee YJ, et al. Erythropoietin protects against 6-hydroxydopamine-induced dopaminergic cell death. J Neurochem. 2006; 96(2):428-443
12. Genc S, Kuralay F, Genc K, Akhisaroglu M, Fadiloglu S, Yorukoglu K, et al. Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. Neurosci Lett. 2001; 298(2):139-141.
13. Margaret OJ, Zimeng Y, Rachel C, Murali VKMJ, George NH, Natalia D, et al. Pharmacokinetics and Metabolism of [14C] Dichloroacetate in Male Sprague-Dawley Rats. Drug metabolism and disposition. 1998; 26(11):1134-1143.
14. Ghosh MN. Fundamentals of experimental Pharmacology. Scientific Book Agency, Calcutta 1984: Second Edition.

15. Latendresse JR, Newbold RR, Weis CC, Delclos KB**.** Polycystic Kidney Disease Induced in F1 Sprague-Dawley Rats Fed para-Nonylphenol in a Soy-Free, Casein-Containing Diet. Toxicological Sciences. 2001; 62:140-147.
16. Paxinos G and Watson C. The Rat Brain in Stereotaxic Coordinates, 1986; 2nd ed. Academic Press.
17. Singh S, Ahmed R Sagar RK, Krishana B. Neuroprotection of the nigrostriatal dopaminergic neurons by melatonin in hemiparkinsonium rat. Indian J Med Res. 2006; 124(4):419-426.
18. Ungerstedt U and Arbuthnott GW. Quantitive recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. Brain Research. 1970; 24:485-493.
19. Nikkhah G, Rosenthal C, Hedrich HJ, Samii M. Differences in acquisition and full performance in skilled forelimb use as measured by the 'staircase test' in five rat strains. Behav Brain Res. 1998; 92(1):85-95.
20. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology. 2000; 39(5):777-787.
21. Olsson M, Nikkhah G, Bentlage C, Bjorklund A. Forelimb akinesia in the rat Parkinson model: Differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. J Neurosci. 1995; 15:3863-3875.
22. Mandel RJ, Brundin P, Bjorklund A. The important of graft placement and task complexity for transplant-induced recovery of simple and complex sensorimotor deficits in dopamine denervated rats. Eur J Neurosci. 1990; 2:888-894.
23. Winkler C, Sauer H, Lee CS, Bjorklund A. Short-term melatonin treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. J Neurosci. 1996; 16:7206-7215.
24. Rozas G, Labandeira García JL. Drug-free evaluation of rat models of parkinsonism and nigral grafts using a new automated rotarod test. Brain Res 1997:749(2):188-199.
25. Saji M, Blau AD, Volpe BT. Prevention of transneuronal degeneration in the substantia nigra reticulata by ablation of the subthalamic nucleus. Expl Neurol. 1988; 141:120-129.
26. Nikkhah G, Falkenstein G, Rosenthal C. Restorative plasticity of dopamine neuranol transplants depends on the degree of hemispheric dominance. The journal of Neuroscience. 2001; 21:6252-6263.
27. Svingos AL, Clarke CL, Pickel VM. Cellular sites for activation of ð-opiod receptors in the rat nuceleus accumbens shell: relationship with met5-enkephalin. J Neurosci. 1998; 18(5):1923-1933.
28. Subbakrishna DK. Statistics for Neuroscientists. Annals of Indian Academy of Neurology. 1988; 3:55-67.
29. Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur J Epidemiol. 2011; 26:S1-S58.
30. Beal MF. Mitochondrial dysfunction in neurodegenerative diseases. Biochim Biophys Acta. 1998;1366:211-223.
31. Schober A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. Cell Tissue Res. 2004; 318:215-224.
32. Kanaan NM, Collier TJ, Marchionini DM, McGuire SO, Fleming MF, Sortwell CE. Exogenous erythropoietin provides neuroprotection of grafted dopamine neurons in a rodent model of Parkinson's

disease. Brain Res. 2006; 1068(1):221-229.
33. McLeod M, Hong M, Mukhida K, Sadi D, Ulalia R, Mendez I. Erythropoietin and DNF enhance ventral mesencephalic fiber outgrowth and capillary proliferation following neural transplantation in a rodent model of Parkinson's disease. Eur J Neurosci. 2006; 24(2):361-370.
34. Xue YQ, Zhao LR, Guo WP, Duan WM. Intrastriatal administration of erythropoietin protects dopaminergic neurons and improves neurobehavioural outcome in a rat model of Parkinson's disease. Neuroscience. 2007; 146(3):1245-1258.
35. Kadota T, Shingo T, Yasuhara T, Tajiri N, Kondo A, Morimoto T, et al. Continuous intraventricular infusion of erythropoietin exerts neuroprotective/rescue effects upon Parkinson's disease model of rats with enhanced neurogenesis. Brain Res. 2009; 1254:120-127.
36. Halliwell B. Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol. 1995; 49:1341-1348.
37. Villa P, Bigini P, Mennini T, Agnello D, Laragione T, Carganotto A et al. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. J Exp Med. 2003; 198:971-975.
38. Liu R Suzuki A, Guo Z, Mizuno Y, Urabe T. Intrinsic and extrinsic erythropoietin enhances neuroprotection against ischemia and reperfusion injury in vitro. J Neurochem. 2006; 96:1101-1110.
39. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature. 2001; 412(6847):641-647.
40. Anton-Tay F, Diaz JL, Fernandez-Guardiola A. On the effect of melatonin upon human brain. Its possible therapeutic implications. Life Sci. 1971; 10:841-850.
41. Shaw KM, Stern GM, Sandler M. Melatonin and parkinsonism. Lancet. 1973; 1:271-279.
42. Willis GL and Armstrong SM. A therapeutic role for melatonin antagonism in experimental models of Parkinson’s disease. Physiol Behav. 1999; 66:785-795.
43. Guarduika-Lemaitre B. Toxicology of melationin. J Biol Rhythms. 1997; 12:697-706.
44. Willis GL and McLennan CA. Pinealectomy and dopamine replacement therapy in models of Parkinson’s disease. A satellite symposium of the 34th International congress of physiological sciences on the theme melatonin and biological rhythms; 2001; 10.

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