CHAPTER-1.1

To study the role of selective and non-selective COX-inhibitors in perphenazine-induced catatonia and their interaction with levodopa/carbidopa in rats

1.1.1. Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the SNpc region of the brain and typified by four cardinal features viz. bradykinesia (slowness of movement), resting tremor, increased muscular rigidity and impaired postural balance (Montastruc et al., 1996). In the pathogenesis of PD, interplay of multifactorial components have been envisaged, in addition to hereditary, ageing and environmental toxins (Langston, 1999). Severity of PD depends on the striatal dopamine levels and for PD symptoms to appear, more than 75% degeneration of dopaminergic neurons has been suggested (Marsden, 1982). Based on the dopamine-deficiency hypotheses, L-DOPA (a dopamine precursor) use was started, which decarboxylates into dopamine in brain and responsible for its therapeutic effectiveness. However, in today’s practice a combination of levodopa with carbidopa (a peripheral DOPA-decarboxylase inhibitor) is used which allows the entry of maximum amount of L-DOPA into the brain.

The chronic use of the L-DOPA is limited now a days due to the development of “on-off” phenomenon which results in decreased efficacy of these agents (Sweet et al., 1975; Fahn, 2005). On the other hand, increased doses may lead to the development of dyskinesias (Lang and Lozano, 1998a; 1998b). Other promising drug therapies include treatment with anticholinergics, dopamine receptor (D2/D3) agonists, monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) inhibitors. However, despite the availability of large number of drugs, the relapse rate in PD patients is significant which has compelled the researchers to search some other alternative therapeutic approaches/targets by which the progressive loss of dopaminergic neurons could be halted.
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Perphenazine, a phenothiazine, known to block dopamine D2 receptors and produces motor disturbance in the form of catatonia (rigidity) (Albin et al., 1989). This exemplifies a very simple and preliminary model for the evaluation of antiparkinson activity of drugs (Khanna and Madan, 1975; Kulkarni et al., 1980; Singh and Kulkarni, 2002; Arzi and Rezaei, 2003; Singh et al., 2003). Although, perphenazine-induced catatonia does not resemble the actual pathophysiological basis of PD; however, it is well known to produce symptoms that mimic the disease phenotype, for example, rigidity measured in the form of catatonia (Kulkarni et al., 1980). This model reflects some of the early symptoms of Parkinson disease and can be employed for the preliminary screening of new molecules proposed to be useful for the treatment of the PD (Singh and Kulkarni, 2002, Singh et al., 2003). It is important to mention here that various dopamine D2 receptor agonists possess antiparkinson properties (Neusch et al., 2000) which justify the importance of this model in antiparkinson drug discovery.

It is now a growing concern that neuroinflammation plays a critical role in the progression of PD (Hernan et al., 2006; Bartels and Leenders, 2007). Studies have revealed a substantial increase in the expression of various inflammatory cascades within the neurons of PD patients (Teismann et al., 2003; Kim and Joh, 2006). Cyclooxygenase (COX) is a rate-limiting enzyme involved in the production of various prostaglandins and thromboxanes. It exists in two isoforms, COX-1 and COX-2. COX isoenzymes which are generally expressed mainly in the peripheral organs (kidneys, stomach, uterus etc.) have also been found to be up-regulated in the brain following neuronal insult (Kulkarni and Dhir, 2009). Out of these two isoforms, evidences have revealed the involvement of COX-2 isoform in several neuropathological conditions particularly neurodegenerative disorders (Teismann et al., 2003; Minghetti, 2004). Studies from our laboratory have also demonstrated the beneficial effect of COX-inhibitors in various neurological disorders including epilepsy, drug addiction, depression and stress related pathologies (Naidu et al., 2002; Dhir et al., 2007; Akula et al., 2008).
Current ongoing research using various animal models has also demonstrated the neuroprotective potential of COX-2 inhibitors in the neurological diseases (Aubin et al., 1998; Reksidler et al., 2007; Sanchez-Pernaute et al., 2004). The mechanisms of antiparkinson-like effect of these COX-inhibitors are not yet clear. So far, some of the proposed hypothesis includes: i) inhibition of nitric oxide free radicals formation, ii) agonistic action for peroxisome proliferator-activated receptor gamma, and/or iii) possible suppressive effects against dopamine quinone formation (Asanuma et al., 2003). Contrary to this, one of the clinical studies has demonstrated the ineffectiveness of COX-inhibitors in the treatment of PD (Bornebroek et al., 2007). Although the experimental and epidemiological studies suggest the beneficial role of COX-inhibitors in PD (Aubin et al., 1998; Esposito et al., 2007; Etminan et al., 2008), still exact mechanism of their protective action is yet to be explored.

With this background, the present study was designed to evaluate the effect of various selective and non-selective COX-inhibitors viz. rofecoxib, celecoxib (both selective COX-2 inhibitors), nimesulide (preferential COX-2 inhibitor) and naproxen (non-selective COX-inhibitor) against perphenazine-induced catatonia in rats.

1.1.2. Materials and Methods

1.1.2.1. Animals

Male Wistar rats (250-300 g) bred in the Central Animal House facility of Panjab University, Chandigarh were used. Animals were acclimatized to laboratory conditions prior to experimentation. Animals were kept under standard conditions of light and dark cycle with food and water ad libitum. All the experiments were carried out between 0900 and 1700 h. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) and was carried out in accordance with the Indian National Science Academy (INSA) Guidelines for the use and care of animals.

1.1.2.2. Drugs and treatment schedule

Perphenazine (PPZ) (5 mg/kg) (Sigma, St. Louis, MO, USA), L-DOPA (50 mg/kg, p.o.) and carbidopa (10 mg/kg, p.o.) (Hi Media, Mumbai, India);
rofecoxib (ROF) (2-8 mg/kg), celecoxib (CEL) (10-40 mg/kg), nimesulide (NIM) (2.5-10 mg/kg) and naproxen (NPX) (7-20 mg/kg) (Panacea Biotec Ltd., New Delhi, India) were used in the present study. All the drugs except perphenazine were suspended in 0.25 % w/v sodium carboxymethyl cellulose (Na-CMC) and administered per orally (p.o.) in a constant volume of 0.5 ml/100 g body weight of rat. Perphenazine was dissolved with the aid of diluted hydrochloric acid (0.1 N), pH adjusted to neutral and volume was made up with distilled water and administered by intraperitoneal (i.p.) injection.

1.1.2.3. Experimental protocol

**Protocol 1** – In this protocol, 6-8 animals were used to study the effect of various COX-inhibitors against perphenazine induced catatonia.

In the present study protocol, assessment of catatonia was done by block or bar test for at 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 12 and 24 h post perphenazine challenge. Area under the curve (AUC) (0 to 24 hrs) was calculated graphically using trapezoidal rule (Bourne, 1995). According to this rule, we calculate the AUC of each segment where individual segment is considered as trapezoid and the area from the first to the last data point is added to get the total AUC. The values for the catatonic score in perphenazine
Protocol 3 – In this protocol, based on protocol 1 results, the effect of rofecoxib or celecoxib was assessed against perphenazine induced oxidative stress at 4 h post perphenazine injection in animals. 5-6 animals were used in each group in this study.

Protocol 2 – The effect of combination of COX-inhibitors with L-DOPA/carbidopa was evaluated wherein COX-inhibitors viz. rofecoxib, celecoxib, nimesulide or naproxen were administered 30 min prior to L-DOPA and 15 min before carbidopa. After 30 min of L-DOPA administration or 45 min of carbidopa administration, animals were challenged with catatonic dose of perphenazine. 6-8 animals were used in each group in this protocol.

Protocol 3 – In this protocol, based on protocol 1 results, the effect of rofecoxib or celecoxib was assessed against perphenazine induced oxidative stress at 4 h post perphenazine injection in animals. 5-6 animals were used in each group in this study.
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All the doses were selected based on the previous studies reported from our laboratory (Naidu et al., 2002; Dhir et al., 2007; Akula et al., 2008).

1.1.2.4. Assessment of catatonia

Two tests were employed in the present study to assess the severity of catatonia following perphenazine administration (5 mg/kg) and the assessment of catatonic response was done in both the tests as per the protocols described below:

1.1.2.4.1. Bar test

This test was conducted as per the procedure previously validated in our laboratory (Singh and Kulkarni, 2002; Singh et al., 2003). In brief, front paws of the rat were gently placed on a horizontal metal bar with 5-6 mm diameter and placed 10 cm above ground level and the length of time, the rats maintained in this abnormal posture with at least one paw was recorded. The test was terminated when the animal withdrew its paw and attained the normal posture or 180 sec had passed. The total time the animals stayed on the bar was recorded. If the animal did not hold on to the bar even after three attempts, zero score was recorded (Singh et al., 2003).

1.1.2.4.2. Block test

In block test, the development and severity of the four stages of catatonia were observed and scored as follows: Stage 1, rat moves when placed on the table, score=0; Stage 2, rat moves only when touched or pushed, score=0.5; Stage 3, rat placed on the table with front paws set alternately on a 3 cm high block fails to correct the posture in 10 sec, score=0.5 for each paw with a total of 1 for this stage; State 4, rat fails to move when the front paws are placed alternately on a 9 cm high block, score= 1 for each paw with a total score 2 for this stage. Thus, the maximum possible score would be 3.5 reflecting full catatonia. Lesser score would mean an apparently lesser degree of catatonia (Kulkarni et al., 1980).

1.1.2.5. Dissection and homogenization

Four hours after perphenazine administration, animals were sacrificed by cervical dislocation, their brain were quickly removed and perfused
immediately with ice-cold normal saline and weighed. A 10% (w/v) tissue homogenates were prepared in chilled 0.1 M phosphate buffer (pH 7.4) using a Potter Elvenhjem homogenizer (Remi, Mumbai, India). The homogenates were centrifuged at 12 000 g for 20 min, 4°C to obtain the post mitochondrial supernatants (PMS), which were used for further enzymatic analysis.

1.1.2.6. Measurement of oxidative stress parameters

1.1.2.6.1. Measurement of lipid peroxidation

The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) (Ohkawa et al., 1979).

In brief, the reaction mixture consisted of 0.2 ml of 8.1% w/v sodium lauryl sulfate, 1.5 ml of 20% v/v acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% w/v aqueous solution of thiobarbituric acid was added to 0.2 ml of 10% (w/v) of homogenate. The mixture was brought up to 4.0 ml with distilled water and heated at 95°C for one hour. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added, shaken well and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm. The MDA levels was calculated as nanomoles of MDA per milligram protein and expressed as % of control.

1.1.2.6.2. Estimation of reduced glutathione

The reduced glutathione (GSH) was measured by the method of Ellman (1959).

In brief, 1.0 ml of PMS (10% w/v) was precipitated with 1.0 ml of sulphosalicylic acid (4% w/v). The samples were then kept at 4°C for at least 1 h and then subjected to centrifugation at 1200 rpm for 15 min at 4°C. The assay mixture contained 0.1ml of filtered aliquot and 2.7 ml of phosphate buffer (0.1 M, pH 7.4) and 0.2ml of DTNB (40 mg/10 ml of 0.1 M phosphate buffer, pH 7.4) in a total volume of 3.0 ml. The yellow color developed by the reduction of Ellman's reagent by –SH group of GSH was read at 412 nm. The –SH group was calculated on the molar extinction coefficient of yellow colored anion, \( 2- \)
nitro mercaptobenzoic acid (1.36 X 10³ M⁻¹ cm⁻¹). The results are calculated as micromoles of GSH/mg protein and as expressed % of control.

1.1.2.6.3. Superoxide dismutase (SOD) activity

Superoxide dismutase activity was assayed according to the method of Kono, 1978 in which the inhibition of the reduction of nitrazobluetetrazolium (NBT) by superoxide dismutase is measured at 560 nm using a Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA).

Briefly, the reaction was initiated by the addition of 20 mM of hydroxylamine hydrochloride to the mixture containing 96 mM of Nitroblue tetrazolium (NBT) and 0.1 ml of the sample. The results were calculated as unit/mg protein and expressed as % of control

1.1.2.6.4. Estimation of nitrite

Nitrite levels were estimated using Griess reagent that served as an indicator of nitric oxide production (Raghavendra et al., 2000). Briefly, 1.0 ml of Griess reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) was added to 1.0 ml of brain homogenate and absorbance was measured at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitrite and nitrite levels were expressed as percentage of control.

1.1.2.6.5. Protein estimation

The protein content was measured according to the method of Lowry et al., (1951) using bovine serum albumin as standard.

1.1.2.7. Statistical analysis

Values are expressed as mean ± SEM. One Way Analysis of Variance (ANOVA) followed by Dunnett's test was employed to calculate the statistical significance between various groups. A value of p<0.05 was considered to be statistically significant. Area under the curve (AUC) (0 to 24 hrs) was calculated graphically using trapezoidal rule.
1.1.3. Results

1.1.3.1. Effect of various COX-inhibitors against perphenazine-induced catatonia

Perphenazine (5 mg/kg) induced significant catatonia in both bar and block tests. The peak response was observed at 4 h which remained constant for 12 h and diminished over a period of 24 h (Fig. 1.1.). Area under the curve (AUC) was calculated starting from 0 h to 24 h and considered to be 100 %. For all the further experiments, the level of significance was calculated at 4 h.

Pretreatment with rofecoxib (4 and 8 mg/kg) decreased the catatonic score on bar (Fig. 1.1.1.A) as well as block tests (Fig. 1.1.1.B), respectively. Celecoxib (10-40 mg/kg) significantly decreased the catatonic score both in bar (Fig. 1.1.2.A) and block tests (Fig. 1.1.2.B). Nimesulide (2.5-10 mg/kg) also significantly decreased the degree of catatonia in both bar (Fig. 1.1.3.A) as well as in block test (Fig. 1.1.3.B). Similarly, naproxen (14 and 20 mg/kg), significantly decreased the catatonic response in both bar test (Fig. 1.1.4.A) as well as block test (Fig. 1.1.4.B).

When the AUC values of each COX-inhibitor were compared, rofecoxib (2-8 mg/kg) and nimesulide (2.5-10 mg/kg) exhibited dose dependent decrease in catatonic response in both tests while significant effects were seen only with celecoxib (40 mg/kg). Naproxen (14-20 mg/kg) significantly decreased the catatonic response in both bar and block test (Table 1.1.1.).

1.1.3.2. Effect of L-DOPA, carbidopa and its combination against perphenazine-induced catatonia

As shown in Table 1.1.2., L-DOPA (50 mg/kg), carbidopa (10 mg/kg) and L-DOPA/carbidopa (50:10) combination significantly decreased the severity of perphenazine induced catatonia in both bar test (Fig. 1.1.5.A) as well as block test as compared to perphenazine treated group (Fig. 1.1.5.B).

1.1.3.3. Effect of combination of COX-inhibitors with L-DOPA/carbidopa against perphenazine-induced catatonia

Pretreatment with lower dose of rofecoxib (2 mg/kg), celecoxib (10 mg/kg), nimesulide (2.5 mg/kg) or naproxen (7 mg/kg), potentiated the motor
stimulatory effect of L-DOPA/carbidopa (50:10) combination as compared to their per se effect (Fig. 1.1.6-1.1.9). (Table 1.1.2.) represents the percent decrease in the AUC values as compared to perphenazine, where the value of perphenazine was taken as 100%. Combination of COX-inhibitors with L-DOPA/carbidopa significantly decreased the catatonic response in both bar [Fig. 1.1.6.(A)-1.1.9.(A)] and block tests [Fig. 1.1.6.(B)-1.1.9.(B)] respectively as compared to lower dose of respective COX-inhibitors.
Fig. 1.1.1. Effect of rofecoxib in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. $^aP<0.05$ versus 0 min; $^bP<0.05$ versus PPZ (5) at 4 h
Fig. 1.1.2. Effect of celecoxib in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. aP<0.05 versus 0 min; bP<0.05 versus PPZ (5) at 4 h.

(A)
Fig. 1.1.3. Effect of nimesulide in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. \(^aP<0.05\) versus 0 min; \(^bP<0.05\) versus PPZ (5) at 4 h
Fig. 1.1.4. Effect of naproxen in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. *P<0.05 versus 0 min; †P<0.05 versus PPZ (5) at 4 h

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Table 1.1.1. Effect of various COX-inhibitors on AUC values in perphenazine-induced catatonia in rats

<table>
<thead>
<tr>
<th>Drug treatment (mg/kg)</th>
<th>AUC (% of Control)</th>
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<tbody>
<tr>
<td></td>
<td>Bar Test</td>
<td>Block Test</td>
<td></td>
</tr>
<tr>
<td>PPZ(5)</td>
<td>100±1.10</td>
<td>100±1.12</td>
<td></td>
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<tr>
<td>ROF(2)</td>
<td>77.2±1.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.0±1.55&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>ROF(4)</td>
<td>65.4±1.55&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>44.4±1.56&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>ROF(8)</td>
<td>33.2±1.91&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>23.0±1.92&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
<td>CEL(10)</td>
<td>61.9±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.65±1.58&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>CEL(20)</td>
<td>60.2±1.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.8±1.59&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>CEL(40)</td>
<td>42.9±1.43&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>24.0±1.35&lt;sup&gt;a,d&lt;/sup&gt;</td>
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<tr>
<td>NIM(2.5)</td>
<td>74.1±2.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.51±1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>NIM(5)</td>
<td>66.2±2.30&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td>60.1±1.98&lt;sup&gt;a,e&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>NIM(10)</td>
<td>50.2±1.56&lt;sup&gt;a,e,f&lt;/sup&gt;</td>
<td>40.6±1.75&lt;sup&gt;a,e,f&lt;/sup&gt;</td>
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<tr>
<td>NPX(7)</td>
<td>72.95±2.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.15±2.30&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>NPX(14)</td>
<td>60.5±3.20&lt;sup&gt;a,g&lt;/sup&gt;</td>
<td>54.1±2.21&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>NPX(20)</td>
<td>40.4±1.91&lt;sup&gt;a,g,h&lt;/sup&gt;</td>
<td>27.9±1.96&lt;sup&gt;a,h&lt;/sup&gt;</td>
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<sup>a</sup>P<0.5 versus PPZ(5), <sup>b</sup>P<0.5 versus ROF(2)+PPZ(5), <sup>c</sup>P<0.5 versus ROF(4)+PPZ(5), <sup>d</sup>P<0.5 versus CEL(20)+PPZ(5), <sup>e</sup>P<0.5 versus NIM(2.5)+PPZ(5), <sup>f</sup>P<0.5 versus NIM(10)+PPZ(5), <sup>g</sup>P<0.5 versus NPX(7)+PPZ(5), <sup>h</sup>P<0.5 versus NPX(14)+PPZ(5)
Fig. 1.1.5. Effect of combination of L-DOPA/carbidopa (50:10) in perphenazine-induced catatonia in rats (A) bar test (B) block test. *P<0.5 versus 0 min; †P<0.5 versus PPZ (S) at 4 h
Fig. 1.1.6. Effect of combination of rofecoxib with L-DOPA/carbidopa (50:10) in perphenazine-induced catatonia in (A) bar test and (B) block test in rats.  

- $^aP<0.05$ versus 0 min; $^bP<0.05$ versus PPZ (5) at 4 h; $^cP<0.05$ versus L-DOPA/carbidopa (50:10) at 4 h; $^dP<0.05$ versus ROF (2) at 4 h.
Fig. 1.1.7. Effect of combination of celecoxib with L-DOPA/carbidopa (50:10) in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. *P<0.05 versus 0 min; †P<0.05 versus PPZ (5) at 4 h; ‡P<0.05 versus L-DOPA/carbidopa (50:10) at 4 h; §P<0.05 versus CEL (10) at 4 h
Fig. 1.1.8. Effect of combination of nimesulide with L-DOPA/carbidopa (50:10) in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. *P<0.05 versus 0 min; **P<0.05 versus PPZ (5) at 4 h; ***P<0.05 versus L-DOPA/carbidopa (50:10) at 4 h; ****P<0.05 versus NIM (2.5) at 4 h
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Fig. 1.1.9. Effect of combination of naproxen with L-DOPA/carbidopa (50:10) in perphenazine-induced catatonia in (A) bar test and (B) block test in rats. \(^a\)P<0.05 versus 0 min; \(^b\)P<0.05 versus PPZ (5) at 4 h; \(^c\)P<0.05 versus L-DOPA/carbidopa (50:10) at 4 h; \(^d\)P<0.05 versus NPX (7) at 4 h.
Table 1.1.2. Effect of combination of various COX-inhibitors with L-DOPA/carbidopa on AUC values in perphenazine-induced catatonia in rats

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>AUC (% of control)</th>
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<tbody>
<tr>
<td></td>
<td>Bar Test</td>
</tr>
<tr>
<td>PPZ(5)</td>
<td>100</td>
</tr>
<tr>
<td>L-DOPA(50)</td>
<td>74.5±2.65(^a)</td>
</tr>
<tr>
<td>CAR(10)</td>
<td>82.2±3.32(^a)</td>
</tr>
<tr>
<td>L-DOPA/CAR(50:10)</td>
<td>69.8±3.1(^a)</td>
</tr>
<tr>
<td>ROF(2)</td>
<td>77.2±1.61(^a)</td>
</tr>
<tr>
<td>ROF(2)+L-DOPA/CAR(50:10)</td>
<td>25.1±2.54(^a,b,c)</td>
</tr>
<tr>
<td>CEL(10)</td>
<td>61.9±1.90(^a)</td>
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<td>25.7±2.1(^a,b,d)</td>
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<tr>
<td>NIM(2.5)</td>
<td>74.1±2.12(^a)</td>
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<tr>
<td>NIM(2.5)+L-DOPA/CAR(50:10)</td>
<td>28.4±2.1(^a,b,e)</td>
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<tr>
<td>NPX(7)</td>
<td>72.95±2.03(^a)</td>
</tr>
<tr>
<td>NPX(7)+L-DOPA/CAR(50:10)</td>
<td>37±2.35(^a,b,f)</td>
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\(^a\)P<0.5 versus PPZ(5); \(^b\)P<0.5 versus L-dopa/carbidopa (50:10)+PPZ(5); \(^c\)P<0.5 versus ROF(2)+PPZ(5); \(^d\)P<0.5 versus CEL(10)+PPZ(5); \(^e\)P<0.5 versus NIM(2.5)+PPZ(5); \(^f\)P<0.5 versus NPX(7)+PPZ(5)
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1.1.3.4. Effect of selective COX-2 inhibitors, rofecoxib or celecoxib on oxidative stress induced by perphenazine

When the animals (n=5-6) were sacrificed 4 h after perphenazine administration (the time when catatonic score was maximum), a significant increase in oxidative damage was observed. As shown in Table 1.1.3, the brain levels of lipid peroxide and nitrite concentration were increased and there was a decrease in the antioxidant pool (reduced glutathione and SOD levels) as compared to vehicle. Pretreatment with COX-inhibitors, rofecoxib (8 mg/kg), celecoxib (40 mg/kg), nimesulide (10 mg/kg) or naproxen (20 mg/kg) significantly attenuated the oxidative stress as evidenced by reduced levels of lipid peroxides and brain nitrite as compared to perphenazine treated group. These COX-inhibitors also restored the depleted levels of brain antioxidants such as reduced glutathione and SOD as compared to perphenazine treated group (Table 1.1.3.).

Table 1.1.3. Effect of selective COX-2 inhibitors, rofecoxib or celecoxib on perphenazine-induced alterations in oxidative stress parameters in rats

<table>
<thead>
<tr>
<th>Drug Treatment (mg/kg)</th>
<th>Lipid peroxidation (n moles of MDA/mg protein) (% of control)</th>
<th>Reduced Glutathione levels (GSH) (µmol/mg protein) (% of control)</th>
<th>SOD levels (% of control)</th>
<th>Nitrite levels (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>100±3.3</td>
<td>100±4.2</td>
<td>100±2.2</td>
<td>100±5.5</td>
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<tr>
<td>PPZ (5)</td>
<td>294±4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82±3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62±4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>137±5.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ROF (8)</td>
<td>149±3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>115±4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>97±7.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88±1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CEL (40)</td>
<td>159±4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98±3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94±5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90±4.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NIM (10)</td>
<td>166±3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94±2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91±4.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NPX (7)</td>
<td>171±3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89±3.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104±4.0&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup>P<0.5 versus Veh; <sup>b</sup>P<0.5 versus PPZ(5)
Chapter 1.1
1.1.4. Discussion

Present study highlights the protective effect of selective (rofecoxib, celecoxib), preferential (nimesulide) and non-selective (naproxen) COX inhibitors in perphenazine induced catatonia in rats. Results demonstrate that: (i) out of selective and non-selective COX inhibitors, rofecoxib (a selective COX-2 inhibitor) exhibited more protective effect in reducing the severity of catatonia (ii) COX-inhibitors in combination with L-DOPA/carbidopa significantly decreased the severity of catatonia in both the tests as compared to their alone effects (iii) COX-inhibitors also attenuated the perphenazine induced oxidative stress in rat brain.

Perphenazine being a dopamine D$_2$ receptor antagonist blocks the action of dopamine on its dopaminergic receptors. Due to this, there is an imbalance between the dopamine and acetylcholine levels and dysregulated movement and muscle control. Thus, when animals were treated with perphenazine, animals displayed Parkinson-like symptoms such as rigidity or postural inabilities in the form of catatonia as assessed by bar or block test. The present results demonstrate that various COX-inhibitors viz. rofecoxib, celecoxib, nimesulide and naproxen significantly attenuated the degree of catatonia induced by perphenazine. Out of all these COX-inhibitors, rofecoxib (8 mg/kg) exhibited pronounced protection in comparison with other COX-inhibitors at their highest dose.

Oxidative stress and inflammatory events play a significant pathological role in the development of PD (Gu et al., 1998). Induction of inflammatory process due to activated microglia generates reactive oxygen species such as superoxide anions which may react with nitric oxide to form peroxynitrite, a potent prooxidant (Cohen and Heikkila, 1974; Li et al., 2011). Since inflammatory process involves many pathways in the dopaminergic cell loss, COX and the subsequent formation of prostaglandins may play a crucial role and represent the potential site in attenuating the progression of PD. Dopamine being an important neurotransmitter involved in locomotion and reward phenomenon is very sensitive to oxidative attack. This is further known to get oxidized to dopamine-quinone due to the enhanced COX-2 expression and further generation of free radicals following neuroinflammation (Hastings, 1995;
Chapter 1.1

Slivka and Cohen, 1985). These observations further demonstrate the involvement of oxidative stress and COX-2 in the motor deficits produced by neuroleptics. It is a known fact that typical antipsychotics like haloperidol produce oxidative stress in brain and result in neurotoxic actions (Martins et al., 2008; Polydoro et al., 2004). Therefore, the present study was designed to evaluate the effect of selective and non-selective COX-inhibitors in perphenazine-induced oxidative stress in rats. Furthermore, the present study was extended to explore the dopaminergic modulatory action of COX-inhibitors where in COX-inhibitors were combined with L-DOPA and carbidopa.

Since the maximum effect of perphenazine induced catatonia was seen at 4 h post perphenazine administration, we also intended to assess the level of oxidative stress in perphenazine treated rat brain. When oxidative stress parameters were measured 4 h after perphenazine administration, there was significant increase in the brain lipid peroxidation and nitrite levels as well as diminished antioxidant pool. It is hypothesized that perphenazine administration probably induced the generation of reactive oxygen species, which further initiate proinflammatory cascades and result in the development of catatonia. Treatment with rofecoxib and celecoxib ameliorated the perphenazine-induced oxidative stress suggesting their antioxidant like effect. Therefore, it is speculated that inhibition of oxidative stress dependent up regulation of inflammatory events appears to attenuate the behavioral deficits produced by perphenazine. Our results are in concurrence with earlier studies where neuroprotective effect of COX-2 selective inhibitors has been reported in animal studies (Aubin et al., 1998; Teismann and Ferger, 2001).

The role of various cellular players involved in the inflammatory cascade (COX and subsequent prostaglandin pathway) in the degradation of dopaminergic system is crucial subject of research. It further represents the potential site which can be inhibited or blocked to attenuate the progression of PD. Therefore, we sought to investigate the modulatory role of COX-inhibitors on dopaminergic pathway in which the combination of sub-effective (lower) dose of different COX-inhibitors with L-DOPA/carbidopa was used, where L-DOPA and carbidopa remains the first line of drug therapy in the management of PD. It is already established that during therapeutic management of PD,
chronic treatment with L-DOPA results in declined efficacy as well as “on-off” phenomenon (Sweet et al., 1975; Fahn, 2005). So, in order to avoid this phenomenon either the reduction in the dose is done or patient undergoes drug free period for some time. Therefore, if a suitable adjunctive is co-administrated with L-DOPA, which can lower its dose and prevent unavoidable side effects, could be a therapeutic alternative in the management of PD. In our study also, L-DOPA at a reduced dose was used and combined with the COX-inhibitors. The present results demonstrate the fact that when rats pretreated with the sub-effective doses of different COX-inhibitors viz. rofecoxib, celecoxib, nimesulide and naproxen were combined with L-DOPA/carbidopa (50:10) combination, COX-inhibitors significantly potentiated the effect of latter and attenuated the severity of catatonia induced by perphenazine. It might be possible the COX-inhibitors prevented the oxidative stress, degradation of dopamine or formation of dopamine quinone and further L-DOPA administration reversed the dopamine levels to normal resulting in the normal motor function. Thus, it is plausible that if COX-inhibitors combined with L-DOPA can be an alternative therapy in the management of PD, but still the clinical studies are warranted before coming to any concrete conclusions.

The findings of the present study indicate the beneficial role of selective COX-2 inhibitors (rofecoxib, celecoxib), preferential COX-2 inhibitor (nimesulide), non-selective COX inhibitor (naproxen) and its combination with L-DOPA/carbidopa in alleviating perphenazine-induced motor deficits in rats. Further, involvement of oxidative pathway in the motor dysfunction and its reversal by COX-inhibitors is speculated. These finding also substantiate the neuroprotective potential of COX-inhibitors especially COX-2 inhibitors in the pathophysiology of PD. Further, this study implicates that COX-inhibitors being known to play a role in neuroprotection could be used as an effective adjunct to L-DOPA in the treatment of extrapyramidal side effects induced by neuroleptics.

Based on the observations discussed above, plausible neuroprotective role of various COX-2 inhibitors has been suggested in neurotoxic model of PD which mimics the clinical symptoms of PD.
CHAPTER-1.2
To study the neuromodulatory potential of selective COX-2-inhibitors in MPTP-induced neurotoxicity in mice

1.2.1. Introduction

Neuroinflammation an important defense mechanism to pathogenic events and environmental toxins, it is the key component in the progression of various neurodegenerative disorders, especially Parkinson’s disease (PD) (Monahan et al., 2008; Vroon et al., 2007). Recent research has shown that oxidative stress, mitochondrial dysfunction and neuroinflammation are the major players involved in the pathogenesis of PD along with the dopaminergic neurodegeneration (Kim et al., 2011; Tufekci et al., 2011; Qian et al., 2010). Therefore, attenuation of neuroinflammation and oxidative stress represents a prime target so as to halt the progression of this disease. Various experimental studies have documented the protective effect of non-steroidal anti-inflammatory drugs (NSAIDs) in MPTP-induced neurotoxicity model of PD (Aguirre et al., 2008; Gupta et al., 2009, Gupta et al., 2010a; 2011; Reiksdler et al., 2007). These drugs exert their anti-inflammatory action by inhibiting cyclooxygenase (COX) isoenzymes which is the rate-limiting enzymes involved in the production of prostaglandins from arachidonic acid (Kulkarni and Dhir, 2009).

Experimental evidence indicates that cellular manifestation of neuroinflammation following MPTP administration takes place in substantia nigra due to microglial activation. Transient activation of microglia precedes the demise of dopaminergic neurons and mimics Parkinson-like symptoms in experimental model of PD (Smeine and Jackson-Lewis, 2005; Liu et al., 2006). These symptoms are the same as demonstrated in the patients afflicted with Parkinson’s disease where increased expression of various inflammatory molecules in the dopaminergic neurons have been observed (Minghetti, 2004). These neuroinflammatory events activate the glial cells viz microglia, which is further speculated to up-regulate the COX expression (Teismann and Ferger, 2001). One of the key features’ of PD pathology, long-term gliosis, has been well documented in monkeys and humans exposed to MPTP (Vroon et al., 2007).
In one of the experimental studies, parecoxib, a selective COX-2 inhibitor exhibited neuroprotection against MPTP-induced Parkinson-like symptoms in rats (Reksidler et al., 2007). Similarly, aspirin, a non-selective COX-inhibitor has been demonstrated to have neuroprotective effect against MPTP-induced dopamine depletion in mice (Aubin et al., 1998). Rofecoxib, valdecoxib and NS-398 used in the present study belong to the second generation of selective COX-2 inhibitors and have >50 fold selectivity in inhibiting COX-2 isoform (Dannhardt and Keifer, 2001; Rao and Knaus, 2008). Valdecoxib, a diarylisoazole class of compound, is metabolized in rodents and dogs to a 5-hydroxymethyl metabolite with additional anti-inflammatory activity (Dannhardt and Keifer, 2001; Rao and Knaus, 2008). Structural modifications of the nimesulide moiety ended up in the development of N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398), with better COX-2 selectivity and anti-inflammatory activity (Huff et al., 1995). NS-398 has shown to reduce the infarct volume and attenuate prostaglandin E₂ elevation in experimental animals (Huff et al., 1995; Nogowa et al., 1997; Nagayama et al., 1999).

During the last few decades, efforts have been made to develop novel anti-inflammatory drugs so as to arrest the progression of PD. With the increasing knowledge about the detrimental effects of neuroinflammatory cascade in the pathogenesis of PD, therapeutic agents that suppress or interfere this pathway represents the probable candidates for the neuroprotection in PD. Based on these observations, the present study has been designed the study to explore the neuromodulatory role of selective COX-2 inhibitors viz. rofecoxib, valdecoxib and NS-398 in MPTP model of PD. The present study is an attempt to unravel the possible cellular mechanisms and to gain more insight into the neuroprotective effect produced by COX-2 inhibitors against MPTP-induced neuronal toxicity in mice.

1.2.2. Materials and Methods

1.2.2.1. Animals

Male Albino Laca mice (25–30 g) bred in the Central Animal House facility of Panjab University were used. The animals were housed under standard laboratory conditions, maintained on a natural light–dark cycle and...
free access of food and water. Animals were acclimatized to laboratory conditions before the test. All the experiments were carried out between 0900 and 1700 h. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the guidelines of Indian National Science Academy for the use and care of experimental animals.

1.2.2.2. Drugs and treatment schedule

The following drugs were used in the present study—MPTP (Sigma Chemicals, St. Louis, MO, USA), rofecoxib, valdecoxib and NS-398 (Panacea Biotec Ltd., New Delhi, India) and sodium carboxy methyl cellulose (Na-CMC) (Himedia, Mumbai). MPTP was dissolved in distilled water and administered intraperitoneally. All drugs except MPTP were suspended in Na-CMC (0.25 % w/v) and administered by per oral route in a constant volume of 1 ml/100 gm.

Study Design

Eleven groups were employed in the present study, consists of 18 animals in each. Study was conducted in different phases. **Group-1** – Vehicle treated group (0.25 % Na-CMC) administered for 7 days; **Group-2** – MPTP-
Chapter 1.2

treated group (40 mg/kg) in divided doses of four injections of 10 mg/kg, i.p at 1 hr interval; **Group-3, 4 & 5** – rofecoxib (8 mg/kg), valdecoxib (10 mg/kg) and NS-398 (10 mg/kg) *per se.* respectively for 7 days; **Group-6 & 7** – rofecoxib (4 and 8 mg/kg, p.o.) + MPTP (40mg/kg); **Group-8 & 9** – valdecoxib (5 and 10 mg/kg, p.o.) + MPTP (40mg/kg); **Group-10 & 11** – NS398 (5 and 10 mg/kg, p.o.) + MPTP (40mg/kg)

**Experimental Protocol**

MPTP was administered only on day 1 in all the groups except vehicle treated group. Drugs treatment was started 1 h before first MPTP injection, followed by three more doses (10 mg/kg, i.p.) of MPTP injections and continued for 7 days.

Animals were then divided into separate groups for biochemical, mitochondrial and cellular estimations.

1.2.2.3. Behavioral assessments

1.2.2.3.1. Assessment of gross behavioral activity (locomotor activity)

The locomotor activity was assessed by using an actophotometer (IMCROP, Ambala, India). The motor activity was detected by infrared beams above the floor of the testing area. Animals were placed individually in the...
activity chamber for a 3-min acclimation period before performing actual activity tasks. Each animal was observed over a period of 5 min and expressed as counts/5 min (Kumar et al., 2007).

1.2.2.3.2. Assessment of catatonia

The severity of catatonia was assessed by employing the bar test as per the procedure previously validated in our laboratory (Gupta et al. 2009; Singh and Kulkarni 2002). In brief, front paws of the mice were gently placed on a horizontal metal bar with 5-6 mm diameter, placed 6 cm above ground level. The test was terminated when animal withdrew its paw and attained the normal posture or cut off time of 180 s had passed. Time latency of the front paws stayed on the bar was recorded. If the animal did not hold on to the bar even after three attempts, zero score was given to the respective animals (Gupta et al. 2009).

1.2.2.4. Dissection and homogenization

On day 8, following behavioral assessments, the animals were randomized into different groups for biochemical analysis, mitochondrial enzyme complex activity and cellular estimations.

For biochemical analysis, animals (n=5-6) were sacrificed by decapitation. The brains were quickly removed and perfused immediately with ice-cold normal saline. The striatum were isolated, pooled and weighed. A 10% (w/v) tissue homogenates were prepared in ice cold 0.1 M phosphate buffer (pH 7.4) using a Potter Elvenhjem homogenizer (Remi, Mumbai, India). The homogenates were centrifuged at 12,000 g for 20 min, 4 °C to obtain the post mitochondrial supernatants (PMS), which were used for further enzymatic analysis. In another set of animals, the brains were removed; stored at −80 °C for measurement of mitochondrial function and estimation of proinflammatory and apoptotic markers.

1.2.2.5. Measurement of oxidative stress parameters

1.2.2.5.1. Measurement of lipid peroxidation

Refer Chapter 1A (Section 1.1.2.6.1.)
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1.2.2.5.2. Estimation of reduced glutathione
   Refer Chapter 1A (Section 1.1.2.6.2.)

1.2.2.5.3. Superoxide dismutase (SOD) activity
   Refer Chapter 1A (Section 1.1.2.6.3.)

1.2.2.5.4. Estimation of nitrite
   Refer Chapter 1A (Section 1.1.2.6.4.)

1.2.2.5.5. Protein estimation
   Refer Chapter 1A (Section 1.1.2.6.5.)

1.2.2.6. Mitochondrial complex estimation

1.2.2.6.1. Isolation of rat brain mitochondria

   Rat brain mitochondria were isolated by the method of Berman and Hastings, 1999. The brain regions were homogenized in isolation buffer with Ethylene Glycol Tetraacetic Acid (EGTA) (215 mM Mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). The homogenates were centrifuged at 13000 \( \times \) g for 5 minutes at 4°C. The pellets were resuspended in isolation buffer with EGTA and spun again at 13000 \( \times \) g for 5 min. The resulting supernatants were transferred to new tubes and topped off with isolation buffer containing EGTA and spun again at 13000 \( \times \) g for 10 min. The pellets containing pure mitochondria were resuspended in isolation buffer without EGTA.

1.2.2.6.2. Complex-I (NADH dehydrogenase activity)

   NADH dehydrogenase is an enzyme located in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q (Co Q) (Fig. 1.1). It is also called the NADH: quinone oxidoreductase or complex I. NADH Dehydrogenase is the first enzyme (complex I) of the mitochondrial electron transport chain. Complex I is one of the main sites at which premature electron leakage to oxygen occurs, thus being one of main sites of production of a harmful free radical called superoxide.
Fig. 1. Electron Transport chain in mitochondria (Adapted from Keane et al., 2011)

\[
\text{NADH} + H^+ + \text{CoQ} + 4H^+_{\text{in}} \rightarrow \text{NAD}^+ + \text{CoQH}_2 + 4H^+_{\text{out}}
\]

Complex-I was measured spectrophotometrically by the method of King and Howard, 1967. The method involves the catalytic oxidation of NADH to NAD\(^+\) with subsequent reduction of cytochrome C. The reaction mixture contained 0.2 M glycyl glycine buffer pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer and 10.5 mM cytochrome C. The reaction was initiated by the addition of a requisite amount of solubilized mitochondrial sample. The change in absorbance was recorded at 550 nm for 2 min.

1.2.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay

The MTT assay is laboratory test for measuring the enzyme activity that reduces MTT + isolated mitochondria to formazan, giving a purple color. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials, since those agents would result in cell toxicity and therefore metabolic dysfunction. Yellow MTT is reduced to purple formazan in living cells. A solubilization solution (dimethyl sulfoxide, an acidified ethanol solution) is
added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring between 500 and 600 nm.

This reduction takes place only when reductase enzymes are active and therefore conversion is often used as a measure of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve.

The MTT assay is based on the reduction of MTT by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess activity of the mitochondrial respiratory chain in isolated mitochondria (Mosmann, 1983). Briefly, 100 µl mitochondrial samples were incubated with 10 µl MTT for 3 hours at 37°C. The blue formazan crystals were solubilized with dimethylsulfoxide and measured by an ELISA reader with a 580 nm filter (Model 680 Microplate Reader, Bio-Rad Japan).

1.2.2.7. Quantification of proinflammatory and apoptotic factors

1.2.2.7.1. Caspase-3 Colorimetric assay

Caspase-3, also known as CPP-32, Yama or Apopain, is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanaline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate/homogenate is directly proportional to the color reaction. The enzymatic reaction for caspase activity was carried out as using Biovision (CA, USA) caspase-3 colorimetric kit.

**Assay Procedure:**

Aliquot enough 2X Reaction Buffer for the number of assays to be performed. Add DTT to the 2X Reaction Buffer immediately before use (10 mM
final concentration: add 10 μl of 1.0 M DTT stock per 1 ml of 2X Reaction Buffer. Protect DEVD-pNA from light.

1. Apoptosis was induced in cells by desired method. Concurrently incubated a control culture without induction.
2. Cells and pellet 1-5 x 106 cells were counted.
3. Resuspend cells in 50 μl of chilled cell lysis buffer and cells were incubated on ice for 10 minutes.
4. Centrifuged for 1 min in a microcentrifuge (10,000 x g).
5. Supernatant (cytosolic extract) was transferred to a fresh tube and put on ice for immediate assay or aliquot and store at –80°C for future use.
6. Protein concentration was assayed.
7. Diluted 50-200 μg protein to 50 μl Cell Lysis Buffer for each assay.
8. 50 μl of 2X Reaction Buffer (containing 10 mM DTT) was added to each sample.
9. 5 μl of the 4 mM DEVD-pNA substrate (200 μM final conc.) added to each well and incubated at 37°C for 1-2 hour.
10. Samples were analyzed at 400- or 405-nm in a microtiter plate reader, or spectrophotometer using a 100-μl micro quartz cuvet (Sigma), or dilute sample to 1 ml with dilution buffer and using regular cuvet.
11. Fold-increase in CPP32 activity was determined by comparing the results with the level of the uninduced control.

1.2.2.7.2. Quantification of NF-κB/p65 levels

The NF-κB/p65 ActivELISA (Imgenex, San Diego, USA) kit was used for the estimation of NF-κB free p65 in the nuclear lysate of the mice striatum. The free p65 was captured by anti-p65 antibody coated plates and the amount of bound p65 was detected by adding secondary anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody for colorimetric detection in a microplate reader at 405nm. The nuclear levels of p65 may correlate positively with the activation of NF-κB pathway.
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Preparation of lysates from Tissue

i) Cytoplasmic Fraction Collection (Tissue Homogenization based on 1 gram rat tissue)

1. Sciatic nerve/brain was weighed and cut into small pieces using clean razor blade and wash in 5 ml of cold 1X PBS-PMSF. Collect cut pieces in a clean homogenizer.

2. 5 μl of 1M DTT and 500 μl of 10% Detergent Solution to 4.495 ml of ice cold 1X hypotonic buffer/ gram of tissue was added and homogenized. The homogenate was incubated on ice for 15 to 30 min (whole cell lysate).

3. The homogenate was centrifuged for 10 min at 10,000 rpm at 4°C and supernatant (cytoplasmic fraction) was transferred into a 15 ml tube and store at 4°C. The pellet is the nuclear fraction.

ii) Nuclear Fraction Collection

1. The nuclear pellet was resuspended in 500 μl nuclear lysis buffer by pipetting up and down. The suspension was vigorously vortexed and incubated at 4°C, for 30 min on a rocking platform.

2. The suspension was centrifuged at 14,000 rpm for 10 min at 4°C in a microcentrifuge.

3. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C until further use.

4. The protein concentration in the nuclear extract was determined using a detergent compatible assay technique.

Assay Procedure:

1. Coating: 100 μl of capture antibody (IMK-503-01) was diluted in 10 ml of coating buffer (KC-104). 100 μl of diluted antibody was pipetted into each well and incubated the plate overnight (12-24 h) at 4°C. The coated wells were washed twice with 300 μl of 1X wash buffer.
2. **Blocking:** 200 μl of prepared blocking buffer was added to each well to block the remaining reactive surface and incubated for 30 min to 1 h at room temperature.

3. **Prepare p65 Standard Curve:** Quick spin down the Recombinant p65 Standard vial and 420 μl of sterile deionized water was added and vortexed to dissolve. A standard curve was set up in duplicate using the following concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.0 (blank) ng/well.

4. **Samples:** 100 μl of positive and negative controls and 100 μl test samples was pipetted into the appropriate wells. Incubate plate at 4°C overnight or 4 h at room temperature. Samples might be diluted or serially diluted using blocking buffer.

5. **Washing:** The samples and control lysates were removed and washed with wash buffer. The plate was tapped several times upside down to remove residual wash buffer after final wash.

6. **Detecting Antibody:** 100 μl of detecting antibody (IMK-503-02) was diluted in 10 ml of blocking buffer and 100 μl diluted detecting antibody was added to each well. Incubate for 1 h at room temperature.

7. **Washing:** The antibody solution was removed and washed wells 4 times with wash buffer. The plate was tapped several times upside down to remove residual wash buffer after final wash.

8. **Secondary Antibody:** 5 μl of AKP-Conjugated secondary Ab (KC-130) was diluted in 10 ml of blocking buffer. 100 μl of diluted secondary antibody was added to each well and incubate for 1 h at room temperature.

9. The secondary antibody was removed and washed thoroughly with wash buffer letting the solution sit briefly between each wash. During the last wash, pNPP substrate was prepared. The plate was tapped several times upside down to remove residual wash buffer after final wash.

10. **pNPP Substrate:** 10 mg pNPP was dissolved into 10 ml of pNPP substrate buffer and mixed. (Note: Prepare substrate mix just before
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use). 100 µl of pNPP substrate was added to each well and incubated
the plate at room temperature for 30 min. The color development was
read at 405 nm

1.2.7.3. Prostaglandin, PGE$_2$ levels

The quantitative determination of PGE$_2$ levels was done by using the
PGE$_2$ assay kit (R&D Systems, USA). This assay is based on the competitive
binding technique in which PGE$_2$ present in a sample competes with a fixed
amount of horseradish peroxidase (HRP)-labeled PGE$_2$ for sites on a mouse
monoclonal antibody. During the incubation, the mouse monoclonal antibody
becomes bound to the goat anti-mouse antibody coated onto the microplate.
Following a wash to remove excess conjugate and unbound sample, a
substrate solution is added to the wells to determine the bound enzyme activity.
The color development is stopped, and the absorbance is read at 450 nm. The
intensity of the color is inversely proportional to the concentration of PGE$_2$ in
the sample.

Range: 39-2500 pg/ml

Assay procedure:

1. Prepare all reagents, working standards and samples as directed in the
   previous sections.
2. Remove excess microplate strips from the plate frame, return them to
   the foil pouch containing the desiccant pack, reseal.
3. Add 150 µL of Calibrator Diluent RD5-39 to the NSB wells.
4. Add 100 µL of Calibrator Diluent RD5-39 to the zero standard (B0) wells.
5. Add 100 µL of Standard or sample to the remaining wells.
6. Add 50 µL of the Primary Antibody Solution to each well (excluding the
   NSB wells). All wells except the NSB wells will now be blue in color.
7. Add 50 µL of PGE2 Conjugate to each well. All wells except the NSB
   wells will now be violet in color. Cover with the adhesive strip provided.
8. Incubate for 2 hours at room temperature on a horizontal orbital
   microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
9. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

10. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the bench top. Protect from light.

11. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

12. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

1.2.2.7.4. Prostaglandin, PGF_2α levels

The quantitative determination of PGF_2α levels was done by using the PGF_2α EIA kit (Cayman, Ann Arbor, MI, USA). The assay is carried out as per the procedure given by the manufacturer. The absorbance of the color development due to the enzymatic reaction is read at 412 nm. The intensity of the color is inversely proportional to the free amount of PGF_2α present in the sample during incubation.

1.2.2.8. Statistical analysis

Values are expressed as mean ± SEM. One Way Analysis of Variance (ANOVA) followed by One-way analysis of variance (ANOVA) followed by post-hoc Tukey’s test was applied to calculate the statistical significance between various groups. A value of P<0.05 was considered to be statistically significant.

1.2.3. Results

1.2.3.1. Effect of rofecoxib, valdecoxib and NS-398 on locomotor activity in MPTP-treated mice

There was no significant difference between the total locomotor activity of vehicle and MPTP-treated animals on day 1. MPTP (40 mg/kg) challenge
gradually decreased the locomotor activity on day 5 to 7 as compared to vehicle (Fig. 1.2.1.). However, seven days treatment with rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) or NS-398 (5 or 10 mg/kg), significantly improved the locomotor activity as compared to MPTP group (Fig. 1.2.1.). However, rofecoxib (8 mg/kg), valdecoxib (10 mg/kg) or NS-398 (10 mg/kg) per se did not modify the locomotor activity as compared to vehicle group.

1.2.3.2. Effect of rofecoxib, valdecoxib and NS-398 on MPTP-induced on severity of catatonia

When the severity of catatonia was measured on the day 7 (Fig. 1.2.2.), MPTP-treated animals exhibited significant rigidity and postural inability as seen by increased time spent on bar as compared to the vehicle-treated mice. Rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) or NS-398 (5 or 10 mg/kg) treatment for 7 days, significantly reduced the degree of catatonia indicated by decrease in the time spent on bar as compared to MPTP-treated group as depicted in Fig. 1.2.2.

1.2.3.3. Effect of rofecoxib, valdecoxib and NS-398 on oxidative stress in MPTP-treated rats

Table 1.2.1. represents the effect of selective COX-2 inhibitors on oxidative damage induced by MPTP. Systemic administration of MPTP significantly increased the striatal lipid peroxidation, nitrite levels and depleted NPSH levels as compared to vehicle-treated group. However, rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) or NS-398 (5 or 10 mg/kg) treatment for 7 days, significantly attenuated the oxidative damage (as seen by decreased levels of lipid peroxidation, nitrite levels and restored the NPSH levels respectively) as compared to MPTP treated group (Table 1.2.1.). However, rofecoxib (8 mg/kg), valdecoxib (10 mg/kg) or NS-398 (10 mg/kg) per se did not modify the biochemical parameters as compared to vehicle group.

1.2.3.4. Effect of rofecoxib, valdecoxib and NS-398 on MPTP-induced mitochondrial enzyme complex activity and cell viability in mice

Fig. 1.2.3.A. & 1.2.3.B depicts the effect of MPTP challenge on mitochondrial complex-I enzyme activity and redox activity respectively. Mitochondrial complex-I activity and redox activity significantly decreased in the
animals challenged with MPTP as compared to vehicle-treated group. Administration of rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) or NS-398 (5 or 10 mg/kg) daily for 7 days respectively, significantly restored the complex-I activity (Fig. 1.2.3.A) as well as redox activity (Fig. 1.2.3.B) in mice as compared to MPTP treated group.

1.2.3.5. Effect of rofecoxib, valdecoxib and NS-398 on caspase-3 activity in MPTP-treated mice

MPTP administration significantly increased caspase-3 (apoptotic factor) (Fig. 1.2.4.) as compared to vehicle group. Rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) and NS-398 (5 or 10 mg/kg) treatment for 7 days significantly attenuated caspase-3 activity as compared to MPTP group (Fig. 1.2.4.). However, rofecoxib (8 mg/kg), valdecoxib (10 mg/kg) or NS-398 (10 mg/kg) per se did not modulate significantly caspase-3 activity as compared to vehicle group.

1.2.3.6. Effect of rofecoxib, valdecoxib and NS-398 on NF-κB/p65 levels in MPTP-treated mice

Challenge with MPTP significantly increased the expression of proinflammatory marker, NF-κB/p65 (Fig. 1.2.4.) as compared to vehicle group. Treatment with rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) or NS-398 (5 or 10 mg/kg) for 7 days significantly attenuated proinflammatory activity, NF-κB in MPTP treated animals (Fig. 1.2.4.). Rofecoxib (8 mg/kg), valdecoxib (10 mg/kg) or NS-398 (10 mg/kg) per se did not modify the NF-κB/p65 levels as compared to vehicle group.

1.2.3.7. Effect of rofecoxib, valdecoxib and NS-398 on PGE2 and PGF2α levels in MPTP-treated mice

MPTP administration significantly increased prostaglandin PGE2 and PGF2α levels in the striatum as compared to vehicle treatment (Fig. 1.2.5.). Rofecoxib (4 or 8 mg/kg), valdecoxib (5 or 10 mg/kg) or NS-398 (5 or 10 mg/kg) treatment for 7 days, respectively, significantly attenuated the prostaglandin levels as compared to MPTP treated group (Fig. 1.2.5.). However, rofecoxib (8 mg/kg), valdecoxib (10 mg/kg) or NS-398 (10 mg/kg) per se treatment did not produce any significant effect on prostaglandin levels (PGE2 and PGF2α) as compared to vehicle treated group.
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Fig. 1.2.1. Effect of rofecoxib, valdecoxib and NS-398 on locomotor activity in MPTP treated animals. *P<0.05 versus vehicle; †P<0.05 versus MPTP(40); ‡P<0.05 versus ROF(4); §P<0.05 versus VAL(5); ¶P<0.05 versus NS-398(5)
Fig. 1.2.2. Effect of rofecoxib, valdecoxib and NS-398 COX-2-inhibitors on severity of catatonia in MPTP treated mice.

*aP*<0.05 versus vehicle;  
*bP*<0.05 versus MPTP(40);  
*cp*<0.05 versus ROF(4);  
*dP*<0.05 versus VAL(5)
### Chapter 1.2

**Table 1.2.1. Effect of rofecoxib, valdecoxib and NS-398 on oxidative stress parameters in striatum of MPTP-treated mice**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Lipid peroxidation (nmol of MDA/mg protein) % of vehicle</th>
<th>Non-protein thiol levels (NPSH) (µmol/mg protein) % of vehicle</th>
<th>Nitrite levels (µmol of nitrite/mg protein) % of vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>100.0±4.15</td>
<td>100.0±5.45</td>
<td>100.0±4.62</td>
</tr>
<tr>
<td>MPTP</td>
<td>223.4±3.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.6±4.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>251.8±6.44&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>ROF(8) per se</td>
<td>98.5±2.65</td>
<td>102.3±3.57</td>
<td>99.0±7.23</td>
</tr>
<tr>
<td>VAL(10) per se</td>
<td>99.1±4.65</td>
<td>101.3±3.57</td>
<td>99.7±5.13</td>
</tr>
<tr>
<td>NS398(10) per se</td>
<td>98.9±3.26</td>
<td>101.9±5.47</td>
<td>99.2±6.32</td>
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<tr>
<td>ROF(4)</td>
<td>162.4±3.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.0±5.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>177.6±5.91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ROF(8)</td>
<td>123.3±2.60&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>94.8±2.56&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>112.4±6.63&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>64.2±4.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>166.4±6.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VAL(10)</td>
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<td>90.4±3.54&lt;sup&gt;b,d&lt;/sup&gt;</td>
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<td>161.6±6.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NS398(10)</td>
<td>118.7±4.60&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>92.3±5.46&lt;sup&gt;b,e&lt;/sup&gt;</td>
<td>116.8±6.32&lt;sup&gt;b,e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05 versus vehicle; <sup>b</sup>P<0.05 versus MPTP(40); <sup>c</sup>P<0.05 versus ROF(4); <sup>d</sup>P<0.05 versus VAL(5); <sup>e</sup>P<0.05 versus NS-398(5)
Fig. 1.2.3. Effect of rofecoxib, valdecoxib and NS-398 on (A) mitochondrial enzyme complex-I activity and (B) MTT assay in MPTP treated mice. 8P<0.05 versus vehicle; 9P<0.05 versus MPTP(40); 6P<0.05 versus ROF(4); 7P<0.05 versus VAL(5); 6P<0.05 versus NS-398(5)
Fig. 1.2.4. Effect of rofecoxib, valdecoxib and NS-398 on caspase-3 and NF-κB/p65 levels in MPTP treated mice. *P<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus ROF(4); dP<0.05 versus VAL(5); eP<0.05 versus NS-398(5)
Fig. 1.2.5. Effect of rofecoxib, valdecoxib and NS-398 on prostaglandin levels in MPTP treated mice. aP<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus ROF(4); dP<0.05 versus VAL(5); eP<0.05 versus NS-398(5)
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1.2.4. Discussion

The present study highlights the therapeutic potential of selective COX-2 inhibitors (rofecoxib, valdecoxib and NS-398) against MPTP-induced neurotoxicity in mice. Results demonstrate that seven days treatment with rofecoxib, valdecoxib and NS-398 significantly reversed the MPTP-induced behavioral alterations (locomotor activity and catatonia); oxidative damage (attenuated lipid peroxidation and nitrite levels, restored NPSH levels). These drugs also restored the mitochondrial enzyme activity as well as viability along with the inhibition of release of prostaglandins, proinflammatory maker (NF-κB) and apoptotic factor (caspase-3).

Systemic MPTP administration significantly induced motor abnormalities as evidenced by reduction in locomotor activity and postural rigidity (increased time spent on bar) which mimics Parkinson-like symptoms very much similar to PD patients. Intriguingly, administration of rofecoxib, valdecoxib or NS-398 for 7 days significantly restored the motor function and postural instability in MPTP-treated mice. Since dopamine is the main neurotransmitter involved in normal motor function, alteration in the brain dopamine levels can produce modification in the motor function. Earlier reports also confirm that COX-inhibitors significantly attenuated the behavioral changes in MPTP-treated animals (Aguirre et al., 2008; Gupta et al., 2009; Gupta et al., 2010a; 2010b). It is likely that these selective COX-2 inhibitors preserved the dopamine levels by preventing the degeneration of dopaminergic neurons by MPTP or generation of free radicals or dopamine-quinone formation. It further improved motor function and coordination in animals.

When animals are challenged with MPTP, it is actively transported by dopamine transporters to dopaminergic neurons and metabolized to its active form MPP⁺ by enzyme monoamine oxidase-B (MAO-B) (Smeaye and Jackson-Lewis, 2005). MPP⁺ blocks electron transport chain, inhibits mitochondrial function and promotes the generation of free radicals such as superoxide anion, hydroxyl ions etc (Halliwell, 1992). These free radicals act a neuroinflammatory insult and activates glial cells especially microglia. Activated microglia stimulates the production of proinflammatory mediators such as cytokines, transcription factors which further regulates the genes required for
the expression of COX and iNOS (Liu et al., 2006; Watanabe et al., 2008; Yokoyama et al., 2008). All these factors further drive dopaminergic neurotoxicity resulting in a vicious, progressive and self-propelling cycle (Halliwel, 1992; Murray et al., 2003). Treatment with rofecoxib, valdecoxib or NS-398 reduced the oxidative damage as seen by decreased levels of lipid peroxides and nitrite levels as well as restored the antioxidant enzymes towards their control values. Thus, it is plausible that rofecoxib, valdecoxib or NS-398 preserved the normal cellular antioxidant pool as well as inhibited the resultant oxidative alterations induced by MPTP.

In the present study, MPTP challenge significantly reduced the mitochondrial complex-I activity as well as the mitochondrial redox activity as assessed by MTT assay. Treatment with rofecoxib, valdecoxib or NS-398 significantly attenuated the reduction of complex-I activity as well as restored the redox activity. In mitochondria derived from the platelets of PD patients, reduced complex-I activity was observed (Berman and Hastings, 1999; Swerdlow et al., 1996; Vila and Przedborski, 2003; Vila et al., 2008; Wu et al., 2003). These observations confirm and suggest that these drugs viz. rofecoxib, valdecoxib or NS-398, have potential neuroprotection property and this could be the possible mechanism by which these drugs restored the cell viability and significantly inhibited the mitochondrial enzyme dysfunction in our study.

Systemic administration of MPTP has been reported to produce neuroinflammation via activation of glial cells especially microglia induces the expression of proinflammatory transcription factors such as NF-κB (Gosh et al., 2007; Yokoyama et al., 2008). This neuroinflammatory signaling cascade upregulates the expression of COX-2 and iNOS which further increases the level of prostaglandins (PGs) and NO. Similarly, in our study MPTP challenge significantly increased levels of PGE2 and PGF2α. This might be due to the up regulation/induction of COX isoenzymes or modulation of COX pathway, suggesting the role of neuroinflammation. However, chronic treatment with rofecoxib, valdecoxib and NS-398 significantly attenuated the increased levels of these prostaglandins. Therefore, it can be speculated that the potent COX inhibitory activity of these drugs might have decreased PGs levels.
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Role of proinflammatory cytokines in the neuropathological conditions is already established (Monahan et al., 2008; Vroon et al., 2007; Rainsford, 2007). In addition to oxidative and nitrosative stress, neurotoxin such as MPTP induces the expression of transcription factor NF-κB/p65 and apoptotic factor caspase-3, both being the markers of inflammation, apoptosis and cellular death (Tipton and Singer, 1993; Vila and Przedborski, 2003). In the present study, MPTP administration significantly increased the activity of these biomarkers. Significant attenuation in the activity of these biomarkers by valdecoxib and NS-398 has been seen in the present study. It is probable that due to their potent anti-inflammatory and antioxidant like effect, these drugs scavenged reactive oxygen and nitrogen species, which otherwise act as inducers of inflammatory response and prevented further up regulation of inflammatory cellular cascades and cell death. In our earlier study, dual COX/LOX-inhibitor, licofelone significantly attenuated the up regulation of caspase-3 activity and NF-κB/p65 levels (Gupta et al., 2010b)

Our findings also point towards the potent neuromodulatory potential of selective COX-2 inhibitors, rofecoxib, valdecoxib and NS-398 in attenuating the behavioral deficits, biochemical and cellular modifications induced by MPTP in mouse model of PD. The potent anti-inflammatory property of these drugs might be responsible for inhibition of the oxidative stress dependent activation of neuroinflammatory cascade and resultant cellular events eventually leading to dopaminergic neurodegeneration. The neuroprotective potential of these drugs is supplemented by the fact that these drugs ameliorated the nitrosative stress, mitochondrial dysfunction and release of proinflammatory biomarkers induced MPTP.

In summary, the present finding implicates that selective inhibition of COX-2 directly or indirectly inhibits the activation microglial-derived proinflammatory cascade and exerts their neuroprotective action. It also indicates the therapeutic potential of COX-2 inhibitors in the management of PD.
CHAPTER 2.1

To investigate the possible neuroprotective effect of caffeic acid (a 5-LOX-inhibitor) and its combination with rofecoxib (a selective COX-2 inhibitor) against MPTP-induced Parkinson-like symptoms in mice

2.1.1. Introduction

Neuroinflammation and oxidative stress theories have been proposed as being a key contributing factor in the genesis of neurodegeneration as well as in age-related neurological disorders (Choi et al., 2010; Farooqui and Farooqui, 2011; Mosley et al., 2006). The arachidonic acid substrate for COX is also the substrate for lipooxygenase (LOX). In the periphery, 5-lipoxygenase (5-LOX) pathway generates leukotrienes which are known to be inflammatory mediators (Crooks and Stockley, 1998; Samuelsson, 1983). The exact role of the 5-LOX enzyme pathway and its interaction with other arachidonic acid mediated enzymes in brain is not fully understood so far. Since 5-LOX and COX are companion pathways using arachidonic acid as their common substrate which indicates that inhibition of 5-LOX could be a promising new way to reduce the production of pro-inflammatory leukotrienes (LT).

Furthermore, NSAIDs, by inhibiting COX isoenzymes, could shunt arachidonic acid generated by lipase action on cell membranes towards leukotriene synthesis via LOX pathways, thus producing a rebound inflammatory effect. To date, much clinical attention has been focused on the action of several NSAIDs and their interaction with COX pathway, but entire cellular interactive mechanism are still poorly understood. 5-LOX products are known to play an important role in allergic inflammation (Bell and Harris, 1999; Peters-Golden and Brock, 2001), and has been suggested in ischemia (Arai et al., 2001; Ohtsuki et al., 1995), stroke (Munsiff et al., 1992), traumatic brain injury (Bazan et al., 1995) and AD pathologies (Manev, 2000) etc. Earlier, NSAIDs have been demonstrated to have beneficial effects in experimental models of PD which correlates with epidemiological evidences that individuals consuming NSAIDs are relatively spared from PD (Etminan et al., 2008; Gupta et al., 2011; Hernan et al., 2006).
Caffeic acid (3,4-dihydroxy cinnamic acid) (CA) is the major subgroup of phenolic compounds with various pharmacological properties (Kumar et al., 2010; Maurya and Devasagyam, 2010; Sato et al., 2011). Caffeic acid is naturally found in many agricultural products, such as fruit, vegetables, wine, olive oil, and coffee, and therefore, significantly present in human diet (Maurya and Devasagyam, 2010). Caffeic acid has been reported to have a wide variety of biological activities, including antioxidants (Maurya and Devasagayam, 2010), antithrombosis (Johnson et al., 2004), antihypertensive (Hudson et al., 2000) and anti-tumor (Morton et al., 2000) properties. Caffeic acid has been shown to produce neuroprotective effects against ischemic brain injuries and β-amyloid neurotoxicity (Kang et al., 2009; Kart et al., 2009; Sul et al., 2009).

The recent techniques and the better understanding in the complexity of etiology and pathogenesis of PD fascinated researchers to identify the novel molecular targets for effective management of PD. Consequently, the therapeutic interventions should target to halt the underlying mechanisms of the degenerative processes rather than attenuating the symptoms of the disease. With this background, the present study was designed to evaluate the neuroprotective effect of caffeic acid against MPTP-induced neurotoxicity in mice. Furthermore, the study also aimed to investigate the modulatory role of dual inhibition of COX and LOX pathway in attenuating the progression of PD. For this, a combination of caffeic acid and rofecoxib was investigated against MPTP-induced neurotoxicity.

2.12. Materials and Methods

2.1.2.1. Animals

Refer chapter 1.2. (Section 1.2.2.1)

2.1.2.2. Drugs and treatment schedule

The following drugs and chemicals were used in the present study-- MPTP (Sigma Chemicals, St. Louis, MO, USA), rofecoxib (Panacea Biotec Ltd., New Delhi, India), caffeic acid (CA) and sodium carboxy methyl cellulose (Na-CMC) (Himedia, Mumbai). MPTP was dissolved in distilled water and administered by i.p. injection. Caffeic acid and rofecoxib were suspended in Na-CMC (0.25 % w/v) and administered by p.o. route in a constant volume of 1 ml/100 gm.
The present study was conducted in different phases: Eight groups were employed in the present study, consists of 15 animals in each.

**Group-I** – Vehicle treated group

**Group-II** – MPTP-treated group (40 mg/kg)

**Group-III** – CA (10 mg/kg) per se

**Group-IV** – CA (2.5 mg/kg) + MPTP (40 mg/kg)

**Group-V** – CA (5 mg/kg) + MPTP (40 mg/kg)

**Group-VI** – CA (10 mg/kg) + MPTP (40 mg/kg)

**Group-VII** – ROF (4 mg/kg) + MPTP (40 mg/kg)

**Group-VIII** – CA (5 mg/kg) + ROF (4 mg/kg) + MPTP (40 mg/kg)

**Study Protocol**

MPTP was administered on first day in all the groups except vehicle group. Caffeic acid or rofecoxib treatment was started 1 hr before first MPTP injection, followed by three more doses (10 mg/kg, i.p.) of MPTP injections as per the scheme mentioned below. Separate groups were used for biochemical and mitochondrial studies.
2.1.2.3. Behavioral assessments

2.1.2.3.1. Assessment of gross behavioral activity (locomotor activity)

Refer Chapter-1.2 (Section 1.2.2.3.1.)

2.1.2.3.2. Assessment of catatonia

Refer Chapter-1.2 (Section 1.2.2.3.2)

2.1.2.4. Dissection and homogenization

Refer Chapter-1.2 (Section 1.2.2.4.)

2.1.2.5. Measurement of oxidative stress parameters

2.1.2.5.1. Lipid peroxidation

Refer Chapter 1.1 (Section 1.1.2.6.1.)

2.1.2.5.2. Non-protein thiols

Refer Chapter 1.1 (Section 1.1.2.6.2)

2.1.2.5.3. Estimation of nitrite

Refer Chapter 1.1 (Section 1.1.2.6.4.)
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2.1.2.5.4. Protein estimation
Refer Chapter 1.1 (Section 1.1.2.6.5.)

2.1.2.6. Mitochondrial complex estimation

2.1.2.6.1. Isolation of rat brain mitochondria
Refer Chapter 1.2 (Section 1.2.2.6.1.)

2.1.2.6.2. Complex-I (NADH dehydrogenase activity)
Refer Chapter 1.2 (Section 1.2.2.6.2.)

2.1.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay
Refer Chapter 1.2 (Section 1.2.2.6.3.)

2.1.2.7. Quantification of apoptosis and inflammatory markers

2.1.2.7.1. Caspase-3 colorimetric assay
Refer Chapter 1.2 (Section 1.2.2.7.1.)

2.1.2.7.2. NFκβ/p65 activity
Refer Chapter 1.2 (Section 1.2.2.7.2.)

2.1.2.8. Statistical analysis
Refer Chapter 1.2 (Section 1.2.2.8.)

2.1.3. Results

2.1.3.1. Effect of caffeic acid or its combination with rofecoxib on total locomotor activity in MPTP-treated animals

Locomotor activities of all the animals were stable on day 1 before MPTP challenge. MPTP (40 mg/kg, i.p.) treatment significantly reduced locomotor activity when observed on day 3, 5 and 7 as compared to vehicle group (Fig. 2.1.1.). Caffeic acid (2.5, 5 and 10 mg/kg) treatment for 7 days significantly improved the locomotor activity in animals as compared to MPTP group (Fig. 2.1.1.). Furthermore, a combination of caffeic acid (5 mg/kg) and rofecoxib (4 mg/kg) produced significant improvement in the locomotor activity as compared
to their individual effects. However, caffeic acid (10 mg/kg) per se treatment did not modify the locomotor activity as compared to vehicle.

2.1.3.2. Effect of caffeic acid or its combination with rofecoxib on MPTP-induced catatonia

MPTP treatment significantly induced postural inability and increased time spent on bar as compared to vehicle treated group on day 7 (Fig. 2.1.2.). Caffeic acid (2.5, 5 and 10 mg/kg) treatment for 7 days significantly attenuated the severity of catatonia as indicated by decrease in time spent on bar as compared to MPTP-treated group (Fig. 2.1.2.). Furthermore, a combination of caffeic acid (5 mg/kg) with rofecoxib (4 mg/kg) produced significant protective effect by reducing the severity of catatonia as compared to their individual effects. However, caffeic acid (10 mg/kg) per se treatment did not modify the postural stability as compared to vehicle.

2.1.3.3. Effect of caffeic acid or its combination with rofecoxib on MPTP-induced oxidative stress

Systemic administration of MPTP significantly increased the lipid peroxidation, nitrite levels and depleted NPSH levels in the striatum as compared to vehicle group (Table 2.1.1.). Treatment with caffeic acid (2.5, 5 and 10 mg/kg) for 7 days, significantly attenuated the oxidative damage (as evidenced by decrease level of lipid peroxidation, nitrite levels and restored NPSH levels) as compared to MPTP-treated group (Table 2.1.1.). Furthermore, a combination of caffeic acid (5 mg/kg) with rofecoxib (4 mg/kg) significantly attenuated the oxidative stress which was significant as compared to their individual effects. However, caffeic acid (10 mg/kg) per se treatment did not influence oxidative stress markers as compared to vehicle group.

2.1.3.4. Effect of caffeic acid or its combination with rofecoxib on MPTP-induced changes in mitochondrial enzyme complex-I activity and MTT assay in mice

MPTP challenge significantly impaired mitochondrial enzyme complex-I activity and MTT assay (Fig. 2.1.3.) as compared to vehicle treated group. Seven days caffeic acid (5 and 10 mg/kg) treatment significantly restored the mitochondrial complex-I activity and redox capacity (MTT assay) as compared to
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MPTP group (Fig. 2.1.3.). Additionally, a combination of caffeic acid (5 mg/kg) with rofecoxib (4 mg/kg) significantly restored the mitochondrial enzyme function as compared to their individual drug effects. However, caffeic acid (10 mg/kg) per se treatment did not influence mitochondrial enzyme complex-I activity and MTT assay as compared to vehicle group.

2.1.3.5. Effect of caffeic acid or its combination with rofecoxib on caspase-3 activity in MPTP-treated mice

MPTP treatment significantly increased caspase-3 activity in the striatum as compared to vehicle treated group (Fig. 2.1.4.). Caffeic acid (2.5, 5 and 10 mg/kg) treatment for 7 days significantly attenuated caspase-3 activity and inhibited the induction of apoptotic pathway as compared to MPTP-treated group (Fig. 2.1.4.). In addition, a combination of caffeic acid (5 mg/kg) with rofecoxib (4 mg/kg) significantly attenuated the increase in caspase-3 activity as compared to their individual effects. Caffeic acid (10 mg/kg) per se treatment did not influence the caspase-3 activity as compared to vehicle group.

2.1.3.6. Effect of caffeic acid or its combination with rofecoxib on NF-κB/p65 activity in MPTP-treated mice

MPTP administration significantly increased NF-κB/p65 levels in the striatum as compared to vehicle treatment (Fig. 2.1.4.). Caffeic acid (2.5, 5 and 10 mg/kg) treatment for 7 days, significantly inhibited the transcription factor, NF-κB/p65 as compared to MPTP treated group (Fig. 2.1.4.). Furthermore, a combination of caffeic acid (5 mg/kg) and rofecoxib (4 mg/kg) significantly decreased the NFκB/p65 levels as compared to their individual drug effects. However, caffeic acid (10 mg/kg) per se treatment did not modulate the NF-κB/p65 activity as compared to vehicle group.
Fig. 2.1.1. Effect of caffeic acid or its combination with rofecoxib on locomotor activity in MPTP treated animals. *P<0.05 versus vehicle; †P<0.05 versus MPTP(40); ‡P<0.05 versus CA(2.5); §P<0.05 versus CA(5); ¶P<0.05 versus ROF(4)
Fig. 2.1.2. Effect of caffeic acid or its combination with rofecoxib on severity of catatonia in MPTP treated animals. \(^aP<0.05\) versus vehicle; \(^bP<0.05\) versus MPTP (40); \(^cP<0.05\) versus CA(2.5); \(^dP<0.05\) versus CA(5); \(^eP<0.05\) versus ROF(4)
Fig. 2.1.3. Effect of caffeic acid or its combination with rofecoxib on mitochondrial enzyme complex-I activity and MTT assay in MPTP treated animals. aP<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus CA(2.5); dP<0.05 versus CA(5); eP<0.05 versus ROF(4)
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Effect of caffeic acid or its combination with rofecoxib on caspase-3 activity and NF-κB/p65 levels in MPTP treated animals. aP<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus CA(2.5); dP<0.05 versus CA(5); eP<0.05 versus ROF(4)
Table 2.1.1. Effect of caffeic acid or its combination with rofecoxib on MPTP-induced biochemical changes in the mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg protein (% of vehicle))</th>
<th>NPSH levels (µmol of NPSH/mg protein (% of vehicle))</th>
<th>Nitrite level (µ mol/mg protein (% of vehicle))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100.0±3.18</td>
<td>100.0±4.92</td>
<td>100.0±6.36</td>
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<td>MPTP(40)</td>
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<td>38.2±4.69a</td>
<td>195.0±3.49a</td>
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<tr>
<td>CA(10) per se</td>
<td>98.3±2.43</td>
<td>100.9±3.42</td>
<td>100.3±5.72</td>
</tr>
<tr>
<td>CA(2.5)</td>
<td>201.4±2.56b</td>
<td>45.3±4.33</td>
<td>181.4±7.67</td>
</tr>
<tr>
<td>CA(5)</td>
<td>167.8±3.44b,c</td>
<td>76.8±3.49b,c</td>
<td>146.5±4.68b,c</td>
</tr>
<tr>
<td>CA(10)</td>
<td>122±4.31b,c,d</td>
<td>89.3±4.12b,c,d</td>
<td>113.9±5.89b,c,d</td>
</tr>
<tr>
<td>ROF(4)</td>
<td>162.4±3.06b</td>
<td>75.0±5.54b</td>
<td>177.6±5.91b</td>
</tr>
<tr>
<td>CA(5)+ROF(4)</td>
<td>131.4±3.10b,d,e</td>
<td>90.2±3.28b,d,e</td>
<td>129.1±5.31b,d,e</td>
</tr>
</tbody>
</table>

\(^a\)P<0.05 versus vehicle; \(^b\)P<0.05 versus MPTP(40); \(^c\)P<0.05 versus CA(2.5); \(^d\)P<0.05 versus CA(5); \(^e\)P<0.05 versus ROF(4)

2.1.4 Discussion

The present study highlights that caffeic acid (a 5-LOX inhibitor) exerted a neuroprotective effect against MPTP-induced behavioral changes, biochemical abnormalities, mitochondrial dysfunction and up regulation of inflammatory mediators. All the abnormalities were attenuated by 7 days treatment with caffeic acid (2.5, 5 and 10 mg/kg) in MPTP challenged animals. Furthermore, a combination of caffeic acid (5 mg/kg) with a selective COX-2 inhibitor, rofecoxib (4 mg/kg) produced significant improvement in the locomotor activity as compared to individual drug treatments. Thus, it is plausible that caffeic acid or its combination with rofecoxib by virtue of their modulatory effect on COX/LOX pathway exhibited their protective effects against MPTP-induced Parkinson-like symptoms in mice.
Many studies have indicated the potential role of metabolites of arachidonic acid pathway (COX and LOX) in the pathophysiology of various neurodegenerative disorders. Inhibitors of COX-isoenzyme, especially COX-2 has demonstrated the neuroprotective effect in experimental models of PD (Aguirre et al. 2008; Gupta et al. 2009; Gupta et al. 2011). Since, COX and LOX pathway generates metabolites of arachidonic acid, so inhibition of the COX pathways or PGs, may upregulate the formation of LTs through LOX pathway (Martel-Pelletier et al. 2003; Rainsford 2007). Therefore, inhibition of the LOX pathway or antagonizing the effects of LT receptor could arrest the neuroinflammatory cascade (Bray et al. 1981; Lewis et al. 1990). Based on these evidences and reported data, the present study was designed to evaluate the effect of caffeic acid (a 5-LOX inhibitor) against MPTP–induced neurotoxicity in mice. Moreover, the study also focused on the modulatory effect of a combination of 5-LOX inhibitor (caffeic acid) and selective COX-2 inhibitor (rofecoxib) in attenuating MPTP-induced neurotoxicity in mice.

When animals were challenged with MPTP, a significant decrease in locomotor activity and catatonia were observed. This is in agreement with earlier studies where MPTP by virtue of its neurotoxic property produced degeneration of the dopaminergic neurons in the nigrostriatal pathway (Smyene and Jackson-Leiws, 1995). Caffeic acid treatment for 7 days significantly attenuated the impaired motor function which suggests that these drugs preserved the dopaminergic neurons from degeneration. The resultant increase in the oxidative stress following MPTP administration increased MDA and nitrite levels, depleted the NPSH levels. Glutathione is one of the potent antioxidant enzymes playing an important role in the neurobiology of PD (Spina and Cohen 1989). Therefore, restoration of the antioxidant enzymes represents a hallmark for preventing the cellular disturbances and the cellular death. In our study, caffeic acid treatment significantly restored the levels of NPSH in MPTP treated mice. Moreover, the combination of caffeic acid and rofecoxib potentiated their neuroprotective effect and significantly attenuated the behavioral and biochemical changes against MPTP. These observations indicate that caffeic acid and rofecoxib exhibited antioxidant-like property and up-regulated the cytoprotective enzyme within the dopaminergic neurons.

The present study besides demonstrating the antioxidant properties of caffeic acid also highlights the involvement of altered mitochondrial enzyme.
complex function and resultant generation of ROS following MPTP administration. The mitochondrial complex-I activity and redox capacity was significantly decreased in the present study which was attenuated by caffeic acid in a significant manner. The protective effect of caffeic acid can be justified by its action on LOX-inhibition and further formation of LTs. Therefore, either inhibiting the formation of LT could leads to the inhibition of the loop feedback mechanism, responsible for overproduction of ROS involved in neuronal cell death. Thus it is plausible that both caffeic acid attenuated the free radicals generation and exerted their neuroprotective effects. In addition, caffeic acid and rofecoxib combination significantly attenuated the mitochondrial dysfunction. In earlier studies, caffeic acid has been reported to produce protective effect against neuronal disorders (Altug et al., 2008; Li et al., 2008; Yang et al., 2008).

MPTP also upregulates the expression of various proinflammatory and apoptotic mediators such as TNF-α, NF-κB, caspases etc either due to the activation of glial cells especially microglia or due to the mitochondrial dysfunction and resultant oxidative stress dependent inflammatory insult (Keane et al., 2011). Similarly, in the present study, elevated levels of NF-κB/p65 and caspase-3 has been observed following MPTP challenge. These proinflammatory markers were significantly decreased by caffeic acid treatment or its combination with rofecoxib for seven days. Fascinatingly, these results present an interesting neuropharmacological profile of caffeic acid and might be helpful in the prevention of neurotoxic events due to excessive inflammatory reactions in Parkinsonian brain. Literature reports have confirmed the anti-inflammatory property of caffeic acid in various disease conditions (Chao et al., 2009; Kalonia et al., 2009). The protective effects of caffeic acid or its combination with rofecoxib on MPTP-induced neurotoxicity can be attributed to their ability to (i) inhibit LT formation (ii) inhibit COX-2 enzyme (iii) inhibit the expression of pro-inflammatory mediators (iv) balance oxidant-antioxidant status and (v) inhibit apoptosis.

In summary, our data suggests that dual inhibition of COX or LOX pathway significantly attenuated the dopaminergic neurotoxicity mediated by MPTP. The use of modulators of COX/LOX pathway in combination with regular drug therapy can be further explored so that these drugs can be available for clinical intervention for PD patients.
CHAPTER-2.2

To elucidate the modulatory effect of licofelone, a dual COX/LOX-inhibitor in MPTP model of PD

2.2.1. Introduction

Neuroinflammation causes an increased activation of microglia and expression of proinflammatory cascades including COX, NF-κB, caspase and iNOS (Knott et al. 2000). It is now well known that COX and LOX isoenzymes catalyze the biosynthesis of arachidonic acid derived lipid mediators involved in the inflammatory process (Martel-Pelletier et al. 2003). The role of COX isoenzymes especially COX-2 has been suggested in the pathophysiology of PD (Teismann et al. 2003). The critical role of neuroinflammation and oxidative stress has been speculated by various researchers in the pathophysiology of PD where induction of glial cells, especially microglia induces the expression of COX-2 in the brain (Kim and Joh 2006; Mosley et al. 2006). Studies also highlighted the beneficial effects of NSAIDs in experimental model of PD (Aguirre et al. 2008; Gupta et al. 2009; Gupta et al. 2011). However, the exact role of dual inhibition of COX/LOX enzyme has been poorly understood so far (Li et al. 2008). Therefore, the present study has been designed to explore the possible role of COX/LOX enzymes underlying the disease mechanisms in PD.

Licofelone ([2,2-dimethyl-6-(4-chloropheny-7-phenyl-2,3-dihydro-1H-pyrrozoline-5-yl] acetic acid), a dual inhibitor of COX/LOX pathway, belongs to the newer generation of anti-inflammatory class of drugs (Laufer et al. 1994; Martel-Pelletier et al. 2003; Tries et al. 2002). Experimental studies from our laboratory also demonstrated the effectiveness of licofelone in various painful and inflammatory conditions (Singh et al. 2005; Singh et al. 2006). Besides, its gastroprotective, cardioprotective, analgesic and antipyretic effects have also been documented in several experimental models (Tries et al. 2002; Tries and Laufer 2001). Based on advancement in the understanding of both COX and LOX pathways, it is now speculated that PGs and LTs together may play an important role in the progression of inflammatory diseases (Fiorucci et al. 2001) and therefore can be exploited to develop suitable drug candidate for neurological
diseases including PD. Since selective COX-2 inhibitors have their own limitations; simultaneous inhibition of both COX/LOX pathways may represent a new class of anti-inflammatory drugs with lesser side effects in neurodegenerative conditions.

Based on the above observations and lacunae of experimental evidence, the present study has been designed to investigate the potential effect of licofelone, a dual COX/LOX-inhibitor against MPTP-induced behavioral, biochemical and cellular alterations in mice.

2.2.2. Materials and Methods

2.2.2.1. Animals Refer chapter 1.2. (Section 1.2.2.1)

2.2.2.2. Drugs and treatment schedule

The following drugs and chemicals were used in the present study– MPTP (Sigma Chemicals, St. Louis, MO, USA), licofelone, LCF (Panacea Biotec Ltd., New Delhi, India) and sodium carboxy methyl cellulose (Na-CMC) (Himedia, Mumbai). MPTP was dissolved in distilled water and administered intraperitoneally. Licofelone was suspended in Na-CMC (0.25 % w/v) and administered by per oral in a constant volume of 1 ml/100 gm.

Six groups were employed in the present study, consists of 20 animals in each. Study was conducted in different phases.

Group-I – Vehicle group (0.25 % CMC)

Group-II – MPTP group (40 mg/kg)

Group-III – LCF (10 mg/kg) per se

Group-IV – LCF (2.5 mg/kg) + MPTP (40 mg/kg)

Group-V – LCF (5 mg/kg) + MPTP (40 mg/kg)

Group-VI – LCF (10 mg/kg) + MPTP (40 mg/kg)
MPTP was administered on first day in all the groups except vehicle treated group. Licofelone treatment was started 1 hr before first MPTP injection, followed by three more doses (10 mg/kg, i.p.) of MPTP injections as per the scheme mentioned above. Separate groups were used for biochemical and mitochondrial studies.

2.2.2.3. Behavioral assessments

2.2.2.3.1. Assessment of gross behavioral activity (locomotor activity)

Refer Chapter-1.2 (Section 1.2.2.3.1.)
2.2.2.3.2. Assessment of catatonia

Refer Chapter-1.2 (Section 1.2.2.3.2)

2.2.2.4. Dissection and homogenization

Refer Chapter-1.2 (Section 1.2.2.4.)

2.2.2.5. Measurement of oxidative stress parameters

2.2.2.5.1. Lipid peroxidation

Refer Chapter 1.1 (Section 1.1.2.6.1.)

2.2.2.5.2. Non-protein thiols

Refer Chapter 1.1 (Section 1.1.2.6.2)

2.2.2.5.3. Superoxide anion levels

The superoxide anion levels were measured by method devised by Babior et al. (1973). The brain samples were homogenized in Tris KCl buffer to produce 10% homogenate. From each homogenized sample, 25 µl of homogenate was taken and mixed with 0.05 mM cytochrome c solution in Tris KCl buffer to make up the volume to 2 ml. This mixture was then incubated for 15 min at 37 °C. The reaction was terminated by placing the mixture in ice. It was then centrifuged at 700 x g for 10 min. Absorbance of each supernatant was measured at 550 nm with Shimadzu UV spectrophotometer. Results were calculated as nmol of cytochrome c reduced/min using molar extinction coefficient of chromophore (2.1×10⁴ M⁻¹ cm⁻¹) and expressed as percentage of vehicle group taking vehicle values as 100%.

2.2.2.5.4. Estimation of nitrite

Refer Chapter 1.1 (Section 1.1.2.6.4.)

2.2.2.5.5. NPSH estimation

Refer Chapter 1.1 (Section 1.1.2.6.5.)

2.2.2.6. Mitochondrial complex estimation

2.2.2.6.1. Isolation of rat brain mitochondria

Refer Chapter 1.2 (Section 1.2.2.6.1.)
Chapter 2.2

2.2.2.6.2. Complex-I (NADH dehydrogenase activity)

Refer Chapter 1.2 (Section 1.2.2.6.2.)

2.2.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay

Refer Chapter 1.2 (Section 1.2.2.6.3.)

2.2.2.7. Quantification of apoptosis and inflammatory markers

2.2.2.7.1. Caspase-3 colorimetric assay

Refer Chapter 1.2 (Section 1.2.2.7.1.)

2.2.2.7.2. NFκβ/p65 activity

Refer Chapter 1.2 (Section 1.2.2.7.2.)

2.2.2.7.3. PGE2 levels

Refer Chapter 1.2 (Section 1.2.2.7.3.)

2.2.2.7.4. PGF2α levels

Refer Chapter 1.2 (Section 1.2.2.7.4.)

2.2.2.8. TTC (2,3,5-Triphenyltetrazolium Chloride) histo-chemical staining

Immediately following sacrifice, 2 mm thick sections of brain were cut and incubated in dark for 5 min at 37 °C in 2% solution of TTC in phosphate saline buffer (pH 7.0) (Bederson et al., 1986). After staining, the brain slices were fixed in 4% buffered formaldehyde solution. Then the slices were photographed and images were used for assessing the damage.

2.2.2.9. Statistical analysis Refer Chapter 1.2 (Section 1.2.2.8.)

2.2.3. Results

2.2.3.1. Effect of licofelone on MPTP-induced on total locomotor activity

Locomotor activities of all the treatment groups were stable on day 1 before MPTP challenge. MPTP (40 mg/kg, i.p.) treatment significantly reduced
locomotor activity on day 3, 5 and 7 as compared to vehicle treated group (Fig. 2.2.1.). Treatment with licofelone (2.5, 5 or 10 mg/kg) for seven days significantly improved locomotor activity as compared to MPTP group (Fig. 2.2.1.). However, licofelone (10 mg/kg) per se treatment did not modify the locomotor activity as compared to vehicle.

Fig. 2.2.1. Effect of licofelone on locomotor activity in MPTP treated animals. aP<0.05 versus vehicle; bP<0.05 versus MPTP (40); cP<0.05 versus LCF(2.5); dP<0.05 versus LCF(5)

2.2.3.2. Effect of licofelone on MPTP-induced on catatonia

MPTP treatment significantly caused rigidity or postural inability and increased time spent on bar on day 7 as compared to vehicle treated group (Fig. 2.2.2.). Seven days licofelone (2.5, 5 and 10 mg/kg) treatment significantly attenuated the severity of catatonia as indicated by decrease in time spent on bar as compared to MPTP-treated group (Fig. 2.2.2.). However, licofelone (10 mg/kg) per se treatment did not modify the catatonia as compared to vehicle.
2.2.3.3. Effect of licofelone on MPTP-induced oxidative stress

Systemic administration of MPTP significantly increased the lipid peroxidation, superoxide anion, nitrite levels and depleted NPSH levels in the striatum region as compared to vehicle group (Table 2.2.1.). Treatment with licofelone (2.5, 5 and 10 mg/kg) for 7 days, significantly attenuated oxidative damage (as evidenced by decrease level of lipid peroxidation, superoxide anion, nitrite levels and restoration of the NPSH levels) as compared to MPTP-treated group (Table 2.2.1.). However, licofelone (10 mg/kg) per se treatment did not alter the oxidative stress parameter as compared to vehicle.
Table 2.2.1. Effect of licofelone on MPTP-induced biochemical changes in the mice striatum

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>MDA (nmol/mg protein (% of vehicle))</th>
<th>NPSH levels (% of vehicle)</th>
<th>Superoxide anion levels (nmoles of cytochrome c reduced/min (% of vehicle))</th>
<th>Nitrite level (μmol/mg protein (% of vehicle))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100.0±2.25</td>
<td>100.0±6.27</td>
<td>100.0±5.78</td>
<td>100.0±3.45</td>
</tr>
<tr>
<td>MPTP (40)</td>
<td>208.5±1.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.0±5.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>175.9±4.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>268.3±2.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCF per se (10)</td>
<td>98.0±1.71</td>
<td>100.1±7.33</td>
<td>100.8±5.23</td>
<td>92.2±4.13</td>
</tr>
<tr>
<td>LCF (2.5)</td>
<td>186.7±1.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.4±5.81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>157.4±3.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>179.0±4.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCF (5)</td>
<td>169.9±4.31&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>69.0±7.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>138.8±2.85&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>151.7±3.90&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCF (10)</td>
<td>115.2±4.07&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>93.8±3.71&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>119.1±3.92&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>113.3±5.04&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05 versus vehicle; <sup>b</sup>P<0.05 versus MPTP (40); <sup>c</sup>P<0.05 versus LCF (2.5); <sup>d</sup>P<0.05 versus LCF (5) 05 versus

2.2.3.4. Effect of licofelone on MPTP-induced mitochondrial enzyme complex-I activity and MTT assay in mice

MPTP challenge significantly impaired mitochondrial enzyme complex-I activity and redox capacity (MTT assay) (Fig. 2.2.3.) as compared to vehicle treated group. Seven days licofelone (2.5, 5 and 10 mg/kg) treatment significantly restored the mitochondrial complex-I activity and redox capacity as compared to MPTP group (Fig. 2.2.3.). However, licofelone (10 mg/kg) per se treatment did not influence mitochondrial enzyme function compared to vehicle group.
Chapter 2.2

![Bar chart]

Fig. 2.2.3. Effect of licofelone on mitochondrial enzyme complex-I activity and MTT assay in MPTP treated animals. 

\(^a\)P<0.05 versus vehicle; \(^b\)P<0.05 versus MPTP(40); \(^c\)P<0.05 versus LCF(2.5); \(^d\)P<0.05 versus LCF(5)
Chapter 2.2

2.2.3.5. Effect of licofelone on caspase-3 activity in MPTP-treated mice

MPTP treatment significantly increased caspase-3 activity in the striatum as compared to vehicle treated group (Fig. 2.2.4.). Seven days licofelone (2.5, 5 and 10 mg/kg) treatment significantly attenuated caspase-3 activity (inhibited the induction of apoptotic pathway) as compared to MPTP-treated group (Fig. 2.2.4.). Licofelone (10 mg/kg) per se treatment did not influence the caspase-3 activity as compared to vehicle group.

2.2.3.6. Effect of licofelone on NF-κβ/p65 activity in MPTP-treated mice

MPTP administration significantly increased NF-κβ/p65 levels in the striatum as compared to vehicle treatment (Fig. 2.2.4.). Licofelone (2.5, 5 and 10 mg/kg) treatment for 7 days, significantly inhibited NF-κβ/p65 (induction of proinflammatory marker) as compared to MPTP treated group in a dose dependent manner (Fig. 2.2.4.). However, licofelone (10 mg/kg) per se treatment did not produce the NF-κβ/p65 activity as compared to vehicle group.

2.2.3.7. Effect of licofelone on PGE2 and PGF2α levels in MPTP-treated mice

MPTP challenge significantly increased PGE2 and PGF2α levels in the striatal homogenates as compared to vehicle treatment (Fig. 2.2.5.). Daily treatment with licofelone (2.5, 5 and 10 mg/kg) for 7 days, significantly inhibited PGE2 and PGF2α levels as compared to MPTP treated group (Fig. 2.2.5.). Licofelone (10 mg/kg) per se treatment did not modulate the prostaglandin levels as compared to vehicle group.

2.2.3.8. Effect of licofelone on striatal degeneration induced by MPTP in mice

Fig. 2.2.6A-D represents the histo-chemical effect of licofelone in MPTP-induced neurodegeneration. MPTP induced significant striatal neurodegeneration as compared to control (vehicle) as assessed by TTC staining where prominent white portion is the marker of neuronal death (Fig. 2.2.6.B). Seven days licofelone treatment (5 and 10 mg/kg) significantly attenuated neuronal death as assessed by striatal lesion volume as compared to MPTP group (Fig. 2.2.6.C and 2.2.6.D).
Fig. 2.2.4. Effect of licofelone on caspase-3 activity and NFκβ/p65 levels in MPTP treated animals. *P<0.05 versus vehicle; 
**P<0.05 versus MPTP(40); ***P<0.05 versus LCF(2.5); ****P<0.05 versus LCF(5)
Fig. 2.2.5. Effect of licofelone on PGE$_2$ and PGF$_{2\alpha}$ levels in MPTP treated animals. $^{a}$P<0.05 versus vehicle; $^{b}$P<0.05 versus MPTP(40); $^{c}$P<0.05 versus LCF(2.5); $^{d}$P<0.05 versus LCF(5)
Fig. 2.2.6. Effect of licofelone treatment on striatal lesions in MPTP-treated brain slices of mice as seen by TTC staining. A. Control section; B. MPTP(40) section; C. LCF(5) section; D. LCF(10) section and (E) Striatal lesion volume.
2.2.4 Discussion

Recent evidences suggest the potential role of COX-inhibitors especially COX-2-inhibitors in the pathophysiology of PD (Aguirre et al. 2008; Gupta et al. 2009; Gupta et al. 2011). However, the inhibition of the COX pathway, while inhibiting the synthesis of prostaglandins, may lead an increased formation of leukotrienes through LOX pathway (Martel-Pelletier et al. 2003; Rainsford 2007). Thus formed leukotrienes may also have a role in the proinflammatory process and their inhibition could arrest the neuroinflammatory cascade (Bray et al. 1981; Lewis et al. 1990). Based on these evidences and reported data, dual inhibition of both COX and 5-LOX pathway may exhibit superior pharmacological profile with lesser adverse effects (Kulkarni and Singh 2007; Singh et al. 2006; Tries et al. 2002). In the present study, we evaluated the effect of licofelone against MPTP-induced PD-like symptoms in mice.

In the present study, MPTP administration produced significant motor and behavior abnormalities along with significant alterations in the normal striatal biochemical, mitochondrial and cellular parameters. These findings are in agreement with earlier reports in which motor dysfunction and biochemical abnormalities were observed following MPTP administration (Gupta et al. 2009; Gupta et al 2011). Licofelone treatment significantly attenuated these behavioral, biochemical and cellular abnormalities induced by MPTP. Thus, it is plausible that licofelone administration by virtue of its dual COX/LOX-inhibitor property exhibited the protective effects in MPTP-induced neuronal toxicity in mice.

When the effect of MPTP challenge was studied in mice, a significantly decrease in locomotor activity was seen. The severity of catatonia was also seen in MPTP treated mice. MPTP administration is known to produce degeneration of the dopaminergic neurons in the nigrostriatal pathway and could be correlated with the impairment of locomotor activity as well as severity of catatonia as observed in the present study. Licofelone administration for 7 days significantly attenuated the impaired motor function, suggesting that licofelone might have neuroprotective action possibly by preventing dopaminergic neuronal loss and thus maintained the proper motor function. Earlier, studies with COX-2 inhibitor have also demonstrated an improved motor function in neurotoxin models of PD (Aguirre et al. 2008; Gupta et al. 2011).
Several evidences point toward the role of oxidative (reactive oxygen species, ROS) and nitrosative (reactive nitrogen species, RNS) stress in the pathophysiology of PD (Halliwel, 1992; Yokoyama et al., 2008). Dopamine, a neurotransmitter present especially in the dopaminergic neurons, is relatively more susceptible to oxidative stress, forms dopamine quinone and reactive oxygen species (hydroxyl radicals), which further damages the cellular lipids (Hastings, 1995; Slivka and Cohen, 1985). In the present study, elevated levels of lipid peroxides were observed following MPTP administration. However, seven days treatment with licofelone significantly ameliorated the increased levels of lipid peroxides. Licofelone exhibited antioxidant-like effect in attenuating the increased level of lipid peroxide (Singh et al., 2005). Another dual COX and LOX inhibitor, phenidone has earlier demonstrated a significant protection from neuroinflammation-mediated oxidative damage in experimental model of LPS-induced dopaminergic neurotoxicity in rats (Li et al., 2008). Therefore, it may be plausible that licofelone, a COX/LOX competitive inhibitor attenuated the dopamine loss by preventing its auto-oxidation and the resultant oxidative damage induced by free radicals.

The resultant increase in the oxidative stress following MPTP administration depletes the level of antioxidant enzymes such as glutathione and superoxide dismutase (which under normal conditions, catalyze the free radicals to neutral molecules) (Drechsel et al., 2007). The striatal level of NPSH significantly decreased in the present study. Glutathione is one of the potent antioxidant enzymes playing an important role in the neurobiology of PD (Spina and Cohen, 1989). It is responsible for the scavenging free radicals such as hydrogen peroxide. But due to the persistent oxidative stress, cellular protective enzymes are not sufficient to ameliorate the generated free radicals and this creates a vicious cycle which results in an uninterrupted generation of free radicals and hence the cellular death. Therefore, restoration of the antioxidant enzymes represents a hallmark for preventing the cellular disturbances and the cellular death. In the present study, licofelone treatment significantly restored the levels of NPSH in the striatum of MPTP treated mice. These results highlight the antioxidant-like potential of licofelone which might be due to the free radical scavenging activity of the compound or the ability to up-regulate the cytoprotective enzymes within the dopaminergic neurons.
NO, a retrograde intracellular second messenger with a very short half-life, is an endogenous modulator of neuronal function (Bredt and Snyder, 1990). There is increase in the expression of iNOS following MPTP administration as well as due to the activation of microglia. Numerous studies have demonstrated the pathologic role of NO in neuronal disorders (Chalimoniuk et al., 2006; Przedborski et al., 1996; Yokoyama et al., 2008). Similarly, in the present study, MPTP challenge significantly increased the levels of NO in the striatum. Generation of free NO, peroxynitrite and nitrotyrosine formation ultimately inhibits the normal cellular functioning and lead to the death of dopaminergic neurons. During neuroinflammation, the levels of NO increase due to the activation of glial cells such as microglia (Hirsch et al., 2003). Licofelone treatment significantly attenuated the elevated NO levels offered protection against MPTP-induced nitrosative stress.

Till now anti-inflammatory drugs have not been formally tested in clinics in PD patients, but there is a vast array of experimental data which suggest the efficacy of such anti-inflammatory drugs especially NSAIDs in preventing dopaminergic degeneration in PD (Aguirre et al., 2008; Gupta et al., 2009). Epidemiological studies also point toward the beneficial role of NSAIDs against PD (Etminan et al., 2008; Wahner et al., 2007). Selective COX-2 inhibitors such as GW637185X, rofecoxib and preferential COX-2 inhibitor such as nimesulide have exhibited significant neuroprotection in MPTP model of PD (Aguirre et al., 2008; Gupta et al., 2009; Gupta et al. 2010a). Transgenic models in which genes responsible for COX-2 and iNOS were deleted displayed significant protection against MPTP-induced neurotoxicity (Feng et al., 2002; Teismann et al., 2003). Activation of microglia up regulates the release of various proinflammatory cytokines such as NF-kB and TNF-α during the course of neuroinflammatory process in PD (Kim and Joh, 2006; Mosley et al., 2006). Minocycline by virtue of its ability to inhibit microglial activation exhibited significant protective effect against neuroinflammation in experimental models (Sriram et al., 2006; Yokoyama et al., 2008).

MPTP is a potent inhibitor of mitochondrial function, which further leads to the inhibition of electron transport chain and results in energy depletion (Tipton and Singer, 1993; Vila et al., 2008). During mitochondrial inhibition, the
production of ROS (such as superoxide anions) increases with the subsequent damage to the cellular proteins that leads to up regulation and transcription of proinflammatory markers such as NF-κB and induction of apoptotic proteins such as caspase-3 (Vila et al., 2008; Vila and Przedborski, 2003). All these processes induce a cascade of events that ultimately lead to the neuronal cell death. Similarly, in the present study, mitochondrial enzyme complex I activity significantly decreased in MPTP treated animals. Supporting studies suggest that due to inhibition of mitochondrial enzyme complex activity, there might be increased production of superoxide anions which further up regulate the levels of NF-κB as well as caspase-3 (Minghetti, 2007; Vila and Przedborski, 2003).

Earlier reports suggest a persistent activation of cytokines and microglia in neurodegenerative conditions, suggesting the involvement of neuroinflammatory mechanism (Wong and Crack 2008; Minghetti, 2007). In the present study, increased level of NF-κB, PGE₂ and PGF₂α has been observed in the striatum of MPTP treated animals, which are in line with earlier reports (Kim and Joh, 2006; Mosley et al., 2006) demonstrating involvement of neuroinflammatory cascades in neuronal degeneration.

Based on these findings, it is conceivable that agents with an ability to prevent the alteration of mitochondrial enzyme activity or generated ROS may afford protection to the dopaminergic neurons from neurotoxins such as MPTP. Therefore, the present study suggests that licofelone might have the therapeutric role in producing cytoprotection. Further, it seems that licofelone might act by some unknown mechanism by which it restores mitochondrial complex-I activity and cell viability against MPTP induced neurotoxicity. Also, it substantiates the potent anti-inflammatory activity of licofelone, which resulted in the attenuation of proinflammatory markers and subsequent activation of cellular death cascade.

Based on the above findings, the study suggests that dual inhibition of COX/LOX pathway by licofelone has potential neuroprotective effect in alleviating the behavioral, biochemical and cellular alterations against MPTP induced neurotoxicity. These findings also point towards the antioxidant-like property and anti-apototic potential of licofelone. Furthermore, the present findings suggest the potential role of COX or LOX inhibitors in the management of PD.
CHAPTER-2.3

To investigate the effect of montelukast (a leukotriene receptor antagonist) against MPTP-induced Parkinson-like symptoms in mice

2.3.1. Introduction

Several studies have well documented the role of the COX as well as LOX isoenzymes in the pathogenesis of neurodegenerative disorders (Bishnoi et al., 2005; Feng et al., 2002; Gupta et al., 2011; Teismann et al., 2003). Experimental reports have established the protective effect of COX/LOX inhibitors in various neurodegenerative conditions including PD (Bishnoi et al., 2005; Gupta et al., 2010; Kalonia et al., 2009; Sul et al., 2009). The LT receptor antagonists have also been reported to have beneficial neuroprotective potential in several neurodegenerative disorders including PD (Ballerini et al., 2005; Tu et al., 2010).

Montelukast sodium is an oral, potent and selective antagonist of cysteinyl LT receptor. Research reports demonstrated its anti-inflammatory, mainly anti-eosinophil property (Diamant and Sampson, 1999). Montelukast has displayed cytoprotection from oxidative insult and proinflammatory mediators in various models of renal injury (Sener et al., 2006; Tugtepe et al., 2007). In 2006, Sener and coworkers demonstrated the effectiveness of montelukast against ischemia-reperfusion injury in rats. Sener and coworkers (2007) have attributed the protective effect of montelukast against ischemia-reperfusion injury in rats. Sener and coworkers (2007) have attributed the protective effect of montelukast in chronic renal failure induced oxidative tissue damage in rats due to its ability to balance oxidant-antioxidant status and inhibition of proinflammatory mediators and apoptotic factors. Similarly, montelukast demonstrated significant cytoprotection in pyelonephritis-induced cellular oxidative stress (Tugtepe et al., 2007). Montelukast has been shown to cross the blood–brain barrier readily (Biber et al., 2009).

CysLTs, the 5-LOX metabolites of arachidonic acid pathway, are the potent inflammatory mediators. A considerable amount of CysLTs is produced
Chapter 2.3

in the brain responding to a variety of brain pathologies, such as cerebral ischemia, brain trauma, epileptic seizures, intracerebral hemorrhage, brain tumor, and aging (Ballerini et al., 2005; Sener et al., 2006). CysLTs can increase the blood–brain barrier permeability and induce brain edema after cerebral ischemia (Yu et al., 2005; Biber et al., 2009). However, their exact status in neurodegenerative conditions including PD is yet to be established.

With this background, the present study was designed to evaluate the modulatory role of montelukast (a LT receptor antagonist) against MPTP-induced neurotoxicity in mice.

2.3.2. Materials and Methods

2.3.2.1. Animals

Refer chapter 1.2. (Section 1.2.2.1)

2.3.2.2. Drugs and treatment schedule

The following drugs and chemicals were used in the present study–MPTP (Sigma Chemicals, St. Louis, MO, USA), Montelukast sodium (MKT) (Ranbaxy Research Labs, Gurgaon, India). MPTP and montelukast were dissolved in distilled water and administered by i.p. injection. Montelukast was administered by i.p. route in a constant volume of 1 ml/100 gm.

The present study was conducted in different phases: Six groups were employed in the present study, consists of 15 animals in each.

**Group-I** – Vehicle treated group

**Group-II** – MPTP-treated group (40 mg/kg)

**Group-III** – MKT (1 mg/kg) per se

**Group-IV** – MKT (0.25 mg/kg) + MPTP (40mg/kg)

**Group-V** – MKT (0.5 mg/kg) + MPTP (40mg/kg)

**Group-VI** – MKT (1 mg/kg) + MPTP (40mg/kg)
MPTP was administered on first day in all the groups except vehicle group. Montelukast treatment was started 1 hr before first MPTP injection, followed by three more doses (10 mg/kg, i.p.) of MPTP injections as per the scheme mentioned below. Separate groups were used for biochemical and mitochondrial studies.
Chapter 2.3

2.3.2.3. Behavioral assessments

2.3.2.3.1. Assessment of gross behavioral activity (locomotor activity)

Refer Chapter-1.2 (Section 1.2.2.3.1.)

2.3.2.3.2. Assessment of catatonia

Refer Chapter-1.2 (Section 1.2.2.3.2)

2.3.2.4. Dissection and homogenization

Refer Chapter-1.2 (Section 1.2.2.4.)

2.3.2.5. Measurement of oxidative stress parameters

2.3.2.5.1. Lipid peroxidation

Refer Chapter 1.1 (Section 1.1.2.6.1.)

2.3.2.5.2. Non-protein thiols

Refer Chapter 1.1 (Section 1.1.2.6.2)

2.3.2.5.3. Estimation of nitrite

Refer Chapter 1.1 (Section 1.1.2.6.4.)

2.3.2.5.4. Protein estimation

Refer Chapter 1.1 (Section 1.1.2.6.5.)

2.3.2.6. Mitochondrial complex estimation

2.3.2.6.1. Isolation of rat brain mitochondria

Refer Chapter 1.2 (Section 1.2.2.6.1.)

2.3.2.6.2. Complex-I (NADH dehydrogenase activity)

Refer Chapter 1.2 (Section 1.2.2.6.2.)

2.3.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay

Refer Chapter 1.2 (Section 1.2.2.6.3.)

2.3.2.7. Quantification of apoptosis and inflammatory markers

2.3.2.7.1. Caspase-3 colorimetric assay

Refer Chapter 1.2 (Section 1.2.2.7.1.)
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2.3.2.7.2. NFκβ/p65 activity

Refer Chapter 1.2 (Section 1.2.2.7.2.)

2.3.2.8. Statistical analysis

Refer Chapter 1.2 (Section 1.2.2.8.)

2.3.3. Results

2.3.3.1. Effect of montelukast on total locomotor activity in MPTP-treated animals

Locomotor activities of all the animals were stable on day 1 before MPTP challenge. MPTP (40 mg/kg, i.p.) treatment significantly reduced locomotor activity on day 3, 5 and 7 as compared to vehicle group (Fig. 2.3.1.). Montelukast (0.25, 0.5 or 1.0 mg/kg) treatment for 7 days significantly improved locomotor activity as compared to MPTP group (Fig. 2.3.1.). However, montelukast (1.0 mg/kg) per se treatment did not modify the locomotor activity as compared to vehicle.

2.3.3.2. Effect of montelukast on MPTP-induced catatonia

MPTP treatment significantly induced postural inability and increased time spent on bar as compared to vehicle treated group on day 7 (Fig. 2.3.2.). Montelukast (0.25, 0.5 and 1.0 mg/kg) treatment for 7 days significantly attenuated the severity of catatonia as indicated by decrease in time spent on bar as compared to MPTP-treated group (Fig. 2.3.2.). However, montelukast (1.0 mg/kg) per se treatment did not modify the postural stability as compared to vehicle.

2.3.3.3. Effect of montelukast on MPTP-induced oxidative stress

Systemic administration of MPTP caused a marked increase in lipid peroxidation, nitrite levels and depleted NPSH levels in the striatum region as compared to vehicle group (Table 2.3.1.). Treatment with montelukast (0.25, 0.5 and 1.0 mg/kg) for 7 days, significantly attenuated the oxidative damage (as evidenced by decrease level of lipid peroxidation, nitrite levels and restored NPSH levels) as compared to MPTP-treated group (Table 2.3.1.).
2.3.3.4. Effect of montelukast on MPTP-induced changes in mitochondrial enzyme complex-I activity and MTT assay in mice

MPTP challenge significantly impaired mitochondrial enzyme complex-I activity and MTT assay (Fig. 2.3.3.) as compared to vehicle treated group. Seven days montelukast (0.25, 0.5 and 1.0 mg/kg) treatment significantly restored the mitochondrial complex-I activity and redox capacity (MTT assay) as compared to MPTP group (Fig. 2.3.3.). However, montelukast (1.0 mg/kg) per se treatment did not influence mitochondrial enzyme complex-I activity and MTT assay as compared to vehicle group.

2.3.3.5. Effect of montelukast on caspase-3 activity in MPTP-treated mice

MPTP treatment significantly increased caspase-3 activity in the striatum as compared to vehicle treated group (Fig. 2.3.4.). Montelukast (0.25, 0.5 and 1.0 mg/kg) treatment for 7 days significantly attenuated the caspase-3 activity as compared to MPTP-treated group (Fig. 2.3.4.). Montelukast (1.0 mg/kg) per se treatment did not influence the caspase-3 activity as compared to vehicle group.

2.3.3.6. Effect of montelukast on NF-κB/p65 activity in MPTP-treated mice

MPTP administration significantly increased NF-κB/p65 levels in the striatum as compared to vehicle treatment (Fig. 2.3.4.). Montelukast (0.25, 0.5 and 1.0 mg/kg) treatment for 7 days, significantly inhibited the transcription factor, NF-κB/p65 as compared to MPTP treated group (Fig. 2.3.4.). However, montelukast (1.0 mg/kg) per se treatment did not modulate the NF-κB/p65 activity as compared to vehicle group.
Fig. 2.3.1. Effect of montelukast on locomotor activity in MPTP treated animals. $^{a}P<0.05$ versus vehicle; $^{b}P<0.05$ versus MPTP(40); $^{c}P<0.05$ versus MKT(0.25)
Fig. 2.3.2. Effect of montelukast on severity of catatonia in MPTP treated animals. \(^a\)P<0.05 versus vehicle; \(^b\)P<0.05 versus MPTP(40); \(^c\)P<0.05 versus MKT(0.25); \(^d\)P<0.05 versus MKT(0.5)
Fig. 2.3.3. Effect of montelukast on mitochondrial enzyme complex-I activity and MTT assay in MPTP treated animals.

*P<0.05 versus vehicle; †P<0.05 versus MPTP(40); ‡P<0.05 versus MKT(0.25)
Fig. 2.3.4. Effect of montelukast on caspase-3 activity and NF-κB/p65 levels in MPTP treated animals. \(^a_P<0.05\) versus vehicle; \(^b_P<0.05\) versus MPTP(40); \(^c_P<0.05\) versus MKT(0.25); \(^d_P<0.05\) versus MKT(0.5)
Table 2.3.1. Effect of montelukast on MPTP-induced biochemical changes in the mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg protein (% of vehicle))</th>
<th>NPSH levels (μmol of NPSH/mg protein (% of vehicle))</th>
<th>Nitrite level (μmol/mg protein (% of vehicle))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100.0±3.18</td>
<td>100.0±4.92</td>
<td>100.0±6.36</td>
</tr>
<tr>
<td>MPTP(40)</td>
<td>242.8±2.22a</td>
<td>38.2±4.69a</td>
<td>195.0±3.49a</td>
</tr>
<tr>
<td>MKT(1) per se</td>
<td>99.3±3.11</td>
<td>101.1±5.71</td>
<td>99.6±6.22</td>
</tr>
<tr>
<td>MKT(0.25)</td>
<td>174.5±3.94b</td>
<td>60.5±7.47b</td>
<td>169.0±5.83b</td>
</tr>
<tr>
<td>MKT(0.5)</td>
<td>151.9±4.75b,c</td>
<td>79.5±8.44b,c</td>
<td>129.0±7.0b,c</td>
</tr>
<tr>
<td>MKT(1)</td>
<td>111.0±3.09bcd</td>
<td>92.1±3.04bcd</td>
<td>110.9±6.0bcd</td>
</tr>
</tbody>
</table>

aP<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus MKT(0.25); dP<0.05 versus MKT(0.5)

2.3.4 Discussion

The present study highlights that montelukast (a LT receptor antagonist) exerted significant neuroprotective effect against MPTP-induced behavioral changes (locomotor activity and catatonia), biochemical abnormalities (lipid peroxidation, reduced glutathione, nitrite), mitochondrial dysfunction (complex-I activity and MTT assay) and up regulation of inflammatory mediators. All these abnormalities supported Parkinson-like symptoms and were attenuated by 7 days treatment with montelukast (0.25, 0.5 and 1 mg/kg) in MPTP challenged animals. Thus, it is plausible that montelukast by virtue of its modulatory effect on LT synthesis exhibited its neuroprotective effects in PD.

Various reports have demonstrated the potential role of arachidonic acid pathway end products such as PG and LT in the pathophysiology of various neurodegenerative disorders where NSAIDs and dual inhibition of COX/LOX
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pathway exerted neuroprotective effect in experimental paradigms (Aguirre et al., 2008; Gupta et al., 2010b; Kalonia et al., 2009). Although, inhibiting COX or LOX pathway may provide an evidence for neuroprotection but it may either increase the formation of PGs or LTs (Martel-Pelletier et al., 2003; Rainsford, 2007). Therefore, antagonizing the effects of LT receptor could arrest the neuroinflammatory cascade (Bray et al., 1981; Lewis et al., 1990). Based on the reported data, the present study was designed to evaluate the modulatory effect of montelukast (a LT receptor antagonist) against MPTP–induced Parkinson-like symptoms in mice.

MPTP has been demonstrated to produce mitochondrial dysfunction in the dopaminergic neurons and increased expression of proinflammatory markers in the glial cells (Liu et al., 2006; Teismann and Ferger, 2001). Moreover, enhanced oxidative stress has been observed in experimental paradigms (Monahan et al., 2008; Vroon et al., 2007). These findings indicate that biochemical and cellular changes induced by MPTP modulate the behavioral parameters in animals which is evident by decreased locomotor activity and catatonia. This is in agreement with earlier studies where MPTP by virtue of its neurotoxic property produced degeneration of the dopaminergic neurons in the nigrostriatal pathway (Smyene and Jackson-Leiws, 1995). Montelukast administration for 7 days significantly attenuated the impaired motor function suggesting that montelukast preserved the dopaminergic neurons from degeneration.

In the present study, systemic administration of MPTP increased the oxidative stress as seen by increased levels of lipid peroxidation and nitrite as well as depleted the NPSH levels. Therefore, restoration of the prooxidant-antioxidant balance remains the characteristic for attenuating the neuronal death. Montelukast treatment significantly attenuated the lipid peroxidation and nitrite levels and restored the NPSH levels in MPTP treated mice. These observations indicate that montelukast exhibited antioxidant-like property and up-regulated the cytoprotective enzyme within the dopaminergic neurons. The mitochondrial complex-I activity and redox capacity was significantly decreased in the present study which was attenuated by montelukast in a significant manner. The protective effect of montelukast can be attributed to its antagonistic property of LT receptors especially LTB₄ receptors. Due to this mechanistic property of montelukast, it attenuated the free radicals generation and exerted neuroprotective effects (Diamant and Sampson, 1999).
Neruoinflammatory markers play a crucial role in the pathophysiology of PD as seen in experimental and clinical practice (Bartels and Leenders, 2007; Gupta et al., 2011; Vroon et al., 2007). In the present study also increased expression of NF-κβ/p65 and caspase-3 were observed following challenge with MPTP in mice. Montelukast treatment for seven days significantly decreased these proinflammatory and apoptotic markers. These findings are in accordance with the earlier published reports where montelukast demonstrated anti-inflammatory property (Chao et al., 2009; Kalonia et al., 2009; Sener et al., 2007). The protective effects of montelukast in MPTP-induced neurotoxicity can be attributed to their ability to (i) inhibit the action of LT on LTB₄ receptors and preserve the antioxidant enzymes. Earlier, montelukast has demonstrated anti-inflammatory property and protection against oxidative tissue damage by a neutrophil-dependent mechanism (Sener et al., 2005).

In summary, our data suggests that inhibiting the action of LT by LTB₄ receptor antagonism significantly attenuated the dopaminergic neurotoxicity mediated by MPTP. The study for the first time demonstrated the therapeutic potential of montelukast against PD-like symptoms and could be used as a protective strategy against neurodegenerative disorders including PD.
Chapter 3

CHAPTER-3

To elucidate the role of peroxisome proliferator-activated receptor-γ (PPARγ) in attenuating neuroinflammatory cascade in experimental model PD

3.1 Introduction

Peroxisome proliferator-activated receptor (PPARs) is ligand activated nuclear hormone receptors and occurs in three isoforms namely PPARα, PPARβ/δ and PPARγ (Green, 1995). Among the three isoforms, PPARγ is significantly expressed in various brain regions including striatum, substantia nigra, mesencephalon, cortex and hippocampus and its beneficial role has been demonstrated in various neurological conditions including cancer, atherosclerosis and inflammation (Mrak and Landreth, 2004; Moraes et al., 2006; Sundararajan et al., 2006). PPARγ agonists inhibit apoptotic pathway because of its anti-inflammatory action and currently being tried against several neurodegenerative disorders (Bordet et al., 2006; Henka et al., 2007). Studies have suggested that activation of glial cells and up regulation of inflammatory processes plays a central role in the pathogenesis of PD (Hunot et al., 1997; Gerhard et al., 2006; McGeer and McGeer, 2008). There is evidence of PPARγ activation and its modulatory influence on inflammatory responses in-vitro and in-vivo, protecting cells from death and toxicity (Jiang et al., 1998; Inestrosa et al., 2005).

Pioglitazone and rosiglitazone are two potent PPARγ agonists belong to thiazolidinedione class of drugs and currently being used in the treatment of type II diabetes. These potential drug candidates are currently being tried in several neurodegenerative conditions (Smith, 2001; Yki-Jarvinen, 2004; Lehrke and Lazar, 2005). Studies have demonstrated the efficacy of PPARγ agonists against various neurological diseases, including amyotrophic lateral sclerosis (Kiaei, 2008), AD (Camacho et al., 2004), PD (Dehmer et al., 2004). PPARγ agonists have also been implicated in adipocyte differentiation and insulin sensitivity (Lemberger et al., 1996) and down-regulate proinflammatory mediators in macrophages and microglia, mainly by inhibiting transcription of NF-κB-dependent inflammatory genes (Ricote et al., 1998).
Previous investigations have also assessed the vasculoprotective effect of pioglitazone independent of their metabolic action (Marx et al., 2004). In 2008, Quinn and coworkers demonstrated the neuroprotective effect of pioglitazone by inhibition of monoamine oxidase (MAO-B) in MPTP experimental model of PD (Quinn et al., 2008). Wright and coworkers demonstrated the neuroprotective effect of rosiglitazone that was antagonized by Bisphenol A diglycidyl ether (BADGE), a PPARy antagonist (Wright et al., 2000). However, none of studies could explain the neuroprotective mechanisms of pioglitazone in neurological disorders.

Based on the above facts, the present study was designed to explore the mechanistic role of PPARy in experimental model of MPTP-induced PD.

3.2. Materials and Methods

3.2.1. Animals

Refer Chapter 1.2. (Section 1.2.2.1.)

3.2.2. Drugs and treatment schedule

The following drugs were used: MPTP and BADGE (Sigma, St. Louis, MO, USA); pioglitazone (Ind-Swift Labs. Ltd., Chandigarh, India). Pioglitazone and BADGE were suspended in 0.25% w/v sodium carboxy methyl cellulose (Na-CMC) solution. MPTP was dissolved in distilled water. All the drugs except MPTP were administered per orally (p.o.). MPTP was administered intraperitoneally (i.p.) in a total dose of 40 mg/kg (four injections of 10 mg/kg at an interval of 1 hr).

Eight groups were employed in the present study, each comprising of 18 animals. Study was conducted in multiple phases.

Group I: vehicle group
Group II: MPTP-treated group – MPTP (40 mg/kg)
Group III: Pioglitazone per se (40 mg/kg)
Group IV: BADGE per se (15 mg/kg)
Group V to VII: Pioglitazone (10, 20 and 40 mg/kg)+MPTP (40 mg/kg)
Group VIII: BADGE (15 mg/kg) + Pioglitazone (40 mg/kg) + MPTP (40 mg/kg)
**Experimental Protocol**

MPTP was administered only on day 1 in the entire groups except vehicle-treated group while pioglitazone was administered 60 min before the first MPTP injection on day 1. BADGE was administered 15 min before
pioglitazone treatment. Drug treatments were continued daily for a total of 7 days as per the scheme mentioned above.

3.2.3. Behavioral assessments

3.2.3.1. Assessment of gross behavioral activity (locomotor activity)
Refer Chapter 1.2 (Section 1.2.2.3.1.)

3.2.3.2. Assessment of catatonia
Refer Chapter 1.2 (Section 1.2.2.3.2)

3.2.4. Dissection and homogenization
Refer Chapter 1.2 (Section 1.2.2.4.)

3.2.5. Measurement of oxidative stress parameters

3.2.5.1. Measurement of lipid peroxidation
Refer Chapter 1.1 (Section 1.1.2.6.1.)

3.2.5.2. Estimation of non protein thiols
Refer Chapter 1.1 (Section 1.1.2.6.2.)

3.2.5.3. Estimation of nitrite
Refer Chapter 1.1 (Section 1.1.2.6.4.)

3.2.5.4. Protein estimation
Refer Chapter 1.1 (Section 1.1.2.6.5.)

3.2.6. Mitochondrial complex estimation

3.2.6.1. Isolation of rat brain mitochondria
Refer Chapter 1.2 (Section 1.2.2.6.1.)

3.2.6.2. Complex-I (NADH dehydrogenase activity)
Refer Chapter 1.2 (Section 1.2.2.6.2.)

3.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay
Refer Chapter 1.2 (Section 1.2.2.6.3.)
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3.2.7. Quantification of proinflammatory and apoptotic factors

3.2.7.1. Caspase-3 Colorimetric assay
Refer Chapter 1.2 (Section 1.2.2.7.1.)

3.2.7.2. Quantification of NF-κβ/p65 levels
Refer Chapter 1.2 (Section 1.2.2.7.2.)

3.2.7.3. Prostaglandin, PGE$_2$ levels
Refer Chapter 1.2 (Section 1.2.2.7.3.)

3.2.7.4. Prostaglandin, PGF$_{2α}$ levels
Refer Chapter 1.2 (Section 1.2.2.7.4.)

3.2.8. Statistical analysis
Values are expressed as mean ± SEM. One Way Analysis of Variance (ANOVA) followed by One-way analysis of variance (ANOVA) followed by post-hoc Tukey’s test was applied to calculate the statistical significance between various groups. A value of $P<0.05$ was considered to be statistically significant.

3.3. Results

3.3.1. Effect of pioglitazone and BADGE on total locomotor activity in MPTP-treated mice

MPTP-treated group did not produce any significant effect on the total locomotor activity as compared to vehicle group treated group on day 1. However, MPTP treatment (40 mg/kg) significantly reduced the locomotor activity on day 7 as compared to vehicle group (Fig. 3.1). Further, pioglitazone (10, 20 and 40 mg/kg) treatment for 7 days, dose dependently improved the locomotor activity as compared to MPTP challenge (Fig. 3.1). Further, BADGE (15 mg/kg) pretreatment significantly reversed the protective effect of pioglitazone (40 mg/kg) in MPTP challenged animals which was significant as compared to their effect per se. However, pioglitazone (40 mg/kg) or BADGE (15 mg/kg) per se treatment did not modify the locomotor activity as compared to vehicle group.
Fig. 3.1. Effect of pioglitazone and BADGE on total locomotor activity in MPTP-treated mice. Total locomotor activity is expressed as % of vehicle group. 

3.1.2. Effect of pioglitazone and BADGE on the severity of catatonia in MPTP-treated mice

Unlike on 1st day, MPTP treatment significantly produced rigidity and postural inability (as seen by increased time spent on bar) as compared to the vehicle-treated mice on the day 7 (Fig. 3.2). Seven days pioglitazone (10, 20 or 40 mg/kg) treatment dose dependently attenuated the degree of catatonia indicated by reduced time latency on bar as compared to MPTP-treated group. BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly reversed the protective effect of pioglitazone (40 mg/kg). Pioglitazone (40 mg/kg) or BADGE (15 mg/kg) treatment per se did not produce any postural abnormality as compared to vehicle group.
Fig. 3.2. Effect of pioglitazone and BADGE on the severity of catatonic response in MPTP-treated mice on day 7. Catatonic response is expressed as % of MPTP group. \( \text{aP}<0.05 \) versus vehicle; \( \text{bP}<0.05 \) versus MPTP; \( \text{cP}<0.05 \) versus PIO(10); \( \text{dP}<0.05 \) versus PIO(20); \( \text{eP}<0.05 \) versus PIO(40); \( \text{fP}<0.05 \) versus BADGE(15)
### 3.3.3. Effect of pioglitazone and BADGE on alterations in oxidative stress parameters in mice challenged with MPTP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA levels (mg/kg)</th>
<th>GSH levels (% of vehicle)</th>
<th>Nitrite levels (% of vehicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100.0±3.0</td>
<td>100.0±6.5</td>
<td>100.0±4.9</td>
</tr>
<tr>
<td>MPTP(40)</td>
<td>234.5±2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.6±4.7&lt;sup&gt;+&lt;/sup&gt;</td>
<td>221.1±4.5&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>PIO(40) per se</td>
<td>99.8±4.3</td>
<td>101.3±5.1</td>
<td>99.2±5.6</td>
</tr>
<tr>
<td>BADGE(15) per se</td>
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<td>101.3±6.1</td>
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<td>187.1±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PIO(20)</td>
<td>148.8±4.8&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>76.4±3.1&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>147.4±5.6&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PIO(40)</td>
<td>115.6±3.4&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
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</tr>
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<td>BADGE(15)</td>
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<td>208±6.7</td>
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<tr>
<td>BADGE(15)+PIO(40)</td>
<td>141.7±3.9&lt;sup&gt;b,e,f&lt;/sup&gt;</td>
<td>71±4.6&lt;sup&gt;b,e,f&lt;/sup&gt;</td>
<td>155.67±5.8&lt;sup&gt;b,e,f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Systemic administration of MPTP caused a significant increase in the striatal lipid peroxidation, nitrite concentration and depleted NPSH levels as compared to vehicle-treated group (Table 3.1.). Treatment with pioglitazone (10, 20 and 40 mg/kg) significantly attenuated the oxidative damage (as seen by decreased levels of lipid peroxidation, nitrite levels and restored the NPSH levels respectively) in MPTP treated animals (Table 3.1.). BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly reversed the antioxidant like effect of pioglitazone (40 mg/kg) in MPTP treated animals. However, pioglitazone (40 mg/kg) or BADGE (15 mg/kg) treatment per se did not modify the biochemical parameters as compared to vehicle group.

### Table 3.1. Effect of pioglitazone and BADGE on oxidative damage induced by MPTP

<sup>a</sup>P<0.05 versus vehicle; <sup>b</sup>P<0.05 versus MPTP; <sup>c</sup>P<0.05 versus PIO(10); <sup>d</sup>P<0.05 versus PIO(20); <sup>e</sup>P<0.05 versus PIO(40); <sup>f</sup>P<0.05 versus BADGE(15)
3.3.4. Effect of pioglitazone and BADGE on mitochondrial enzyme complex-I activity in MPTP-treated mice

Fig. 3.4. depicts the effect of MPTP challenge on mitochondrial complex-I enzyme activity. MPTP treatment significantly decreased mitochondrial complex-I activity as compared to vehicle-treated group. Administration of pioglitazone (10, 20 and 40 mg/kg) daily for 7 days respectively, significantly restored the complex-I activity (Fig. 3.4.) as compared to MPTP treated group. BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly reversed its protective on mitochondrial dysfunction. However, pioglitazone (40 mg/kg) or BADGE (15 mg/kg) treatment per se did not modify mitochondrial enzyme complex as compared to vehicle group.

Fig. 3.4. Effect of pioglitazone and BADGE on mitochondrial enzyme complex-I activity in MPTP-treated mice. All values are expressed as % of vehicle. aP<0.05 versus vehicle; bP<0.05 versus MPTP; cP<0.05 versus PIO(10); dP<0.05 versus PIO(20); eP<0.05 versus PIO(40); fP<0.05 versus BADGE(15)
3.3.5. Effect of pioglitazone and BADGE on mitochondrial redox activity (MTT assay) in MPTP-treated mice

MPTP treatment significantly decreased mitochondrial redox activity (MTT assay) as compared to vehicle-treated group. Administration of pioglitazone (10, 20 and 40 mg/kg) daily for 7 days respectively, significantly restored the redox activity (Fig. 3.5.) as compared to MPTP treated group (p<0.05). BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly reversed its protective on mitochondrial redox activity as compared to MPTP-treated animals. However, pioglitazone (40 mg/kg) or BADGE (15 mg/kg) treatment per se did not modify mitochondrial enzyme complex and redox activity as compared to vehicle group.

![Graph showing the effect of pioglitazone and BADGE on mitochondrial redox activity in MPTP-treated mice.](image)

**Fig. 3.5. Effect of pioglitazone and BADGE on mitochondrial redox activity in MPTP-treated mice.** All values are expressed as % of vehicle. aP<0.05 versus vehicle; bP<0.05 versus MPTP; cP<0.05 versus PIO(10); dP<0.05 versus PIO(20); eP<0.05 versus PIO(40); fP<0.05 versus BADGE(15)
3.1.5. Effect of pioglitazone and BADGE on caspase-3 activity in MPTP-treated mice

MPTP administration significantly increased caspase-3 activity (marker of apoptosis) as compared to vehicle group (Fig. 3.6.). However, pioglitazone (10, 20 and 40 mg/kg) treatment for 7 days significantly attenuated caspase-3 activity (marker of apoptosis) as compared to MPTP treated group (Fig. 3.6.). Further, BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly reversed its protective effect (increased the caspase-3 level) as compared to MPTP treated animals. Pioglitazone (40 mg/kg) or BADGE (15 mg/kg) treatment per se did not modify the caspase-3 activity as compared to vehicle group.

**Fig. 3.6. Effect of pioglitazone and BADGE on caspase-3 activity in MPTP-treated mice.** All values are expressed as % of vehicle. aP<0.05 versus vehicle; bP<0.05 versus MPTP; cP<0.05 versus PIO(10); dP<0.05 versus PIO(20); eP<0.05 versus PIO(40); fP<0.05 versus BADGE(15)
3.1.6. Effect of pioglitazone and BADGE on NF-κB/p65 activity in MPTP-treated mice

Challenge with MPTP significantly increased the expression of NF-κB/p65 (proinflammatory marker) as compared to vehicle group (Fig. 3.7.). Pioglitazone (10, 20 and 40 mg/kg) treatment for 7 days significantly attenuated the NF-κB (indicator of proinflammation) in animals challenged with MPTP (Fig. 3.7.). BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly reversed the protective effect of pioglitazone and increased NFκB expression which was significant as compared to their effect per se. However, pioglitazone (40 mg/kg) or BADGE (15 mg/kg) per se treatment did not modify the NF-κB/p65 levels as compared to vehicle group.

![Graph showing effect of pioglitazone and BADGE on NF-κB/p65 activity in MPTP-treated mice.](image)

**Fig. 3.7.** Effect of pioglitazone and BADGE on NF-κB/p65 activity in MPTP-treated mice. All values are expressed as % of vehicle. aP<0.05 versus vehicle; bP<0.05 versus MPTP; cP<0.05 versus PIO(10); dP<0.05 versus PIO(20); eP<0.05 versus PIO(40); fP<0.05 versus BADGE(15)
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3.1.7. Effect of pioglitazone and BADGE on PGE$_2$ and PGF$_{2\alpha}$ levels activity in MPTP-treated mice

MPTP challenge significantly increased the levels of proinflammatory marker, PGE$_2$ and PGF$_{2\alpha}$ levels as compared to vehicle group (Fig. 3.8.). Chronic treatment with pioglitazone (10, 20 and 40 mg/kg) for 7 days, significantly attenuated the levels of PGE$_2$ and PGF$_{2\alpha}$ in animals challenged with MPTP (Fig. 3.8.). BADGE (15 mg/kg) pretreatment with pioglitazone (40 mg/kg) significantly abolished the protective effect of pioglitazone on PGE$_2$ and PGF$_{2\alpha}$ levels in animals challenged with MPTP. However, pioglitazone (40 mg/kg) or BADGE (15 mg/kg) treatment per se did not modify the PGs levels as compared to vehicle group.

Fig. 3.8. Effect of pioglitazone and BADGE on (A) PGE$_2$ and (B) PGF$_{2\alpha}$ levels in MPTP-treated mice. All values are expressed as % of vehicle. $^a$P<0.05 versus vehicle; $^b$P<0.05 versus MPTP; $^c$P<0.05 versus PIO(10); $^d$P<0.05 versus PIO(20); $^e$P<0.05 versus PIO(40); $^f$P<0.05 versus BADGE(15)
3.4 Discussion

The present study highlights the therapeutic potential of pioglitazone in the pathogenesis of MPTP-induced behavioral, biochemical and molecular alterations in mice. Results of the present study demonstrate that pioglitazone treatment significantly attenuated MPTP induced behavioral alterations (locomotor activity and severity of catatonia), oxidative damage and neuroinflammation in animals. Further, neuroprotective effect of pioglitazone is reversed by BADGE, a PPARγ antagonist suggesting the role of PPARγ in the experimental model of MPTP-induced PD-like symptoms.

Increasing evidence suggests that oxidative stress and mitochondrial dysfunction play a central role in the pathogenesis of PD (Chung et al., 2010; Gupta et al., 2011; Lee et al., 2011). Mitochondrial dysfunction has been implicated in the pathogenesis of several neurological disorders with direct neuronal damage linked to the energy impairment and depletion (Banerjee et al., 2009; Naoi et al., 2009). The complex pattern of MPTP induced degeneration of dopaminergic neurons includes mitochondrial dysfunction, free radical generation and up regulation of neuroinflammatory cascade (Berman and Hastings, 1999). Earlier studies have also suggested the role of free radical scavengers; anti-inflammatory agents and antioxidants in attenuating MPTP induced neurotoxicity in experimental models (Gupta et al., 2011; 2010b; Lee at al., 2011).

In the present study, MPTP challenge significantly induced motor abnormalities as observed by decrease in locomotor activity and postural rigidity (catatonia). MPTP induced neurotoxicity primarily involves degeneration of dopaminergic neurons in the nigrostriatal pathway. This further explains pharmacological basis of Parkinson-like symptoms such as catatonia and impaired motor function as observed in the present study. Biochemical investigations of the striatal region also revealed augmented level of lipid peroxides and nitrite concentration as well as depleted levels of glutathione in MPTP treated animals, suggesting oxidative damage. Fascinatingly, seven days pioglitazone treatment significantly attenuated behavioral changes; biochemical alterations in MPTP treated animals suggesting its therapeutic potential in PD-like symptoms.
Earlier reports demonstrated that activation of PPARγ effectively decreased neuroinflammation processes in neurodegenerative diseases (Gracia-Bueno et al., 2005; Culman et al., 2007; Quinn et al., 2008). It has been shown that PPARγ suppresses the expression of IL-6 in ischemic brain tissue during the initial phase of ischemic stroke (Patzer et al., 2008). Neuroprotection with pioglitazone against lipopolysachrides insult on dopaminergic neurons may be due to its inhibitory potential on many proinflammatory transcription factors, JNK activation and suppression of COX-2 activity (Xing et al., 2007). It also inhibited microglial activation and astrogliosis and exerted neuroprotection by targeting several downstream destructive mechanisms which act as neuronal insult (McTigue et al., 2007; Park et al., 2007).

Interestingly, PPARγ plays a direct role on energy metabolism due to its modulatory effect on the transcription of insulin-sensitive genes involved in the control of glucose and lipid metabolism in muscle, adipose tissue and liver (Smith, 2001). Recent studies demonstrate that activation of PPARγ stimulated the brain mitochondrial biogenesis thereby up-regulating the key mitochondrial and anti-apoptotic proteins and increasing the defense mechanisms against oxidative stress and mitochondrial damage (Fuenzalida et al. 2007; Wang et al., 2010). It is clearly evident from our study that MPTP challenge significantly decreased the mitochondrial complex-I activity and redox capacity which was significantly restored by pioglitazone treatment. Thus, it is plausible that treatment with pioglitazone plays a key role in restoring the mitochondrial function and this phenomenon could be vital in affording protection against neurotoxic stressors such as MPTP.

It is well documented that elevated oxidative stress subsequently damages cellular proteins, which further induces various apoptotic pathways that cause cell death of dopaminergic neurons (Klivenyi et al., 2000; Gupta et al., 2011). Recent reports suggest that persistent oxidative stress act as a neuroinflammatory insult and leads to the activation of glial cells especially microglia which stimulates the release of proinflammatory mediators such as cytokines, interleukins etc (Lin and Beal, 2006; Banarjee et al., 2009; Chung et al., 2010). In the present study, MPTP challenge upregulated the expression of
transcription factor NF-κβ and apoptotic factor (caspase-3), both being the markers of proinflammation and apoptotic cellular death. Treatment with pioglitazone significantly attenuated proinflammatory and apoptotic process. This action of pioglitazone could be attributed due to its protective effect on mitochondrial function and further release of free radicals. In support, rosiglitazone, a PPARγ agonist has been demonstrated to decrease the apoptotic markers (pro-apoptotic genes caspase-3 and Bax) and number of TUNEL positive neurons in the cortical area adjacent to the injury core in the injured cerebral cortex (Yi et al., 2008).

Up-regulation of cyclooxygenase (COX) enzyme has been implicated in the various neuronal disorders such as PD etc (Naidu and Kulkarni, 2002; Teismann et al., 2003; Gupta et al., 2009). The COX enzyme catalyzes the arachidonic acid pathway to form the end product, prostaglandins (PGs) mainly, PGE₂ and PGF₂α. Elevated levels of PGs have been observed during neuroinflammation and tumor genesis which promote apoptosis and angiogenesis (Hazra et al., 2007). Previous studies have demonstrated that activation of PPARγ by its agonists regulated the COX-2 expression and exerted their inhibitory effect on PGE₂ production via PPARγ independent pathway (Hazra et al., 2007; Cao et al., 2009). Supporting to the present study also MPTP challenge significantly increased the PGE₂ and PGF₂α levels that has been attenuated significantly by pioglitazone treatment. Therefore, the probable mechanism could be because of its anti-inflammatory effect responsible for attenuation of PGs. This further might be due to either modulation of COX-2 expression or any other pathway independent of PPARγ (Hazra et al., 2007; Cao et al., 2009).

Since there is no earlier study to investigate the role of antagonism of PPARγ in experimental model on PD, the present study explored the effect of BADGE, a PPARγ antagonist in MPTP-induced PD-like symptoms. Pretreatment of BADGE with the highest dose of pioglitazone significantly reversed its protective effect on behavioral parameters, oxidative damage and release and expression of proinflammatory markers (NF-κβ, PGE₂, PGF₂α). These findings strongly suggest the involvement of PPARγ in the pathogenesis of PD as well as the up regulation of inflammatory mediators in MPTP induced neurotoxicity. BADGE has earlier reported to antagonize the protective effect of
rosiglitazone and inhibited the adipocyte differentiation (Wright et al., 2000). In recent studies, BADGE has been used as a tool to study the role of PPARγ in various experimental conditions including neurodegeneration (Kalonia et al., 2010; Oster et al., 2010; Weismann et al., 2010).

Therefore, all the above findings and observations strongly points toward the potent neuromodulatory effect of activation of PPARγ in brain by pioglitazone in attenuating the behavioral, biochemical and molecular alterations induced by neurotoxic challenge. It is probable that pioglitazone inhibited the oxidative-nitrosative stress dependent activation of neuroinflammatory events that possibly result in neurodegeneration. The neuroprotection afforded by PPARγ agonist was reversed by treatment with PPARγ antagonist which highlights the therapeutic potential of PPARγ in neurodegenerative diseases.

In summary, the present finding implicates the potential role of PPARγ receptors in the pathogenic mechanism of neurodegenerative processes and modulating their activity can be beneficial in the management of PD.
CHAPTER-4.1

Effect of Vitamin E and its analogs on the oxidative stress and neuroinflammatory cascade against MPTP-induced neurotoxicity in mice

4.1.1. Introduction

Vitamin E is a generic term used to designate tocopherols (T) and tocotrienols (T3). Structurally, natural Vitamin E includes different chemical molecules: T (α, β, γ, δ) and T3 (α, β, γ, δ) (Aggarwal et al., 2010; Sen et al., 2006). Vitamin E is a lipophilic antioxidant and protects membrane from oxidative damage. There are a several reports on the effect of Vitamin E against MPTP induced oxidative damage on dopaminergic neurons in experimental animals (Odunze et al., 1990; Meredith et al., 2002; Itoh et al., 2006). Itoh and its colleagues (2006) compared the effect of γ-T in MPTP-induced damage of dopaminergic neurons in mice as compared to α-T where authors used α-T transfer protein knockout mice and demonstrated the neuroprotective effects of γ-T than that of α-T (Itoh et al., 2006). Furthermore, Vitamin E deficient mice displayed more susceptibility for dopaminergic neurotoxicity of MPTP in nigrostriatal region of the brain as compared to control (Odunze et al., 1990). It is further speculated that MPTP has dual synergistic action in causing PD either by direct initiation of oxidative damage leading to Vitamin E deficiency in cells or by accumulation of lipofuscin (Meredith et al., 2002).

With isolation of other structurally and functionally similar members of the Vitamin E family such as T3, recent research has now been focused on the possible roles of these moieties in halting the progression of various diseases such as diabetes; renal toxicity; neuroprotection etc (Aggarwal et al., 2010; Gupta and Chopra, 2009; Sen et al., 2000; Sen et al., 2006). T3 are less abundant in human diet as compared to α-T and differs by possessing an isoprenoid, rather than a saturated phytyl side chain. This unsaturated side chain of T3 affords far more efficient penetration into tissues (brain, liver) having more saturated fatty layers (Suzuki et al., 1993). Studies conducted on T3 by various research groups also demonstrated a potent antioxidant and free
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radical scavenging activity (Sen et al., 2000; Khanna et al., 2003; Gupta and Chopra, 2009). In one of the studies, α-T3 has been demonstrated more potent antioxidant effects than α-T where it prevented glutamate-induced death of T4 hippocampal neuronal cells even at nanomolar concentration but α-T did not show any effect at such a low concentration (Sen et al., 2000). There are many other studies also which indicate the more potent antioxidant effects of T3 as compared to α-T (Gupta and Chopra, 2009; Khanna et al., 2003; Khanna et al., 2005). Now a days, researchers have focused to evaluate the exact role of T3 in neurodegenerative conditions.

Thus, the present study is aimed to investigate the neuroprotective potential of T3 against MPTP induced Parkinson-like symptoms in mice. The effect of T3 on various behavioral, biochemical and cellular parameters were evaluated in mice.

4.1.2. Materials and Methods

4.1.2.1. Animals

Refer chapter 1.2. (Section 1.2.2.1)

4.1.2.2. Drugs and treatment schedule

The following drugs and chemicals were used in the present study: MPTP and α-tocopherol (T) (Sigma Chemicals, St. Louis, MO, USA) and Tocotrienol (T3) (Carotech Inc., Malaysia). MPTP was dissolved in distilled water and administered i.p. α-T and different doses of T3 were freshly prepared by triturating with 5% Tween 80 in distilled water and administered daily by oral route.

Eight groups were employed in the present study, each comprising of 20 animals. Study was conducted in multiple phases.

- **Group-I** – Vehicle treated group
- **Group-II** – MPTP (40 mg/kg)
- **Group-III** – T (100 mg/kg) per se
- **Group-IV** – T3 (100 mg/kg) per se
- **Group-V** – T (100 mg/kg) + MPTP (40)
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**Group-VI** – T3 (25 mg/kg) + MPTP (40)

**Group-VII** – T3 (50 mg/kg) + MPTP (40)

**Group-VIII** – T3 (100 mg/kg) + MPTP (40)

MPTP was administered only on day 8 in all the groups except vehicle group. Animals were pretreated with α-T and T3 for 7 days before MPTP.
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challenge on 8th day and continued till day 14. Behavioral observations were done as per the schematic representation mentioned below. Separate groups were used for biochemical, mitochondrial and proinflammatory marker studies.

4.1.2.3. Behavioral assessments

4.1.2.3.1. Assessment of gross behavioral activity (locomotor activity)

Refer Chapter-1.2 (Section 1.2.2.3.1.)

4.1.2.3.2. Assessment of catatonia

Refer Chapter-1.2 (Section 1.2.2.3.2)

4.1.2.4. Dissection and homogenization

Refer Chapter-1.2 (Section 1.2.2.4.)

4.1.2.5. Measurement of oxidative stress parameters

4.1.2.5.1. Measurement of lipid peroxidation

Refer Chapter 1.1 (Section 1.1.2.6.1.)

4.1.2.5.2. Estimation of non protein thiols

Refer Chapter 1.1 (Section 1.1.2.6.2)

4.1.2.5.3. Estimation of nitrite

Refer Chapter 1.1 (Section 1.1.2.6.2)

4.1.2.5.4. Protein estimation

Refer Chapter 1.1 (Section 1.1.2.6.4.)

4.1.2.6. Mitochondrial complex estimation

4.1.2.6.1. Isolation of rat brain mitochondria

Refer Chapter 1.2 (Section 1.2.2.6.1.)

4.1.2.6.2. Complex-I (NADH dehydrogenase activity)

Refer Chapter 1.2 (Section 1.2.2.6.2.)

4.1.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay

Refer Chapter 1.2 (Section 1.2.2.6.3.)
4.1.2.7. Quantification of proinflammatory and apoptotic factors

4.1.2.7.1. Caspase-3 Colorimetric assay

Refer Chapter 1.2 (Section 1.2.2.7.1.)

4.1.2.7.2. Quantification of NF-κβ/p65 levels

Refer Chapter 1.2 (Section 1.2.2.7.2.)

4.1.2.7.3. Prostaglandin, PGE_2 levels

Refer Chapter 1.2 (Section 1.2.2.7.3.)

4.1.2.7.4. Prostaglandin, PGF_2α levels

Refer Chapter 1.2 (Section 1.2.2.7.4.)

4.1.2.8. Statistical analysis

Refer Chapter 1.2 (Section 1.2.2.8.)

4.1.3. Results

4.1.3.1. Effect of α-T and T3 on total locomotor activity in MPTP-treated mice

There was no significant change in the locomotor activity on day 1 or day 8 (before challenge with MPTP). Administration of MPTP (40 mg/kg) significantly reduced locomotor activity as seen on day 10, 12 and 14 as compared to vehicle treated group (Fig. 4.1.1.). α-T (100 mg/kg) and T3 (25, 50 or 100 mg/kg) drug treatments for 14 days significantly improved locomotor activity as compared to MPTP group (Fig. 4.1.1.). However, T3 (100 mg/kg) produced significant effect on 14 as compared to α-T (100 mg/kg) in attenuating motor dysfunction in MPTP treated group. α-T (100 mg/kg) and T3 (100 mg/kg) treatment per se did not alter the locomotor activity as compared to vehicle group.

4.1.3.2. Effect of α-T and T3 on severity of catatonia in MPTP-treated mice

MPTP (40 mg/kg) treatment significantly caused rigidity or postural inability (as observed by increased time spent on bar) as compared to vehicle treated group on day 14 (Fig. 4.1.2.). 14 days treatment with α-T (100 mg/kg) and T3 (25, 50 and 100 mg/kg) significantly attenuated severity of catatonia (as indicated by decrease in time spent on bar) as compared to MPTP group (Fig. 4.1.2.).
4.1.2. But T3(100 mg/kg) produced significant anti-catatonic effect as compared to α-T (100 mg/kg). However, α-T (100 mg/kg) and T3 (100 mg/kg) treatment *per se* did not produce postural changes as compared to vehicle group.

4.1.3.3. Effect of α-T and T3 on oxidative stress parameters in MPTP treated animals

MPTP challenge caused a mark increase in lipid peroxidation, nitrite levels and depleted NPSH levels as compared to vehicle group (*Table 4.1.1.*). α-T (100 mg/kg) and T3 (25, 50 and 100 mg/kg) treatment for 14 days, significantly attenuated lipid peroxidation and nitrite levels as well as restored the levels of NPSH as compared to MPTP-treated groups (*Table 4.1.1.*). This effect was more significant in T3 (100 mg/kg) as compared to α-T (100 mg/kg). α-T (100 mg/kg) and T3 (100 mg/kg) treatment *per se* did not alter the biochemical parameters as compared to vehicle group.

4.1.3.4. Effect of α-T and T3 on mitochondrial enzyme complex-I activity and cell viability in MPTP-treated mice

MPTP challenge significantly impaired mitochondrial complex-I activity and cell viability (*Fig. 4.1.3.*) as compared to vehicle treated group. However, α-T (100 mg/kg) and T3 (25, 50 and 100 mg/kg) treatment for 14 days significantly restored mitochondrial complex-I activity and cell viability as compared to MPTP treated group. This effect was more significant with T3 (100 mg/kg) as compared to α-T (100 mg/kg) pretreatment (*Fig. 4.1.3.*). However, α-T (100 mg/kg) and T3 (100 mg/kg) *per se* treatments did not produce any significant effect on the mitochondrial enzyme functions as compared to vehicle group.

4.1.3.5. Effect of α-T and T3 on caspase-3 activity in MPTP-treated mice

MPTP treatment significantly increased caspase-3 activity as compared to vehicle treated group (*Fig. 4.1.4.*). Administration of α-T (100 mg/kg) and T3 (25, 50 and 100 mg/kg) treatment for 14 days significantly reduced caspase-3 activity (marker of apoptotic pathway) as compared to MPTP treated group (*Fig. 4.1.4.*). T3 (100 mg/kg) treatment produced more significant effect as compared to α-T (100 mg/kg). However, α-T (100 mg/kg) and T3 (100 mg/kg) *per se* treatments did modify the caspase-3 activity as compared to vehicle group.
4.1.3.6. Effect of α-T and T3 on NF-κB/p65 activity in MPTP-treated mice

Administration of MPTP significantly increased proinflammatory transcription factor, NF-κB/p65 as compared to vehicle treatment (Fig. 4.1.5.). Chronic treatment with T3 (25, 50 and 100 mg/kg) for 14 days, significantly inhibited NF-κB/p65 (induction of proinflammatory marker) in MPTP treated groups while α-T (100 mg/kg) did not show any significant effect on NF-κB/p65 as compared to MPTP treated group (Fig. 4.1.5.). However, α-T (100 mg/kg) and T3 (100 mg/kg) treatment per se did not alter the NF-κB/p65 activity as compared to vehicle group.

4.1.3.7. Effect of α-T and T3 on PGE$_2$ and PGF$_{2α}$ levels activity in MPTP-treated mice

Challenge with MPTP significantly increased PGE$_2$ and PGF$_{2α}$ levels in the striatum as compared to vehicle treatment (Fig. 4.1.6.). Treatment with T3 (25, 50 and 100 mg/kg) for 14 days, significantly inhibited the PGE$_2$ and PGF$_{2α}$ levels in MPTP treated groups (Fig. 4.1.6.). α-T (100 mg/kg) did not show any effect in attenuating PGE$_2$ and PGF$_{2α}$ levels as compared to MPTP treated group. However, α-T (100 mg/kg) and T3 (100 mg/kg) treatment per se did not alter the PGs as compared to vehicle group.
**Fig. 4.1.1.** Effect of α-T and T3 on total locomotor activity in MPTP-treated mice. *aP<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus T(100)
Fig. 4.1.2. Effect of α-T and T3 on the severity of catatonic response in MPTP-treated mice on day 14. ⁶P<0.05 versus vehicle; ⁴P<0.05 versus MPTP(40); ³P<0.05 versus T(100)
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Table 4.1.1. Effect of α-T and T3 on oxidative damage induced by MPTP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA levels (% of vehicle)</th>
<th>NPSH levels (% of vehicle)</th>
<th>Nitrite levels (% of vehicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100.0±3.1</td>
<td>100.0±4.2</td>
<td>100.0±5.1</td>
</tr>
<tr>
<td>MPTP (40)</td>
<td>244.3±4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.7±5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>267.6±6.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T(100) <em>per se</em></td>
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<td>98.9±4.9</td>
<td>100.4±5.8</td>
</tr>
<tr>
<td>T3(100) <em>per se</em></td>
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<td>101.4±5.2</td>
<td>99.3±6.1</td>
</tr>
<tr>
<td>T(100)</td>
<td>181.3±4.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.6±3.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>191.3±6.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T3(25)</td>
<td>173.1±3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.4±5.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>179.4±7.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>T3(50)</td>
<td>145.7±4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.2±4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>139.8±5.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T3(100)</td>
<td>123.4±3.4&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>90.2±4.8&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>116.6±6.8&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05 versus vehicle; <sup>b</sup>P<0.05 versus MPTP(40); <sup>c</sup>P<0.05 versus T(100)
Fig. 4.1.3. Effect of α-T and T3 on mitochondrial function in MPTP-treated mice. *P<0.05 versus vehicle; **P<0.05 versus MPTP(40); ***P<0.05 versus T(100)
Fig. 4.1.4. Effect of α-T and T3 on caspase-3 activity in MPTP-treated mice. aP<0.05 versus vehicle group; bP<0.05 versus MPTP group; cP<0.05 versus T(100)
Fig. 4.1.5. Effect of α-T and T3 on NF-κB/p65 activity in MPTP-treated mice. *P<0.05 versus vehicle; **P<0.05 versus MPTP(40); ***P<0.05 versus T(100)
Fig. 4.1.6. Effect of α-T and T3 on (A) PGE$_2$ and (B) PGF$_{2α}$ levels in MPTP-treated mice. *P<0.05 versus vehicle; *P<0.05 versus MPTP(40); *P<0.05 versus MPTP(40); *P<0.05 versus T(100)
4.1.4 Discussion

Although the role of oxidative stress has already been well established in experimental models of PD, various antioxidants such as melatonin, ebselen and α-T have been reported to exhibit significant neuroprotective property against MPTP-induced neurodegeneration (Itoh et al., 2006; Ma et al., 2009; Moussaoui et al., 2000). Laboratory reports compared different T (α- or γ-) suggesting potent antioxidant profile of γ-T as compared to α-T (Itoh et al., 2006). In comparison to T, T3 has been poorly evaluated for their biological activity especially in neuronal disorders. Recent experimental evidence suggests that T3 (structural and functional analogues of α-T) demonstrate more potent antioxidant and free radical scavenging activity as compared to α-T (Gupta et al., 2009; Maniam et al., 2008; Selvaduray et al., 2010). Based on these evidences and published reports, inhibition of oxidative damaging cascade exhibits potential site in attenuating dopaminergic neurodegeneration induced by MPTP (Gupta et al., 2010b, Gupta et al., 2011; Yokoyama et al., 2008). Thus in the present study, we investigated the neuroprotective mechanism of T3 in animal model of PD, where MPTP was used to induce neurodegeneration in mice.

Studies have documented the evidence pointing toward the role of reactive oxygen species, ROS and reactive nitrogen species, RNS in the pathophysiology of PD (Gupta et al., 2011; Halliwell, 1992; Yokoyama et al., 2008). The main causative factor in the pathogenesis of PD is oxidative stress and dopamine, one of the neurotransmitters present especially in the dopaminergic neurons (Slivka and Cohen, 1985). Due to attack by reactive oxygen species, dopamine forms dopamine quinone, which further damages the cellular lipids and results in the degeneration of dopaminergic neurons in the nigrostriatal pathway (Hastings, 1995; Slivka and Cohen, 1985). MPTP administration also results in the generation of free radicals and promotes degeneration of dopaminergic neurons specifically. Thus, decreased locomotor activity and catatonia (postural inability) have been observed in the present study following MPTP administration. Treatment with α-T (100 mg/kg) and T3 (25, 50 or 100 mg/kg) significantly attenuated these behavioral abnormalities induced by MPTP. Thus, it is plausible that tocotrienol administration by virtue
Chapter 4.1

of its potent antioxidant property exhibited significant neuroprotective effects in MPTP-induced neuronal toxicity in mice.

Biochemical investigations also revealed a significant increase in TBARS levels in the present study. Since TBARS is an index to measure lipid peroxidation, increased TBARS levels suggested a significant lipid peroxidation following MPTP administration. However, pretreatment with α-T or T3 significantly attenuated TBARS level. Supplementing to the earlier published reports (Gupta et al., 2009; Khanna et al., 2005; Maniam et al., 2008), T3 presented better attenuation of TBARS levels as compared to α-T. Therefore, it may be plausible that due to its better ability to penetrate cell membrane, T3 attenuated the resultant oxidative damage induced by free radicals generated following challenge with MPTP (Suzuki et al., 1993).

Reduced glutathione, one of the important antioxidant defense systems present in living tissues plays a significant role in resisting neuronal damage and neurodegeneration as it catalyzes free radicals to neutral molecules (Maetzler et al., 2010; Spina and Cohen, 1989). In the present study, MPTP administration significantly depleted the levels of protective antioxidant enzymes such as reduced glutathione. A vicious cycle is build up by the continual generation of free radicals which sustains oxidative stress and cellular protective enzymes become insufficient to detoxify the generated free radicals. It ultimately leads to the degeneration of dopaminergic neurons and neuronal death. Therefore, compensation of antioxidant enzymes signifies hallmarks for ameliorating cellular disturbances and neuronal death. Chronic T3 administration restored the levels of NPSH of MPTP treated mice in a significant manner as compared to α-T pretreatment. These results highlight the potential of T3 to up regulate cytoprotective enzyme, reduced glutathione and its ability to scavenges free radicals within the dopaminergic neurons.

Recently, numerous studies have demonstrated the pathologic involvement of NO in various neuronal disorders such as PD etc (Bredt and Snyder, 1990; Gupta et al., 2010a; Yokoyama et al., 2008). Experimental studies conducted using MPTP confirmed the probable role of iNOS in degeneration of the dopaminergic neurons and neuronal toxicity (Chalimoniuk et al., 2006; Yokoyama et al., 2008). Similarly, in the present study MPTP
challenge significantly increased the levels of NO in the striatum. Neuroinflammatory cascade also up regulates the level of iNOS in glial cells and further increases the levels of nitric oxide (Dehmer et al., 2000; Hirsch et al., 2003). Treatment with T3 significantly reversed elevated NO levels towards their normal values and offered protection against MPTP-induced nitrosative stress as compared to α-T. T3 have been already demonstrated to exhibit a potent anti-inflammatory activity in *in-vivo* studies (Aggarwal et al., 2010; Khanna et al., 2003; Khanna et al., 2005; Suzuki et al., 1993). Thus, it is plausible that T3 exhibited a potent anti-inflammatory effect by attenuating these cellular processes in our experimental model and offered neuroprotection against MPTP.

Systemic administration of MPTP has been reported to produce neuroinflammation (Gupta et al., 2011; Hirsch et al., 2003; Mosley et al., 2006). MPTP via activation of glial cells especially microglia induces the expression of proinflammatory transcription factors such as NF-κB (Gupta et al., 2010b; Gupta et al., 2011; Vroon et al., 2007). This neuroinflammatory signaling cascade upregulates the expression of COX-2 and iNOS which further increases the level of PGs and NO. Supporting to the present study, MPTP challenge significantly increased levels of PGE$_2$ and PGF$_{2α}$. This might be due to the up regulation/ induction of COX isoenzymes or modulation of COX pathway, suggesting the role of neuroinflammation. However, chronic treatment with T3 significantly attenuated the increased levels of PGE$_2$ and PGF$_{2α}$, but this effect was not seen with α-T pretreatment. Therefore, it seems that T3 exhibits potent anti-inflammatory activity in the attenuating the PGs levels or this might be due to their modulatory effect on COX-2 expression or COX pathway.

Till now various *in-vitro* and *in-vivo* studies have demonstrated neuroprotective potential of T3. Osakada and coworkers (2004) have demonstrated the effectiveness of T3 against hydrogen peroxide-induced neuronal death in rat striatal cultures as compared to α-T. Similarly, T3 were more effective than α-T in attenuating the effect of oxidative stress on rat brain mitochondria (Kamat and Devasagayam, 1995). The remarkable cytoprotective activity of T3 relates to the differential cellular uptake of Vitamin E analogs (Sen
et al., 2000; 2006). Several other evidences indicate towards non-antioxidant effects of T3 such as cholesterol-lowering activity, anticarcinogenic activity and cytoprotective properties (Nesaretnam et al., 1998; Parker et al., 1993).

Since MPTP is a potent inhibitor of mitochondrial function and energy depletion (Gupta et al., 2010b; Gupta et al., 2011; Vila and Przedborski, 2003). In the present study MPTP challenge significantly induced mitochondrial dysfunction as indicated by decrease in the complex-I activity and mitochondrial viability. Mitochondrial dysfunction also leads to the production of ROS which up regulates the transcription of proinflammatory and apoptotic markers (Vila and Przedborski, 2003). This evidence is in line with the present study where we observed increased levels of NF-κβ levels as well as caspase-3 following MPTP administration. Therefore, agents with their ability to attenuate mitochondrial function or ROS generation can offer neuroprotection to dopaminergic neurons from neurotoxins. T3 treatment for 14 days significantly restored the mitochondrial complex-I activity and viability as well as inhibited the up regulation of NF-κβ and caspase-3 levels as compared to α-T. Our results suggest the potential protective action of T3 may be due to the restoration of mitochondrial function. Since T3 attenuated the proinflammatory markers and subsequent activation of cellular death cascade, it substantiates their potent anti-inflammatory activity. Earlier, studies have confirmed the potential antitumor and antiangiogenic effect of T3 (Aggarwal et al., 2010; Selvaduray et al., 2010)

These results provide a valuable observation and explain the potential neuroprotective effect of T3 in comparison to α-T. The better efficacy and antioxidant property of T3 may be attributed to the structural differences between these Vitamin E analogs. These results represents a novel approach of using natural products such as T3 as neuromodulators in attenuating oxidative damage and decreased antioxidant pool along with mitochondrial dysfunction and energy production as well as induction of inflammatory processes involved in neurodegeneration. The probable neuroprotective mechanism exerted by T3 against MPTP-induced dopaminergic neurodegeneration is schematically depicted in Fig. 4.1.7
In conclusion, the finding of the present study implicates the potential role of oxidative stress in the pathogenesis of PD and suggests that antioxidant supplementation with T3 may provide a therapeutic alternative for attenuation of neuroinflammation and oxidative stress in PD.
CHAPTER-4.2

To explore the neuroprotective potential of lycopene against MPTP-induced neurotoxicity in mice

4.2.1. Introduction

Over the past decade, researchers have focused on the role of nutritional supplements in various neurodegenerative diseases. Strong evidence points towards the potential role of oxidative stress and defective energy metabolism in the pathogenesis of many neurodegenerative disorders, such as PD, AD and HD (Aria et al., 2006; Banerjee et al., 2009; Kalonia et al., 2010). Various reports have established the role of mitochondrial dysfunction and resultant oxidative insult in experimental models of PD (Gupta et al., 2011; Gu et al., 1998). MPTP, a neurotoxin also produces Parkinson-like symptoms in animals via generation of ROS and inhibition of mitochondrial enzyme complex-I activity leading to energy depletion (Smeyne and Jackson-Lewis, 2005). Thus, there is a robust scientific rationale for testing suitable antioxidants’ for potential neuroprotective therapy in such disorders.

One such nutritional supplement is lycopene, a red pigment belonging to carotenoid family that is mostly found in tomatoes and tomato products, is a powerful antioxidant. Among naturally occurring carotenoids, lycopene has the strongest ability to scavenge free radicals; being 10-fold, 47-fold and 100-fold more effective in quenching singlet oxygen than α-tocopherol, β-carotene and vitamin E respectively (Conn et al., 1991; Miller et al., 1996). Epidemiological studies have evaluated the role of lycopene as a potential antioxidant. Lycopene acts as a potent hypocholesterolemic agent by controlling the serum insulin-like growth factor (IGF)-1 levels in healthy subjects (Riso et al., 2006), in treatment of osteoporosis and diabetes (Rao et al., 2006; Rao and Rao, 2007), antiproliferative and anticancerous in rat prostate carcinoma cells (Gunasekera et al., 2007), in the treatment of cardiovascular diseases (Kong et al., 2010). In addition, pretreatment with lycopene offers protection to normal lymphocytes against gamma-radiation-induced cellular damage (Srinivasan et al., 2009).

Recent interest has focused on lycopene as a potential useful agent in the management of neurodegenerative disorders. Lycopene has been found to
be neuroprotective against microglial activation in focal cerebral ischemia in rats (Hsiao et al., 2004), cognition enhancer in elderly population (Akbaraly et al., 2007), conditions that involve impaired mitochondrial functions. Lycopene has recently been shown to mediate its protective effect in 3-NP induced HD by targeting nitric oxide pathway (Kumar et al., 2009; Kumar and Kumar, 2009). Due to its chemical structure containing eleven conjugated double bonds, lycopene is a powerful antioxidant and free radical quencher.

Based on the unmet need for information focusing on the chemopreventive potential of naturally occurring compounds, the current study was designed to evaluate the role of lycopene treatment against MPTP-induced Parkinson-like symptoms in animals.

4.2.2. Materials and Methods

4.2.2.1. Animals

Refer Chapter 1.2. (Section 1.2.2.1.)

4.2.2.2. Drugs and treatment schedule

The following drugs and chemicals were used in the present study: MPTP (Sigma Chemicals, St. Louis, MO, USA), Lycopene (LYP) (Mankind Pharma, Ltd., Mumbai, India). MPTP was dissolved in distilled water and administered by i.p. route. LYP was freshly prepared by triturating with 5% Tween 80 in distilled water and administered daily by oral route.

The present study was conducted in different phases:

Six groups were employed in the present study, each comprising of 18 animals.

**Group-I** – vehicle treated group  
**Group-II** – MPTP group (40 mg/kg)  
**Group-VII** – LYP (10 mg/kg) *per se*  
**Group-VIII** – LYP (2.5 mg/kg) + MPTP (40 mg/kg)  
**Group-IX** – LYP (5 mg/kg) + MPTP (40 mg/kg)  
**Group-X** – LYP (10 mg/kg) + MPTP (40 mg/kg)
Study Design

MPTP was administered only on day 8 in all the groups except vehicle group. LYP treatment was started 7 days before MPTP challenge and continued till 14th day. Behavioral observations were done as per the schematic representation mentioned below. Separate groups were used for biochemical, mitochondrial and proinflammatory marker studies.

Experimental Protocol

4.2.2.3. Behavioral assessments

4.2.2.3.1. Assessment of gross behavioral activity (locomotor activity)

Refer Chapter-1.2 (Section 1.2.2.3.1.)
4.2.2.3.2. Assessment of catatonia
   Refer Chapter-1.2 (Section 1.2.2.3.2)

4.2.2.4. Dissection and homogenization
   Refer Chapter-1.2 (Section 1.2.2.4.)

4.2.2.5. Measurement of oxidative stress parameters
   4.2.2.5.1. Measurement of lipid peroxidation
       Refer Chapter 1.1 (Section 1.1.2.6.1.)
   4.2.2.5.2. Estimation of non protein thiols
       Refer Chapter 1.1 (Section 1.1.2.6.2)
   4.2.2.5.3. Estimation of nitrite
       Refer Chapter 1.1 (Section 1.1.2.6.2)
   4.2.2.5.4. Protein estimation
       Refer Chapter 1.1 (Section 1.1.2.6.4.)

4.2.2.6. Mitochondrial complex estimation
   4.2.2.6.1. Isolation of rat brain mitochondria
       Refer Chapter 1.2 (Section 1.2.2.6.1.)
   4.2.2.6.2. Complex-I (NADH dehydrogenase activity)
       Refer Chapter 1.2 (Section 1.2.2.6.2.)
   4.2.2.6.3. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay
       Refer Chapter 1.2 (Section 1.2.2.6.3.)

4.2.2.7. Quantification of proinflammatory and apoptotic factors
   4.2.2.7.1. Caspase-3 Colorimetric assay
       Refer Chapter 1.2 (Section 1.2.2.7.1.)
   4.2.2.7.2. Quantification of NF-κB/p65 levels
       Refer Chapter 1.2 (Section 1.2.2.7.2.)
4.2.2.7.3. Prostaglandin, PGE$_2$ levels

Refer Chapter 1.2 (Section 1.2.2.7.3.)

4.2.2.7.4. Prostaglandin, PGF$_{2\alpha}$ levels

Refer Chapter 1.2 (Section 1.2.2.7.4.)

4.2.2.8. Statistical analysis

Values are expressed as mean ± SEM. One Way Analysis of Variance (ANOVA) followed by One-way analysis of variance (ANOVA) followed by post-hoc Tukey’s test was applied to calculate the statistical significance between various groups. A value of $P<0.05$ was considered to be statistically significant.

4.2.3. Results

4.2.3.1. Effect of lycopene on total locomotor activity in MPTP-treated mice

There was no significant change in the locomotor activity on day 1 or day 8 (before challenge with MPTP). Administration of MPTP (40 mg/kg) significantly reduced locomotor activity seen on day 10, 12 and 14 as compared to vehicle treated group (Fig. 4.2.1.). Lycopene (2.5, 5 and 10 mg/kg) treatment for 14 days significantly improved locomotor activity as compared to MPTP group (Fig. 4.2.1.). However, lycopene (10 mg/kg) per se treatment did not alter the locomotor activity as compared to vehicle group.

4.2.3.2. Effect of lycopene on the severity of catatonia in MPTP-treated mice

MPTP (40 mg/kg) treatment significantly caused rigidity or postural inability (as observed by increased time spent on bar) as compared to vehicle treated group on day 14 (Fig. 4.2.2.). Lycopene (2.5, 5 and 10 mg/kg) significantly attenuated the severity of catatonia (as indicated by decrease in time spent on bar) on day 14 as compared to MPTP group (Fig. 4.2.2.). However, lycopene (10 mg/kg) per se treatment did not produce postural abnormality as compared to vehicle group.
4.2.3.3. Effect of lycopene on alterations in oxidative stress parameters in mice challenged with MPTP

Systemic administration of MPTP significantly increased the lipid peroxidation, nitrite levels and depleted NPSH levels as compared to vehicle group (Table 4.2.1.). Treatment with lycopene (2.5, 5 and 10 mg/kg) for 14 days, significantly mitigated the oxidative damage induced by MPTP by attenuating the increased levels of lipid peroxidation and nitrite as well as restored the levels of NPSH as compared to MPTP-treated group (Table 4.2.1.). Lycopene (10 mg/kg) per se treatment did not alter the biochemical parameters as compared to vehicle group.

Table 4.2.1. Effect of lycopene on oxidative damage induced by MPTP

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>MDA (nmol/mg protein (% of vehicle))</th>
<th>NPSH levels (µmol of NPSH/mg protein (% of vehicle))</th>
<th>Nitrite level (µmol/mg protein (% of vehicle))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100.0±3.2</td>
<td>100.0±4.9</td>
<td>100.0±6.6</td>
</tr>
<tr>
<td>MPTP(40)</td>
<td>242.8±2.2a</td>
<td>38.2±4.6a</td>
<td>229.0±5.9a</td>
</tr>
<tr>
<td>LYP(10) per se</td>
<td>99.3±3.1</td>
<td>101.1±5.7</td>
<td>99.6±6.2</td>
</tr>
<tr>
<td>LYP(2.5)</td>
<td>174.5±3.9b</td>
<td>61.8±7.4b</td>
<td>162.0±5.8b</td>
</tr>
<tr>
<td>LYP(5)</td>
<td>151.9±4.8bc</td>
<td>79.5±8.4bc</td>
<td>126.0±7.0bc</td>
</tr>
<tr>
<td>LYP(10)</td>
<td>111.0±3.9b,c,d</td>
<td>92.1±3.4b,c,d</td>
<td>110.9±6.0b,c,d</td>
</tr>
</tbody>
</table>

aP<0.05 versus vehicle treated, bP<0.05 versus MPTP, cP<0.05 versus LYP(2.5), dP<0.05 versus LYP(5)
4.2.3.4. Effect of lycopene on mitochondrial enzyme complex-I activity and cell viability in MPTP-treated mice

MPTP challenge significantly impaired mitochondrial enzyme function by decreasing the complex-I activity and cell viability (Fig. 4.2.3.) as compared to vehicle group. Chronic lycopene (2.5, 5 and 10 mg/kg) treatment for 14 days significantly restored the mitochondrial function in MPTP treated animals (Fig. 4.2.3.). However, lycopene (10 mg/kg) per se treatment did not influence the mitochondrial enzyme function as compared to vehicle group.

4.2.3.5. Effect of lycopene on caspase-3 activity in MPTP-treated mice

MPTP treatment significantly increased caspase-3 activity as compared to vehicle treated group (Fig. 4.2.4.). Treatment with lycopene (2.5, 5 and 10 mg/kg) for 14 days significantly reduced the caspase-3 activity as compared to MPTP treated group (Fig. 4.2.4.). However, lycopene (10 mg/kg) per se treatment did not modify the caspase-3 activity as compared to vehicle group.

4.2.3.6. Effect of lycopene on NF-κB/p65 activity in MPTP-treated mice

Administration of MPTP significantly increased the proinflammatory transcription factor, NF-κB/p65 levels as compared to vehicle treatment (Fig. 4.2.4.). Chronic treatment with lycopene (2.5, 5 and 10 mg/kg) for 14 days, significantly inhibited NF-κB/p65 levels in MPTP treated group (Fig. 4.2.4.). However, lycopene (10 mg/kg) per se treatment did not alter the NF-κB/p65 activity as compared to vehicle group.

4.2.3.7. Effect of lycopene on PGE₂ and PGF₂α levels activity in MPTP-treated mice

Challenge with MPTP significantly increased the PGE₂ and PGF₂α levels as compared to vehicle treatment (Fig. 4.2.5.). Treatment with lycopene (2.5, 5 and 10 mg/kg) for 14 days, significantly inhibited the PGE₂ and PGF₂α levels in MPTP treated group (Fig. 4.2.5.). However, lycopene (10 mg/kg) per se treatment did not alter the PG level as compared to vehicle group.
Fig. 4.2.1. Effect of lycopene on total locomotor activity in MPTP-treated mice. *P<0.05 versus vehicle; †P<0.05 versus MPTP(40); ‡P<0.05 versus LYP(2.5); §P<0.05 versus LYP(5)
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Fig. 4.2.2. Effect of lycopene on the severity of catatonic response in MPTP-treated mice on day 14. aP<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus LYP(2.5); dP<0.05 versus LYP(5)
Fig. 4.2.3. Effect of CoQ10 and lycopene on mitochondrial enzyme function in MPTP-treated mice. *P<0.05 versus vehicle; bP<0.05 versus MPTP(40); cP<0.05 versus LYP(2.5); dP<0.05 versus LYP(5)
Fig. 4.2.4. Effect of CoQ10 and lycopene on caspase-3 activity and NF-kB/p65 levels in MPTP-treated mice. *P<0.05 versus vehicle; †P<0.05 versus MPTP(40); ‡P<0.05 versus LYP(2.5); §P<0.05 versus LYP(5)
Fig. 4.2.5. Effect of CoQ10 and lycopene on PGE$_2$ and PGF$_{2\alpha}$ levels in MPTP-treated mice. 

- $a$P<0.05 versus vehicle;
- $b$P<0.05 versus MPTP(40);
- $c$P<0.05 versus LYP(2.5);
- $d$P<0.05 versus LYP(5)

Veh
MPTP
LYP(10) per se
LYP(2.5)
LYP(5)
LYP(10)
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4.2.4 Discussion

The exact molecular mechanism of progressive loss of neuromelanin containing nigrostriatal dopaminergic neurons in PD remains unknown (Chinta and Andersen, 2006; Paris et al., 2009), yet reports suggest that free radicals might play an important role in PD pathology by inducing oxidative and nitrosative stresses on dopaminergic neurons (Ebadi et al., 2001; Ebadi and Sharma, 2003). Additionally, studies have also shown that failure of the ubiquitin-proteosome system due to impaired iron homeostasis might also play a major role in the pathogenesis of PD (Zhang et al., 2005). It has been shown that MPTP generated ROS involved in accelerating the progressive nigrostriatal neurodegeneration (Berg et al., 2001) via apoptotic or necrotic mechanisms in PD (Kannan and Jain, 2000).

In the present study, MPTP produced significant neurodegeneration as confirmed by various behavioral (locomotor activity, catatonia), biochemical (lipid peroxidation, NPSH, nitrite), mitochondrial (complex-I activity, redox capacity), proinflammatory (NFκB, PGE$_2$, PGF$_{2α}$) and apoptotic (caspase-3) markers. Treatment with lycopene significantly mitigated these alterations and afforded neuroprotection against MPTP in mice. It is assumed that lycopene acted by enhancement of mitochondrial function and/or by reduction of the levels of ROS in the damaged tissue and exerted its neuroprotective effects in attenuating MPTP-induced neurotoxicity in mice.

MPTP acts by impairing the mitochondrial complex-I activity, interferes with electron transport chain and increases the production of ROS (Singer et al., 1987; Sriaram et al., 1997). Oxidative stress is believed to be one of the critical events that lead to NF-κB induction (Schreck et al., 1991). Excessive ROS production might damage lipids, proteins, and DNA in the cell. Free radicals can promote macrophage activation and TNF-α-mediated cytotoxicity (Coutier et al., 1999). TNF-α is a key mediator of mitochondrial oxidative stress (Goossens et al., 1995), because its expression is regulated by NF-κB. The translocation of NF-κB in the nucleus and its binding with target genes requires phosphorylation and proteolysis of inhibitory protein IκB (Baeuerle, 1991). Free radical-induced apoptosis can occur through extrinsic pathways involving a complex interaction of death receptors from the TNF receptor family and...
through intrinsic pathways involving the release of cytochrome c from the mitochondrial membranes as a result of aberrant ionic flux, changes in pH, osmolarity, and transmembrane potential (Buki et al., 2000; Burek and Oppenheim, 1996). Nuclear translocation of NF-κβ in the dopaminergic neurons of PD postmortem brains suggests that apoptotic cell death in PD might occur through NF-κβ-related signaling transduction pathways (Hunot et al., 1997).

There is a large amount of evidence to show that the production of reactive species occurs at the site of inflammation and contributes to tissue damage (Salvemini et al., 1996; Bartels and Leenders, 2007). Moreover, ROS have been involved in many inflammatory-related diseases. The study of ROS and oxidative stress in inflammation is difficult due to the transient nature of ROS, the number and complexity of ongoing processes, and the capacity of ROS to alter a large number of cellular components. Epidemiological studies show that populations consuming a tomato-rich diet, containing high levels of lycopene, exhibit lower incidence of chronic inflammation-related diseases, such as coronary heart disease and certain types of cancers (Rao and Agarwal, 2000). Moreover, increasing investigations have proven that lycopene molecule possesses anti-inflammatory activity on various cellular and animal models of inflammation (Lee et al., 2008; Bignotto et al., 2009).

Several studies have indicated that lycopene is able to react with free radical species, acting as an effective antioxidant and free radical scavenger (Palozza et al., 2010). Lycopene has also demonstrated to have neuroprotective and hypocholesterolemic effects in experimental animals (Fuhrman et al., 1997; Kumar et al., 2009; Sandhir et al., 2010). Therefore, it has been suggested that lycopene can exert modulatory action on inflammation by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. At the moment, the possible molecular mechanisms involved in the anti-inflammatory activities of lycopene include: i) the inhibition of ROS production and the modulation of redox sensitive molecular pathways such as COX-2, LOX, iNOS, NADPH oxidase and transcription factors, NF-κβ; ii) the modulation of cytokine levels (Fig. 4.2.6.). In the present study, lycopene attenuated the oxidative damage in MPTP treated mice may by scavenging the free radicals and preventing the further up regulation of cellular cascades.
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Lycopene has been reported to counteract the effects of iNOS by inhibiting nitric oxide production and/or by decreasing iNOS at protein and mRNA levels (Palmer et al., 1988; Rafi et al., 2007). In an in-vitro study, lycopene demonstrated to inhibit the expression of COX-2 and iNOS genes in RAW 264.7 macrophages (De Stefano et al., 2007). These finding agrees with two recent studies showing that tomato lycopene extract or purified lycopene blocked NF-κB signaling (Kim et al., 2004; Joo et al., 2009). These observations indicate that ROS play an important role in NF-κB activation and inflammatory gene expression. Therefore, keeping in view the in-vitro and in-vivo effects of lycopene, it is plausible that the potent antioxidant and free radical scavenging activity of lycopene may be responsible for attenuating the biochemical and cellular changes in MPTP treated animals (Fig. 4.2.6.). This further might be responsible for the improvement in the behavioral activity of mice such as increased locomotion and decreased severity of catatonia. These findings strengthen the role of ROS and oxidative stress in the pathophysiology of PD.

Fig. 4.2.6. Possible effects of lycopene on oxidative stress during inflammatory processes

A clear understanding of the molecular mechanisms of action of lycopene is crucial in the valuation of this molecule as a potential preventive and therapeutic agent in neuronal disease in which inflammation is the hallmark factor in its progression.