

Neuroprotection of the nigrostriatal dopaminergic neurons by melatonin in hemiparkinsonium rat

S. Singh, R. Ahmed, R.K. Sagar & B. Krishana

Department of Physiology, Maulana Azad Medical College, New Delhi, India

Received January 24, 2005

Background & objectives: Several lines of evidence point to a significant role of antioxidants in Parkinson's disease (PD). Few studies report that melatonin, a neurohormone, is one of the best physiological antioxidants. Review of literature indicates that none of the drugs so far studied for preventing the PD was found to be promising for use. Therefore in the present study the effect of neuroprotectory melatonin was tested against 6-hydroxydopamine (6-OHDA) neurotoxicity for striatal dopaminergic neurons in the rat.

Methods: Thirty animals were randomly divided into two groups. Animals of group 1 received saline (melatonin vehicle) daily 1 ml ip for seven days. Melatonin (500 µg/kg body weight dissolved in 1 ml saline ip) was administered in rats of group 2 for seven days. Then all animals of groups 1 and 2 were lesioned unilaterally with 8 µg 6-OHDA into the lateral striatum on 8th day. Various behaviour and histological tests were used to evaluate the neuroprotective effect of melatonin.

Results: Statistically significant difference in various behaviour tests was found between post lesion values of group 1 and group 2 ($P < 0.001$ in apomorphine-induced rotational behaviour, staircase test (success rate), disengage time and $P < 0.05$ in stepping test, initiation time, postural balance test).

Interpretation & conclusion: Our results demonstrated that melatonin acted as an effective neuroprotective agent for striatal dopaminergic neurons in 6-OHDA lesioned rat model of Parkinson's disease.

Key words 6-hydroxydopamine - melatonin - Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra par compacta that results in a significant decrease of dopamine level in the striatum. In the last three

decades, levodopa (L-dopa) has remained the best drug for PD. It dramatically improves morbidity and mortality. But various adverse reactions such as dyskinesia, on-off phenomenon and psychiatric effects (agitation, visual hallucination, psychosis,

paronia, hyper sexuality *etc.*) are seen with long term L-dopa therapy. In addition, it actually speeds the progression of disease. This led to a search for alternative treatment¹.

Melatonin is a neurohormone that was first reported in 1993 by Tan *et al*² as an efficient endogenous antioxidant. Since then, it has been reported as one of the best physiological antioxidants and *in vivo* cell protectors³. It is secreted from the pineal gland and inhibits DNA adduct formation induced by the chemical carcinogen safrole *in vivo*². Conversely, it protects the oxidative damage in the central nervous system³. Melatonin treatment following kainic acid administration has a protective effect on antioxidant enzyme activities and thus supports the role of melatonin and oxidative stress in the regulation of antioxidative enzyme activity⁴. It prevents learning and memory deficits caused by thinner exposure possibly by reducing oxidative stress and regulating neural plasticity⁵. Recently it has been reported that melatonin not only plays an important role in the regulation of circadian rhythms, but also acts as an antioxidant and neuroprotector that may be important in aging and Alzheimer's disease⁶. In addition, melatonin has been proposed as a drug for the treatment of cancer⁷.

Melatonin is unique potent antioxidant because it is highly soluble in both aqueous⁸ and lipid medium⁹; it can easily cross the blood-brain barrier and lacks if any, side effect¹⁰; it can also enter in both glial and neuronal cells¹¹; and it has been proven to protect neuronal cells from neurotoxic induced damage in a wide spectrum of neuronal culture system³.

Therefore the present study was undertaken to determine the ability of malatonin to protect the striatal dopaminergic loss induced by 6-hydroxydopamine (6-OHDA) in rat model of PD. Both functional and morphological changes were examined after animals were pretreated with melatonin and subsequently administered the neurotoxin 6-OHDA into the striatum. Information

available so far on protective potential of malatonin in PD is controversial.

Material & Methods

Animals: Thirty Sprague-Dawley female adult rats, weighing 200-250 g at the beginning of experiment, were maintained under 12 : 12 h light: dark cycle with food and water provided *ad libitum*. They were housed four per cage in a temperature-controlled room. The study protocol was approved by the Institutional Animal Ethics Committee for the care and use of laboratory animals. All efforts were made to reduce the number of animals used and their suffering.

The animals were randomly allocated to two groups of 15 rats each. Group 1 (vehicle group) received first saline (melatonin vehicle) 1 ml i.p. for seven consecutive days daily and then 8 µg 6-OHDA into lateral striatum on day 8. Group 2 (melatonin group) received first melatonin (Sigma, USA) dissolved in 1ml saline at a dose of 500 µg/kg body weight per day ip for seven consecutive day daily and then 8 µg 6-OHDA into lateral striatum on day 8.

Lesion by 6-hydroxydopamine: Unilateral striatal lesion was produced by stereotaxic injection of 8 µg 6-OHDA into the lateral striatum according to the atlas of Paxinos and Watson¹².

The animal was anaesthetized with pentobarbital anesthesia (Sigma, 45 mg/kg ip) and placed in David Kopf stereotaxis frame Instruments and Chemicals (P) Ltd., Ambala, India (INCO). The solution was injected into lateral striatum with a Hamilton syringe at a rate of 1 µl/min. The stereotaxic co-ordinates were 0 mm anterior to bregma, 3.5 mm lateral from midline and 5.5 mm below the dura, with the incisor bar located 3.3 mm below the interaural line on the nondominant side.

Quantitation of rotational behaviour: The animals were tested for rotations in response to apomorphine (Sigma, USA, 0.05 mg/kg sc) two days before

6-OHDA (Sigma, USA) and five weeks after stereotaxic injection in both groups. Apomorphine was injected first to observe apomorphine induced rotational behavior immediately after apomorphine administration for 30 min duration¹³.

Staircase test: The food restriction was started seven days before 6-OHDA injection. After two days of food restriction, the animals were tested for staircase test. The animals performed the staircase test five days before (pre-lesion) and five weeks after 6-OHDA injection (post-lesion). The food restriction was also done for two days prior to staircase test after five weeks of 6-OHDA injection. Five pellets were placed on both sides of every step, giving a total of 30 pellets available on each side. As staircase apparatus consists of six steps in both sides. The test score corresponds to the number of pellets taken and eaten. Analysis was then performed using the results of the last three days during the performance plateau¹⁴.

Stepping test: This is a test for akinesia. The rat was held with one hand by the experimenter fixing the hind limbs (slightly raising the torso) and with the other hand fixing the forelimb that was not to be monitored. In this way the other forepaw had to bear the weight. The rat was moved slowly sideways in both forehand and backhand directions. This was done for both the contralateral and ipsilateral forepaw. The number of adjusting steps for both directions and both paws were counted¹⁵.

Initiation time: Initiation time, the time to actively initiate a forelimb movement was determined in the same test sessions as for the stepping test. The rats were pre-trained for two days to turn up a wooden ramp (1.1 m) into their home cage. During the test, the rat was held as described for the stepping test and placed with its unrestrained paw (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 toward the home cage was recorded. The test was performed once a day for each

forelimb on three consecutive days and the mean of the three test sessions was calculated¹⁶. All 15 rats were tested daily.

Postural test: In the same test sessions as for the stepping test, postural balance was examined in a side-falling test. The rat was held in the same position as described for the stepping test and then in a fast movement tilted toward the side of the paw touching the table, which caused a loss of balance. The animal's attempt to regain balance with an adjusting step toward the side was monitored by a scoring system ranging from 0 to 3: (0) no detectable muscle reaction, the rat falls onto the side; (i) clear forelimb reaction, but the rat cannot move limb under the body toward the center of gravity and thus still falls onto the side; (ii) incomplete recovery of balance *i.e.*, the rat moves its limb under the body but not yet fully into the center of gravity, and thus the forelimb is not aligned vertically to the body; further, the forepaw might not be placed in a plain position on the table and digits might be crossed over each other; (iii) complete recovery of balance. The test was repeated six times every day on both sides giving a maximum daily score of 18. Final results were expressed as average of the three-test days score¹⁷. Fifteen animals repeated 6 times every day for 3 days then mean calculated from 270 values for 15 rats.

Disengage time: A blunt wooden probe touched the perioral region beneath the vibrissae of the rat repeatedly at 1 s intervals when the rat was engaged in eating a piece of milk chocolate. The latency of the orienting response, *i.e.*, turning of the head toward the stimulus, was recorded; an immediate response was scored as 1 s. Stimulation was discontinued if the animal did not respond within a period of 180 s. As for the stepping test, a blinded experimenter who was not aware of the identity of the animals performed this test. The test was performed once a day on each side over 2 days and the mean of the two subtests was calculated¹⁷.

Brain histology: After the completion of behaviour test, all animals were anaesthetized with lethal dose

of sodium pentobarbital. The rats were perfused with 4 per cent paraformaldehyde in 0.1 M, pH 7.4 phosphate buffer solution (PBS). The brains were removed and placed in the same fixative for 24 h. Then they were transferred to 15 per cent sucrose in 0.1 M PBS until they sank. Brains sections (5 μ m cryostat coronal sections) were cut using a microtome, and were stained with haematoxylin & Eosin (H & E) and observed for neurons under ordinary microscope¹⁸.

Statistical analysis: The significance of difference between pre-lesion and post-lesion within the group was determined by paired student's t test. Unpaired student's t test was used to find the significance of difference between the values of Groups 1 and 2 at base line and post lesion. $P < 0.05$ was regarded as being statistically significant.

Results

Rats subjected to stereotaxic injection of 6-OHDA and receiving melatonin had significant effect

in various behaviour tests *i.e.*, apomorphine-induced rotational behaviour, staircase test, stepping test, initiation time, postural balance test and disengage time (Table). The difference between pre- and post-lesion values of group 1 was found statistically significant in apomorphine-induced rotational behaviour, staircase test, initiation time and disengage time ($P < 0.001$) and in stepping test and postural balance test ($P < 0.05$). Comparative analysis between pre- and post-lesion values of group 2 was found significant in apomorphine-induced rotational behaviour and disengage time ($P < 0.001$) and in staircase test, initiation time and postural balance test ($P < 0.05$). Statistically significant difference was found between post-lesion values of groups 1 and 2 [$P < 0.001$ in apomorphine-induced rotational behaviour, staircase test (success rate) and disengage time; $P < 0.05$ in stepping test, initiation time and postural balance test]. No significant differences were seen between pre-lesion values of groups 1 and 2.

Marked losses of DA neurons were observed in group 1 animals. The animals of group 2 did not

Table. Behavioural tests for groups 1 and 2 rats before and after 6-OHDA lesion

Behaviour tests	Group 1		Group 2	
	Pre-lesion	Post-lesion	Pre-lesion	Post-lesion
Apomorphine-induced rotational behaviour contralateral full body turns/30 min	13 \pm 2	268 \pm 18**	11 \pm 1	140 \pm 10***##
<i>Staircase test:</i>				
No. of pellets taken	28 \pm 2	12 \pm 1**	25 \pm 2	19 \pm 3 *#
No. of pellets eaten	25 \pm 1	4 \pm 1**	23 \pm 2	13 \pm 2*#
Success rate (%) $\left(\frac{\text{Pellets eaten}}{\text{Pellets taken}} \times 100 \right)$	89 \pm 2	33 \pm 2**	92 \pm 3	68 \pm 3***
<i>Stepping test:</i>				
No. of steps in forehand direction	10 \pm 2	6 \pm 1*	12 \pm 1	10 \pm 1#
No. of steps in backhand direction	14 \pm 1	7 \pm 1*	12 \pm 2	11 \pm 2#
Initiation time (sec)	2 \pm 1	16 \pm 2**	3 \pm 1	9 \pm 1*#
Postural balance test score	17 \pm 1	5 \pm 1*	15 \pm 2	11 \pm 2*#
Disengage time (sec)	7 \pm 1	120 \pm 10**	6 \pm 1	80 \pm 4***##

Data expressed as mean \pm standard error of the mean (S.E.M.) (n=15 in each group)

* $P < 0.05$ vs pre-lesion value of same group ** $P < 0.001$ vs pre-lesion value of same group

$P < 0.05$ vs post-lesion value of group 1; ## $P < 0.001$ vs post-lesion value of group 1

exhibit any significant neuronal loss on macroscopic and microscopic examination of rat striatum section (Figs 1, 2). Injected 6-OHDA alone resulted in almost complete loss of DA neurons in group 1 rats compared to the group 2. Partially preserved DA neurons were observed in melatonin treated animals of group 2.

Discussion

The findings of the present study demonstrated that systemic administration of melatonin corrected a hemi-Parkinson's condition in rats caused by intra striatal application of the neurotoxin 6-OHDA. Various behaviour tests used in the present study,

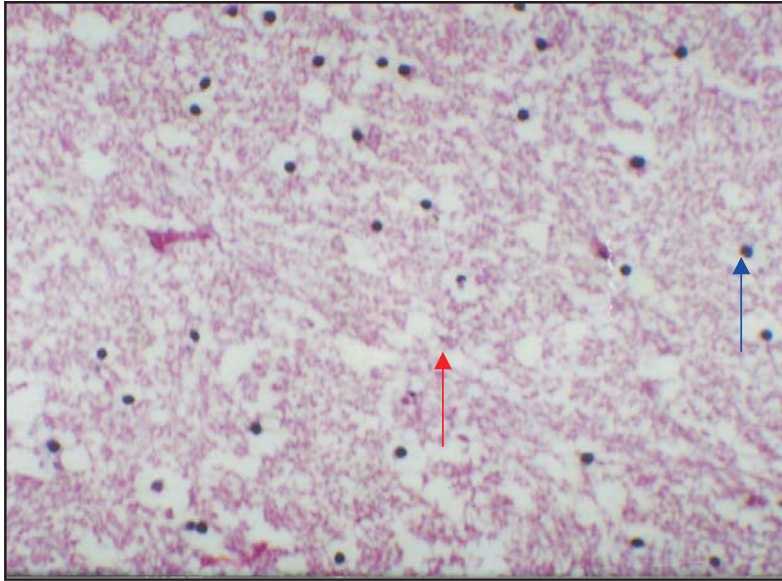


Fig. 1. Photomicrograph of striatal areas showing a significant neuronal loss (red arrow) on the lesioned side concomitant with enhanced astroglial profile (blue arrow) density in the group 1. 250 x haematoxylin and eosin.

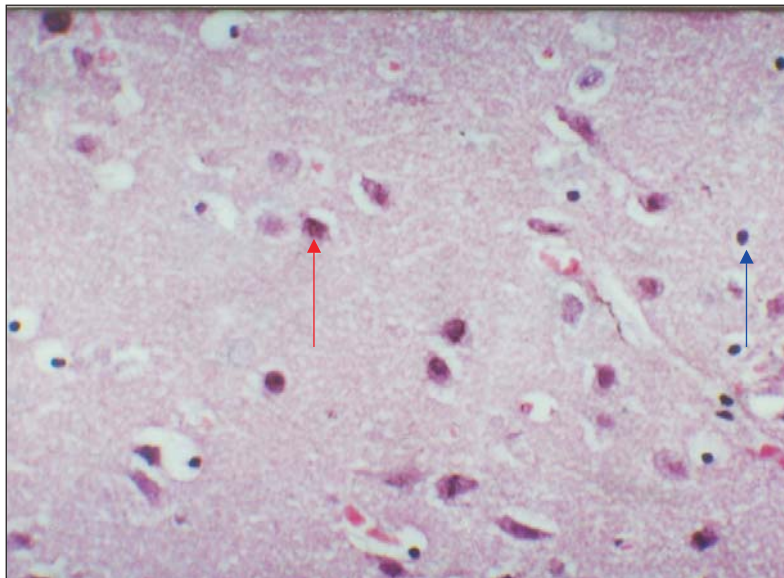


Fig. 2. Photomicrograph illustrating different cell type on haematoxylin and eosin stained sections of striatal tissue of rats of group 2. Note the typical neuronal profile (red arrow) with large cytoplasm, polygonal shape and emanating processes, astroglial profile (blue arrow) with small, round and intensely stained nuclei. 250 x haematoxylin and eosin.

acted as an index of striatal dopaminergic function. Melatonin improved these behaviour tests in 6-OHDA-induced rat model of PD. Our findings were consistent with earlier reports of improved motor activity in PD^{19,20}. But there is controversy in earlier reports regarding the protective potential of melatonin in PD. Anton-Tay *et al*²¹ suggested improved motor activity in PD patients given high doses of melatonin. Later replication of studies employing melatonin administration either failed to confirm such therapeutic effects²² or actually demonstrated worsening by melatonin²³. In the original clinical study where melatonin was administered to PD patients²¹, there were difficulties with experimental design and interpretation. Firstly, the definition of each patient's condition prior to treatment and their response after the treatment commenced and during drug withdrawal was not standardized²⁴. Secondly, the doses of melatonin employed were large (1.2 g/day) making a meaningful interpretation as to the therapeutic mechanism difficult to decipher²⁴. Thirdly, the melatonin was mixed with 2 per cent alcohol solution, a combination that will enhance the sedative effect of melatonin²⁵. Fourthly, a bolus of melatonin (5 mg/kg) given immediately after 6-OHDA stereotaxic injection failed to modify apomorphine-induced contralateral rotation. This is consistent with the short (20 min) biological half-life of melatonin¹⁹. These factors were improved in later studies. Melatonin was administered one hour after 6-OHDA lesion and thereafter daily for seven days²⁶. Osmotic minipumps filled with a solution of melatonin were placed in the subcutaneous tissue between the scapulae. The delivery rate was constant at $1 \pm 0.15 \mu\text{l/h}$ ($50 \pm 7.5 \mu\text{g melatonin/h}$). This produced a plasma concentration of $1660 \pm 240 \text{ pg melatonin/ml}$ for at least 7 days¹⁹. In the present study also melatonin was injected for seven days to increase melatonin bioavailability.

In fact, recent findings suggest that melatonin may improve motor system function. Administration of melatonin is shown to reduce the apomorphine-

induced rotational behaviour^{26,27}, reversed the dopamine (DA), 3, 4, dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) level²⁶, restored the dopamine, tyrosine hydroxylase enzyme activity and malondialdehyde (MDA) level²⁸, resulted in the survival of dopaminergic neurons in substantia nigra (SN) and tyrosine hydroxylase (TH)-immunoreactive terminal in the dorsolateral striatum²⁷.

In the present study, we used the partial lesion rat model of PD, which involves 6-OHDA administration to striatal dopaminergic terminals. This induces rapid degeneration of striatal dopaminergic fibers, followed by the protracted death of their cell bodies in the SN, which begins after a delay of about 1 wk and progresses over several weeks. This partial lesion is a more appropriate model of the early stages of PD, when neurodegeneration is still ongoing, compared to the complete lesion of the medial forebrain bundle (MFB), which is a good model of the end stage of the disease. This reflects more accurately the pathological process of neurodegeneration that occurs in PD than the complete lesion model does, making the intrastriatal lesion a suitable model¹⁸.

6-OHDA causes the selective and almost complete destruction of dopaminergic neurons in the substantia nigra or nigrostriatal pathway. It is accumulated by dopaminergic containing nerves and subsequently causes their degeneration. 6-OHDA is extremely unstable in aqueous solution and auto-oxidizes to form the super oxide radical, the hydroxyl radical and hydrogen peroxide. One or a combination of these chemicals is responsible for 6-OHDA induced neurotoxicity¹³. Elevated reactive oxygen species (ROS) may participate in 6-OHDA neurotoxicity, as evidenced by reductions in brain glutathione (GSH) and loss in superoxide dismutase (SOD) activity²⁹. Melatonin stimulates antioxidant enzyme such as SOD, glutathione peroxidase (GPX) and glutathione reductase (GR)³⁰. Recently the increment in antioxidant enzyme activities induced

by melatonin is shown to involve the inhibition of the retinoid-related orphan receptor α (ROR α) pathway³⁰.

Melatonin has been reported to be highly effective endogenous free radical scavenger, and increases the mRNA levels and the activity of various antioxidant enzymes²⁰. Taken together our results support the hypothesis that melatonin, as an antioxidant, may have beneficial effect on therapeutic approaches for the animal model of PD.

In conclusion, our findings demonstrated that systemic administration of melatonin protected striatal dopaminergic neurons against 6-OHDA neurotoxicity in the rat. The effect was accompanied by a significant recovery in behaviour tests. Further studies need to be done to confirm these findings.

Acknowledgment

One of the authors, Shalini Singh acknowledges Indian Council of Medical Research, New Delhi, for providing financial support.

References

1. Agid Y, Chase T, Marsden D. Adverse reaction to levodopa drug toxicity or progression of disease. *Lancet* 1998; *351* : 851-2.
2. Tan DX, Poeggeler B, Reiter RJ, Chen CD, Chen S, Manchester LC, *et al.* The pineal hormone melatonin inhibits DNA adduct formation induced by the chemical carcinogen safrole *in vivo*. *Cancer Lett* 1993; *70* : 65-71.
3. Reiter RJ. Oxidative damage in the central nervous system: protection by melatonin. *Prog Neurobiol* 1998; *56* : 359-84.
4. Akcay YD, Yalcin A, Sozmen EY. The effect of melatonin on lipid peroxidation and nitrite/nitrate levels, and on superoxide dismutase and catalase activities in kainic acid-induced injury. *Cell Mol Biol Lett* 2005; *10* : 321-9.
5. Baydas G, Ozveren F, Akdemir I, Tuzcu M, Yasar A. Learning and memory deficits in rats induced by chronic thinner exposure are reversed by melatonin. *J Pineal Res* 2005; *39* : 50-6.
6. Wu YH, Swaab DF. The human pineal gland and melatonin in aging and Alzheimer's disease. *J Pineal Res* 2005; *38* : 145-52.
7. Leon J, Castroviejo DA, Escames G, Tan DX, Reiter RJ. Melatonin mitigates mitochondrial malfunction. *J Pineal Res* 2005; *38* : 1-9.
8. Shida CS, Castrucci AMI, Lamy-Freund MT. High melatonin solubility in aqueous medium. *J Pineal Res* 1994; *16* : 198-201.
9. Costa EJX, Lopez RH, Lamy-Freund MT. Permeability of pure lipid bilayers to melatonin. *J Pineal Res* 1995; *19* : 123-6.
10. Reiter RJ. Melatonin: that ubiquitously acting pineal hormone. *New Physiol Sci* 1991; *6* : 223-7.
11. Menendez-Pelaez A, Poeggeler B, Reiter RJ, Barlow-Walden LR, Pablos MI, Tan DX. Nuclear localizations of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. *J Cell Biochem* 1993; *53* : 373-82.
12. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. 2nd ed. Sydney, Australia: Academic Press; 1986.
13. Ungerstedt U, Arbuthnot GW. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res* 1970; *24* : 485-93.
14. Montoya CP, Campbell-Hope LJ, Pemberton KD, Dunnett SB. The "Staircase test": a measure of independent forelimb reaching and grasping abilities in rats. *J Neurosci Methods* 1991; *36* : 219-28.
15. Schallert T, Ryck MD, Wishaw IQ, Ramirez VD, Teitelbaum P. Excessive bracing reactions and their control by atropine and L-dopa in an animal analog of parkinsonism. *Exp Neurol* 1979; *64* : 33-43.
16. Olsson M, Nikkiah 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* : 3863-75.
17. Winkler C, Sauer H, Lee CS, Bjorklund A. Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. *J Neurosci* 1996; *16* : 7206-15.
18. Sauer H, Oertel WH. Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: A combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience* 1994; *59* : 401-15.
19. Sala FD, Santo SD, Franceschini D, Skaperr SD, Giusti P. Melatonin protects against 6-OHDA-induced neurotoxicity

- in rats: a role for mitochondrial complex I activity. *FASEB J* 2001; 15 : 164-70.
20. Mayo JC, Sainz RM, Uria H, Antolin I, Esteban MM, Rodriguez C. Melatonin prevents apoptosis induced by 6-hydroxydopamine in neuronal cells: implications for Parkinson's disease. *J Pineal Res* 1998; 24 : 179-92.
21. Anton-Tay F, Diaz JL, Fernandez-Guardiola A. On the effect of melatonin upon human brain. Its possible therapeutic implications. *Life Sci* 1971; 10 : 841-50.
22. Shaw KM, Stern GM, Sandler M. Melatonin and parkinsonism. *Lancet* 1973; 1 : 271-9.
23. Willis GL, Armstrong SM. A therapeutic role for melatonin antagonism in experimental models of Parkinson's disease. *Physiol Behav* 1999; 66 : 785-95.
24. Guarduika-Lemaitre B. Toxicology of melatonin. *J Biol Rhythms* 1997; 12 : 697-706.
25. Willis GL, 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 p. 10.
26. Aguiar LMV, Vasconcelos SMM, Sousa FCF, Viana GSB. Melatonin reverses neurochemical alterations induced by 6-OHDA in rat striatum. *Life Sci* 2002; 70 : 1041-51.
27. Mayo JC, Sainz RM, Antolin I, Rodriguez C. Ultrastructural confirmation of neuronal protection by melatonin against the neurotoxin 6-hydroxydopamine cell damage. *Brain Res* 1999; 13 : 221-7.
28. Joo WS, Jin BK, Park CW, Maeng SH, Kim YS. Melatonin increases striatal dopaminergic function in 6-OHDA-lesioned rats. *Neuroreport* 1998; 9 : 4123-6.
29. Kumar R, Agarwal AK, Seth PK. Free radical-generated neurotoxicity of 6-hydroxydopamine. *J Neurochem* 1995; 64 : 1703-7. Erratum in: *J Neurochem* 1995; 65 : 1906.
30. Zapico T, Montes C. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. *J Pineal Res* 2005; 39 : 99-104.

Reprint requests: Dr B. Krishana, Professor, Department of Physiology, Maulana Azad Medical College
New Delhi 110002, India
e-mail: dr.balkrish_mamc@yahoo.co.in