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A comparative study of neurobehavioral changes induced by rotenone through oral and intraperitoneal administration

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ABSTRACT

Parkinson's disease is one of the major neurological disorders seen worldwide and associated with many motor and nonmotor symptoms. Rotenone-induced motor and behavior impairments could involve the gut-brain axis which is emphasized in several recent works. To explore how oral and intraperitoneal rotenone toxicity impacts neurobehavioral alterations in Wistar rats. The effect of intraperitoneal (3 mg/kg body weight 21 days) and oral (50 mg/kg body weight for 28 days) rotenone toxicity in male Wistar rats on various neurobehavioral parameters viz., rotarod, actophotometer, rearing behavior and elevated plus maze was comparatively studied. The neurobehavioral studies such as the Rota-rod test, rearing behavior, Elevated plus maze (EPM), and Actophotometer were performed by following standard published protocols and by utilizing the facilities available at the Institution. Kruskal-Wallis one-way ANOVA on ranks with Student-Newman-Keul's multiple comparisons was used. It was found that both oral and intraperitoneal rotenone treatments had a significant impact on neurobehavioral parameters when compared to their respective controls. Although there were few differences observed in the study set behavioral parameters between intraperitoneal and oral rotenone treatments, it was not significant. The study findings suggest that neurobehavioral alterations caused by oral or intraperitoneal rotenone toxicity are found to be significant. The fact that oral rotenone toxicity has gut-brain axis-related problems can also have a role in PD-related motor/movement and anxiety behavior dysfunctions.

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INTRODUCTION

Parkinson's disease is the second most common neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and also due to the accumulation of cytoplasmic proteinaceous inclusion bodies known as Lewy bodies [1, 2]. Severe physical and mental impairments occur in PD. Dysfunction of the central motor system in PD results in bradykinesia, resting tremors, muscular instability and muscular rigidity [1, 3]. The pathogenesis of PD involves mitochondrial dysfunction, oxidative stress, aggregation of misfolded and damaged proteins, inflam-

mation and exposure to various environmental toxins [1, 3]. Epidemiological studies revealed exposure to environmental toxins has been associated with an increase in the incidence of PD [4, 5].

PD severely affects motor functions thereby leading to problems associated with movement and stability. In up to 49% of PD, anxiety-like behavior is reported to occur and it is suggested that episodes of anxiety can occur prior to motor impairments [6, 7]. Moreover, the events of depression and anxiety propagating in PD are believed to be highly complicated and perhaps not explored in detail [7]. Importantly, the involvement of the gut-brain axis in the progression of PD is very much emphasized in many consecutive research works [8–10]. It was hypothesized that the gastrointestinal (GI) tract and the olfactory system are the areas associated with early PD signs before any key motor symptoms could be seen after many years [8]. In addition, developing a PD model using the intraperitoneal route of administration is a bit complicated and invasive whereas the oral route of administration is non-invasive. While ample previous works had reported motor and cognitive dysfunctions in rotenone-induced PD models, studies on elevated plus maze and actophotometer tests in rats induced with oral or intraperitoneal rotenone toxicity are still unexplored. Moreover, it could be seen that Parkinson's disease induced by oral and intraperitoneal route administrations might have a different pathological sequel of events in propagating PD [11]. Especially oral rotenone has lower toxicity and might involve non-motor complications such as gastrointestinal abnormalities with dysfunctional microbiota-gut-brain axis [12]. However the results of [11] Pan-Montojo et al; 2010 provided strong evidence favoring the intragastric effect of rotenone on the ENS that mimicked brain anatomical features of PD as well as alpha-synuclein accumulation in the ENS [13].

Interestingly, this intragastric mode of PD induction didn't reveal mitochondrial Complex I inhibition. Therefore the present study aimed to explore if there could be any difference in neurobehavioral alterations between oral and intraperitoneal rotenone toxicity in Wistar rats.

MATERIALS AND METHODS

Male Wistar rats weighing approximately 250-300g were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in rooms with a 12hrs light / dark cycle.

The animals were maintained in standard environmental conditions and provided with a commercial rat pellet and water *ad libitum* as per the experimental protocol.

This study was confined to the guiding principles of the Institutional Animal Ethics Committee (IAEC) and was approved by the Institutional Animal Ethics Committee of the Institution (IAEC Approval Number: SU/CLAR/RD/014/2015).

Experimental protocol

The randomly selected male Wistar rats were equally divided into four groups. Each group consisted of six animals as follows:

Group I (n = 6): Rats received daily olive oil (2.0 mL/Kg b.w) as vehicle i.p for 21 days

Group II (n = 6): Rats received daily rotenone (3 mg/kg b.w) i.p for 21 days.

Group III (n = 6): Rats received daily 0.5 % HPC (5 mL/Kg b.w) p.o as vehicle for 28 days

Group IV (n = 6): Rats received daily rotenone (50 mg/Kg b.w) p.o for 28 days

Rotenone Treatment

For the intraperitoneal dose, the rotenone was prepared by the method of [14] Cannon et al (2009) with many modifications. The medium-chain triglyceride Miglyol 812 was replaced with olive oil in the present work. To prepare solutions for Rot-3-ip injections, 0.0315 g of rotenone (TCI America), respectively, was first dissolved in 250 μL dimethyl sulphoxide (DMSO) and subsequently diluted with 21 mL of olive oil. Rat weights were recorded daily, and the volume of injected rotenone was adjusted to provide the appropriate dose. Control rats (Con-OO) were injected with 1.5 mL/kg b.w olive oil. The rotenone solution and the vehicle olive oil were administered intra-peritoneal once daily at 1 mL/kg b.w. to PD and control group rats respectively for 21 days. The solutions were prepared fresh weekly in amber-colored bottles and vortexed before each IP injection to avoid the possibility of settling. The solutions were stored at 4°C.

For po administration of Rot-50-po, 0.525 g of rotenone was dissolved in 63 ml of 0.5% hydroxypropyl cellulose (HPC) procured from HiMedia, Mumbai, India to obtain 50 mg/kg bw concentrations, respectively. The solutions were made fresh weekly and vortexed several times before each po administration to avoid the possibility of settling.

Control rats (Con-HPC) were administered only po HPC (0.5%) for 28 days. The solution was made fresh every week, kept in amber septa vial to protect from light and vortexed several times before each oral administration to avoid the possibility of settling. The rotenone solution was administered

orally at 1.5 mL/kg.b.w. and the control animals received 0.5 % HPC (1.5 mL/kg.b.w) only for 28 days.

Behavioral studies

Rota-rod test

The rota-rod apparatus is a three-panel techno device with a timer. The Rota-rod test was used to assess motor coordination and balance [15]. Rats have to maintain their balance on a rotating rod. To perform the rotarod test, the animal was placed with all four paws on a rod with a diameter of 7cm and a height of 25cm above the floor. The rod was rotated at 4 rpm and gradually increased to 11 rpm. Animals were placed on a rod rotating at a speed of 11 rpm. Before being tested, the rats were trained in three sessions of 180sec each for habituation and after being trained, the rats were tested three times. A cut-off time of 180sec was maintained throughout the test. The latency time required for the rat to fall off from the rotating rod, either three falls or a total of 180sec on the rotarod was recorded. The average results in each group were recorded at the time of fall. The apparatus was cleaned with 5% ethanol before each behavioral test to eliminate bias due to odors left by the previous rat.

Rearing behavior

Rats explore and rear when they are placed in a clear cylinder. In this test [14], the rats were placed in a clear chamber with a height of 30cm and diameter of 20cm for 5 min. The rat had to raise forelimbs above shoulder level and make contact with the wall of the cylinder with both forelimbs. Removal of both the forelimbs from the wall and contact with the surface was required before another rear was scored. The test was performed as described by [14] Cannon et al., and the number of rears was recorded and quantified.

Elevated plus maze (EPM)

It is a four-armed platform with two closed arms and two open arms with 50cm X 10cm dimensions that are elevated 50cm above the ground. The animal was placed in the centre and allowed to explore all the arms for 5min [16, 17]. The time spent in each arm was measured. The EPM was cleaned by 5% ethanol before each animal was tested to eliminate bias. Increased activity in the open arms e.g. proportion of time spent in the open arms (time in open arms/total time in open or closed arms) and an increase in the proportion of entries into the open arms (entries into open arms/total entries into open or closed arms) reflects anti-anxiety behavior. By contrast, animals showing anxious-like behavior tend to spend more time in enclosed arms.

Actophotometer

The animal locomotor activity was monitored using an actophotometer [15]. It is an activity cage consisting of six inbuilt photo sensors and digits digital counter. It is a digital device that measures the indicated and spontaneous activity. It works on the principle of photoelectric cells that are connected in a circuit with a counter. Animals were placed in an actophotometer individually and basal activity was recorded over a period of 180sec. It records a count when a beam of light falling on the photocell is cut off by the animal.

Statistical Analysis

Results were expressed as Median and percentile and the statistical significance of the data obtained from the studies were analyzed and determined by Kruskal-Wallis one-way ANOVA on ranks with Student-Newman-Keul's multiple comparisons. p-Value < 0.05 was considered significant.

RESULTS

The behavioral parameters (rotarod, actophotometer, rearing behavior and elevated plus maze) results of con-OO, Rot-3-ip, con-HPC and Rot-5-po groups are depicted in Tables 1, 2 and 3 & Figure 1 respectively. The data suggest that either intraperitoneal or oral rotenone toxicity caused significant behavioral alterations in rats as compared with their controls.

However, in the Elevated plus maze test, the evaluation of time spent in either the open arm or closed arm also showed a level of statistical significance between Con-OO and Con-HPC groups which is quite interesting. The comparison between groups (Con-OO Vs Con-HPC; Rot-50-po Vs Rot-3-ip) for rotarod, actophotometer tests and rearing behavior didn't reveal any statistical level of significance. The overall assessment of behavioral responses indicates PD-related problems especially motor and movement-related functions in oral and intraperitoneal rotenone toxicity.

DISCUSSION

Rotenone administration in Wistar rats induces motor symptoms of PD including gait and flexed posture, tremors, rigidity and even catalepsy [18]. In the present investigation, we explored how oral and intraperitoneal rotenone toxicity altered various behavioral parameters in rats through a comparative study. It was found that the motor activity was significantly decreased/altered after intraperitoneal or oral rotenone administration.

Table 1: The effect of rotenone on Rotarod in rats

S. No.	Parameter	Groups	Median	25 % - 75 %	Kruskal-Wallis statistical information	Student-Newman-Keuls Statistical information
1.		Con-OO	180.0	35.5 - 180.0		Con-OO Vs Rot-3ip : S
	Fall I	Rot-3ip	6.0	4.250 – 31.50	H – 15.747	Con-HPC Vs Rot-50po : S
		Con-HPC	180.0	163.0 – 180.0	P – 0.001	Con-OO-Con-HPC : NS
		Rot-50-po	14.50	7.250 – 25.0		Rot-50poVs Rot-3ip : NS
2.		Con-OO	180.0	152.50 – 180.0		Con-OO Vs Rot-3ip : S
	Fall II	Rot-3-ip	19.0	1.750 – 24.250	H – 15.883	Con-HPC Vs Rot-50po : S
		Con-HPC	180.0	135.25 – 180.0	P – 0.001	Con-OO-Con-HPC : NS
		Rot-50-po	9.5	5.0 – 18.0		Rot-50poVs Rot-3ip : NS
3.		Con-OO	180.0	152.25 – 180.0		Con-OO Vs Rot-3ip : S
	Fall III	Rot-3ip	5.500	2.750 – 12.750	H – 13.519	Con-HPC Vs Rot-50po : S
		Con-HPC	180.000	137.25 – 180.0	P – 0.004	Con-OO-Con-HPC : NS
		Rot-50p0	11.000	2.500 – 17.750		Rot-50poVs Rot-3ip : NS

Table 2: The effect of rotenone on movements in actophotometer and rearing behaviour in rats

S. No.	Parameter	Groups	Median	25 % - 75 %	Kruskal-Wallis statistical information	Student-Newman-Keuls Statistical information
1.	Movements on Acto-photometer	Con-OO	250.0	242.75 – 258.25		Con-OO Vs Rot-3ip : S
		Rot-3ip	94.50	78.00 – 109.25	H – 17.667	Con-HPC Vs Rot-50po : S
		Con-HPC	228.50	219.25 – 256.25	P – < 0.001	Con-OO Vs Con-HPC : NS
		Rot-50p0	99.00	86.25 – 112.75		Rot-50poVs Rot-3ip : NS
2.	Rearing behaviour	Con-OO	9.50	8.5 – 15.750		Con-OO Vs Rot-3ip : S
		Rot-3ip	1.00	0.00 – 3.250	H – 17.309	Con-HPC Vs Rot-50po : S
		Con-HPC	8.00	5.25 – 11.25	P – < 0.001	Con-OO Vs Con-HPC : NS
		Rot-50p0	1.00	0.00 – 2.00		Rot-50poVs Rot-3ip : NS

The rearing activity significantly decreased in rats after oral or intraperitoneal rotenone administration [19, 20]. The rotarod performance test which measures balance, grip strength, and motor coordination suggests that the administration of either Rot-3-ip or Rot-50-po had an almost similar effect in altering those behavioral functions.

The EPM test that measured the anxiety-like behavior in various groups suggests that rats from either Rot-3-ip or Rot-50-po group had spent more time in the enclosed arms as compared with their respective control groups.

Based on our preliminary published results, Kavuri et al., 2020 [21] , it was found that Rot-3-ip induction for 21 days in Wistar rats showed significant upregulation of α-synuclein expression (Immunohistochemistry) with Lewy bodies in brain striatum; whereas Rot-50-po treatment for 28 days in rats caused a moderate increase of α-synuclein with no detectable Lewy bodies. The brain sections from the control groups showed normal architecture. Having found these results and connecting them with present work, it appears that the marked neurobehavioral changes witnessed in Rot-50-po administered rats irrespective of α-synuclein pathology in the brain are quite interesting.

Works from other groups had shown that either PQ or 6-OHDA treatment could induce anxiety-like behavior in Wistar rats [22]. In another recent work, it was shown that even a low dose of rotenone when injected by i.p route (1mg/kg/day in sunflower oil) to mice for 45 days caused anxiety-

Table 3: The effect of rotenone on elevated plus maze in rats

S. No.	Parameter	Groups	Median	25 % - 75 %	Kruskal- Wallis statistical information	Student-Newman-Keuls Statistical information
1.	Time spent in Open Arm	Con-OO Rot-3ip Con-HPC Rot-50p0	146.5 20.50 172.0 36.50	120.75 – 160.0 6.75 – 45.25 151.50 – 212.0 10.0 – 60.0	H – 18.793 P – < 0.001	Con-OO Vs Rot-3ip : S Con-HPC Vs Rot-50po : S Con-OO-Con-HPC : S Rot-50poVs Rot-3ip : NS
2.	Time spent in Closed Arm	Con-OO Rot-3ip Con-HPC Rot-50p0	122.0 276.0 81.0 263.0	114.75 – 142.0 254.75 – 286.5 69.75 – 112.0 235.25 – 288.5	H – 18.857 P – < 0.001	Con-OO Vs Rot-3ip : S Con-HPC Vs Rot-50po : S Con-OO-Con-HPC : S Rot-50poVs Rot-3ip : NS
3.	Time spent in centre	Con-OO	33.0	26.25 – 38.5	H – 15.882 P - 0.001	Con-OO Vs Rot-3ip : S Con-HPC Vs Rot-50po : S Con-OO-Con-HPC : NS Rot-50poVs Rot-3ip : NS

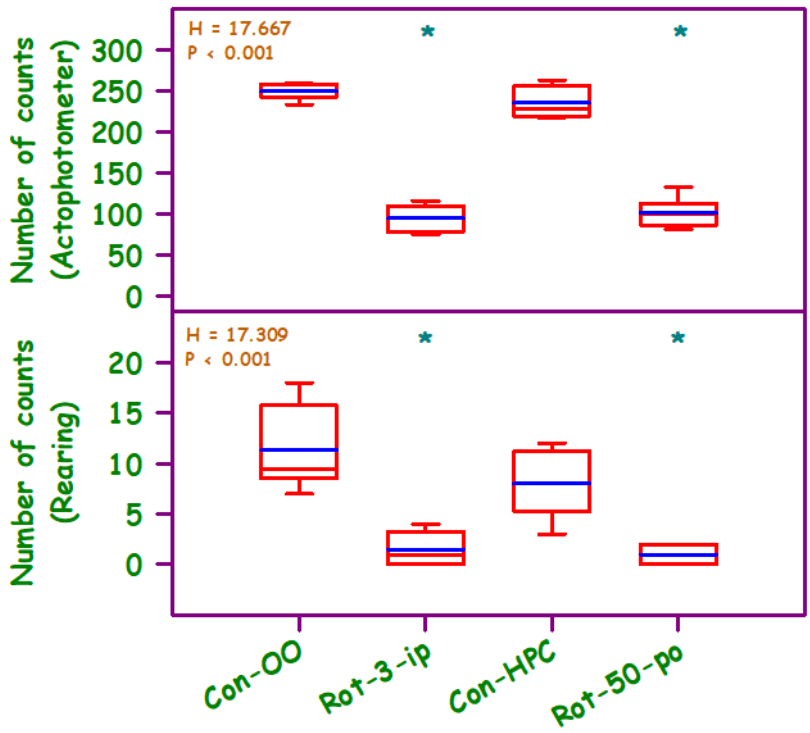

Figure 1: The effect of rotenone on movements in actophotometer and rearing behavior in rats. The middle red line is the median and the blue line is the mean. (n = 6 each).
Con= control; OO = olive oil, i.p.; HPC = hydroxypropyl cellulose, p.o.
Rot= rotenone. 3 and 50 are mg/kg dose.
The 'H' and 'P' values are by Kruskal Wallis one-way ANOVA on ranks with Student-Newman-Keul's multiple comparison test.
***Significantly different from the respective control groups.**

like behavior [23]. Using open field test, it was shown that paraquat-induced anxiety-like behaviors of mice have a relation with altered neurotransmitter activity in various brain regions [24, 25]. Likewise, such pesticide-induced anxiety-like behaviors in rats were also revealed through elevated plus maze experiments which paralleled mitochondrial dysfunction and oxidative stress in prefrontal cortex regions [26].

Khatri and Juvekar, 2016 [27] performed actophotometer and open field tests and showed that chronic administration of rotenone (1mg/kg i.p) for a period of three weeks significantly reduced the cognitive functions in mice. In the present study, we have injected threefold higher rotenone i.p doses in rats which showed striking alterations of movement and motor coordination-associated behavioural parameters.

As many recent works provide support for the involvement of the microbiota-gut-brain axis in Parkinson's disease (PD) pathogenesis [8, 9], it was suggested that PD pathology could be propagated through plethora of factors such as pro-inflammatory intestinal milieu, intestinal hyper-permeability and/or microbial dysbiosis.

In fact, intestinal hyper-permeability and dysbiosis are mainly attributed to stress-mediated neuronal degeneration and motor deficits occurring in Parkinsonism rodent models. As oral rotenone toxicity could lead to the above-mentioned complications, it is important to assess the movement and motor-associated complications to understand PD development.

Importantly, studies emphasizing oral rotenone-induced behavioural complications in rats could be rarely seen in the literature. Therefore the present work fills this gap and provides a better understanding of neurobehavioral alterations occurring in PD progression.

CONCLUSION

A comparative assessment of intraperitoneal and oral rotenone toxicity in rats in the present work reveals that the neurobehavioral alterations of oral toxicity are on par with the intraperitoneal induction model. Oral rotenone induction in rats can develop PD symptoms slowly and progressively so that it could imitate human PD progression for a better understanding of the clear-cut pathogenesis in order to develop more focused and targeted therapeutics.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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