

Original Article

Role of pramipexole in delaying L-dopa induced dyskinesia and enhancing the response to L-dopa in 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine-parkinsonian mice

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A B S T R A C T

L-dopa remains the most effective treatment for Parkinson's disease (PD). However, its effectiveness declines on continuous use, leads to motor complications, and does not modify the progression of the neurodegenerative process. Medical therapy that provides the benefits of l-dopa without motor complications and enhances cell proliferation would be a major advance in the treatment of Parkinson's disease (PD). The aim of the current study was to evaluate the role of pramipexole in delaying l-dopa induced dyskinesia and providing neuroprotective effect for the substantia nigra neurons. Additionally, the study was extended to evaluate the impact of this neuroprotective effect in enhancing the response to l-dopa in the 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) model of PD in mice. Mice were allocated to five groups as follows: saline group, MPTP group, l-dopa group, pramipexole group and l-dopa + pramipexole group. Co-administration of pramipexole with l-dopa delayed the priming of l-dopa-induced dyskinesia, improved the motor performance of MPTP-parkinsonian mice throughout the entire period of the experiment and promoting cell proliferation. Therefore, pramipexole seems to be a useful adjunct to l-dopa in the current model of experimental parkinsonism, not only to gain more therapeutic effect and to delay the priming of l-dopa induced dyskinesia but also to reduce dopaminergic neuron loss and promote cell proliferation.

Key Words: Parkinsonism, MPTP, l-dopa, dyskinesia, pramipexole, cell proliferation.

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1. INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by slowly progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Haleagrahara and Ponnusamy, 2010). Many of the neurotoxic agents that are used to produce animal models of PD are mitochondrial electron transport chain inhibitors, such as MPTP, 6-hydroxydopamine (6-OHDA), rotenone and paraquat (Fleming et al., 2004).

Since its introduction in the late 1960s, l-dopa (L-3,4-dihydroxyphenylalanine) has been the cornerstone of the treatment of PD (Abdin and Hamouda, 2008). However, chronic l-dopa therapy is complicated by the development of motor complications as motor fluctuations, dyskinesias and drug-induced involuntary movements (Pham and Nogid, 2008; Kerstin and Boris, 2009); which can be disabling, difficult to treat, and limit the usefulness of the drug.

The process, by which the brain becomes sensitized such that each administration of dopaminergic therapy modifies the response to subsequent dopaminergic treatments, is called priming. The priming process is associated with changes in receptors for dopamine or other several neurotransmitters (Gerfen et al., 1990; Nash and Brotchie, 2000; Spinnewyn et al., 2011).

New neurons continue to be produced in adult mammals, including humans, predominantly in the anterior subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus. This update focuses on the emerging concept that adult central nervous system (CNS) neurogenesis can be regulated by targeting neurotransmitter receptors in neurological disorders. Dopamine is known by its effects on neurogenesis which in turn drive expression of crucial neurotrophic and growth factors (Hagg, 2009). Such an approach might enable the development of pharmacological treatments which harness the

endogenous potential of the CNS to replace the lost cells and regulate adult CNS neurogenesis (Winner *et al.*, 2009).

Many physicians routinely use dopamine agonists in early disease because they provide symptomatic benefits with a low risk of motor complications. Pramipexole, a non-ergoline azepine derivative, is a D₂-like family selective dopamine agonist, having about 8-fold higher selectivity for D₃ receptors compared to D₂ receptors, and minimal activity at D₄ receptors (Hubble, 2000; Kvernmo *et al.*, 2008). Pramipexole has been recently shown to be endowed with neuroprotective activity, neurogenic potential and cell proliferating capacity (Merlo *et al.*, 2011; Winner *et al.*, 2009).

As the most important challenge in the research on PD is to develop a neuroprotective therapy that can be supplemented with l-dopa in the early course of disease to slow its progression. The present study aimed to explore if pramipexole can reduce dopaminergic neuron loss and promote cell proliferation in the MPTP mouse model of PD, and whether concurrent use of pramipexole with l-dopa may reduce and/or delay l-dopa-induced dyskinesia.

2. MATERIALS AND METHODS

2.1. Animals

Fifty male albino mice, weighing 20-25 g, were used in this study. Mice were purchased from The National Centre of Research (Cairo, Egypt) and housed in clean polyethylene cages in groups of five and maintained in standard hygienic conditions in a reversed light-dark cycle and temperature range of 22 ± 3 °C. Mice were acclimatized for 7 days prior to conduction of the experiment. Standard rodent chow and water were provided *ad libitum*. The care and handling of the animals were approved by the animal care and use committee at the Suez Canal University.

2.2. Chemicals and drugs

MPTP hydrochloride was purchased from Sigma-Aldrich (MO, USA) and was dissolved in normal saline. L-dopa methyl ester and carbidopa were kindly provided by Global Napi Pharmaceuticals (Cairo, Egypt) and was given in a dose of 100/10 mg/kg (Paille *et al.*, 2004). Pramipexole dihydrochloride monohydrate was supplied as a white powder from Eva Pharma for Pharmaceutical and Medical Appliances (Giza, Egypt) and was given in a dose of 1 mg/kg (Winner *et al.*, 2009). Both l-dopa and

pramipexole were freshly prepared by dissolution in distilled water.

2.3. Induction of experimental parkinsonism

Experimental parkinsonism was induced by 4 doses of MPTP hydrochloride (20 mg/kg, i.p.) at 2 hours intervals (Serra *et al.*, 2008). Pharmacological treatment started from day 8 after MPTP administration, i.e. when dopamine cell loss stabilizes (Jackson-Lewis and Przedborski, 2007) and continued until the end of week 8.

2.4. Experimental Protocol

Mice were randomly divided into 5 groups; 10 animals each. They were assigned to saline group (received 4 intraperitoneal injections of saline parallel to MPTP and served as a normal control group); MPTP control, l-dopa, pramipexole, and l-dopa + pramipexole-treated groups (received firstly MPTP and thereafter received in respective distilled water, l-dopa/carbidopa, pramipexole and a combination of l-dopa/carbidopa and pramipexole in the same aforementioned doses). L-dopa and pramipexole were administered by using an oral tube, twice daily, at 7 a.m. and 7 p.m. The motor tests were performed at 8-10 a.m. to minimize the circadian influence on motor activity.

2.5. Assessment of abnormal involuntary movements (AIMs)

At the end of the 2nd, 4th, 6th and 8th weeks, mice were placed individually in transparent plastic cages and scored every 30 min for 4 hours following a single dose of l-dopa/carbidopa. According to this scale, AIMs were classified into 4 subtypes: locomotive dyskinesias i.e. increased circular locomotion; axial dystonia i.e. twisted posturing of the neck and upper body; orolingual dyskinesias i.e. stereotyped jaw movements and tongue protrusion and forelimb dyskinesias i.e. repetitive rhythmic jerks of dystonic posturing. Enhanced manifestations of normal behaviours such as grooming, rearing and sniffing were not included in the rating. AIMs severity was assessed using a score from 0 to 4 for each of the four AIM subtypes according to the time per monitoring period during which the AIM is present: 0, absent; 1, occasional i.e. present during < 50% of the observation time; 2, frequent i.e. present during > 50% of the observation time; 3, continuous but interrupted by strong sensory stimuli i.e. sudden noise, opening of the cage lid; 4, continuous not interrupted by strong sensory stimuli (Lundblad *et al.*, 2004). The maximum AIMs score and the AIMs duration (sec) registered for each group during each observation were compared.

2.6. Tests for assessment of motor impairment

At the end of the 2nd, 4th, 6th and 8th weeks, the following tests were performed for assessment of the motor function of the mice. Motor function was assessed one hour after administration of the morning dose of the drugs.

2.6.1. Spontaneous activity

Spontaneous movement was measured by placing animals in a small transparent cylinder (height, 15.5 cm; diameter, 12.7 cm). Spontaneous activity was evaluated for 3 min. The number of rears and number of forelimb and hindlimb steps were measured. A rear was counted when an animal made a vertical movement with both forelimbs removed from the ground. Forelimb and hindlimb steps were counted when an animal moved either both forelimbs or both hindlimbs across the floor of the cylinder. Number of rears and hindlimb/forelimb steps ratio were compared (Fleming *et al.*, 2004).

2.6.2. Pole test

The pole test has been used previously to assess basal ganglia related movement disorders in mice (Fernagut and Chesselet, 2003). Briefly, mice were placed head-up on top of a vertical wooden pole 50 cm long (1 cm in diameter). The base of the pole was placed in the home cage. When placed on the pole, animals orient themselves downward and descend the length of the pole back into their home cage. Mice received 2 days of training that considered of 5 trials for each session. On the test day, animals received 5 trials, and time to orient downward (t-turn) and total time to descend (t-total) were measured. The best performance over the 5 trials was used.

2.7. Processing of the brains

After assessment of the motor performance, mice were anesthetized using thiopental sodium (50 mg/kg) (Vogler, 2006) and sacrificed by cervical decapitation. After that, the brains were quickly dissected and washed with ice cold phosphate-buffered saline (PBS) and one hemisphere from each brain was rapidly frozen at -80 °C and the striata was used for extraction of dopamine as described later. The second hemisphere of all the brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) by immersion for 48 h. Brains were sectioned at 30 µm thickness at the substantia nigra on a sliding microtome. Further, these sections were subjected to staining with hematoxylin & eosin (H&E) as well as immunostaining for tyrosine hydroxylase and Ki-67 (a marker for cell proliferation).

2.7.1. Determination of striatal dopamine level

For these studies, striatum processed and stored at -80 °C was used. The content of dopamine was determined using an HPLC apparatus with an electrochemical detector (Model 5600A CoulArray Detector System ESA, Brighton, MA, U.S.A.). Tissues were homogenized in 0.2 M ice-cold perchloric acid using a teflon homogenizer (Glas Col homogenizer system, Vernon hills, USA). The homogenate was placed in an ice bath for 60 min. Subsequently, the sample was centrifuged at 15000 × g for 20 min at 4 °C and the supernatant was transferred to a clean tube and measured for volume. One-half volume of a solution containing 0.02 M potassium citrate, 0.3 M potassium dihydrogen phosphate, and 0.002 M Na₂EDTA was added and mixed in thoroughly to deposit perchloric acid. After incubation in an ice bath for 60 min, the mix was centrifuged at 15000 × g, for 20 min at 4 °C. The supernatant was filtered and then injected into the HPLC system for analysis. The mobile phase was 0.125 M sodium citrate buffer containing 20% methanol, 0.1 mM Na₂EDTA, 0.5 mM 1-octanesulfonic acid sodium salt (Acros Organics, Morris Plains, NJ, U.S.A.) adjusted to pH = 4.3. The flow rate was set at 1.2 ml/min. Dopamine level was expressed in pMol/g tissues.

2.7.2. Assessment of dopaminergic neuronal survival

Dopaminergic neuronal survival was assessed using rabbit monoclonal tyrosine hydroxylase primary antibodies (R&D systems[®]) and rat monoclonal Ki-67 primary antibodies (Abcam[®]). Sections were fixed in a 65 °C oven for 1 hour and then the slides were placed in a coplin jar filled with 60 ml of triology (Cell Marque[®], CA-USA) working solution and the jar was securely positioned in an autoclave. The autoclave was adjusted at 120 °C and maintained for 15 minutes after which pressure is released and the coplin jar was removed to allow slides to cool for 30 minutes. Sections were then washed and immersed in triton-buffered saline (TBS) to adjust the pH, this was repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 minutes. Background staining was blocked by putting 2-3 drops of 10% goat non immune serum blocker on each slide to be incubated in a humidity chamber for 10 minutes. Without washing, excess serum was drained from each slide. The slides were then subjected to either tyrosine hydroxylase primary antibodies or Ki-67 primary antibodies and were incubated in the humidity chamber for 1 hour. Henceforward, suitable biotinylated secondary antibody was applied on each slide for 20 minutes,

followed by 20 minutes incubation with the enzyme conjugate. DAB chromogen was prepared and 2-3 drops were applied on each slide for 2 minutes. After that, DAB was rinsed and the slides were counterstaining with Mayer's hematoxylin and cover slipping were performed as the final step before slides were examined under a light microscope (Olympus CX21, Tokyo, Japan).

2.8. Quantification of dopaminergic neuronal survival profiles

Slides were viewed using a light microscope and cell numbers were quantified stereologically on three regularly spaced sections covering the entire surface of the substantia nigra stained with H&E as described (Hoglinger *et al.*, 2004). Each section was viewed at low power ($\times 10$ magnification) and the SNpc was outlined. The cell numbers were counted at high power ($\times 40$ magnification). Neurons were counted only when their nuclei were clearly visualized within one focal plane. After determination of the cell number in each slide, the percentage increase in the cell number relative to MPTP group was calculated and compared.

The SNpc slides were examined to measure the TH immunopositive cells using a computer assessed image analysis system "Image J 1.45F" (National Institute of Health, USA) as described previously (Bezard *et al.*, 2001). The boundaries of the SNpc were chosen on three consecutive sections corresponding to a representative median plane of the SNpc by examining the size and shape of the different TH-immunoreactive neuronal groups. Cells that were clearly stained for TH with a visible nucleus were counted. After determination of the TH positive cells in all tissue sections, the percentage increase in TH positive cells relative to MPTP group was calculated. In a similar way, number of Ki-67 positive nuclei were counted and calculated as percentage increase from MPTP group.

2.9. Statistical analysis

Obtained results were expressed as mean \pm SEM. For multiple comparisons of behavioural ratings, quantitative data were compared using a repeated measures one-way analysis of variance (ANOVA), followed by Bonferroni's *post-hoc* test. Qualitative variables were compared using Chi square test. Statistical significance was set at P value <0.05 . Results were analyzed using The Statistical Package for the Social Sciences, version 17 (SPSS Software, SPSS Inc., Chicago, USA).

3. RESULTS

In the present study, saline treated and MPTP-treated mice did not show any signs of dyskinesia. However, l-dopa group showed the highest AIMS scores among all the experimental groups all over the study period. The total AIMS scores in l-dopa group increased gradually until the end of the experiment (the end of week 8) (Fig. 1-A) and the total AIMS scores at the end of week 8 were significantly higher than those obtained at the end of week 2 (Fig.1-B). On the other hand, pramipexole group and the combination group (l-dopa + pramipexole) showed significantly lower AIMS scores (Fig. 1-B) and AIMS duration (Fig. 1-C) as compared to l-dopa group at week 4, 6 and 8 ($P < 0.05$).

Data also showed that, mice treated with 4 doses of MPTP (20 mg/kg, i.p.) showed parkinsonian-like syndrome. MPTP-treated mice exhibited low number of rears and low hindlimb/forelimb steps ratio in the spontaneous activity test, as compared to saline-treated mice (Fig. 2A&B). Treatment with l-dopa, pramipexole or their combination enhanced the number of rears and hindlimb/forelimb steps ratio in the spontaneous activity test, as compared to MPTP group throughout the entire course of the experiment (Fig. 2A&B). However, the response to l-dopa declined gradually until the end of the experiment; the no. of rears in the l-dopa group at the end of week 8 was significantly lower than that at the end of week 2; indicating decreased response to l-dopa (Fig 2-A). Further, monotherapy with pramipexole or the combination group (l-dopa + pramipexole) showed a higher number of rears in comparison to l-dopa treatment at all time points ($P < 0.05$, Fig. 2-A).

In addition, time to orient downward (t-turn) and total time to descend (t-total) in the pole test were significantly increased in MPTP-treated mice, as compared to saline-treated mice ($P < 0.05$, Fig. 3A&B). Treatments with l-dopa, pramipexole as well as their combination improved the t-turn and t-total in the pole test, as compared to MPTP group (Fig. 3-A&B). The combination of pramipexole and l-dopa improved the t-turn in comparison to l-dopa treatment at week 8 (Fig. 3-A); further, the combination group showed lower t-total at all time points (Fig. 3-B).

Striatal dopamine level was significantly lower in MPTP group as compared to saline group ($P < 0.05$, Fig. 4). At the end of the experiment, monotherapy with l-dopa or pramipexole as well as the combination therapy significantly increased striatal dopamine level as compared to MPTP group. The combination group showed higher striatal dopamine level when compared to single l-dopa therapy (Fig. 4).

Histopathological examination of the SNpc neurons stained with H&E indicated that number of neurons in the MPTP group was significantly lower in comparison to saline group (Fig. 5-A&B). Monotherapy with l-dopa did not improve the number of neurons; however, monotherapy with pramipexole as well as the combination therapy induced a significant increase in the number of neurons in comparison to MPTP group (Fig. 5-D-G).

Immunostaining for TH in the SNpc revealed high immunoreactivity for TH in saline treated group and lower number of TH positive neurons in MPTP group (Fig 6-A&B). Single treatment with l-dopa did not

improve TH immunostaining; meanwhile, single treatment with pramipexole or its combination with l-dopa significantly increased the percentage of TH-positive neurons in comparison with both MPTP group ($P < 0.05$, Fig. 6-C-F).

In the present study, immunostaining for Ki-67 indicated low number of immunopositive nuclei in MPTP group as compared to saline group (Fig 7-A&B). Treatment with l-dopa did not ameliorate the number of Ki-67 positive neurons. Monotherapy with pramipexole or its combination with l-dopa significantly increased the Ki-67 positive nuclei as compared to MPTP group (Fig 7-C-F).

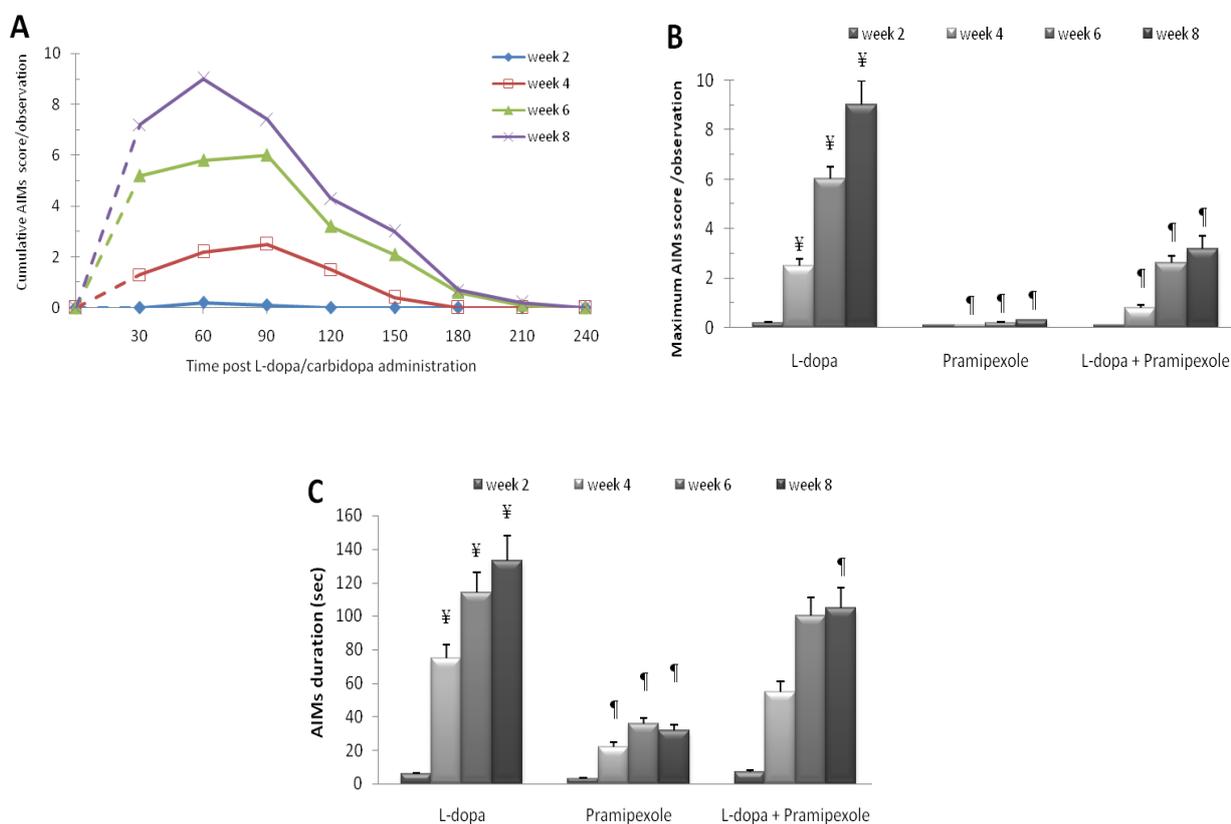


Figure 1. AIMs score during chronic l-dopa/carbidopa treatment in MPTP-parkinsonian mice. Mice were placed individually in transparent plastic cages and scored every 30 min for 4 hours following a single dose of l-dopa/carbidopa. AIMs were classified into 4 subtypes: locomotive dyskinesias, axial dystonia, orolingual dyskinesias and forelimb dyskinesias. AIMs severity was assessed using a score from 0 to 4 for each of the four AIM subtypes. Assessment of AIMs was performed at the end of the 2nd, 4th, 6th and 8th weeks. Treatment with l-dopa/carbidopa (100/10 mg/kg/twice/day, p.o.) induced a significant increase in AIMs score at the end of the 4th, 6th and 8th weeks (A). Co-treatment with pramipexole (1 mg/kg/twice/day, p.o.) significantly decreased the maximum AIMs score (B) and AIM duration (C). MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine, AIMs: abnormal involuntary movements. Values are expressed as mean \pm SEM. (n=10), analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. [#] $P < 0.05$ compared to l-dopa group at the same time point, [¥] $P < 0.05$ compared to l-dopa group at week 2.

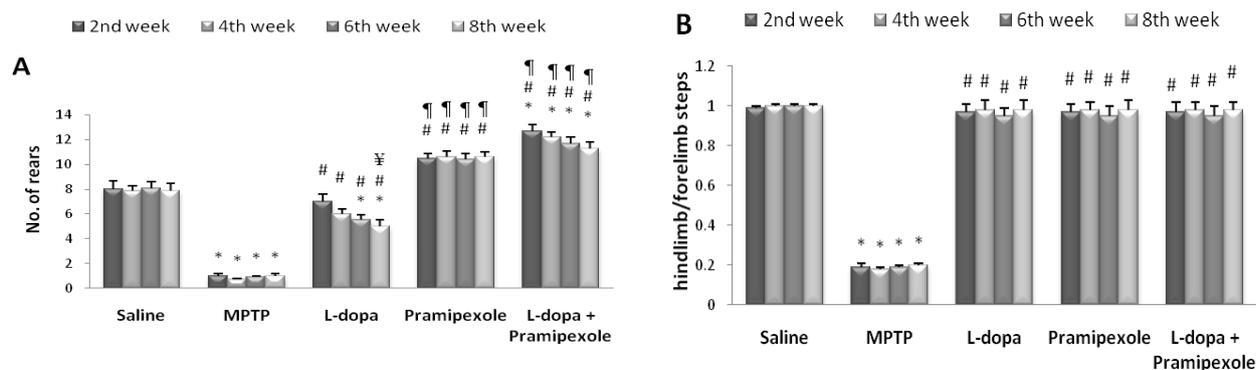


Figure 2. Time-course of: number of rears (A) and hindlimb/forelimb steps ratio (B) for the mice in the spontaneous activity test. Mice were treated with 4 doses of MPTP (20 mg/kg, i.p.) to induce experimental parkinsonism. Treatment with l-dopa/carbidopa (100/10mg/kg/twice/day), pramipexole (1 mg/kg/twice/day) or their combination increased the rearing frequency and hindlimb/forelimb steps ratio as compared to MPTP group. MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. Values are expressed as mean \pm SEM. (n=10), analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. * $P < 0.05$ compared to saline group at the same time point, # $p < 0.05$ compared to MPTP group at the same time point, ¶ $P < 0.05$ compared to l-dopa group at the same time point, ¥ $P < 0.05$ compared to l-dopa group at week 2.

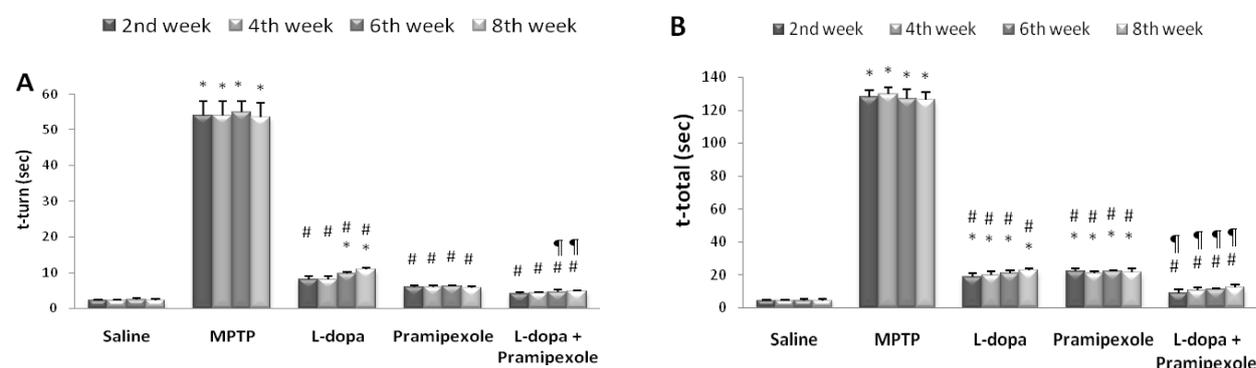


Figure 3. Time course of: the time taken to orient downward (t-turn) (A) and the total time to descend (t-total) (B) in the pole test. The best performance over 5 trials was used. Mice were treated with 4 doses of MPTP (20 mg/kg, i.p.) to induce experimental parkinsonism. Treatment with l-dopa/carbidopa (100/10 mg/kg/twice/day), pramipexole (1 mg/kg/twice/day) or their combination improved the t-turn and t-total in the pole test as compared to MPTP group at all time points. MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. Values are expressed as mean \pm SEM. (n=10), analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. * $P < 0.05$ compared to saline group the same time point, # $P < 0.05$ compared to MPTP group at the same time point, ¶ $P < 0.05$ compared to l-dopa group at the same time point, ¥ $P < 0.05$ compared to l-dopa group at week 2.

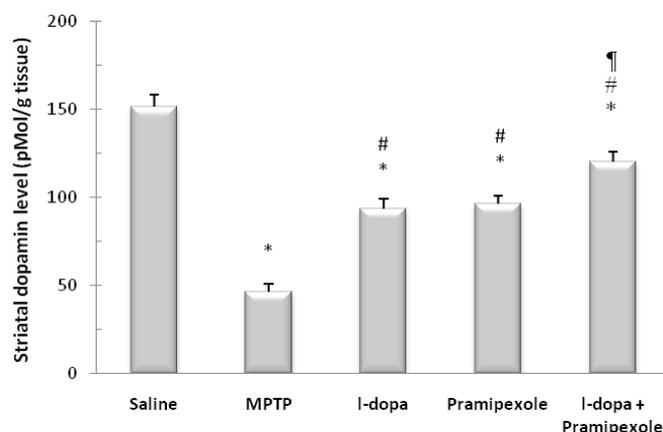


Figure 4. Striatal dopamine level in the experimental groups at the end of the experiment (week 8). Mice were injected with 4 doses of MPTP (20 mg/kg, i.p.) to induce experimental parkinsonism. Striatal dopamine level was markedly decreased in MPTP group as compared to saline group. Treatment with l-dopa/carbidopa (100/10 mg/kg/twice/day), pramipexole (1 mg/kg/twice/day) or their combination significantly increased the striatal dopamine level as compared to MPTP group. MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. Values are expressed as mean \pm SEM. (n=10), analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. * $P < 0.05$ compared to saline group. # $P < 0.05$ compared to MPTP group. † $P < 0.05$ compared to l-dopa group.

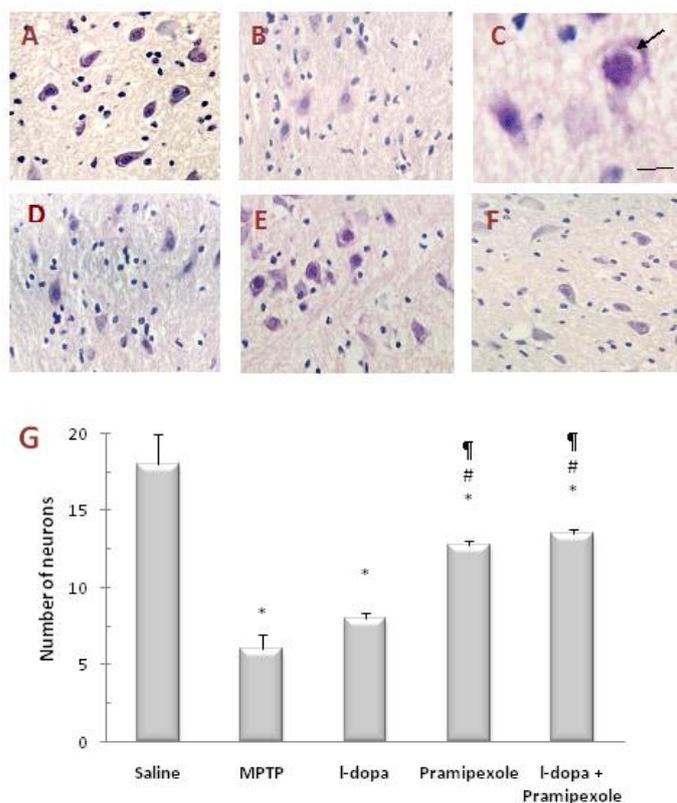


Figure 5. The histopathological picture of the substantia nigra pars compacta (SNpc) neurons stained with hematoxylin & eosin (H&E) at the end of the experiment (week 8). Saline-treated group showed normal histological appearance of the SNpc neurons (A). MPTP group (20 mg/kg/4 doses, i.p.) showed marked decrease in the number of the neurons (B). Large vacuolations in the cytoplasm of the neurons (arrow) in MPTP group (x240) (C). (D) a histopathological picture for the SNpc in l-dopa/carbidopa group, Treatment with pramipexole (1 mg/kg/twice/day) (E) or its combination with l-dopa (F) improved the histopathological picture of the SNpc neurons. G-The number of intact neurons in the experimental groups. Cell numbers were quantified stereologically on three regularly spaced sections covering the entire surface of the SNpc. MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. Values are expressed as mean \pm SEM. (n=10), analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. * $P < 0.05$ compared to saline group. # $P < 0.05$ compared to MPTP group. † $P < 0.05$ compared to l-dopa group. H&E \times 40, (scale bar = 40.87 μ m).

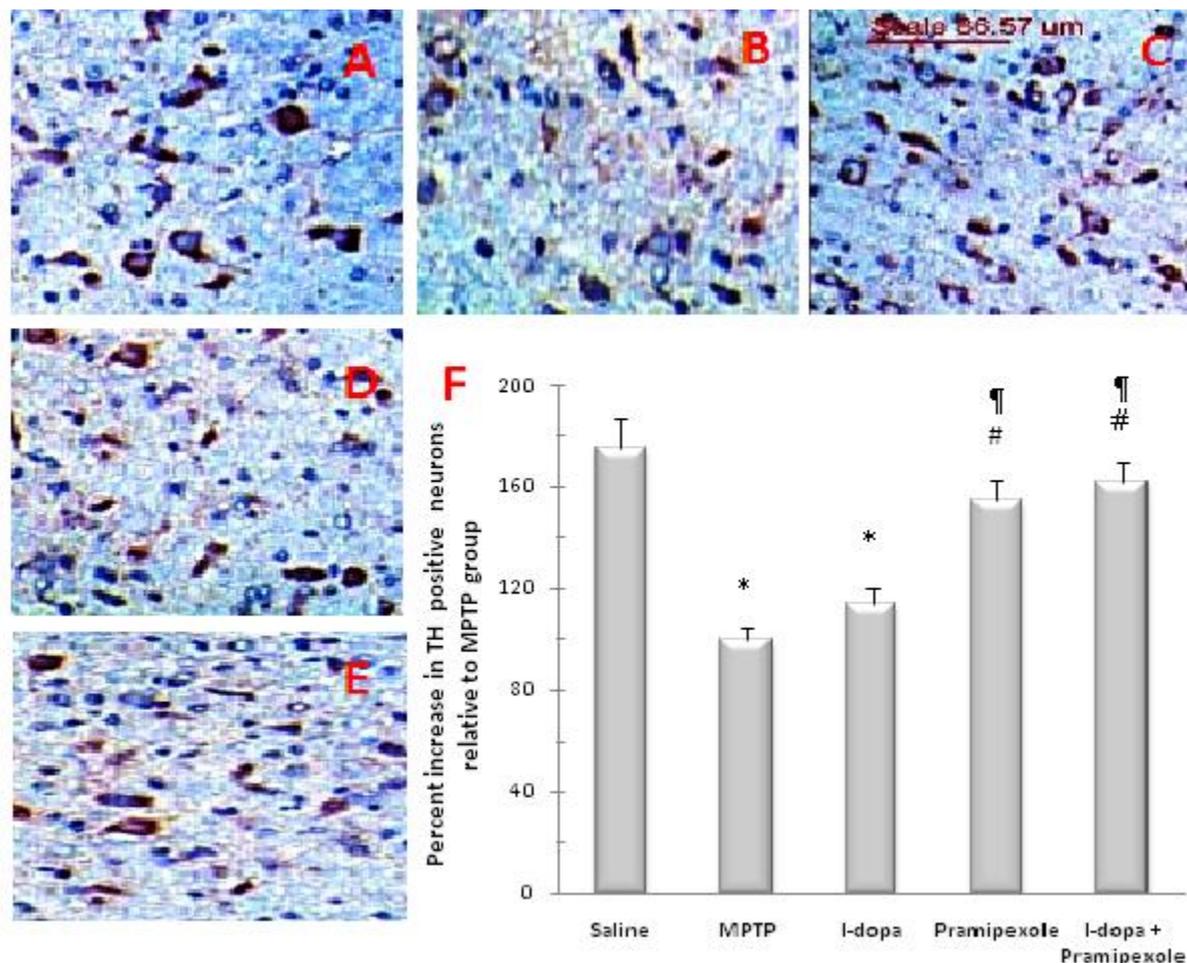


Figure 6. Immunohistochemical staining for tyrosine hydroxylase (TH) in the substantia nigra neurons in the experimental groups. Saline treated mice shows higher number (A) of TH positive neurons in comparison to MPTP treated mice (B). MPTP (20 mg/kg/2 h/4 doses, i.p.) was used to induce experimental parkinsonism in mice. Single treatment with l-dopa/carbidopa (100/10 mg/kg/twice/day) did not enhance the immunoreactivity to TH (C). Single treatment with pramipexole (1 mg/kg/twice/day) (D) or its combination with l-dopa/carbidopa (E) significantly increased the immunoreactivity for TH in dopaminergic neurons (F). MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. Values are expressed as percentage increase from MPTP group and analyzed using Chi square test (n=10). * $P < 0.05$ compared to saline group. # $P < 0.05$ compared to MPTP group. † $P < 0.05$ compared to l-dopa group. Scale par = 86.57 μ m.

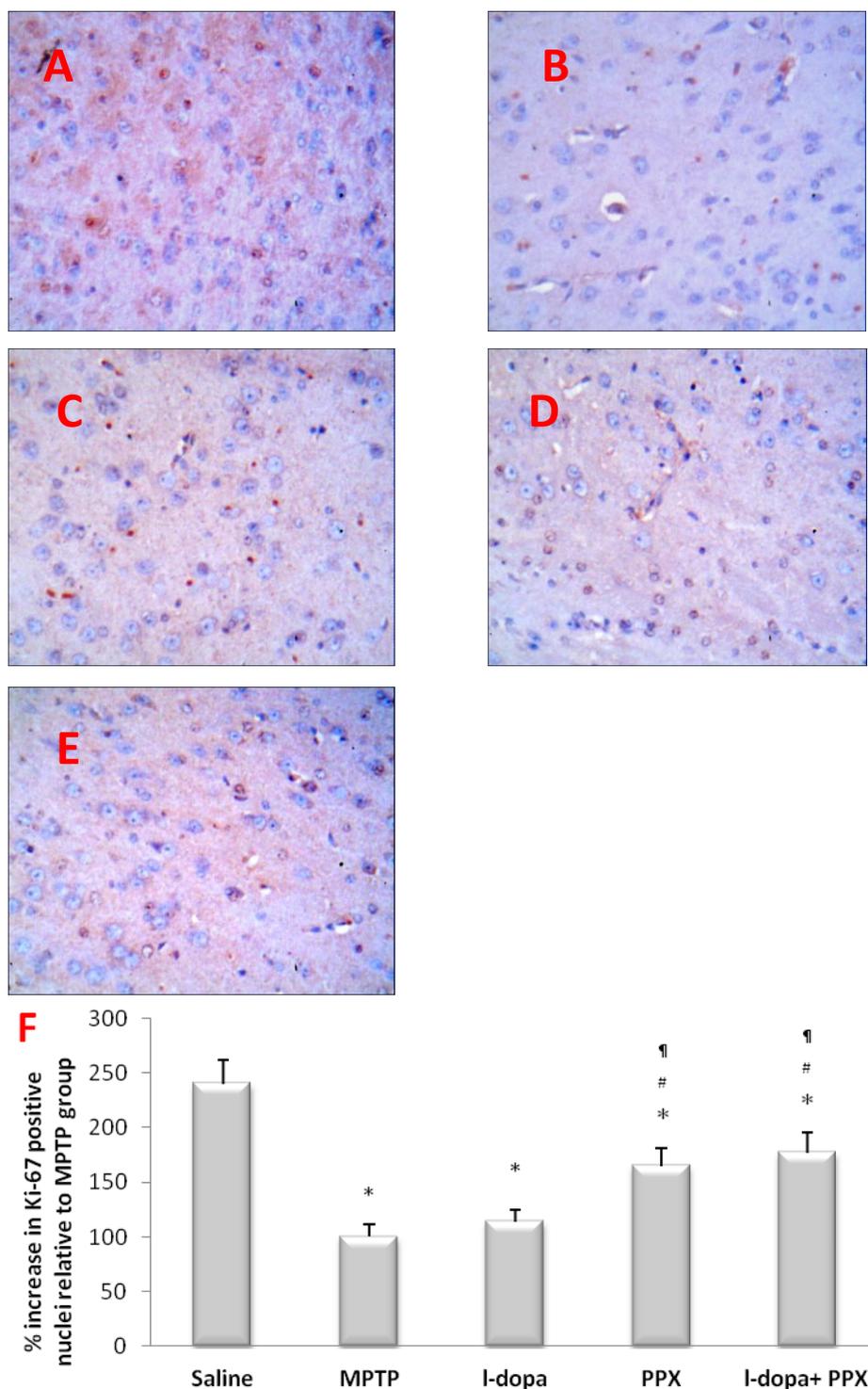


Figure 7. Immunohistochemical staining for Ki-67 in the substantia nigra neurons in the experimental groups. Saline group showed higher number of Ki positive nuclei compared to MPTP group (A&B). Single treatment with l-dopa/carbidopa (100/10 mg/kg/twice/day) did not enhance the immunoreactivity to Ki-67 (C). Treatment with pramipexole (1 mg/kg/twice/day) alone or in combination with l-dopa significantly ameliorated the number of immun-positive nuclei as compared to MPTP group (D-F). MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. Values are expressed as percentage increase from MPTP group and analyzed using Chi square test (n=10). * $P < 0.05$ compared to saline group. # $P < 0.05$ compared to MPTP group. ¶ $P < 0.05$ compared to l-dopa group. Scale par = 92.05 μ m.

4. DISCUSSION

In the present study, intraperitoneal injection of MPTP induced parkinsonian-like hypokinetic behaviour in mice, as indicated in spontaneous activity test and pole test, coupled with a decrease in striatal dopamine level and loss of nigral TH immunoreactivity. These were in line with Serra et al., 2008; Sy et al., 2010 and Viaro et al., 2008 who reported that, MPTP induces motor deficits in mice together with loss of striatal dopamine and neurodegeneration.

In the present study, chronic treatment with l-dopa produced a progressive increase in AIMS score in MPTP-parkinsonian mice. Consistently, mice treated with l-dopa for 5 weeks showed increasing phenotypic expression of l-dopa-induced dyskinesia (Ding et al., 2010). One of the central prerequisites for the unstable motor response to l-dopa is the severe nigrostriatal denervation in advanced disease. Clinical experience showed that l-dopa given to non-parkinsonian patients produces no dyskinesias in the absence of nigral neuronal degeneration (Chase et al., 1993). MPTP-lesioned monkeys with upwards of 95% loss of dopamine neurons develop dyskinesias within days of starting l-dopa therapy (Pearce et al., 1998). Dopaminergic denervation and dopaminergic priming induce a series of anatomical and functional changes in the striatum; leading to further and more persistent dysfunction (Santini et al., 2008).

It has been accepted that non-dopaminergic neurotransmitters, such as glutamate, adenosine and serotonin, are involved in the control of motor symptoms (Spinnewyn et al., 2011; Tani et al., 2010). Although l-dopa administration will continue to enhance dopamine synthesis and release by the surviving dopaminergic neurons, exogenous l-dopa is also decarboxylated and released as dopamine by serotonergic terminals, striatal capillaries, noradrenergic neurons, and non-aminergic striatal interneurons (Mercuri and Bernardi, 2005). The unregulated release of dopamine from these non-dopaminergic terminals leads to more dysfunctional dopamine receptor stimulation resulting in l-dopa-induced dyskinesia (Iciar et al., 2010; Lindgren et al., 2010; Tani et al., 2010).

In the present study, single treatment with l-dopa induced dyskinesia and the severity of the AIMS increases throughout the course of the experiment. However, single treatment with pramipexole showed very little dyskinesia. The combination of pramipexole with l-dopa resulted in delaying of the priming of l-dopa-induced dyskinesia while keeping the motor benefits for longer time. Concurrent administration of

pramipexole attenuated AIMS score in response to l-dopa along with improving the l-dopa's therapeutic anti-parkinsonian benefits. In accordance, administration of a selective D₂ agonist should correct this imbalance (Boraud et al., 2000) and long acting dopaminergic drugs (bromocriptine, ropinorole) were found to provide comparable motor benefits with little or no dyskinesia (Pearce et al., 1998; Grondin et al., 1996; Jenner, 2000). The therapeutic effects of dopamine agonists are derived from binding of the dopamine agonists to the postsynaptic dopamine receptor subtypes in the striatum and the reduction of dopamine turnover in presynaptic dopaminergic neurons in the SN (Olanow et al., 1998). Similarly, RGS9-2, a GTPase accelerating protein that inhibits dopaminergic D₂ receptor-activated G proteins, negatively modulates l-dopa-induced dyskinesia in experimental PD (Gold et al., 2007).

While dopamine agonist therapy per se is associated with less dyskinesias, parkinsonian patients who start with a dopamine agonist first and then switch to l-dopa develop fluctuations at about the same time as those who start l-dopa from the outset. These findings propose that the degree of neuronal loss during disease progression is an important element in the genesis of motor complications (Parkinson-study group, 2000&2002; Whone et al., 2003). Our results could support this theory, as the administration of pramipexole, both alone and in combination with l-dopa, induced a significant increase in striatal dopamine level and improved immunostaining for TH in the SNpc dopaminergic neurons. Hence, the release of functionally regulated dopamine from dopaminergic terminals may account, at least partially, for the anti-dyskinetic effect of pramipexole.

It was also found that daily treatment with l-dopa for seven weeks resulted in a gradual decrease in the therapeutic response. It was known that, oxidative stress in the SN could result from overactivity of surviving dopamine neurons with increased hydrogen peroxide production. Both l-dopa and dopamine undergo oxidative metabolism and can thereby generate cytotoxic free radicals (Mytilineou et al., 2003). Long-term intermittent l-dopa administration is another prerequisite for the development of motor fluctuations in PD (Bibbiani et al., 2005; Cenci et al., 2007), a phenomenon that is dependent on the severity of dopamine neurons loss (Papa et al., 1994). Motor complications due to l-dopa arise from the combined effects of nigrostriatal dopamine degeneration and l-dopa treatment (Rascol and Fabre, 2001). The plasticity of the basal ganglia circuitry that underlie the central pharmacodynamic changes associated also with long-term intermittent dopaminergic stimulation appears to be reversible, at least to some extent.

Persistent drug delivery, such as with intravenous infusion of l-dopa, ameliorated motor fluctuations of the unpredictable 'on-off' type. Sudden switching back to intermittent therapy did not revert the patients to their baseline severity of motor fluctuations (Mouradian *et al.*, 1987). The latter observation suggests that continuous therapy had produced plastic changes in the basal ganglia, which lasted well beyond the removal of the physiologic stimulation and reintroduction of intermittent therapy (Mouradian *et al.*, 2006).

The current study verifies that monotherapy with pramipexole in MPTP-parkinsonian mice improved the locomotor deficits, increased striatal dopamine level and improved the histopathological picture of the SNpc neurons in addition to reducing dopaminergic neuron loss and promoting cell proliferation as evident by immunostaining for Ki67. Pramipexole alleviates PD symptoms by mimicking dopamine's effects in the striatum (Ferrari *et al.*, 2010). Pramipexole was found to increase adult neurogenesis by increasing proliferation and cell survival of newly generated neurons; specifically increased mRNA levels of epidermal growth factor receptors (Winner *et al.*, 2009). Recently, pramipexole produced a marked induction of cell proliferation, assessed by enhanced cell number and S phase population at cell cycle analysis. Merlo and his colleagues in 2011 concluded that, pramipexole increased brain-derived neurotrophic factors release with a mechanism involving D2 but not D3 receptors.

Our results were in line with Imamura *et al.*, (2008) ; Inden *et al.*, (2009) and Ferrari *et al.*, (2010) who emphasized that, pramipexole provided neuroprotection in rodent models of PD. Additionally, constantinescu in 2008 concluded that, dopamine agonists present several advantages over l-dopa such as direct stimulation of striatal dopaminergic neurons, longer half life, providing a more continuous stimulation at the dopamine receptors, lack of oxidative metabolites and more reliable absorption and transport.

Overall, our results showed that administration of pramipexole, both alone and in combination with l-dopa, induced a significant increase in striatal dopamine level and improves immunostaining for TH in the SNpc dopaminergic neurons with enhancement of cellular proliferation. L-dopa administration will continue to enhance dopamine synthesis and release by the surviving dopaminergic neurons. Therefore, increasing the number of surviving dopaminergic neurons by using pramipexole may, at least in part, account for the increase in striatal dopamine level and enhancing the motor function in comparison to single l-dopa therapy.

In conclusion, the present results verify that using pramipexole ahead of l-dopa resulted in a reduction in the expression of l-dopa-induced dyskinesia. This was accompanied with further improving the motor performance of MPTP-parkinsonian mice throughout the entire period of the experiment, reducing dopaminergic neuron loss and promoting cell proliferation. Further studies are needed to ensure the present findings in different models of PD. The neuroprotective effect of pramipexole combined with its long duration of action and low propensity for dyskinesia expression are salient features suggest pramipexole to be a promising candidate to l-dopa therapy in clinical settings.

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