# Assessment of gait dynamics in rotenone-induced rat model of Parkinson's disease by footprint method

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**Abstract**: Rotenone (organic pesticide and inhibitor of mitochondrial complex I) is used to generate an experimental model of Parkinson's disease (PD). In the present study, we investigated rotenone-induced locomotor deficits, gait dynamics and muscular weakness in rats. The study also determined dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) levels following rotenone administration. In the study, adult male rats were administered subcutaneously (s.c.) with rotenone (1.5 mg/kg/day) for 8 days. Motor activities were monitored by the Kondziela's inverted screen test, beam walking test and footprint test. Animals were decapitated after behavioral analysis and brains were dissected out for neurochemical estimation. Results showed that the levels of DA and DOPAC were significantly decreased, which further supported by significant impaired motor coordination in rotenone treated rats. In conclusion, the behavioral and neurochemical findings of our study further strengthen the previous report and emphasizes on short term administration of rotenone producing PD-like symptoms in rats.

**Keywords**: Parkinson's disease, rotenone, gait dynamics, dopamine, motor impairment.

## INTRODUCTION

Rotenone is widely used as an organic pesticide and a specific inhibitor of mitochondrial complex I. It is lipophilic in nature and can readily cross the blood brain barrier, entering into dopaminergic neurons because it does not depend on dopamine (DA) transporters, resulting in complex I inhibition (Alam and Schmidt, 2002). Rotenone causes systemic inhibition of mitochondrial complex I, which results in the production of reactive oxygen species (ROS) that block the electron transport chain, and cause neurodegeneration (Moon *et al.,* 2005). Rotenone exposure recapitulates most of the mechanism thought to be important in PD pathogenesis such as oxidative damage, the selective degeneration of nigrostriatal dopaminergic neurons, and movement disorders (Betarbet *et al.,* 2002). It has been reported that administration of rotenone in rats developed behavioral, neurochemical and biochemical changes similar to that observed in PD (Betarbet *et al.,* 2002; Betarbet *et al.,* 2000). Although, rotenone is widely distributed in the brain, it can cause selective neurodegeneration in specific brain regions (Greenamyre *et al.,* 2011), and has been directly associated with the onset of PD.

PD is a chronic, progressive and the second most common age-related neurodegenerative disorder (Tsouli and Konitsiotis, 2010). Degeneration of dopaminergic neurons results in the depletion of DA neurotransmitter, which results in extrapyramidal motor dysfunction (Calne, 2001). Tremors, muscular rigidity (stiffness), bradykinesia (slowness of movement) and impairment of balance are most common motor symptoms of PD. The precise cause of PD is remaining unknown but PD is accepted as multifactorial disease. Familial PD has been linked to mutation in genes and the cause of sporadic PD is unknown but both environmental and genetic factors contribute in sporadic PD (Tieu, 2011). Perhaps, there is not one single factor responsible for neurodegeneration (Auluck *et al.,* 2002). A number of mechanisms are involved in PD such as oxidative stress, mitochondrial defects, proteolytic stress and neuroinflammation (Blandini and Armentero, 2012). Evidence showed that mitochondrial dysfunction mainly to the respiratory chain of complex I results in oxidative stress which underlies the pathology of PD (Betarbet *et al.,* 2000; Tieu, 2011).

In order to develop therapeutic strategies for PD it is important to understand its etiology and mechanism of neurodegeneration. A perfect model of PD should consist of pathology and clinical features of PD in which the dopaminergic and non dopaminergic systems, the central and peripheral systems, in addition motor and non motor symptoms should be involved (Alam and Schmidt, 2002). Various helpful animal models of PD are present, among the different animal models the rotenone model mimics the classical features of PD and provides certain advantages in modeling pathogenesis of PD (Betarbet *et al.,* 2002). Various environmental factors have been involved in PD pathogenesis (Freire and Koifman, 2012). Studies suggested that subjects with heavy exposure to pesticides have a higher risk of developing PD (Ahlskog and Muenter, 2001; Blandini and Armentero, 2012). In this perspective, various studies have suggested the involvement of pesticide such as rotenone in contributing

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to the higher incidence of sporadic Parkinsonism in rural populations (Priyadarshi *et al.,* 2001; Vanacore *et al.,* 2002).

Previous studies have employed the chronic use of rotenone to generate an experimental model of PD that mimics and elicits PD-like symptoms, such as motor dysfunction but have used less specific behavior to assessed PD-like symptoms. In the present study, therefore, we investigated whether short term administration of rotenone also induces significant PD-like symptoms in rats. So, this study was further extended to find out the rotenone-induced behavioral changes associated with neurochemical abnormality in rotenone treated PD model. Deficits in gait are a hallmark of symptoms of PD. Although, gait dynamics in various rat models have been assessed but data focusing on gait in rotenone-induced rat model of PD is not available. We therefore assessed the gait changes in rotenone model of PD using footprint method.

## MATERIALS AND METHODS

### *Experimental protocol*

Fourteen *locally* bred Albino-Wistar rats weighing 150-200g were purchased from Dow University of Health Sciences, Ojha Campus, Karachi. After three days of acclimation period, rats (n=14) were randomly divided into two groups; control (*n*=6) and test (*n*=8). Rotenone was purchased from Sigma Chemical Co. (St. Louis, USA). Rotenone was dissolved in sunflower oil. 1.5 mg/kg rotenone was injected subcutaneously (s.c.) to the test rats whereas controls were treated with vehicle (sunflower oil) simultaneously. Treatment was continued during which rats were subjected to Kondziela's inverted screen test to determine the muscular strength and beam walking test to observe the motor coordination and balance following rotenone administration. Footprint test was used to obtain the foot step pattern of control and rotenone injected rats. All experiments were carried out between 8:00 and 17:00 h. Rats were decapitated after behavioral analysis to collect their brains. Whole brain was removed from the skull within 30 s after decapitation. All brain samples were immediately stored at -70°C for neurochemical assays (fig. 1a). Dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) were estimated in whole brain of rat.

### *Behavioral analysis*

#### *Kondziela's inverted screen test*

Kondziela the inverted screen test has been used previously for measure of muscular strength of animals using all four limbs (Kondziela, 1964). The inverted screen is a 43 cm square of wire mesh consisting of 12 mm squares of 1 mm diameter wire. It is bordered by a 4 cm deep wooden beading (which prevents animals from climbing on to the other side). The test was done by placing the rat in the centre of wire mesh screen which was rotated to an inverted position over 120 seconds with the rats head declining first. The time when the rat fell off from the screen was noted.

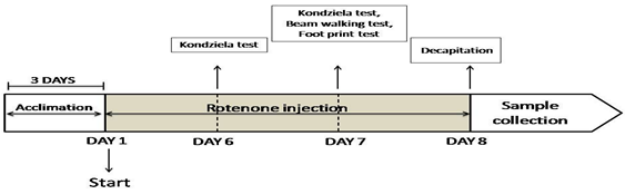


**Fig. 1a**: Schematic representation of experimental protocol.

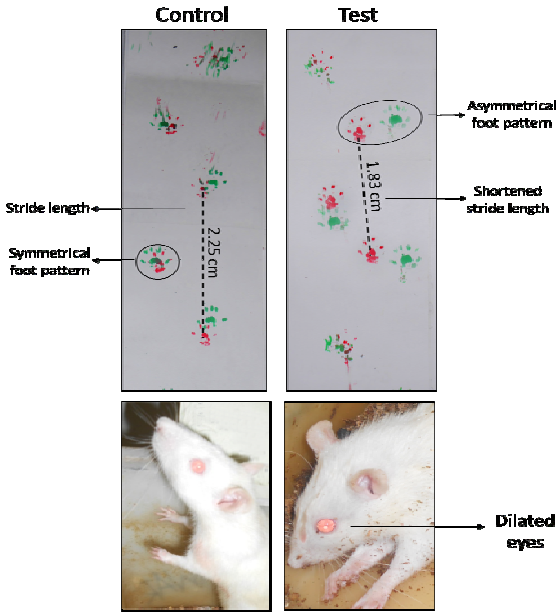


**Fig. 1b**: Representation of footprint pattern and dilated eyes. Forepaws and hindpaws were coated with red and green ink, respectively. The dotted lines indicate stride length. The irregular distance between the fore- and hind paws in control and test rats is encircled. The prominent dilated eyes were also observed in rotenone treated rats in current study.

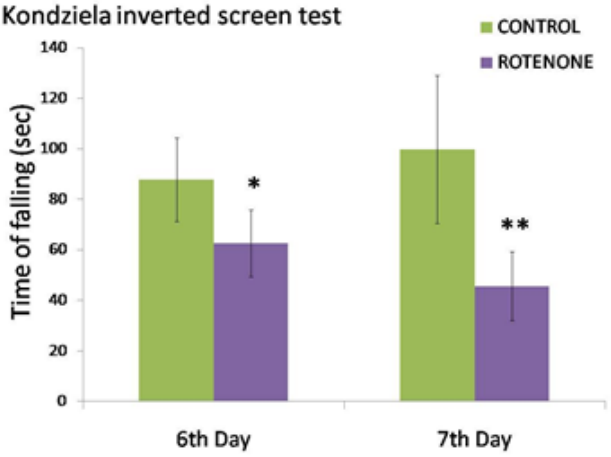


**Fig. 2**: Effect of rotenone on muscular strength for all four paws was assessed by Kondziela test. Rotenone impaired the muscular strength of rats as observed by

significantly decreased time to hold the inverted screen as compared to controls (**$p$<0.01). Significant differences were obtained by independent sample-$t$ test. Values are mean±SD ($n$=6 controls; $n$=8 test rats).

***Beam walking test***

Beam walking is a test of motor coordination (Goldstein, 1993). The rats have to cross a beam which is suspended between a start platform and their home cage at a height of 50 cm and is supported by two pillars. A cushion was placed under the beam in order to protect the animals from the bang into the floor. The difficulty of this task can be assorted by using beams with different shapes and widths (Jover *et al.*, 2006). Motor coordination and balance was assessed by the ability of a rat to crossways a graded series of beams. Three circular beams of different diameters were used in this study such as 3cm, 2cm, 1cm and length of 100 cm. In the training phase, animals were trained to traverse the three beams (from widest to narrowest) directly into the animal's home cage. This helps to make certain that the behavior during testing is more stable and more precisely reflects motor coordination as opposed to the rodent's natural aversion to crossing over unprotected spaces. After training session testing phase was done and the time taken to cross the beam and number of foot slips off the beam were recorded.

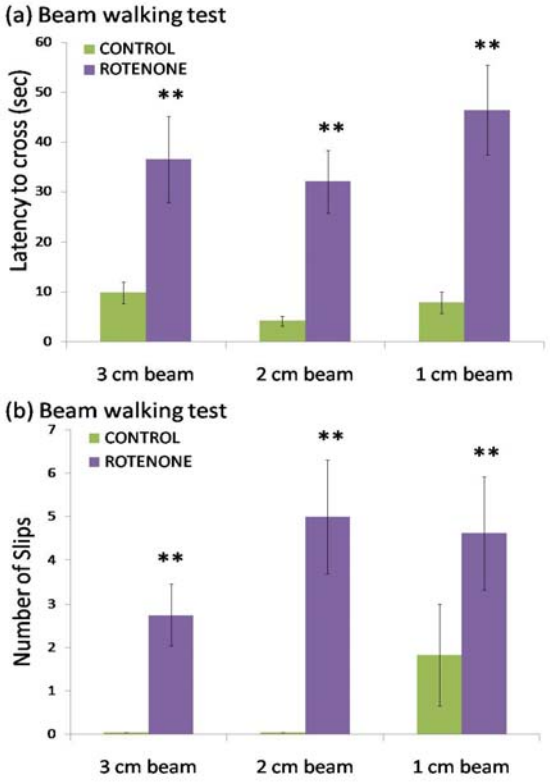


**Fig. 3**: Beam test was performed to assess the motor coordination particularly of the hind limbs. Rotenone significantly increased the (a) time to cross the beam and (b) number of slips in all three beams of varying diameters as compared to control rats, confirming the loss of motor skills. Values are mean ±SD ($n$=6 controls; $n$=8 test rats).

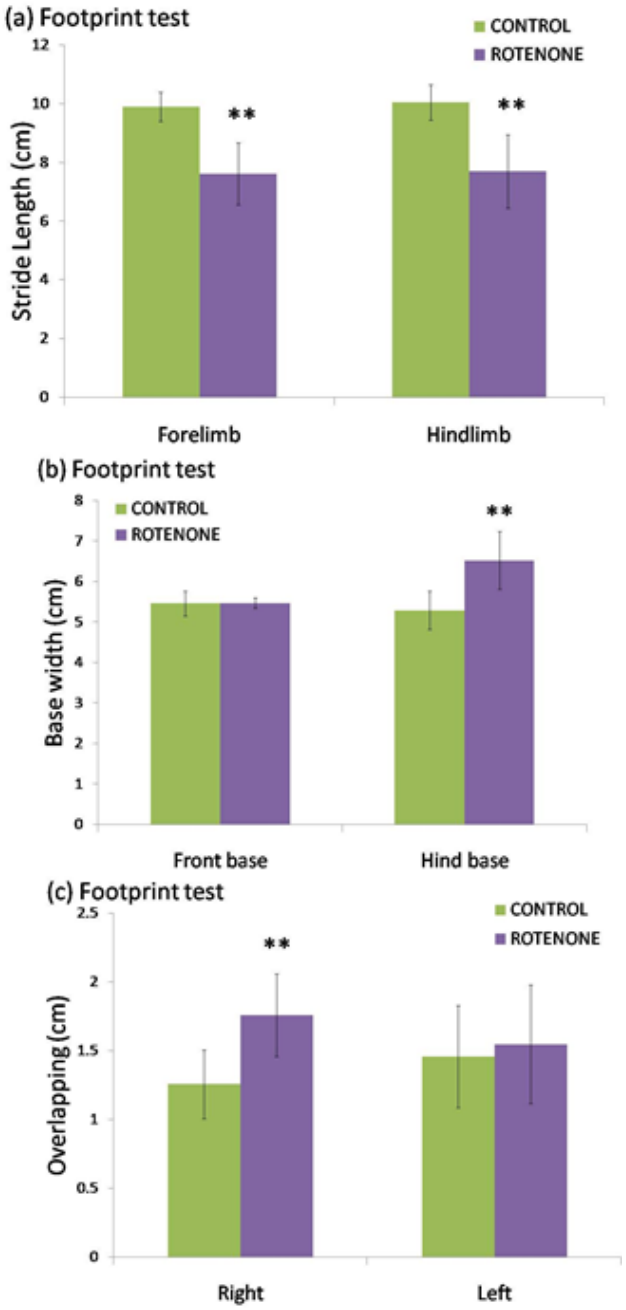


**Fig. 4**: Analysis of foot step pattern by footprint test. The quantification of (a) strides, (b) base width, and (c) overlap in control ($n$=6) and test ($n$=8) groups. Values are mean±SD. Significant data was analyzed by independent sample-$t$ test versus controls (**$p$<0.01).

***Footprint test***

To obtain the footprints, the rat hindlimb and forelimb were coated with green and red non-toxic paints. The animals were then allowed to walk along a 100 cm long, 10cm wide runaway (with 20 cm high walls). A fresh sheet of white paper was placed on the floor of the

runaway for each rat run. The footprint patterns were analyzed for three step parameters (all measured in centimeters): stride length, base width and overlap between forelimb and hind limb. A sequence of four consecutive steps was chosen for evaluation, footprints made at the start and end of the run where the animal was initiating and finishing movements were not included (Nascimento-Ferreira *et al.,* 2013).

***Neurochemical analysis***

***Determination of biogenic amines***

For determination of biogenic amines homogenization of frozen brains was carried out in an extraction medium using an electrical homogenizer (Polytron; Kinematica). The neurochemical analysis was done to assess concentrations of, DA, and its metabolites DOPAC in brain as described by Haider *et al.* (2014).

## STATISTICAL ANALYSIS

Data are presented as mean ±SD. Mean differences for data were evaluated by independent samples *t*-test using SPSS version 20. Values $p<0.05$ was defined as statistically significant.

## RESULTS

***Behavioral assessment***

***Kondziela's inverted screen test***

Kondziela's inverted screen test (fig. 2) was conducted to assess the motor coordination and muscular strength, respectively, of all paws of rats following the injection of rotenone. Rotenone affected the muscle strength of treated rats which is also one of the symptoms of PD. On 6$^{th}$ day time of falling was significantly reduced following rotenone injection (62.59±13.2 sec; $p<0.05$) in Kondziela's inverted screen test as compared to controls (87.85±16.51). These significant results were more prominent on 7$^{th}$ day of rotenone injection(45.6±13.5 sec; $p<0.01$) as compared to control rats (99±29.32 sec), so the other tests were also conducted after seven days of rotenone administration. These data revealed that rotenone affected the muscular strength to the extent that rats were unable to hold the inverted screen and fell down immediately, demonstrating the development of PD following eight days of rotenone injection.

***Beam walking test***

Rotenone administration exhibited a prominent loss of motor coordination in beam walking test. In this test three beams of varying diameters (3, 2, 1 cm) were used and time to traverse the beam (fig. 3a) and number of slips (fig. 3b) were monitored after the training session. Rotenone significantly increased the time to cross the beams (3cm=36.53±8.6 sec; $p<0.01$, 2cm=32.07±6.2 sec; $p<0.01$, 1cm=46.43±9.02 sec; $p<0.01$) as compared to controls (3cm=9.8±2.15 sec, 2cm=4.1±0.98 sec, 1cm=7.8±2.1 sec). Number of slips were also significantly increased during beam crossing following rotenone injection (3cm=2.75±0.7; $p<0.01$, 2cm=5.0±1.3; $p<0.01$, 1cm=4.6±1.30; $p<0.01$) as compared to controls (3cm=0±0, 2cm=0±0, 1cm=1.83±1.8). These observed parameters are much simulate to the symptoms of PD.

***Footprint test***

At the end of motor behavioral assessment, walking pattern was also monitored by footprint test (fig. 1b, fig. 4 (a-c). Rotenone injected rats showed impaired walking pattern as evident by significant shortened stride length for both forelimb (7.62±0.5cm; $p<0.01$) and hindlimb (7.72±1.25cm; $p<0.01$) as compared to controls (9.91±0.5cm, 10.06±0.6cm respectively) indicating gait abnormalities. Impaired movement was also evident by significant increased hind base width (6.53±0.71cm; $p<0.01$) and increased right overlap (1.76±0.3cm; $p<0.01$) as compared to controls (5.30±0.48cm, 1.26±0.25cm respectively).

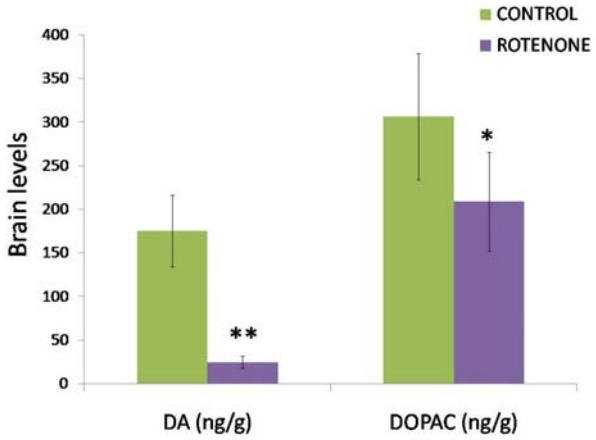


**Fig. 5**: Estimated levels of neurotransmitters in whole brain of controls ($n$=6) and rotenone treated rats ($n$=8). DA (ng/g of brain tissue) and DOPAC (ng/g of brain tissue) levels were analyzed at the end of present study. Significant difference were obtained following independent sample-*t* test (**$p<0.01$, *$p<0.05$). Values are mean ±SD.

***Neurochemical estimations***

Assessment of neurotransmitter levels in whole brain of rat revealed significant changes in the levels of DA and its metabolite following rotenone administration (fig. 5). We found a strikingly decreased levels of DA (24.92±7.03 ng/g; $p<0.01$) and DOPAC (209.28±56.4ng/g; $p<0.05$) in rotenone injected rats as compared to controls (175.7±41.01 ng/g and 306.67±72.3ng/g respectively).

## DISCUSSION

Rotenone model mimics the pathological and biochemical changes like PD which is helpful in studying mechanisms involved in the etiogenesis of PD and also in searching for

possible therapeutics for clinical use. The main goal of this study was to test whether rotenone treatment for 8 days could be used to establish a rat model of PD. Previously; different durations for the administration of rotenone have been reported to induce PD in animals (Sherer *et al*., 2002; Leung *et al*., 2007). In the present study, daily exposure of rat to rotenone for 8 days induced symptoms like bradykinesia, abnormality of balance and unsteady gait (Lapointe *et al.,* 2004; Casarrubea *et al.,* 2010). We demonstrated that acute exposure of rats to rotenone epitomized some key features of Parkinsonism including gait impairments with the help of different behavioral methods and neurochemical estimations. Neurochemical analysis showed a decline in levels of DA and DOPAC following rotenone administration.

PD is a neurodegenerative disorder which is mainly associated with motor dysfunction. On the behavioral level emphasis was given to the hypokinetic behavior. It is a well known fact that reduced DA level has been linked to human rigidity and akinesia PD-like symptoms (Dauer and Przedborski, 2003). Behavioral tests for monitoring locomotor activity such as beam walking test and Kondziela inverted screen test showed that rotenone treated rats exhibited locomotor deficits. Previously it has been shown that 50% neuronal loss in SNpc occurred when 50-70% DA level is depleted in the striatum and causes spontaneous loss of motor activities (Alam and Schmidt, 2002; Fearnley and Lee, 1991). In initial stages of PD, DA depletion affects striatal and cerebral cortex function which play important role in muscle response, movement control and balance which results in motor dysfunction (Stevenson *et al.,* 2011; Alam and Schmidt, 2004). In PD, gait disturbances are commonly observed due to the degeneration of dopaminergic neurons (Bjorklund and Dunnett, 2007). Gait abnormalities are characteristics feature of PD and are used to determine the early stage of PD. Gait dynamics in rotenone model of PD in rats has not been described yet. However, in the present study footprint analysis showed that gait dynamics were significantly different in rotenone treated rats than compared to control rats evident by a decreased stride length in rotenone treated rats. Our results are in agreement with previous findings showing that rats with unilateral PD lesion were found to exhibit a shuffling gait, motor asymmetries and short stride length that resemble the key features of human PD gait (Metz *et al.,* 2005; Klein *et al.,* 2009). Therefore, all these locomotor deficits observed in our study closely correlated with declined levels of DA in rotenone treated rat brain. Administration of rotenone caused a significant decline in DA and its metabolite DOPAC as compared to control rats. The present findings were further supported by former study reports showing decline in DA level following rotenone exposure (Alam and Schmidt, 2002). Various studies reported that rotenone administration in rats caused the selective degeneration of dopaminergic neurons and resulting in movement disorders (Heikkila *et al.,* 1985; Betarbet *et al.,* 2000; Sherer *et al.,* 2003). Therefore, in our study the motor deficits may be attributed to the observed DA deficiency produced by rotenone (Nehru *et al.,* 2008; Erbaş *et al.,* 2016) which is the main cause of PD.

## CONCLUSION

In conclusion, the findings of the present study further provides evidence that rotenone administration produces PD-like symptoms in rats which is evident by gait abnormalities and neurochemical changes after a rotenone administration. Moreover, the study also highlights the fact that environmental toxin such as rotenone contributes to the pathogenesis of PD. However, histopathological examination in short-term rotenone-induced rat model of PD and region specific estimation of DA is needed for further validation.

## ACKNOWLEDGEMENT

The project was funded by a grant from university of Karachi, Karachi, Pakistan.

## REFERENCES

Ahlskog JE, Muenter MD (2001). Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. *Mov. Disord.,* **16**: 448-458.

Alam M and Schmidt WJ (2002). Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behav. Brain Res.,* **136**: 317-324.

Alam M and Schmidt WJ (2004). L-DOPA reverses the hypokinetic behaviour and rigidity in rotenone-treated rats. *Behav. Brain Res.,* **153**: 439-446.

Auluck PK, Chan HY, Trojanowski JQ, Lee VM and Bonini NM (2002). Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. *Science,* **295**: 865-868.

Betarbet R, Sherer TB and Greenamyre JT (2002). Animal models of Parkinson's disease. *Bioessays.,* **24**: 308-318.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV and Greenamyre JT (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neurosci.,* **3**: 1301-1306.

Bjorklund A and Dunnett SB (2007). Dopamine neuron systems in the brain: An update. *Trends. Neurosci.,* **30**: 194-202.

Blandini F, Armentero MT (2012).Animal models of Parkinson's disease. *FEBS. J.*, **279**: 1156-1166.

Calne DB (2001). Parkinson's disease is not one disease. *Parkinsonism. Relat. Disord*., **7**: 3-7.

Casarrubea M, Sorbera F, Santangelo A and Crescimanno G (2010). Microstructure of rat behavioral response to anxiety in hole-board. *Neurosci. Lett.,* **481**: 82-87.

Dauer W and Przedborski S (2003). Parkinson's disease: Mechanisms and models. *Neuron*., **39:** 889–909.

Erbaş O, Yılmaz M and Taşkıran D (2016). Levetiracetam attenuates rotenone-induced toxicity: rat model of Parkinson's disease. *Environ Toxicol Pharmacol.,* **42**: 226-30.

Fearnley JM and Lee AJ (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain.,* **114**: 2283-2301.

Freire C and Koifman S (2012). Pesticide exposure and Parkinson's disease: Epidemiological evidence of association. *Neuro Toxicology*., **33**: 947-971.

Goldstein LB (1993). Rapid reliable measurement of lesion parameters for studies of motor recovery after sensorimotor cortex injury in the rat. *J. Neurosci. Methods.,* **48**: 35-42.

Greenamyre JT, Cannon JR, Drolet R and Mastroberardino PG (2011). Lessons from the rotenone model of Parkinson's disease. *Trends Pharmacol Sci*., **31**: 141-142.

Haider S, Saleem S, Perveen T, Tabassum S, Batool Z, Sadir S, Liaquat L and Madiha S (2014). Age-related learning and memory deficits in rats: Role of altered brain neurotransmitters, acetyl cholinesterase activity and changes in antioxidant defense system. *Age (Dordr.)*., **36**: 1291-1302.

Heikkila RE, Nicklas WJ, Vyas I and Duvoisin RC (1985). Dopaminergic toxicity of rotenone and the 1-methyl-4-phenyl-pyridiniumion after their stereotaxic administration to rats: implication for the mechanism of 1-methyl-4- phenyl-1, 2, 3, 6-tetrahydropyridine toxicity. *Neurosci. Lett.,* **62**: 389-394.

Jover R, Rodrigo R, Felipo V, Insausti R , Sáez-Valero J, García-Ayllón MS, Suárez I, Candela A, Compañ A,Esteban A, Cauli O, Ausó E, Rodríguez E, Gutiérrez A, Girona E, Erceg S, Berbel P, Pérez-Mateo M (2006). Brain edema and Inflammatory activation in bile duct ligated rats and diet-induced hyperammonemia: A model of Hepatic encephalopathy in cirrhosis. *Hepatology.,* **43**: 1257-1266.

Klein A, Wessolleck J, Papazoglou A, Metz GA and Nikkhah G (2009). Walking pattern analysis after unilateral 6-OHDA lesion and transplantation of foetal dopaminergic progenitor cells in rats. *Behav. Brain Res*., **199**: 317-325.

Kondziela W (1964). A new method for the measurement of muscle relaxation in white mice. *Arch. Int. Pharmacodyn. Ther.,* **152**: 277-284.

Lapointe N, St-Hilaire M, Martinoli MG, Blanchet J, Gould P, Rouillard C and Cicchetti F (2004). Rotenone induces non-specific central nervous system and systemic toxicity. *FASEB J.,* **18**: 717-719.

Leung KW, Yung KK, Mak NK, Chan YS, Fan TP and Wong RN (2007). Neuroprotective effects of ginsenoside-Rg1 in primary nigral neurons against rotenone toxicity. *Neuropharmacology*., **52**: 827-835.

Metz GA, Tse A, Ballermann M, Smith LK, Fouad K (2005). The unilateral 6-OHDA rat model of Parkinson's disease revisited: An electromyographic and behavioural analysis. *Eur. J. Neurosci.*, **22**: 735-744.

Moon Y, Lee KH, Park JH, Geum D and Kim K (2005). Mitochondrial membrane depolarization and the selective death of dopaminergic neurons by rotenone: protective effect of coenzyme Q10. *J. Neurochem.,* **93**: 1199-1208.

Nascimento-Ferreira I, Nóbrega C, Vasconcelos-Ferreira A, Onofre I, Albuquerque D, Aveleira C, Hirai H, Déglon N and Pereira de Almeida L (2013). Beclin 1 mitigates motor and neuropathological deficits in genetic mouse models of Machado-Joseph disease. *Brain.,* **136**: 2173-2188.

Nehru B, Verma R, Khanna P and Sharma SK (2008). Behavioral alterations in rotenone model of Parkinson's disease: Attenuation by co-treatment of centrophenoxine. *Brain Res.,* **1201**: 122-127.

Priyadarshi A, Khuder SA, Schaub EA, Priyadarshi SS (2001). Environmental risk factors and Parkinson's disease: a meta-analysis. *Environ. Res.,* **86**: 122-127.

Sherer TB, Betarbet R, Stout AK, Lund S, Baptista M, Panov AV, Cookson MR and Greenamyre JT (2002). An in vitro model of Parkinson's disease: Linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J. Neurosci.,* **22**: 7006-7015.

Sherer TB, Kim JH, Betarbet R and Greenamyre JT (2003). Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and α-synuclein aggregation. *Exp. Neurol.,* **179**: 9-16.

Stevenson, JK, Oishi MM, Farajian S, Cretu E, Ty E and McKeown MJ (2011). Response to sensory uncertainty in Parkinson's disease: A marker of cerebellar dysfunction?. *Eur. J. Neurosci.,* **33**: 298–305.

Tieu K (2011). A Guide to Neurotoxic Animal Models of Parkinson's disease. *Cold. Spring. Harb. Perspect. Med.*, **1**: a009316.

Tsouli S and Konitsiotis S (2010). How should we treat a patient with early Parkinson's disease? *Int. J. Clin. Pract.,* **64**: 1210-1219.

Vanacore N, Nappo A, Gentile M, Brustolin A, Palange S, Liberati A, Di Rezze S, Caldora G, Gasparini M, Benedetti F, Bonifati V, Forastiere F, Quercia A and Meco G (2002). Evaluation of risk of Parkinson's disease in a cohort of licensed pesticide users. *Neurol Sci*., **23**: S119-S120.