Impaired Mitochondrial functions and Energy Metabolism in MPTP-induced Model of Parkinson’s Disease in Mice: Comparison of Strains and Dose Regimens

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Abstract

Parkinson’s disease is a multiplexed disease involving diverse symptoms and progression rate. Heterogenous diseases need an efficient animal model to enhance the understanding of the underlying mechanism. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin, has been widely used to replicate the pathophysiology of PD in rodents, but its effect on energy metabolic perturbation is limited. Moreover, susceptibility to different dosage regime of MPTP varies among mice strains. Thus, herein the present study compares the effect of acute and sub-acute MPTP dosage regimes on mitochondrial functions in terms of mitochondrial respiratory chain enzymes, mitochondrial swelling and membrane potential in C57BL/6 and Balb/c mice. In addition, activities of enzymes involved in energy metabolic pathways were also studied along with behaviour and neurochemical alterations. The results showed that acute dose of MPTP in C57BL/6 mice had more profound effect on the enzyme activities of electron transport chain complexes. Further, the activity of MAO-A and MAO-B was increased following acute and sub-acute MPTP administration in C57BL/6 mice. However, no significant change was observed in Balb/c strain. Acute MPTP treatment resulted in decreased mitochondrial membrane potential along with swelling of mitochondria in C57BL/6 mice. In addition, perturbations were observed in hexokinase and pyruvate dehydrogenase of glycolysis pathway and citrate synthase, aconitase, isocitrate dehydrogenase and fumarase of TCA cycle. Moreover, acute MPTP led to pronounced depletion in neostriatal dopamine levels in C57BL/6 than in Balb/c mice. Behavioral tests such as open field, Narrow beam walk test and footprint test showed that locomotor activity was drastically reduced as an acute effect of MPTP in C57BL/6 mice strain. Therefore, these results consistently showed that acute MPTP treatment in C57BL/6 strain had severe mitochondrial dysfunctions, perturbed energy metabolic pathways, altered neurotransmission and motor defects as compared to Balb/c strain. Thus, the findings suggest that the dose and strain of mice need to consider for pre-clinical studies targeting mitochondrial dysfunctions in MPTP-induced Parkinson’s disease model.

Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease (Gibrat et al., 2009). It is mainly characterised by resting tremors, rigidity, slowness of movement and postural instability. The main pathological hallmark of PD involves marked loss of nigrostriatal dopaminergic neurons and a depletion of their neurotransmitter, dopamine (DA), in the striatum. It normally affects the people aged 65 years and above and estimates indicate over 60,000 people are diagnosed each year (DeMaagd and Philip, 2015). PD involves complex multi-factorial etiology, developing from environmental, genetic or complex gene-environment interaction (Braak and Del Tredici, 2008). Animal models of human disorders are indispensable tool for basic research and are prerequisite to investigate therapeutic strategies (Cenci et al., 2002). PD can be developed in animals using various procedures that recreate pathological events. The genetic model of PD comes up with few challenges. Firstly, they have not been reported with DA nigral cell loss and are not suitable for testing therapeutic strategies. Secondly, the genetic models do not consider the environmental triggers of the disease that
are predominant in PD (Masliah et al., 2000; Hashimoto et al., 2003). Over the last few years, environmental toxins are taken into account for the development of PD, for example pesticides such as paraquat, maneb and rotenone (McCormack et al., 2002; Perier et al., 2003). These models do not completely replicate the neuropathological conditions of the disease and also lack consistency and specificity. The 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD model is the most widely used animal model in PD research (Meredith and Rademacher, 2011). It has been applicable to wide variety of animal species, ranging from fruit flies and worms to mammals. The most frequently used animals have been monkeys and mice (Przedborski et al., 2001). MPP+, an active metabolite of MPTP inhibits Complex-I of electron transport chain. As a result, a rapid decrease in ATP content occurs in the striatum and SNpc, the brain regions most sensitive to MPTP-induced neurotoxicity. Interestingly, a significant ATP depletion can result from as little as 25% inhibition of complex I (Davey et al., 1998). This leads to loss of DA nerve terminals in striatum and DA cell bodies in SNpc, which is responsible for the loss of dopamine inside the brain. In order to establish a rodent model that closely resembles PD, it is crucial that it exhibits as many of the phenotypic features of the disease as possible. For instance, mice inbred strains C57BL/6 and Balb/c have been shown to replicate PD symptoms when treated with different doses of MPTP but they represent different degrees of responses. In addition to this there are many reports that showed dopaminergic neuronal degeneration and behavioural changes in MPTP based C57BL/6 and Balb/c model of PD, none of the studies have shown the energy metabolic perturbation in MPTP mice model of PD.

Mitochondria are organelles that maintain cellular energetic status and are known to play key role in energy metabolism. Oxidative phosphorylation plays an essential role in the production of ATP from ADP in the mitochondria, which drives the neurons to exert variety of physiological functions including neurotransmission and osmoregulation. Alterations in energy metabolic pathway have been observed in PD patients (Ahmed et al., 2009). It includes diminished activities of hexokinase and pyruvate dehydrogenase complex, two very important enzymes of glucose metabolism yielding reduced levels of acetyl-Coenzyme A. Furthermore, it has been reported that aconitase activity was inhibited by PINK 1 mutation and complex I inhibitor, paraquat (Anandhan et al., 2017). Additionally, glucose hypometabolism induced by dysfunction of oxidative phosphorylation is also linked to PD pathophysiological alterations (Eberling et al., 1994). Therefore, there is a necessity to understand the role of energy metabolism in the development of PD.

In the present study, we investigated the perturbed energy metabolism in both acute and sub-acute MPTP mice model and compared it in C57BL/6 and Balb/c mice strains. The study also provided additional evidence for the validity of the C57BL/6 strain in MPTP model of PD through behavioural and neurochemical studies.

Materials And Methods

Animals and treatment schedule
Eight- to ten-week-old male, C57BL/6 and Balb/c strain mice weighing 22-25 g were procured from the Central Animal House of the Panjab University, Chandigarh. The animals were acclimatized to the local vivarium before the study and were fed with standard pellet diet (Ashirwad Industries, Kharar, Punjab, India). The procedures followed for the study were approved by the Institutional Animal Ethics Committee (PU/IAEC/S/15/107) and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The mice were randomly segregated into the following three groups with each group having 5-6 animals:

**Control:** Animals received normal saline (Vehicle for MPTP) intra-peritoneally throughout the study.

**Acute:** Animals received four injections of MPTP (20mg/kg b.w) intraperitoneally at 2h interval in a single day.

**Sub-acute:** Animals received MPTP (20mg/kg b.w) intraperitoneally daily for 5 consecutive days.

The treatment paradigm followed for the study is illustrated in [Figure 1](#).

**Mitochondrial functions**

**Preparation of homogenate and mitochondrial fractions**

Mitochondrial fractions were prepared from the midbrain region of the brain as described by Puka-Sundvall *et al.*, 2000. Briefly, 10% (w/v) homogenate was prepared in ice-cold buffer A [10 mM Tris-HCl (pH 7.4), 0.44 M sucrose, 10 mM ethylene diamine tetra-acetic acid (EDTA) and 0.1% (w/v) bovine serum albumin (BSA)] by using Potter-Elvehjam-type glass homogenizer and the homogenate was centrifuged at 2100 g for 15 min at 4 °C. The supernatant was further centrifuged at 14000 g for 15 min at 4 °C. The crude mitochondrial pellet was separated and washed with buffer A and again centrifuged at 7000 g for 15 min at 4 °C. The final mitochondrial pellet was re-suspended in buffer B [10 mM Tris-HCl (pH 7.4) and 0.44 M sucrose]. Various biochemical assays were performed in homogenate or mitochondrial fractions. The protein concentration was estimated by Lowry’s method using BSA as standard (Lowry et al., 1951).

**Mitochondrial respiratory chain complexes**

The activity of NADH dehydrogenase (EC 1.6.99.3, Complex-I) was measured colorimetrically as described by King and Howard, 1967. Succinate dehydrogenase (EC 1.3.5.1, Complex-II) activity was assayed in the mitochondrial samples according to the method of King *et al.*, 1976. Cytochrome c oxidase (EC 1.9.3.1, Complex-IV) activity was assayed as described by Sottocasa *et al.*, 1967. F1-F0 ATP synthase (EC 3.6.3.14, Complex-V) activity was assayed according to the method of (Griffiths and Houghton, 1974)

**Monoamine oxidases activity**

MAO-A & B activities were assayed by the method as described by Gupta *et al.*, 2014 with slight modifications. The assay mixture contained 0.1 ml of 4mM of 5-HT and 0.1ml of 4mM of benzyl-amine
as the specific substrate for MAO-A and MAO-B respectively, mitochondrial fraction and 2.75ml of sodium phosphate buffer (pH 7.4). The change in absorbance was recorded at 280 nm and 249.5 nm for MAO-A and MAO-B respectively for 5 minutes against the blank containing sodium phosphate buffer and 5-HT or benzyl-amine.

**Mitochondrial swelling**

Permeability transition pore opening was assayed spectrophotometrically as described by Kristián *et al.*, 2000. Freshly isolated mitochondria were pre-incubated at 37 °C for 5 min in the reaction mixture [125 mM sucrose, 50 mM KCl, 2 mM KH₂PO₄ and 10 mM 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES)]. The swelling was initiated by the addition of 40–200 μM CaCl₂ to the reaction mixture. Mitochondrial swelling was monitored as changes at 540 nm at 1 min interval over 5 min. The results were expressed as A₅₄₀ nm (arbitrary units)/mg protein.

**Mitochondrial membrane potential**

Mitochondrial membrane potential was measured through flow cytometry according to the method described by Jogdand *et al.*, 2012. Briefly, the midbrain tissues were minced with sterile surgical blades in Dulbecco's Modified Eagle's Medium (DMEM) containing 2% (v/v) fetal bovine serum (FBS), 2mg/ml sodium carbonate, 100g/ml streptomycin, 100 units/ml penicillin and prepared in 1M HEPES buffer. The entire content was centrifuged at 200 x g for 5 min at room temperature to collect the pellet. Pellet was then re-suspended in complete DMEM. The resuspended pellet was homogenised followed by collagenase digestion (0.2 mg/ml in DMEM without FBS). The entire suspension was then subjected to DNase digestion (0.015 mg/ml in 0.1M PBS) and allowed to filter by 100μm cell strainer. The complete filtration and collection in a 50 ml falcon tube was done by centrifuging it at 500 x g for 10 min along with cell strainer. The cells were then washed and re-suspended in only DMEM before counting the cell number using Bright-Line™ Hemocytometer (Sigma-Aldrich Co., St. Louis, MO, USA).

The isolated cells were stained by Mitotracker Red CMXRos and Hoechst 33258 according to the protocol described by Cottet-Rousselle *et al.*, 2011; Jogdand *et al.*, 2012. Briefly, Cells at a concentration of 10⁶ cells/ml were incubated in a final concentration of 5nM for Mitotracker Red CMXRos dye and 5μg/ml of Hoechst 33258 for 30min. in dark at 37°C. The results in flowcytometer were recorded at an excitation/emission of 579/599 nm for Mitotracker Red CMXRos and 352/481 nm for Hoechst 33258.

**Energy metabolism**

**Glycolytic Enzymes**

**Hexokinase activity**

Hexokinase catalyzes the important irreversible step of glucose metabolism. Hexokinase is present in the cytosol of the cell. Consequently, its catalytic activity was assayed spectrophotometrically in the cytosolic fraction as described by Tegge, 1987. The reaction mixture (final volume of 1ml) contained 50 mM of
TEA buffer, 555 mM of D-glucose solution, 100 mM of magnesium chloride, 19 mM of ATP, 14 mM of NADP$^+$. The reaction was initiated by the addition of mitochondrial preparation. The reagents were mixed and equilibrated to 25°C. Absorbance was read at 340nm to measure the production of NADPH for 10 mins. Enzyme activity was calculated using the molar extinction coefficient of NADPH (6.22 mM$^{-1}$ cm$^{-1}$) and the results were expressed as nmoles/min/mg of protein.

**Pyruvate dehydrogenase complex (PDHc)**

PDHc plays an indispensable part in glucose metabolism. Therefore, the activity of PDHc was measured in post mitochondrial supernatant as described by Ke et al., 2014. Briefly, the reaction was initiated by addition of phosphate buffer (50mM, pH 7.6), MgCl$_2$ (1.0mM), TPP (0.2mM), MTT (0.5mM), PMS (6.5 mM), Sodium pyruvate (2.0 mM) to the test sample and absorbance read at 560nm. Enzyme activity was calculated using molar extinction coefficient of PMS-MTT (18.65mM$^{-1}$ cm$^{-1}$) and the results were expressed as nmoles /min/mg protein.

**TCA Cycle Enzymes**

**Citrate synthase (CS)**

CS activity was measured by the method described by Spinazzi et al., 2012 with slight modifications. Adequate amount of sample was added to a cuvette containing 300 µl of distilled water, 500 µl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (vol/vol)), 100 µl of DTNB, 30 µl of acetyl CoA (10 mM) and adjusted the final volume with distilled water. The absorption at 412 nm was followed for 3 minutes to measure baseline activity (acetyl-CoA deacylase activity). The citrate synthase reaction is then started by the addition of 50 µl of 10 mM oxaloacetic acid and then absorbance was read at 412nm. Linear rates were obtained for at least 3 minutes. CS activity was calculated using molar extinction coefficient of TNB (13.6 mM$^{-1}$ cm$^{-1}$) and the results were expressed as nmole/min/mg protein.

**Aconitase**

The activity of aconitase enzyme was measured spectrophotometrically as described by Morton et al., 1998. Briefly, the mitochondrial sample was added to Tris-MnCl$_2$- citrate buffer (50 mM Tris·HCl, pH 7.4, 0.6 mM MnCl$_2$, and 5 mM sodium citrate). The reaction was initiated by the addition of NADP$^+$ (0.2mM) and Isocitrate dehydrogenase (0.03U/mg). The activity was measured as the change in absorbance at 340 nm for 10min and results were expressed as nmol/min/mg protein using molar extinction coefficient of NADPH (6.22 mM$^{-1}$ cm$^{-1}$).

**Isocitrate dehydrogenase**

NADP$^+$-linked isocitrate dehydrogenase was measured spectrophotometrically at 37°C by method given by Ellis and Goldberg, 1971. The final reaction medium, in a volume of 1ml, contained 0.1 mM TEA buffer (pH 7.4), 0.1 mM NADP$^+$, 100 mM MnCl$_2$ and the requisite amount of mitochondrial preparation. The
contents of the reference cuvette were the same as the test cuvette. The reaction was initiated by the addition of a requisite amount of 67 mM of isocitrate in test cuvette and distilled water in reference cuvette. An increase in NADPH production was monitored at 340 nm for 5 min and results were expressed as nmol/min/mg protein using molar extinction coefficient of NADPH (6.22 mM⁻¹ cm⁻¹).

**Fumarase**

Fumarase activity was assayed spectrophotometrically as described by Kanarek and Hill, 1964. Briefly, mitochondrial preparation was added to the reaction mixture containing 50 mM malate, 50 mM potassium phosphate buffer. An increase in absorbance was monitored at 250 nm for 10 min and results were expressed as nmol/min/mg protein using molar extinction coefficient of fumarate (2.44 mM⁻¹ cm⁻¹).

**Estimation of neurotransmitter levels**

The mice were killed by cervical dislocation 1 week after MPTP treatments. The striatum was then rapidly dissected out and stored at −80°C for further analysis. A 10% tissue homogenate was prepared in 0.1 M perchloric acid using a Potter Elvehjem type homogenizer, centrifuged at 12,000 g for 5 minutes and the supernatant was filtered through 0.25 μm nylon filters. Dopamine, DOPAC, and serotonin were estimated by HPLC with an electrochemical detector by the method of (Church, 2005). Briefly, 20 μl of the sample was injected into the injector valve of HPLC having a high-pressure isocratic pump, C18 reverse-phase column (type: Waters Symmetry C18 (5 μm); length and diameter: 4.6 × 250 mm), and electrochemical detector. The mobile phase consisted of 2% citric acid, 2% KHPO4, 1 mM EDTA, 1.2% methanol and 70 mg/ml of sodium octyl sulfate. The pH of the mobile phase was adjusted to 3.0 with the help of 6N HCl. Electrochemical conditions for the experiment were set at +0.800 V and sensitivity ranged from 5–50 nA. The separation was carried out at a flow rate of 1 ml/minute. Data were recorded and analyzed using Empower software (Waters, Milford, MA, USA) and results were expressed as ng/mg protein.

**Neurobehaviour studies**

Open field test, Narrow beam walk test, Footprint test, and Catalepsy were performed to assess the motor coordination, muscular rigidity, and locomotor activity. All animals were acclimatized to the behaviour room for 30 min prior to the tests. The behaviour data were acquired and analyzed using ANY-maze tracking software (Stoelting Co., Wooddale, IL, USA).

**Open field test**

Spontaneous locomotor activity of animals was assayed using the open-field test. An enclosed square test chamber (50 cm x 50 cm with 40 cm height) was used to assess the locomotor and anxiety-like behavior (Seibenhener and Wooten, 2015). Animals were placed in the center of the arena and observed for 3 min. Apparatus was cleaned with 95% (v/v) ethanol before use and in between each test to remove
odour cues. The data in terms of total distance travelled (m) and total immobile time (s) were recorded to evaluate the horizontal activity. The rearing behavior in terms of vertical activity was also recorded.

*Narrow beam walk test*

Motor coordination and balance were evaluated by the ability of mice to traverse a narrow beam of 1-meter length resting 50cm above the tabletop on two poles. Mice were trained to traverse a beam of 12 mm in diameter and then of 6 mm in diameter. Latency to traverse each beam and paw slips were recorded (Carter *et al.*, 2001; Southwell *et al.*, 2009).

*Footprint test*

This test was used to assess the gait abnormalities in animals as described previously by (Cirillo *et al.*, 2010). To obtain footprints, the hind- and forefeet of the mice were coated with green and red nontoxic paints, respectively. The animals were then allowed to walk along a 50-cm-long, 10-cm-wide runway (with 10-cm-high walls) into an enclosed box and a sheet of white paper was placed on the floor of the runway. To characterize the walking pattern of each mouse we measured the average distance between each stride (stride length, SL), step or stride width and paw overlap (all measured in centimetres).

*Catalepsy bar test*

Catalepsy bar test is used to measure muscular rigidity (Fischer *et al.*, 2002). Animals were tested on a horizontal bar 15 cm long, had a diameter of 0.5 mm and were maintained horizontal 5.5 cm above the surface level. This was done by lifting the mouse by the tail and placing it with its front paws on a steel bar and the hind legs on the plane surface. Descent latency was measured as the time span from placing the animal on the bar until one forepaw touched the floor or the hind legs left the floor to climb onto the bar. A cut-off time of 180 s was used, i.e. the trial was terminated when the animal did not make an active paw movement within that time.

*Statistical analysis*

All values are expressed as mean ± standard error mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA) followed by post hoc test for multiple pairwise comparisons between groups. Values with \( p < 0.05 \) were considered as statistically significant.

*Results*

*Effect on mitochondrial functions*

*Mitochondrial respiratory chain complexes*

Table 1 depicts the activity of mitochondrial electron transport chain complexes. In the present study, both acute and sub-acute MPTP treatment resulted in a decrease in NADH dehydrogenase activity in C57BL/6 mice while no significant change was observed after both MPTP regimes in Balb/c mice.
Further, in C57BL/6 mice, the activity of succinate dehydrogenase has also been observed to be diminished in acute as well as sub-acute groups. Additionally, acute MPTP treatment did not show any effect on the activity of cytochrome c oxidase in both the strains. However, a significant increase in cytochrome c oxidase activity was observed in sub-acute treated C57BL/6 mice as compared to Balb/c. Moreover, ATP synthase i.e. Complex V activity was found to be reduced in acute group as compared to the control group in both the strains. Interestingly, the sub-acute group showed a significant increment in Complex V activity as compared to the acute group in both C57BL/6 and Balb/c strains.

**Monoamine oxidases**

As dopamine gets metabolized by both MAO-A and B enzymes, the activities of both were measured spectrophotometrically. It was observed that acute and sub-acute treatment increased the activities of both MAO-A and MAO-B in C57BL/6 mice as compared to control whereas Balb/c mice with both acute and sub-acute MPTP intoxication did not show any alterations in MAO-A and B activities (Figure 2A and B).

**Mitochondrial swelling**

In C57BL/6 mice, mitochondrial swelling was significantly increased (64.39%) in acute MPTP group as compared to control. In contrast, mitochondrial swelling was not affected by the sub-acute MPTP administration. In Balb/c mice, no significant change was observed in mitochondrial swelling in both acute and sub acute group as compared to the control group (Figure 3).

**Mitochondrial membrane potential**

Mitochondrial membrane potential is established by the proton pump of ETC. Maintenance of the $\Delta \Psi_m$ is necessary for cell survival. However, the disruption or collapse of the $\Delta \Psi_m$ was consistently observed in cells following treatment with mitochondrial toxin, MPTP. The $\Delta \Psi_m$ was quantified by staining cell with fluorochrome MitoTracker Red (CMXRos). In C57BL/6 strain, acute MPTP administration resulted in significant decrease in membrane potential, while no significant change was observed in Balb/c strain as compared to control. However, a significant change was observed in acute MPTP group in between the two strains. Surprisingly, 1.5 fold increased in membrane potential was found in sub-acute treated C57BL/6 strain as compared to the control. The increment of 1.8 fold was found following sub-acute treatment in C57BL/6 strain as compared to acute MPTP group. However, no significant difference was found in Balb/c strain treated with sub-acute MPTP as compared to their respective control (Figure 4).

**Effect on energy metabolism**

**Glycolytic Enzymes**

*Table 2* depicts the activity of enzymes involved in energy metabolic pathways-

*Hexokinase*
Hexokinase initiates the energy metabolic pathway by catalyzing the transfer of phosphate group from ATP to glucose to form glucose-6-phosphate. In C57BL/6 mice, a significant decrease in hexokinase activity was observed in acute (31.35%) as well as the sub-acute group (25.24%) as compared to the control group. Similarly, in Balb/c mice, hexokinase activity was also observed to be declined in both acute (35.44%) and sub-acute groups (27.21%).

**Pyruvate dehydrogenase complex**

The glycolysis and TCA cycle are connected by pyruvate dehydrogenase complex that forms acetyl CoA from pyruvate. Both acute (36.13%) and sub-acute MPTP (16.9%) treatment significantly reduced the activity of pyruvate dehydrogenase in C57BL/6 when compared to control group. In Balb/c mice, both treatment regimes did not affect the enzymatic activity. However, a significant difference was observed in acute as well as sub-acute group in between the two strains.

**TCA Cycle Enzymes**

**Citrate synthase**

Citrate synthase initiates the TCA cycle by catalyzing the condensation of oxaloacetate and acetyl CoA to form citrate. Both acute (49.91%) and sub-acute (76.38%) MPTP treatment significantly reduced the activity of citrate synthase as compared to control in C57BL/6 strain. Similarly, Balb/c mice also exhibited a significant decrease in citrate synthase activity in the acute group (13.06%). However, sub-acute treatment did not affect the enzymatic activity in Balb/c mice.

**Aconitase**

Aconitase catalyzes the isomerization of citrate into isocitrate in two-step reaction which involves dehydration step followed by rehydration step. Aconitase activity was found to be declined in the C57BL/6 strain in acute (19.18%) as well as sub-acute group (28.3%) as compared to control group. In Balb/c mice, acute treatment significantly diminished the activity of aconitase (43.3%) while sub-acute treatment did not show any effect. Moreover, while comparing the two strains, a significant difference was observed in the acute group.

**Isocitrate dehydrogenase**

The activity of isocitrate dehydrogense was found to be unaffected in acute and sub-acute MPTP regimes in both the strain.

**Fumarase**

Fumarase catalyzes the reversible hydration/dehydration of fumarate to malate which then provides cells with NADH. Both acute (49.54%) and sub-acute MPTP (42.62%) treatment significantly increased the activity of fumarase when compared to control group in the C57BL/6 strain. However, in Balb/c strain, both treatment regimes did not alter the enzymatic activity.
**Neurotransmitter levels**

Dopamine, serotonin and DOPAC, a metabolized product of dopamine was presently measured in the striatal brain region of C57BL/6 and Balb/c mice strains after MPTP administration by HPLC. Table 3 depicts that acute MPTP intoxication in the C57BL/6 strain resulted in an approximately 37% decrement of dopamine however sub-acute MPTP intoxication did not statistically affect the dopamine levels. In balb/c strain, both acute and sub-acute MPTP intoxication did not affect the dopamine levels. Surprisingly, a significant difference was observed in dopamine content in the control group of both strains. Furthermore, acute MPTP administration significantly increased the levels of DOPAC and serotonin in the C57BL/6 strain, whereas levels were observed to be unaffected in sub-acute treatment as compared to the control group. In contrast to these results, both acute as well as sub-acute regime did not alter serotonin and DOPAC levels in Balb/c mice.

Ratio DOPAC/DA which represents dopamine turnover was also determined. In C57BL/6 mice, acute MPTP intoxication significantly increased the dopamine turnover whereas sub-acute regime did not affect dopamine turnover. However, a significant difference was observed between the two regimes. Contrary to this, in Balb/c mice, no change was observed in dopamine turnover in acute as well as the sub-acute group.

**Motor behaviour**

**Open field test**

Locomotor activity evaluated using open field test on the 7th day after MPTP administration. Horizontal activity and vertical activity in terms of total distance travelled and number of rearing was assessed which was also evident from the track plot reports (Figure 5A). In C57BL/6 mice, acute treatment significantly decreased the horizontal (75%) and vertical activity (92%) whereas, the time in which mice remain immobile was shown to be elevated by acute treatment (Figure 5 B and C). Sub-acute treatment did not affect the total distance travelled while it significantly declined the vertical movements (80%) and increased the immobility time. Moreover, an increase in immobility time was more in the acute group than the sub-acute group (Figure 5D). In contrast to C57BL/6 mice, Balb/c exhibited a significant decline (30%) in immobility time in the sub-acute group as compared to the control group. However acute and sub-acute treatment did not affect the horizontal and vertical movement. Furthermore, while comparing both the strains, a significant difference was observed in distance travelled and immobility time in the acute group. Additionally in the sub-acute group, immobility time was observed to be significantly different in both strains.

**Narrow beam walk test**

Motor function and hind limb impairment were assessed by the time taken to traverse the beam and paw slips in the narrow beam walk test. Figure 6A depicts that acute MPTP treatment in C57BL/6 mice significantly increased the time taken by the animals to cross the beam (92.8%) whereas, it was found to
be unaffected by sub-acute treatment. However, both acute and sub-acute regimes in Balb/c mice did not affect the time taken to traverse the beam. Moreover, the number of paw slips was unaffected in acute as well as sub-acute regimes in both the strain (Figure 6B).

**Catalepsy bar test**

Catalepsy bar test is used to assess muscular rigidity in rodents. Catalepsy is a complex motor inhibition in which rodents are unable to correct externally imposed abnormal posture and revert to a normal position for initiation of exploratory behaviour. Thus, acute MPTP intoxication in C57BL/6 strain increased muscular rigidity, while Balb/c strain did not show any cataleptic behaviour in both acute and sub-acute regimes (Figure 7).

**Footprint test**

Gait abnormalities were assessed by the footprint test. Representative footprints showed the parameters analysed such as stride length, stride width and paw overlap (Figure 8A). Stride length was significantly decreased in the acute group (46.66%) of C57BL/6 strain however sub-acute MPTP administration did not affect the stride length (Figure 8B). In contrast to this, Balb/c mice does not exhibited any change in the stride length in both the groups as compared to control. The impaired movement was also evident by significant increase in paw overlap (50%) in the acute group of C57BL/6 mice whereas both treatment regimes did not affect the stride width in both strains (Figure 8 C and D, respectively).

**Discussion**

In the animal model of PD, various agents are used in inducing PD symptoms in mice. Out of these, MPTP intoxication is a well established method to mimic PD symptoms (Meredith and Rademacher, 2011). However, to decipher the mechanism of PD and study the effect of anti-parkinsonism drugs, a specific neurotoxin based animal model is required. The factors such as neurotoxin dose, time of dose, strain selectivity are very important while considering the animal model. In this study, we compared the C57BL/6 and Balb/c mice strains in response to different doses of MPTP in terms of behavioural tests, dopamine depletion and metabolic changes. It has been found that C57BL/6 mice are more susceptible to MPTP than the Balb/c mice. Firstly, the effect of MPTP on the nigrostriatal pathway showed that acute MPTP administration in C57BL/6 mice reduces endogenous dopamine levels in the striatum which was followed by an increase in the DOPAC and DOPAC/Dopamine ratio. Similarly, Lee et al. (2013) reported that MPTP caused a severe reduction of dopamine in substantia nigra and striatum of C57BL/6 mice when compared with Balb/c and ICR strains. But MPTP susceptibilities between different mice strains in terms of mitochondrial functions and bioenergetics are not known that hinders research concerning mitochondria in the MPTP model of PD.

In this study, we further evaluated the enzymatic activity of monoamine oxidases as well as the activity of Electron Transport Chain (ETC) enzymes. MAO-B levels are known to increase with age and in association with neurodegenerative disease in both humans and mice (Saura et al., 1994; Fowler et al.,
MAO-B catalyzes ROS production through substrate oxidation and subsequent reduction of oxygen to $\text{H}_2\text{O}_2$. Therefore the age-related increase in MAO-B activity leads to cellular degeneration in the brain. Presently, MAO-B activity was also found to be elevated in C57BL/6 strain after acute and subacute MPTP intoxication while no significant results were found in Balb/c strain. MAO-B enzyme also metabolizes the non-toxic MPTP into the toxic cation $1\text{-methyl-4-phenylpyridinium}$ (MPP+) inside the brain. MPP+ inhibits complex I of the ETC in the dopaminergic neurons that leads to the production of toxic free radicals and ultimately causes cell death. In PD patients, mitochondrial complex I activity has been reported to decrease in the SN, striatum and frontal cortex (Schapira et al., 1990; Mizuno et al., 1989; Parker et al., 2008). Therefore the status of mitochondrial complexes has been investigated to unveil the underlying mechanism of MPTP-induced dopaminergic neurotoxicity in the mice model of PD among two strains. There was severe impairment of mitochondrial complexes (I, II and V) activity in acute treated C57BL/6 mice. Moreover, the activity of complex IV and V were increased in sub-acute treated C57BL/6 mice. In parallel, mitochondrial membrane potential was also evaluated in the midbrain region of C57BL/6 and Balb/c animals. Acute MPTP intoxication in both C57BL/6 and Balb/c strain resulted in significant decrease in membrane potential. Thus, these findings showed mitochondrial abnormalities in MPTP mice model of PD.

Energy metabolism is very important in maintaining the anti-oxidant ability of neurons to protect them from internal and external damage. Studies of post-mortem brain tissue from PD patients and animal models have provided evidence for increased levels of oxidative stress, mitochondrial dysfunction and impaired glucose uptake in vulnerable neuronal populations (Mattson et al., 1999). In the present study, we evaluated the effect of acute and sub-acute MPTP administration on some enzymes involved in different metabolic pathways. Acute and sub-acute treatment severely altered the activities of enzymes such as hexokinase, pyruvate dehydrogenase, citrate synthase, aconitase, fumarase in C57BL/6 strain mice. The observed reduction in hexokinase activity is associated with impaired glucose uptake Mattson et al., 1999. This showed that the glucose metabolic pathway is inhibited at first step in PD. Reduced PDHc activity may correspond to an increased concentration of pyruvate. Similarly, Ahmed et al. (2009) reported the accumulation of pyruvate in plasma of PD patients. Moreover, increased pyruvate in CSF has already been reported in Alzheimer’s patients (Parnetti et al., 1995). Furthermore, reduced citrate synthase activity are also supported by Schapira (2007) in the substantia nigra region of multiple system atrophy (Parkinsonian- like disorder) patients. As aconitase is an iron-sulfur (Fe-S) protein, it is believed that MPTP causes superoxide radical toxicity via inactivation of mitochondrial aconitase (Liang and Patel, 2004). Presently, we also found the reduced activity of aconitase after acute as well as sub-acute MPTP administration. Surprisingly, the current study has shown the increment in fumarase activity in both the groups which was not reported until now. Unlike C57BL/6, PDHc, fumarase was found to be unaltered in both the groups of Balb/c mice while a decrease in activities of CS, aconitase was observed in the acute group only. Interestingly, acute as well as sub-acute MPTP treatment resulted in the reduction of hexokinase activity. Furthermore, the increase in mitochondrial swelling also reflects mitochondrial dysfunction which was observed in the acute group of C57BL/6 strain only while the rest of the groups
were unaltered. Previous studies on PD have also shown an increase in mitochondrial swelling (Darios et al., 2003, Berman and Hastings 2001).

In addition to these results, acute MPTP administration severely affected locomotor and co-ordination abilities of C57BL/6 mice which were tested through Open field test, Footprint test, Narrow beam walk, and Catalepsy test. In the Open field test, all the parameters (total distance, Rearing, Immobility time) were significantly altered by acute MPTP treatment. Motor coordination was also affected by acute treatment in the Narrow beam walk test. Further, in the footprint test, reduction in stride length and increased paw overlap was observed only in the acute group of C57BL/6 strain while stride width was unaffected and the Catalepsy test displayed an insignificant increase in latency time after acute MPTP treatment. On the contrary, Balb/c mice strain exposed to acute and sub-acute treatment didn't show significant changes in the behavioral tests. Similarly, Sedelis et al. (2000) also showed that acute MPTP exposure to C57BL/6 and Balb/c exhibited reduced locomotor activity but Balb/c mice recover faster to control levels than C57BL/6 mice. It is also related to the dopamine levels which showed no significant differences in both groups of Balb/c mice.

In conclusion, the current study compares the two strains of mice- C57BL/6 and Balb/c in terms of mitochondrial dysfunctions and motor functions induced by the DAergic toxin MPTP. It was found that acute MPTP treatment had more pronounced effect on mitochondrial functions, neurotransmitter levels and motor functions in the C57BL/6 strain than in the Balb/c strain. The results clearly demonstrate that C57BL/6 strain of mice is more susceptible than Balb/c strain to MPTP toxin. Thereby, suggesting their efficacy in evaluating potential anti-PD drugs.

Declarations

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Author’s contribution

AP and RS conceptualized and designed the study. AP and PG conducted experiments and analyzed the data. All authors contributed in drafting and editing the manuscript.

Conflict of Interest

The authors declare no competing conflict of interest.

Availability of data and material
The data that support the findings of this study are available on reasonable request from the corresponding author.

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Tables

Due to technical limitations, the tables are only available as downloads in the supplementary files section.

Figures

![Figure 1](image-url)
Experimental design used for acute and sub-acute MPTP administration.

**Figure 2**

Effect of acute and sub-acute MPTP administration on MAO-A (A) and MAO-B (B) enzyme activity in C57BL/6 and Balb/c mice. Values are expressed as mean ± SEM; n=5. *significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p<0.05), $significant difference between acute and sub-acute MPTP treatment (p<0.05).
Figure 3

Effect of acute and sub-acute MPTP administration on mitochondrial swelling in C57BL/6 and Balb/c mice. Values are expressed as mean ± SEM; n=5. *significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p< 0.05), $significant difference between acute and sub-acute MPTP treatment (p< 0.05).
Effect of acute and sub-acute MPTP administration on mitochondrial membrane potential in C57BL/6 and Balb/c mice: Representative plots for mitotracker CMXRos and Hoechst 33342 positive cells isolated from midbrain region of C57BL/6 (A) and Balb/c (B) assessed by Flowcytometric analysis. Mean Fluorescence intensity for mitotracker CMXRos (C). Values are expressed as mean ± SEM; n=5.
*significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p< 0.05), $significant difference between acute and sub-acute MPTP treatment (p< 0.05).

**Figure 5**

Effect of acute and sub-acute MPTP administration on open field test in C57BL/6 and Balb/c mice: Representative track plots of animals from various groups (A); Total distance travelled (B); Rearing (C); Immobility time (D); Values are expressed as mean ± SEM; n=5. * significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p< 0.05), $significant difference between acute and sub-acute MPTP treatment (p< 0.05).
Figure 6

Effect of acute and sub-acute MPTP administration on narrow beam walk test by C57BL/6 and Balb/c mice. Time to traverse the beam (A); Paw slips (B). Values are expressed as mean ± SEM; n=5.

*significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p< 0.05), $significant difference between acute and sub-acute MPTP treatment (p< 0.05).
Figure 7

Effect of acute and sub-acute MPTP administration on descent latency assessed by catalepsy bar test for C57BL/6 and Balb/c mice. Values are expressed as mean ± SEM; n=5. *significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p<0.05), $significant difference between acute and sub-acute MPTP treatment (p<0.05).
Figure 8

Effect of acute and sub-acute MPTP administration on gait analysis for C57BL/6 and Balb/c mice. Representative footprints indicating parameters analysed such as stride length, stride width and paw overlap (A); stride length (B); paw overlap (C); stride width (D); Values are expressed as mean ± SEM; n=5. * significantly different from control group (p<0.05), #significant difference between C57BL/6 and Balb/c strain (p< 0.05), $significant difference between acute and sub-acute MPTP treatment (p< 0.05).

Supplementary Files

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