Neuroendocrinology Letters ISSN 0172–780X

ORIGINAL ARTICLE

Protective role of melatonin against MPTP-induced mouse brain cell DNA fragmentation and apoptosis in vivo

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Submitted: *January 20, 2001*
Accepted: *February 4, 2001*

Key words: **melatonin; apoptosis; DNA fragmentation; Parkinson's disease; MPTP**

*Neuroendocrinology Letters 2001; **22**:101–108 pii: NEL220201A04 Copyright © Neuroendocrinology Letters 2001*

Abstract

OBJECTIVES: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that induces a Parkinsonian-type syndrome in animals which is similar to Parkinson's disease in humans. MPTP toxicity partially depends on the production of free radicals which in turn play a key role in the apoptotic death of neurons. In the present study melatonin, a potent free radical scavenger with antiapoptotic properties, was given to determine whether it would reduce oxidative stress in mice treated with MPTP.

MATERIALS AND METHODS: Male mice were given MPTP with or without melatonin and the brain was studied either 6h, 24h, 7 days or 15 days after the last MPTP injection. RESULTS: The results show that melatonin counteracted in vivo MPTP-induced apoptosis in midbrain neurons at 6 and 24 h after MPTP treatment, and partially prevented apoptosis at 7 and 15 days after MPTP administration. MPTP treatment also produced time-dependent cell damage, whereas melatonin reduced the percentage of damaged cells at all time points, the effect being most evident at 15 days after treatment. Moreover, melatonin counteracted MPTP-dependent DNA fragmentation in the midbrain and striatum at 7 and 15 days after drug administration.

CONCLUSION: These results support a role for melatonin in protecting neurons against MPTP toxicity in vivo, and suggest that its antiapoptotic action is one of the mechanisms by which melatonin protects neuronal cells from neurotoxic insults.

Introduction

Parkinson's disease is a multifactorial condition mainly characterized by degeneration of the dopaminergic nigrostriatal projection. The consequent decrease in striatal dopamine content leads to an uncontrolled cortico-striatal motor circuit dysfunction. The symptoms of Parkinson's disease require years to become apparent since 80% of the cells in the substantia nigra must be lost before signs of the illness are detectable.

Several studies *in vitro* have shown that neurotoxins which produce experimental signs of Parkinson's disease induce apoptosis [1–3]. In *in vivo* studies, acute damage to nigral neurons may also induce apoptosis [1].

The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is normally used to induce Parkinson disease-like neurodegeneration in experimental animal models. MPTP administration to animals [4] produces neural deficits similar to those found in human Parkinsonian subjects [5]. In both cases a massive loss of dopaminergic neurons in the zona compacta of the substantia nigra is apparent. One of the proposed mechanisms to explain the development of Parkinson's disease depends on the production of free radicals. In fact, the presence of high amounts of iron in the substantia nigra of Parkinsonian patients [6], the elevated levels of lipid peroxides in the midbrain of these patients [7], and the reduction in the enzymes involved in metabolically removing free radicals such as glutathione peroxidase, catalase, and superoxide dismutase [8] support a role for oxidative stress in the pathophysiology of the Parkinson's disease. Most of the changes associated with this disease have been also described in MPTP toxicity [9].

Melatonin is synthesized in the pineal gland and in several other tissues. Earlier studies related to melatonin have mainly focused on its actions at the level of the hypothalamo-hypophyseal-gonadal axis, whereby it controls seasonal reproduction. More recently, other physiological roles for melatonin have been reported including neuroimmunomodulation, antineoplasic properties and gene transcription regulation [10]. Additionally, melatonin possesses significant antioxidant capacity. Besides directly scavenging several reactive oxygen species [11, 12], it has been shown to prevent oxidative damage under a wide variety of experimental conditions [13–16]. Thus, a large number of conditions, in which free radicals are involved [12, 13, 15], may benefit from melatonin treatment. The aim of the present work was to investigate the possible protective effect of melatonin on MPTP-induced apoptosis in ventral mesencephalic-striatal areas *in vivo*.

Material and Methods

Animals and drugs

All reagents were of the highest quality available. Tyrosine hydroxylase rabbit antiserum was purchased from Pel Freez. Melatonin and MPTP were purchased from Sigma (St. Louis, MO). Other reagents were obtained from commercial sources.

Twelve to 14 week old male C57/B6 mice were housed in clear plastic cages under a light:dark cycle (12:12h, lights on 07:00h) and in a temperature controlled (22 ± 2°C) room. The mice received standard laboratory chow and water *ad libitum*.

Treatments

MPTP was dissolved in saline and administered subcutaneously (sc) in two doses of 20 mg/kg each separated by 24h. Melatonin was dissolved in 3% ethanol:saline and injected intraperitoneally (ip) at a dose of 10 mg/kg every 6h. Group 1 (control) received two sc and corresponding ip injections of vehicles for MPTP and melatonin, respectively. Group 2 (melatonin) received ip injections of melatonin and two sc injections of MPTP vehicle. Group 3 (MPTP) was injected ip with melatonin vehicle and received two sc injections of MPTP. Group 4 (MPTP+melatonin) received both melatonin and MPTP. The animals were killed by decapitation at 6h, 24h, 7 days and 15 days after the last dose of MPTP.

Tissue preparation

Brains from 8 animals of each group were collected immediately after the animals were killed; the striatum and midbrain were removed and frozen on solid CO_2 for DNA fragmentation assay. From another 8 animals, the brain was removed and fixed in 2% paraformaldehyde for the apoptosis assay. Eight animals of the same groups were anesthetized with equithesin and perfused transcardiacally for 2 min with PBS, pH 7.4, containing 1000 μl/L heparin and 1 g/L procaine. Thereafter, the animals were perfused for an additional 10 min with 4% paraformaldehyde in 0.1M PBS. The brains were removed and postfixed for 24h in the same fixation solution. They were then blocked in Paraplast-Plus and five-micrometer-thick sections were cut, stained with hematoxylin-eosin and examined with a light microscope. Cellular damage was measured on each slide by examining 10 high power fields (500x), using an image analysis system (analySIS 2.0).

Immunohistochemical procedures

Tyrosine hydroxylase (TH) activity was identified using the streptavidin-biotin-peroxidase complex. Sections were rinsed in TBS and incubated successively with: 1) normal sheep serum (20%) to reduce

nonspecific binding for 30 min in a moisture-saturated chamber at 37°C; 2) antityrosine hydroxylase rabbit antiserum at 1/300 overnight in a saturated chamber at room temperature; 3) streptavidin/peroxidase complex (Vectastasin) at 1:100 for 30 min in a moisture-saturated chamber at room temperature. Sections were rinsed after each step with TBS (3 x 5 min). During step 2, control sections were incubated with an equal volume of TBS rather than antiserum. The streptavidin-biotin-peroxidase activity was revealed using 3-3-diaminobenzidine tetrachloride (50 mg/100 ml) in the presence of 0.5% H_2O_2 in TBS.

DNA extraction and gel electrophoresis

After removing the midbrain and striatum, a small piece of each tissue was used for DNA extraction following a modification of the method of Gustincich *et al.* [17]. Briefly, minced tissue was resuspended in 300 μl of 0.5% trypsine solution and incubated with shaking at 37°C for 30 min. Samples were then mixed with 600 μl of lysis buffer (8% DTAB, 1.5M NaCl, 100 mM Tris-HCl pH 8.6, 50 mM EDTA) and incubated at 68°C for 15 min. Chloroform (900 μl) was then added, and after mixing by inversion, the samples were centrifuged at 10,000 g for 15 min. The aqueous phase was recovered into a new tube. 100 μl of Dnase/free Rnase were added and the samples were incubated at 37°C for 1h. Nine hundred μl of H_2O and 100 μl of CTAB (5% in 0.4M NaCl) were added and mixed gently. The pellet was then separated by centrifugation and resuspended in 300 μl of NaCl 0.2M. Finally, DNA was recovered by ethanol precipitation. DNA was analyzed electrophoretically in order to observe DNA fragmentation in a 2 % agarose gel containing 1 μg/ml ethidium bromide. DNA ladder patterns were visualized by UV light and photographed with a Polaroid camera.

Apoptosis assay

The level of apoptosis in midbrain (substantia nigra, pars reticularis and pars compacta) was assayed *in situ* using the terminal deoxynucleotidyl transferase assay (Apoptag^{TM} Kit, Oncor, Inc. USA). Apoptotic cells were counted using a light microscope at a magnification of 300x. A total of 32 high power fields (4 per animal, 8 animals per group) were examined using a image analysis system (analySIS 2.0).

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student-Newman-Keuls test was applied to compare the groups. The level of significance was accepted at $p<0.05$.

Results

Immunohistochemical examination

Since the results were similar at all time points, the photographs corresponding to 15 days of treatment only are shown. The TH-immunopositive fibers were clearly observed in the nigrostriatal system of control mice (Figs. 1A and 1B). After MPTP treatment the number of these fibers was substantially reduced (Fig. 1C). In MPTP + melatonin treated animals, the number of TH-immunoreactive fibers was greater than in MPTP-treated mice, suggesting a protective effect of melatonin against MPTP (Fig. 1D).

DNA fragmentation

DNA fragmentation was not observed at 6 and 24 hours after MPTP treatment. At 7 and 15 days, MPTP administration caused an increase of DNA fragmentation, with the typical appearance of DNA laddering in both the midbrain and striatum (Figs. 2A and 2B). The lanes with DNA from cells treated with melatonin (lanes 3 and 4 for 7 and 15 days, respectively) showed a less visible ladder than those treated only with MPTP only (lanes 5 and 6 for 7 and 15 days, respectively) in both midbrain (Fig. 2A) and striatum (Fig. 2B).

Apoptosis

To investigate whether melatonin prevents apoptosis of mice treated with MPTP, we measured the number of apoptotic cells in midbrain (substantia nigra, pars reticularis and compacta) (Fig. 3). Treatment with MPTP increased the number of apoptotic cells compared with control and melatonin groups in a time-dependent manner (animals sacrificed 15 days after treatment had the highest level of apoptotic cells). The number of apoptotic cells was always lower in the groups treated with melatonin + MPTP than in the MPTP-treated only group.

Total number of cells and damaged cells

Figure 4 shows the total number of cells in midbrain at 15 days of treatment (in 10 high power fields). There was no variation in the total number of cells after 6h, 24h and 7 days of treatment. At 15 days, the total number of cells in the MPTP group diminished significantly, whereas treatment with melatonin prevented, at least in part, this decrease.

To determine the percentage of damaged cells in the midbrain, the following factors were considered: cloudy swelling, hyperchromatic nuclei and what appeared to be degenerating cells. The percentage of damaged cells increased with time after treatment with MPTP (Fig. 5). In general, treating MPTP-injected animals with melatonin diminished the per-

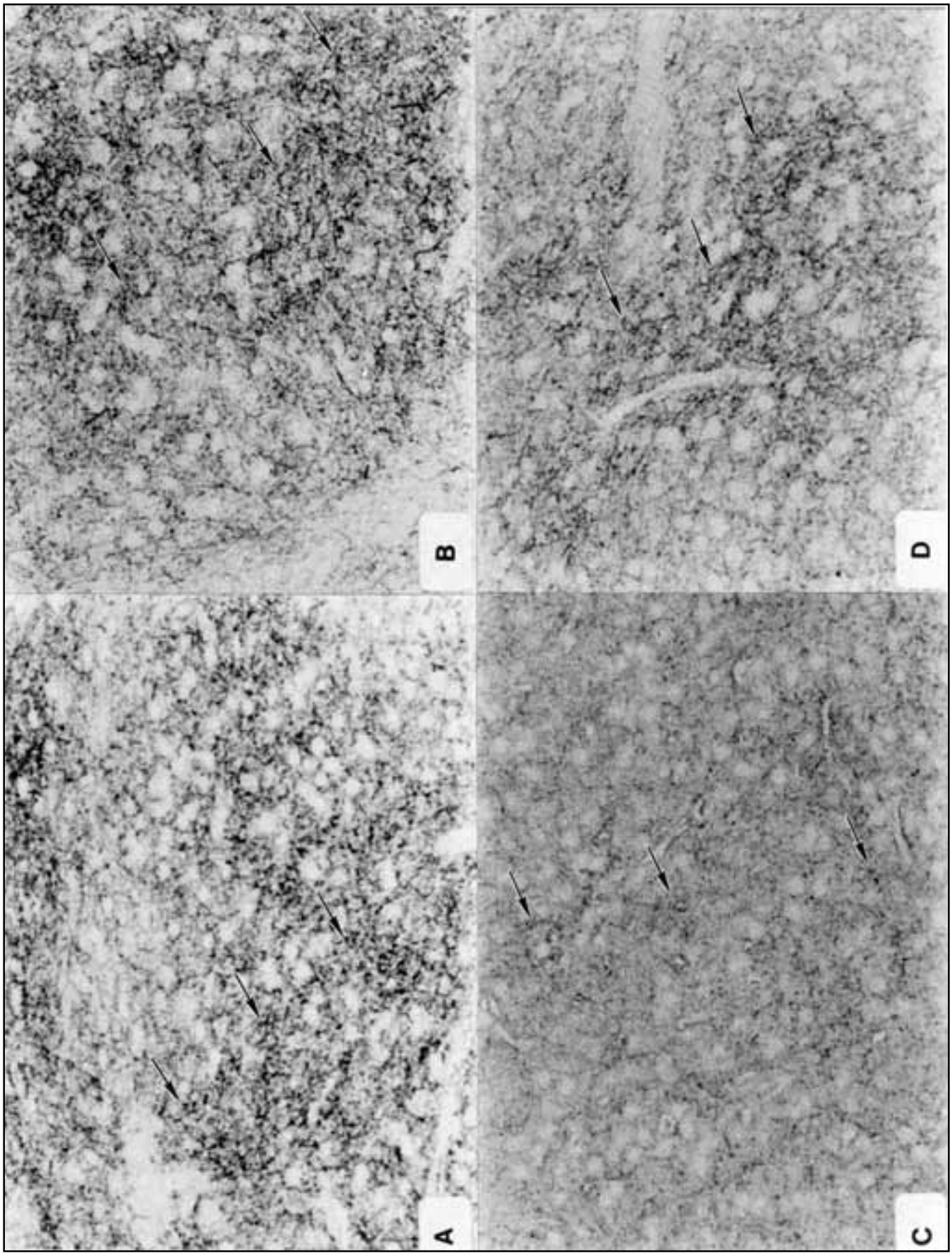

Fig. 1. Tyrosine hydroxylase immunoreactivity in mouse striatum at 15 days after treatment. A) control, B) melatonin, C) MPTP, D) melatonin + MPTP.

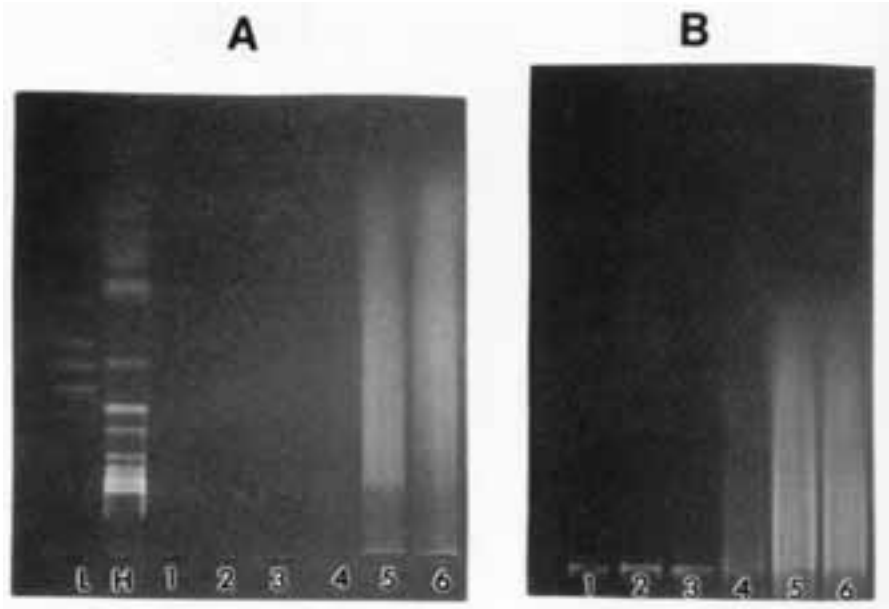

Fig. 2. Melatonin protection against DNA damage induced by MPTP. Figures A (mid brain) and B (striatum): lanes L and H = low and high molecular weight DNA markers; lane 1 = control; lane 2 = melatonin; lanes 3 and 4 = melatonin + MPTP at 7 and 15 days, respectively; lanes 5 and 6 = MPTP at 7 and 15 days, respectively.

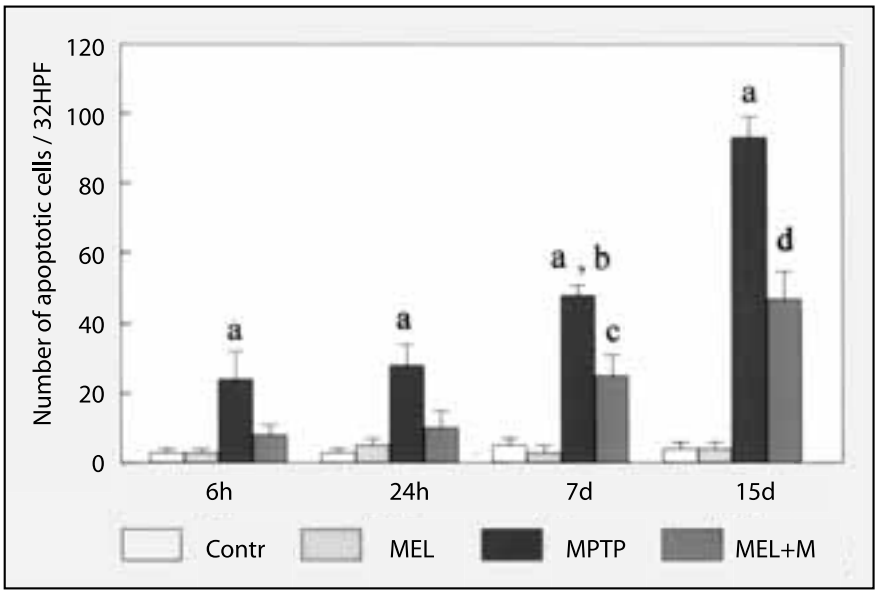

Fig. 3. Melatonin protection against apoptosis induced by MPTP. a: P < 0.001 versus control, melatonin and melatonin + MPTP at the same time; b: P < 0.001 versus MPTP at 6h, 24h and 15d; c: p < 0.001 versus Mel+MPTP at 6h, 24h and 15d, and control at 7d; d: P < 0.001 versus control at 15d. Data are means ± S.D.

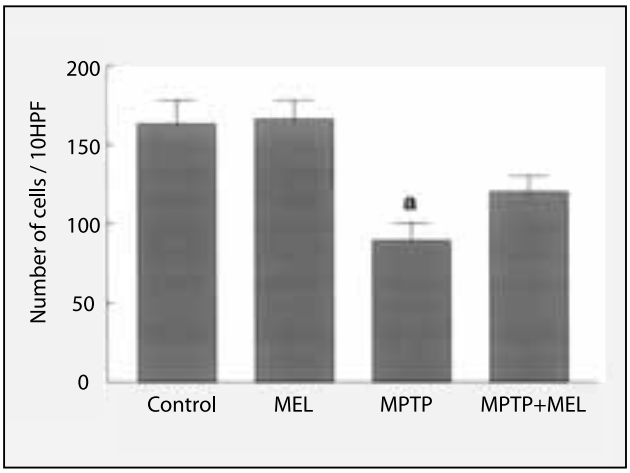

Fig. 4. Total number of cells presented in the midbrain at 15 days after treatment. a: p < 0.01 versus control, melatonin and melatonin + MPTP. Data are means ± S.D.

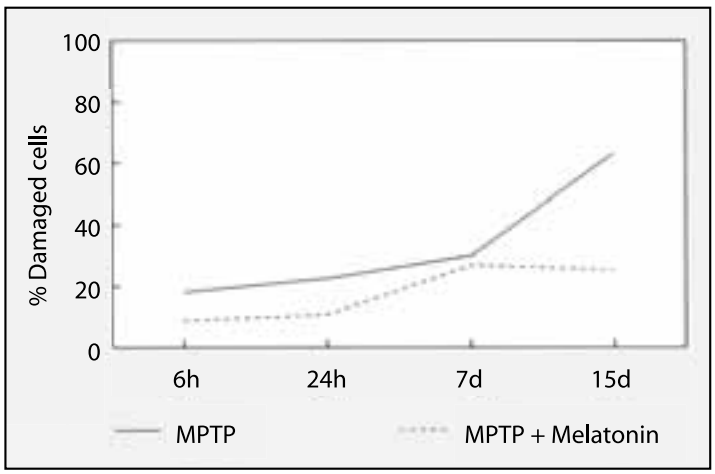

Fig. 5. Melatonin protection against damaged cells (cloudy swelling, hyperchromatic nuclei and dying cells) induced by MPTP in midbrain.

centage of damaged cells in comparison with MPTP-treated only group.

Discussion

This study demonstrates that melatonin inhibits apoptosis and DNA fragmentation induced by MPTP in striatum and midbrain of C57/B6 mice. Also, melatonin reduced cellular loss due to MPTP.

Apoptosis is a controlled form of cell death, although the genetic machinery involved in this process is not yet understood. There are several genes involved in apoptosis including the p53 tumor suppressor gene, which delays cell cycle progression before the initiation of replicative DNA synthesis, and the bcl-2 cell death suppressor gene [18–20]. A number of signalling events are related to apoptosis: cytosolic Ca^{2+} rises, cAMP accumulation, activation of protein kinase C, tyrosine kinases, etc. [21]. However, in some cells these signals promote apoptosis whereas in others they block the apoptotic response.

Neuronal cell death occurs in a number of neuropathological states in humans such as Alzheimer's and Parkinson's diseases [21, 22]. Recently, non-physiological agents such as radiation, toxic substances and drugs, have also been found to induce apoptosis [2, 3, 21]. The neurotoxin MPTP, and its metabolite1-methyl-4-phenylpyridinium ion (MPP+), are known to cause selective loss of dopaminergic neurons in the CNS, thereby mimicking a major clinical sign of idiopathic Parkinson's disease [22]. MPP+ and MPTP induce apoptosis of cerebellar granular cells, ventral mesencephalic-striatal co-culture cells, SH-SY5Y neuroblastoma cells, and PC12 cells [1–3]. The present findings suggest that apoptosis is involved in MPTP-induced neurotoxicity. During metabolism, MPTP produces oxygen radicals [23], and the resulting oxidative stress is thought to play a key role in the apoptotic death of neurons. Thus, oxidative stress is proposed as one of the likely mechanisms of MPTP toxicity [3, 23].

Our work shows that melatonin counteracted the *in vivo* MPTP-induced apoptosis in midbrain at 6h and 24h after MPTP administration, but only partially at 7 and 15 days after MPTP. We also observed that at 15 days after MPTP treatment, the total number of cells diminished significantly, indicating massive cellular death; this was also prevented in part by melatonin treatment. MPTP induced a time-dependent increase in damaged cells (cloudy swelling, hyperchromatic nuclei and degenerative cells). Melatonin reduced the percentage of damaged cells in all cases, the effect being most evident at 15 days after treatment.

MPTP administration produced DNA fragmentation in both midbrain and striatum at 7 and 15 days of treatment, and melatonin reduced this DNA damage in all cases. Melatonin also reduced the loss of TH-immunoreactive fibers in striatum of MPTP-treated mice. Collectively, these data suggest a protective effect of melatonin against MPTP neurotoxicity.

The MPTP-induced Parkinson's disease model has provided some answers regarding the mechanisms of degeneration of the neurons in this disease [24, 25]. MPTP exerts its toxic effect through its transformation by monoamine oxidase B (MAO B) to MPP+. MPP^+ accumulates in the mitochondria and inhibits complex I of the electron transport chain. This produces a rapid decrease of the ATP levels, an accumulation of reduced nicotinamide-adenine-dinucleotide (NADPH) and lactic acid, and a significant alteration in calcium homeostasis.

There are reports claiming that several factors show either an *in vivo* and *in vitro* protection against MPTP damage. These agents include neurotrophic factors [26, 27], other antioxidants [28, 29], inhibitors of MAO B [30, 31], iron chelators [32], blockers of the NMDA receptors [33], and melatonin [34]. The proposed protective mechanisms are thought to be related in each case to their respective antioxidant actions.

The oxidative stress hypothesis of MPTP neurotoxicity is based on the assumption that the damage caused by free radicals accumulates slowly until it interferes with neuron function and eventually kills them. The major free radical implicated in this mechanism is nitric oxide (NO) [35–37]. Increased levels of NO produce pro-apoptotic effects. NO donors can trigger apoptosis and DNA fragmentation in human neuroblastoma SH-SY5Y cells in a concentration- and time-dependent manner, mainly due to the activation of caspase-3-like protease activity [38]. In addition, it has also been shown that specific NO scavengers and nitric oxide synthase (NOS) inhibitors block apoptosis, indicating that NO and/or peroxynitrite anion ($ONOO^-$) are involved in this mechanism of cellular death [39, 40].

Melatonin has been identified as a potent free radical scavenger. Melatonin efficiently scavenges a variety of radicals [39–41] including NO [42] and $ONOO^-$ [43]; it is believed to act in this capacity in all subcellular compartments [44] to decrease tissue damage due to free radical attack [45, 46]. Melatonin treatment prevents MPP^+ neurotoxicity by counteracting lipid peroxidation in the midbrain; this action, like the present findings, indicate it functions as an antioxidant to reduce MPTP neurotoxicity [3, 41, 47].

Recent studies have shown that in cerebellum [48], hypothalamus [49] and striatum [50] physiological levels of melatonin reduce the activity of the constitutive isoform of the NOS. Treatment with melatonin also decreased the tissue levels of nitrite (a

metabolite of nitric oxide) produced by the inducible isoform of the enzyme and inhibited the *in vivo* activity of the enzyme and the expression of its mRNA [14]. Thus, both antioxidant and inhibitory NOS [14, 51] activity of melatonin appears to be involved in the protective effect of the indoleamine. Finally, melatonin may reduce MPTP toxicity at the mitochondrial level by increasing the efficiency of oxidative phosphorylation [52–54].

This work demonstrates that melatonin protects DNA from fragmentation and reduces cellular apoptosis due to MPTP administration. Melatonin's multiple antioxidative actions [11, 12, 51, 55] presumably explain its ability to reduce MPTP toxicity.

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