Pramipexole protective effect on rotenone induced neurotoxicity in mice

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Abstract

Introduction: Pramipexole is a new dopaminergic drug which has been approved for PD treatment. However, we tried to find new capacity for this drug rather than symptomatic effect. *Materials and Methods:* A chronic rotenone model with daily oral dose of 30mg/kg was induced in mice. Pramipexole was tried in a new approach where the treatment began in the middle of rotenone course with oral dose 1mg/kg /day of pramipexole. *Results:* Further analysis of behavioral tests and immunohistochemistry revealed success of pramipexole in improving the rotenone intoxicated mice. *Conclusion:* These results showed possible beneficial effect of pramipexole against rotenone induced neurotoxicity.

Key words: Rotenone; Pramipexole; neurotoxicity; mice.

1- Introduction:

Parkinson's disease (PD) is a major health concern to the population**.** It has been reported in all countries and in all races **(Obeso et al., 2010).** Parkinsonism results primarily from abnormalities of basal ganglia function **(Galvan and Whichmann, 2008)**. In PD, the degeneration of dopaminergic SNc neurons and their projections to the striatum is a slowly evolving process that may take decades to develop **(Arias-Carrión et al., 2009).** PD is considered a multifactorial disease resulting from the effect of environmental factors and genetic susceptibility, however, due to delay of manifestations it is difficult to attribute - with certainty- PD to certain risk factor **(Caldwell et al., 2009)**. Various treatment modalities are used for PD however, due to the lack of understanding of PD etiology and pathogenesis, there is still no treatment that can prevent or retard the progression of the disease **(Lohle and Reichmann, 2010)**.

The NIH Committee to Identify Neuroprotective Agents in Parkinson's (CINAP) published the result of a systematic assessment of currently available pharmacologic neuroprotective agents. The CINAP members stated the importance of using PD models in testing drugs **(Emborg 2004).** Rotenone model can be used as an alternative to other classical PD models e.g. MPTP and 6-OHDA specially when testing the neuroprotective effects of novel therapeutic modalities **(Monti et al. 2009)**. A limiting factor of rotenone models is the high mortality rate of the examined animals **(Dawson et al. 2002).** However, oral rotenone model seems to be devoid of the previous drawbacks which promote its use for assessing candidate antiparkinson drugs **(Takeuchi et al. 2009).**

The introduction of second-generation dopamine agonists appeared as new promising approach for symptomatic treatment **(Kano et al., 2008)**. These new dopamine receptor agonists are potent in controlling primary motor symptoms, with little side effects as compared with the old generations (**Toulouse and Sullivan 2008)**. Moreover, the so called 'direct' dopamine receptor agonists can function in the absence of host dopamine neurons, as they act directly on post synaptic dopamine receptors **(Hedlund and Perlmann 2009)**. May be, through releasing dopamine, pramipexole has shown capacity to direct neural stem cells (NSCs) differentiation towards dopaminergic neurons **(Riaz and Bradford 2005; Winner et al. 2009)**.

In the present work we tried pramipexole in the middle of toxic exposure course in a way similar to the natural disease history. In clinical trials pramipexole is given after the manifestations of PD appears (i.e. losing

80% of DA neurons). In such scenario the regenerative capacity is more important. Assessing the neuro-regenrative effects of pramipexole *(besides its known neuroprotective effects)* was our main question.

2- Materials and methods:

2-1. Rotenone mouse model and drug administration:

Eight-month-old male BALB/c mice (20–25 g) were purchased from vacsera animal house (Cairo, Egypt). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Ethical Committee for Research at Mansoura University.

The mice were divided into 3 groups (10 mice each):

Group (1) [control group] Received only carboxymethyl cellulose orally (through gavage) once daily at a volume of 10 ml/kg body weight

Group (2) [Rotenone group] in this group:
Rotenone (Sigma, St. Louis, MO, USA) was administered orally (through gavage) once a day at a dose of 30 mg/kg for 28 days, as described previously **(Inden et al. 2007).** Rotenone was suspended in 0.5% carboxymethyl cellulose sodium salt (CMC, El Nasr company, Cairo, Egypt) and administered once daily at a volume of 10 mL/kg body weight.

Group (3) [Pramipexole group]: in this group Pramipexole (1 mg / kg body weight / day) was administrated via the drinking water to the mice starting 14 days after beginning of rotenone course.

2-2. Behavioural test: (**Vertical grid test (Kim et al. 2010))**

The vertical grid apparatus is an open box of 8 cm×55 cm×5 cm, set vertically. The back side of the vertically standing box is made of a wire mesh of 0.8 cm×0.8 cm, the front side is open, and the other four sides are made of black plexiglass.

The mice were pretrained on the apparatus before applying the test. During the test, a mouse was carefully placed inside the apparatus at 3cm from the top, facing upward, and was allowed to turn around and climb down. The trials were videotaped.

The videos were replayed for analysis of the total time taken for the mouse to make a turn, climb down, and reach the floor by its forepaw.

The test was made beginning from the 14th day and repeated weakly till the 28th day. The repeated behavioral tests aimed at monitoring the progress of PD manifestations.

2-3. Immunohistochemistry:

A prior pilot study was made, where the immunopathology revealed decrease in TH neuron number in SN. The damage was increased with progress of time from the 14th day till reaching maximum degeneration in the 28th day. According to the pilot study treated mice were perfused in the 28th day through the aorta with 50 mL of 10 mM phosphate-buffered saline (PBS), followed by 150 mL of a cold fixative consisting of 4% paraformaldehyde, 0.35% glutaraldehyde and 0.2% picric acid in 100 mMphosphate buffer (PB), under deep anesthesia with pentobarbital (100 mg/ kg, i.p.). After perfusion, the brain was quickly removed and postfixed for 2 days with paraformaldehyde in 100 mMPB and then transferred to 15% sucrose solution in 100 mMPB containing 0.1% sodium azide at 4 ^{o}C. The brain pieces were cut using a cryostat and collected in 100 mM PBS containing 0.3% Triton X-100 (PBS-T). After

several washes, the sections were stored until use in a free-floating state at 4 ^{o}C for immunohistochemical analysis.

Brain slices were incubated with primary mouse monoclonal anti-TH antibody (diluted 1:1,000 Sigma) over the night at 4 ^{o}C. After several washes, sections were incubated with biotinylated secondary antibody (1:500), as appropriate, for 1 h at room temperature. The sections were then incubated with ABC solution 1:200 for 1 h at room temperature. All of the sections were washed several times with PBS-T between each incubation, and labeling was then revealed by 3,3'-diaminobenzidine (DAB).

2-4. Stereological analysis of DA neurons in the ventral midbrain:

TH-immunopositive neurons in the substantia nigra pars compacta (SNpc) were estimated using stereological counts of cell numbers, on a Stereo-investigator system and optical density measurements on a Leica Q-win system). Six sections (30 μm-thick), from the anterior to the posterior midbrain, were used for counting in each case. The total number of TH-immunopositive neurons was estimated using the optical fractionator method.

2-5 Statistical methods:

All data were given as the mean $\pm$ standard error of the mean (SEM). Two groups of data where analyzed by the Student's t-test. Three groups of data were analyzed by ANOVA with a Tukey post hoc test. For all tests, $p<0.05$ was deemed significant.

3-Results:
3-1. development of progressive PD model:

Regarding the locomotor affection of the mice, the control mice usually take about 10 seconds to complete the vertical grid test. On the other hand, the period increased with progress of time from 14-21-28th day where the rotenone group has taken more than 80 seconds to complete the test (Table 2A). This progressive deterioration of locomotor functions of the mice points to the progressive nature of the induced PD model.

As shown in (Fig. 1B), the oral administration of rotenone at 30 mg/kg for 28 days obviously reduced the number of TH-immunopositive neurons in the SNpc. Stereological analysis of nigral TH-immunopositive neurons showed that rotenone caused a significant loss of DA neurons (Table 1). It is important to mention that the pilot study showed that the number of TH +ve neurons was decreased with progress of time (after 14 and 21 days), denoting the progressive nature of this model.

3-2. Effect of pramipexole on nigrostriatal DA neurons in rotenone mice

On investigating whether treatment with pramipexole (oral 1 mg/kg/ day for 14 days) can offers neuroprotection against the effects caused by the chronic oral administration of rotenone. The rotenone-induced loss of TH-immunopositive neurons in the SNpc was significantly improved by the pramipexole treatment (Fig. 1B and 1C).

3-3. Effect of pramipexole on locomotor coordination in rotenone mice.

In our study the control mice usually took about 10 seconds to complete the vertical grid test. On the other hand the rotenone group has taken more than 80 seconds to complete the test (Table 2A). On studying the effect of treatment we can see that the pramipexole group took about 18 seconds to complete the test which represents a significant improvement in their activity (Table 2B).

4-Discussion:

One of the major drawbacks of rotenone models for PD was the high mortality rates associated with their parenteral administration **(Lapointe et al. 2004)**. This high mortality may lead to inconsistency of results **(Cicchetti et al. 2010).** Despite this major drawback, rotenone models are characterized by many advantages making them more related to the pathogenesis and pathology of idiopathic PD so mimicking the clinical reality **(Moussa et al. 2008; Greene et al. 2009; Verma and Nehru 2009)**. Moreover, rotenone models have been found ideal to test the proposed therapies due to the chronic course they have and the similarity with PD pathology to great extent **(Mao et al. 2007; meurers et al. 2009**; **Greenamyre et al. 2010)**. In the present study we have got a rotenone model with 0% mortality and on the other hand induced effective progressive PD manifestations as can be seen from the deterioration in behavioral tests and immunohistochemical examination results with progress of time.

Usually PD therapies were tested in two ways. The first is to test the effect of the therapy after complete development of the injury **(Fu et al. 2006; Weiss et al. 2006; Hall et al. 2007; Kong et al. 2008; Blandini et al. 2010).** The other, is testing the neuroprotectant effects e.g. nicotine or pramipexole against toxic models (**Inden et al. 2009 and Takeuchi et al. 2009).** In this way, the therapy is given in the beginning of the toxic model seems an attractive point of research. However, in the present study we tried to explore the role of pramipexole in a way similar to clinical real life, where the drug is given in the middle of the disease course (i.e. regenerate damaged neurons and at the same time offers neuroprotection for the rest of the others). Our results show that pramipexole improved the disease both functionally (behavioral tests) and

structurally (immunohistochemistry). **Inden and colleagues** have suggested multiple mechanisms for pramipexole effects on PD pathology e.g. inhibition of oligomerization of human wild-type ά-synuclein by H_2O_2 plus cytochrome c, directly scavenged hydroxyl radical (OH) generated from H_2O_2 and Fe^{2+} and increased Bcl-2 immunoreactivity in DA neurons in the SNpc. In the present study we suggest new mechanism which is regeneration of damaged dopaminergic neurons in the SN. This damage is caused by 14 day treatment of rotenone before we began the pramipexole therapy. The regenerated neurons points to the fact that pramipexole can be valuable as neuroregenerative therapy and not only neuroprotectant as suggested by previous studies.

5-Conclusion:

In conclusion, the chronic oral administration of rotenone induced DA neuronal death and was associated with a motor deficit. This pathology was improved by the dopaminergic agent pramipexole that was given in the middle of the toxic model. These results suggest that pramipexole can improve the pathological condition and not just a symptomatic therapy for PD.

Acknowledgement:

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Tables and Figure Legends:
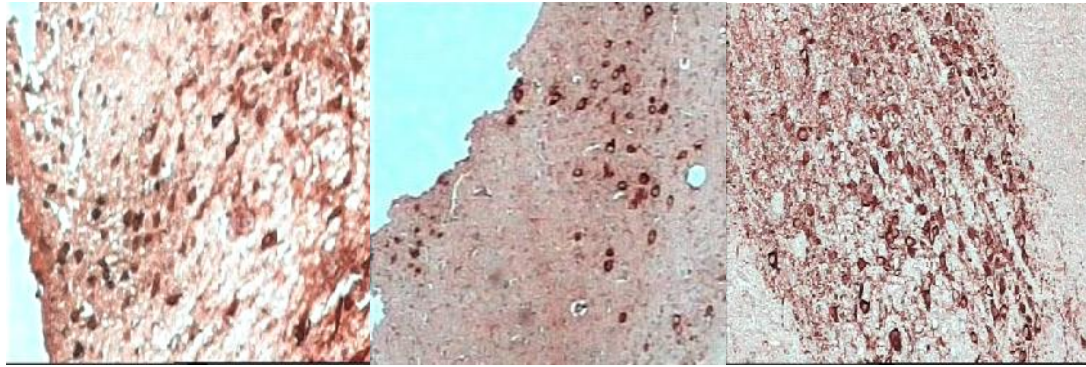
Table 1: Stereological cell counts in substantia nigra

A)Control group	B)Rotenone group	C)Rotenone+pramipexole group
1970± 120	1000 ± 56[*]	1930 ± 88

[*]$p < 0.05$ compared to the control group.

Table 2: vertical grid test results:

Groups	A)Control group	B) rotenone group.	C) rotenone + pramipexole group.
Total time to climb down	10.9 ± 2.4	83.7± 6.8[*]	18.7±3.4

[*]$p < 0.05$ compared to the control group.

1-A   **1-B**   **1-C**

Fig. 1: Tyrosine hydroxylase immunohistochemistry in the control group (1-A), the rotenone group (1-B) and the rotenone+ pramipexole group (1-C). Medium magnification images in dorsolateral region of nigra show cell loss in this particularly vulnerable area in the rotenone group with regeneration in the rotenone+pramipexole group.