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Evaluation of Haematological Alterations in Intraperitoneal and Oral Rotenone Induced Parkinson's Disease Wistar Rats

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ABSTRACT

In the present work, we examined intraperitoneal (3 and 6 mg/kg body weight [bw]; Rot-3-ip and Rot-6-ip, respectively) and oral (50 and 100 mg/kg bw; Rot-50-po and Rot-100-po, respectively) rotenone induced PD modeled Wistar rats to evaluate changes in various haematological parameters. While Rot-3-ip for 21 days and Rot-50-po for 28 days caused major Parkinson's disease (PD)-related alterations, Lewy bodies were only detected in Rot-3-ip rats. The Rot-6-ip and Rot-100-po treatments caused lethal toxicity. The ip and po rotenone doses significantly ($p < 0.05$) and dose dependently altered several haematological variables (haemoglobin [Hb], red blood cells [RBCs], white blood cells [WBCs] and packed cell volume [PCV]).

Key words: *Rotenone, Parkinson's disease, intraperitoneal and oral toxicity, haematology*

INTRODUCTION

The second most common neurodegenerative disorder is Parkinson's disease (PD) after Alzheimer's disease (AD). Intracytoplasmic inclusions known as Lewy bodies are key characteristic hallmarks of PD containing α-synuclein (α-syn) leading to loss of dopaminergic neurons mainly in the substantia nigra region [1-3]. While the exact cause of PD is still not very clear, many risk factors such as oxidative stress, free radical formation, metabolic factors, inhibition of mitochondrial electron transport chain (ETC) complex I and exposure to environmental toxins are associated with PD. The etiology of PD is still unclear; however, the risk factor that has gained considerable importance which cannot be excluded is both pre- and post-natal exposures to environmental factors [4]. The prolonged exposure to lead, manganese, solvents and some pesticides has been related to certain PD hallmarks such as mitochondrial dysfunction, alterations in metal homeostasis and aggregation of α-synuclein (α-syn) seen in Lewy bodies (LB), are considered to be key events in PD pathogenesis [4, 5].

Rotenone induced PD in laboratory animals is widely employed for understanding the pathogenesis as well as to test many therapeutic agents [6-8]. In the past decades, several studies that involved rotenone-induced PD complications have focused on many pathological and molecular aspects to derive key mechanistic insights. Therefore, studies on haematological and biochemical variables are scant in PD animal models. Knowledge about how rotenone administration alters these parameters could increase the understanding of rotenone-induced PD. There are no comparative studies on different routes of rotenone delivery and toxicity in animal models that report haematological variables. Thus, we aimed to determine whether peripheral alterations e.g WBCs, RBCs, Haemoglobin (Hb), and platelets are altered in rotenone-induced PD rats. Here, we determined changes in haematological parameters following ip and po rotenone administration in Wistar rats.

MATERIALS AND METHODS

Animals

Male Wistar rats between 250-300g were housed in solid-bottom polypropylene cages and maintained with a 12:12 hrs natural light:dark cycle. The animals were housed in standard environmental conditions and provided with commercial rat pellets (Biogen Laboratory Animal Facility, Bangalore) and water *ad libitum* during the experimental protocol. This study was confined to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) which comply with National Institutes of Health guide for the care and use of Laboratory animals and was approved by the Institutional Animal Ethics Committee of Saveetha Medical College (IAEC Approval Number: SU/CLAR/RD/014/2015).

Rotenone preparation and PD induction in rats

For intraperitoneal dose, the rotenone was prepared [7] with many modifications. Two ip rotenone doses were involved in this study: 3 and 6 mg/kg body weight (bw) per day (Rot-3-ip and Rot-6-ip, respectively). To prepare solutions for Rot-3-ip and Rot-6-ip injections, 0.0315 or 0.0630 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 bw olive oil. The solutions were prepared fresh weekly in amber coloured 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 or Rot-100-po, 0.525 or 1.050 g of rotenone was dissolved in 63 ml of 0.5% hydroxypropyl cellulose (HPC) procured from HiMedia, Mumbai, India to obtain 50 and 100 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.

Animal groups and experimental protocol

The rats were randomly assigned to six groups (n = 6 each group) as follows:

Group 1 (Con-OO): rats received olive oil (1 mL/kg bw ip) once daily for 21 days.
Group 2 (Rot-3-ip): rats received rotenone (3 mg/kg bw ip) once daily for 21 days.
Group 3 (Rot-6-ip): rats received rotenone (6 mg/kg bw ip) once daily for 2 days.
Group 4 (Con-HPC): rats received 0.5% HPC (5 mL/kg bw po) once daily for 28 days.
Group 5 (Rot-50-po): rats received rotenone (50 mg/kg bw po) once daily for 28 days.
Group 6 (Rot-100-po): rats received rotenone (100 mg/kg bw po) once daily for 7 days.

During the experimental period, all animals were carefully observed twice daily for clinical and distress signs. After completing the treatment protocol, the rats were anaesthetized with isoflurane and blood samples were collected from a retro-orbital puncture into vacutainer tubes that either contained the anti-coagulant ethylene diaminetetraacetic acid (K3 EDTA).

Haematological parameters

Chemical and reagents

Hayem's fluid, Drabkin's reagent and WBC diluting fluid were purchased from Sisco Research Laboratories (Mumbai, India). Haematological measurements included RBCs [9], WBCs [10], haemoglobin [11], platelets and PCV per cent [12]; procedures are described below in detail.

Red blood Cell (RBC) count

The total erythrocyte count was determined by the method of Huxtable (13). Exactly 0.5 mL blood was drawn into a RBC pipette followed immediately by Hayem's fluid. The blood and Hayem's fluid were thoroughly mixed and the solution was incubated for 2-3 min. A Neubauer counting chamber was placed with its cover slip in position. The capillary stem of the pipette was emptied of the diluting fluid and then one drop of blood was released into the groove of the Neubauer counting chamber. The cells were allowed to settle for 2-3 min. The counting chamber was put under the microscope and the ruled area was located. Erythrocytes were counted in the 1 mm square counting area. The number of cells in the 4 corner squares and 1 central square was counted. The total number of cells found in 5 groups of 16 squares were multiplied by 10,000 to give the number of cells in millions/mm^3 blood.

White Blood Cell (WBC) count

WBC number was determined by the method of Raghuramulu *et al.* (10). The method was similar to RBC counting except that the count was made in 4 large (1 mm) corner squares of a Neubauer counting chamber. The total number of cells in the 4 squares was multiplied by a factor of 2,500 to give count/mm^3 blood.

Hb estimation

Hb was measured according to Drabkin and Austin (9). Blood (0.02 mL) was diluted with 5 ml of Drabkin's reagent. The diluted blood was mixed well and allowed to stand for 10 min to ensure reaction completion. The solution was read at 540 nm together with the standard solution of cyanomethemoglobin. Blood Hb levels were expressed as g/dL.

Determination of PCV (haematocrit)

PCV was determined as described by Wintrobe (1990). Blood (0.6 mL) was collected in a wintrobe tube and centrifuged for 30 min in a relative centrifugal field (RCF) of 2,000 to 2,300 *g*. Subsequently, the volume of the packed cells was noted and expressed as percentage.

Estimation of platelets

Air-dried thin peripheral blood smears were prepared for all samples and stained with Leishman stain. The smears were examined with a light microscope using a 100x oil-immersion lens. In a monolayer zone of the smear, platelets were counted using platelet diluting fluid. The number of platelets per 1,000 RBCs was multiplied by an automated RBC count (10^6/µL) to get an estimated platelet count (10^3/µL).

Statistical Analysis

The results are expressed as mean ± standard error (SE). Statistical significance of the data was determined by one-way analysis of variance (ANOVA). The Student–Newman–Keuls (SNK) post-hoc test was used for multiple comparisons to identify sample means that were significantly different from each other. A p-value < 0.05 was considered significant.

RESULTS

Rotenone treatment led to marked changes in many haematological parameters. Blood Hb and RBCs were significantly decreased in Rot-3-ip, Rot-6-ip, Rot-50-po and Rot-100-po groups compared to the corresponding controls, Con-OO and Con-HPC (Figure 1 i and ii; $p < 0.001$). These reductions could indicate increased RBC lysis caused by rotenone and potential anemia in the treated rats. WBCs were also significantly decreased in Rot-3-ip, Rot-6-ip, Rot-50-po and Rot-100-po compared to control groups (Figure 1 iii; $p < 0.001$). This decrease indicates cellular inflammation due to rotenone toxicity. Finally, total platelet count and PCV per cent were significantly decreased in Rot-3-ip, Rot-6-ip, Rot-50-po and Rot-100-po treatment groups compared to their corresponding controls (Figure 1 iv; $p < 0.001$). Overall, ip and po rotentone administration markedly altered haematological parameters, findings that provide further indications of significant systemic toxicity. To confirm whether rotenone administration caused PD associated complications, α-Synuclein (immunohistochemistry) and H&E stained sections were examined (data not shown). The presence of Lewy bodies and pyknotic nuclei in brain samples of rot-3-ip group was histopathologically confirmed by H&E staining (data not shown). Lewy bodies could not be detected in rot-50-po group but showed cytoplasmic vacuolisation and neuronal degeneration.

DISCUSSION

Various PD animal models have been utilized to understand PD mechanisms and pathogenesis. Numerous dysfunctions, including excessive free radical production, oxidative stress, inflammation and metabolic risk factors (e.g., mitochondrial dysfunction, metabolic energy failure due to mitochondrial complex I inhibition and exposure to environmental toxins), have been associated with PD. In rotenone-induced PD animal models, the main reported pathological sequel involve mitochondrial ETC complex I inhibition, oxidative stress, Lewy body formation and marked neuronal damage and degeneration. Although myriad works contribute to understanding PD pathogenesis, we still lack precise therapeutics for its treatment. Yet, there could be many unexplored early cellular factors and causes accountable for the PD-associated complications.

In this work, we went on to explore how hematological parameters are altered in rats after oral or intraperitoneal rotenone induced PD complications. Based on the findings, good correlation appears to exist

between PD/rotenone toxicity and changes in blood cellular components, new revelations which could have relevance to the complications of early disease progression.

Similar to rotenone, exposure to the non-selective herbicide paraquat (PQ) is also associated with PD development [13]. PQ, a mitochondrial energy inhibitor and potent neurotoxin, is a source of superoxide anions and can cause extensive ROS formation, lipid peroxidation and cellular and tissue damage [14, 15]. In an earlier work [16], PQ induced a significant fall in blood erythrocyte count at 10 mg/kg bw ip injections to C57Bl/6 mice and increased ROS. PQ treatment also led to loss of young circulating erythrocytes and thereby induced transient anemia in the mice. Similar to PQ, in this study rotenone reduced RBCs, perhaps indicative of anemia that may complicate and/or exacerbate the tissue pathology and perhaps PD in general. Additionally, WBCs were markedly diminished by rotenone treatment. Indeed, peripheral WBC loss through apoptosis is thought to be an important PD biomarker [17]. Thus, a compromised or confused immune system could be partly responsible for PD progression. Additional studies that examine whether rotenone induces WBC apoptosis, and the specific WBC populations it affects, could provide additional insights into PD pathogenesis.

In a recent work, the nervous system complications that result from treatment of animals with rotenone are well reported, but less is known about how rotenone administration alters haematological measures. We observed significant declines in RBCs, WBCs, Hb, platelet count and PCV per cent after ip and po rotenone administration. Indeed, after only 2 days of treatment with Rot-6-ip, these haematological measures were markedly altered. In another PD-induced rat model, namely unilateral substania nigra lesion with direct injection of 6-hydroxydopamine, haematological parameters including RBCs, WBCs and Hb were all significantly reduced compared to control mice [18]. Although PD was induced in this model by direct nervous system administration, haematological changes similar to our model were still apparent. Thus, the central dopaminergic system appears to be important in regulating erythrocyte and leukocyte dynamics. Assessing haematological parameters could be a useful method for examining PD-induced animal models and may lead to insights into PD pathogenesis in humans.

We observed a dose-dependent decrease in Hb upon rotenone administration. There are several studies that suggest Hb is associated with PD in humans, although the results are somewhat conflicting. While Hb declines with age in men, when it remained high there was an association with PD [19]. However, in a recent study, a lower Hb level after PD onset was associated with greater disease severity and iron metabolism dysfunction [20]. Hb is found in dopaminergic neurons and helps maintains iron and mitochondrial homeostasis. Altered neuronal Hb and/or changes in its catabolism could impair mitochondrial homeostasis and contribute to neurodegeneration in PD [21]. Indeed, abnormal Hb levels in the brain could contribute to iron accumulation, oxidative stress, mitochondrial dysfunction and Lewy body formation [21]. With regards to circulating Hb, one group hypothesized that PD patients may have high-affinity Hb that tightly binds oxygen and thus less efficiently transfers it to tissues. Consequently, reduced tissue oxygenation could underlie at least some of the oxidative damage in PD [22]. Although the exact role of Hb in PD pathogenesis remains unclear, it could represent a potentially useful marker for the disease.

Taken altogether, the overall study findings revealed a similar trend line between oral and inperitoneal rotenone toxicity induced alterations in Hb, RBCs, WBCs and Platelets in rats. No marked difference was seen between rot-3-ip and rot-50-po induced hematological alterations. Notably, the lethal doses of ip and oral rotenone (rot-6-ip and rot-100-po) revealed drastic and abrupt blood cellular component abnormalities within shorter duration which is quite interesting. Therefore early blood cellular alterations seen in PD progression could manifest certain pathological features that could serve as marker for early diagnosis and subsequent treatment approaches for early intervention. To the best of our knowledge, studies on oral rotenone induced hematological changes are rarely found and the present work provides plausible insights into it.

CONCLUSION

In conclusion, peripheral (ip or po) rotenone administration to Wistar rats markedly altered various haematological parameters such as RBCs, WBCs, Hb and Platelets. The data support that this environmental toxin disrupts normal cellular homeostasis and underscores blood dysfunctions as important and potentially early indications of PD pathogenesis. Our findings encourage additional exploration of the mechanisms responsible for rotenone-induced changes that could eventually lead to novel biomarker tests to assess PD risk. Hence, pharmacological interventions aimed to target such early consequences per se could potentially ameliorate some of the neurological complications in PD progression.

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Disclosure Statement

Authors declare no conflict of interest in this study.

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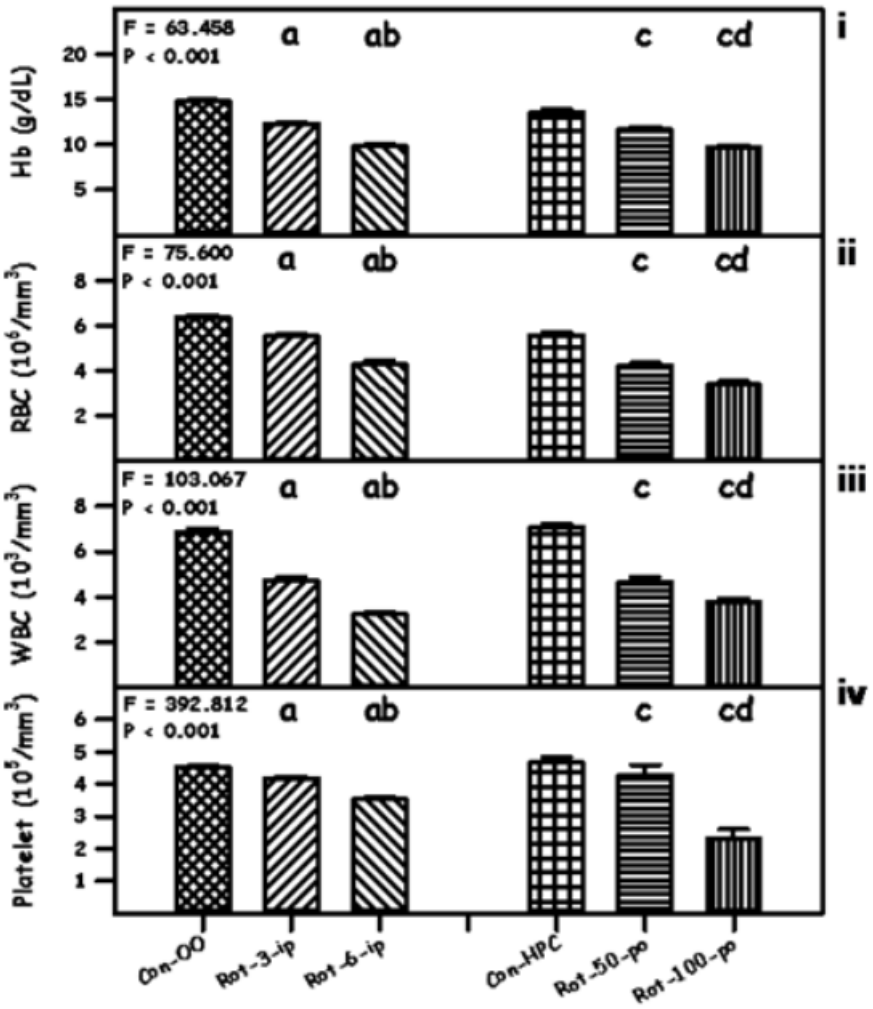

Figure 1: Comparison between of oral and intraperitoneal rotenone toxicity in rats on whole blood (i) haemoglobin (Hb), (ii) red blood cells (RBCs), (iii) white blood cells (WBCs) and (iv) platelets.
All values are mean ± SE (n = 6 each). Con = control; OO = olive oil, ip; HPC = hydroxypropyl cellulose, po; Rot = rotenone. Numbers indicate doses in mg/kg dose.
The 'F' and 'P' values are by one way ANOVA with Student-Newman-Keul's multiple comparison test.
[a]Significantly different from Con-OO.
[b]Significantly different from Rot-3-ip.
[c]Significantly different from Con-HPC.
[d]Significantly different from Rot-50-po