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DEVELOPMENT OF PARKINSON'S DISEASE RAT MODEL IN TWO WEEKS USING CORN OIL AND ROTENONE INDUCTION

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ABSTRACT: Parkinson's disease has been a challenging neurodegenerative disorder for researchers and clinicians alike for decades. Though L-dopa has changed the management of PD to a considerable extent, effective solutions remain elusive since L-dopa, after a few years, partly loses its efficacy and develops dyskinesias as a complication, incapacitating the patient. Also, existing PD animal models using MPTP, rotenone, paraquet, *etc* cause significant suffering to the animals and also require a minimum of 22–25 days for induction. Hence, a better PD animal model was conceived considering the synergistic oxidative stress lipids, and rotenone induce on striatal neurons. The experiment compared the existing rotenone induction with combined rotenone and high-fat diet induction, carefully watching for motor and behavioural symptoms of PD in the Wistar rats in addition to measuring the biochemical profiles for toxicity, strictly following the ARRIVE guidelines for animal research. This study found that using HFD+Rot for induction not only shortened the duration of induction to a mean of 14 days but also reduced the suffering by reducing the number of intraperitoneal injections needed without significant derangement of safety biochemical parameters. This study also proves the effectiveness by demonstrating the reduced dopamine, serotonin levels in striatal neurons and increased oxidative stress parameters. To the best of our knowledge, no animal study shows a better PD model with all the neurobehavioural and biochemical features of PD with less time for induction. This new HFD+Rot model can therefore be employed to test therapeutics and neurobehavioural studies.

INTRODUCTION: Parkinson's disease (PD) animal models are widely studied for the development of new therapies. An array of existing rodent models ranges from pharmacological models involving disruption of the dopaminergic nigrostriatal system to genetic models of PD.

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The currently used rodent models of PD involve neurotoxic chemicals *viz.*, MPTP, 6-OHDA, rotenone, paraquat and manaeb [1, 2]. The use of neurotoxin-induced PD models has led to a better understanding of the pathophysiology of PD.

High doses of MPTP are required to block dopaminergic (DA) receptors to display the signs of Parkinsonism, which results in higher mortality. 6-OHDA lesion in rats does not induce the formation of Lewy body-like inclusions and does not show the clinical symptoms of PD pathology [3, 4]. Paraquat and manaeb models are also similar to MPTP as the pathophysiology of PD induction

remains the same [5]. The rotenone model needs about 25 days for PD induction, which causes considerable mortality, suffering, and prolongation for the animals [6].

The rotenone-induced animal model reproduces the behavioural and pathological features of typical human PD [7]. This model replicates many aspects of PD, such as iron accumulation in the substantia nigra, systemic mitochondrial dysfunction, oxidative damage, microglial activation, selective nigrostriatal dopaminergic degeneration, L-dopa-responsive motor deficits, impaired ubiquitin-proteasome function, acidification and mitochondrial translocation of PARK7 (DJ-1) and gastrointestinal dysfunction associated with Alpha-synuclein (α-Syn) aggregation and accumulation [8, 9, 10]. Since PD is mostly sporadic in humans with rare mutations, a study by Xicoy suggested that animal models were preferable to cell models like SH-SY5Y as clinical evidence correlated biochemically also [11].

L-dopa, used in the treatment of PD, becomes only partly useful after a few years, as dyskinesias often develop in addition to problems of availability in the central nervous system. Many studies suggest that fat content alone in the diet has no significant role in the genesis of PD [12, 13, 14]. But a recent study also reported that a high-fat diet (HFD) with more than 41% saturated fat increased the incidence of PD [15]. A case-control study reported that high cholesterol intake, increased total fat, and saturated fat were associated with a significant risk of PD [16, 17]. However, a weak positive association between PD and omega-6 (ω6) PUFA (polyunsaturated fatty acid), especially linoleic acid, was reported in an observational study [18].

Though lysosomal cholesterol has been found to offer protection from cell death due to oxidative stress in PD, high cholesterol levels were found to stimulate the formation of α-Syn, leading to neuronal cell death [19]. Thus, high cholesterol levels play a contrasting role in determining the progression of PD. Furthermore, statins have been shown to reduce oxidative damage in neurons while having no effect on lysosomal cholesterol, which acts as a barrier against lysosomal membrane permeability and the formation of α-Syn [20]. PUFA was associated with low cell damage and omega6:3 (ω6:ω3) content in it has been found to be important in cellular metabolism, influencing oxidative damage. ω3 PUFA is associated with decreased inflammation, with an ideal ratio for homeostasis at a ω6:3 of about 4:1 [21]. Corn oil has a ω6:3 of 46:1 with a possibility of pro-inflammatory ω6 producing more damage in high energy utilizing areas like Substantia Nigra(SN), damaging the DAergic neurons [22].

Furthermore, the hypercholesterolemic effect of corn oil could be due to hypertriglyceridemia rather than the role played by low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [23]. Hence, this study aimed to test whether corn oil (HFD) administration and rotenone could reduce PD induction time. While rotenone would induce PD, synergistic oxidative damage of neurons by corn oil will result in early induction of PD. A recent study showed that corn oil with more ω6:3 will induce more oxidative damage at cellular levels [21, 22] and this would help in the early induction of PD in rats with less suffering for the animal.

MATERIALS AND METHODS:

Animals and Ethical Approval: Male Wistar rats weighing between 250-300g were housed in polypropylene cages and maintained at 23 °C ± 2 °C with a relative humidity of 50–60% under a 12:12 hour light: dark cycle with a commercial solid diet (Biogen Laboratory Animal Facility, Bengaluru) and water *ad libitum*. This study was conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India), which complied with the National Institutes of Health guide for the care and use of laboratory animals and was approved by the Institutional Animal Ethics Committee (SU/CLAIR/RD/002/2022) and followed ARRIVE guidelines for animal research.

Rotenone Preparation: The mean weight of rotenone needed for each group was estimated after calculating the mean weight of animals in each group. Accordingly, 2.5 mg/kg body weight of Rotenone was dissolved in 0.3 mLDi methyl Sulphoxide (DMSO), made up to the final volume (20 mL) with olive oil, and was administered at 0.5 mL/ animal intra-peritoneally (ip), which is equivalent to 2.5 mg/kg of Rotenone.

HFD Preparation: The mean weight of animals in HFD group was calculated, and 10 mL/kg of corn oil was given as HFD diet orally.

Animal Grouping and Experimental Protocol: The animals were randomly divided into 4 groups with minimal weight variation (± 2 gm) with 5animals/group: Group 1 received DMSO at 0.3 mL dissolved in 0.2 mL of olive oil intraperitoneally and served as a control. Group 2 received HFD (corn oil) at 10 mL/kg orally. Group 3 received 0.5mL of rotenone intraperitoneally at a dose of 2.5 mg/kg DMSO in olive oil daily. Group 4 received rotenone 0.5mL intraperitoneally at a dose of 2.5 mg/kg/day in DMSO in olive oil along with corn oil at a high-fat diet (HFD) orally at the rate of 10mL/kg. All the groups were to receive drugs for at least 21 days. Animal experimentation and grouping were performed per approved institutional guidelines [6, 24].

All animals were carefully screened twice daily for motor impairment, *i.e.*, the appearance of Parkinsonian features like bradykinesia, postural instability/gait disturbances, and rigidity. When the behavioural changes became debilitating, *i.e.*, limiting mobility, feeding, or grooming, the rats were anesthetized with isoflurane (Raman and Weil, Mumbai). Groups 1, 2, 3 and 4 were to receive the respective drugs for 21 days, or until they develop full behavioural changes, whichever is earlier. Blood samples were collected by retro-orbital puncture into vacutainer tubes that contained the anti-coagulant potassium ethylenediamine tetraacetic acid (K3 EDTA). Since 2 rats from group 3 and 1 from group 4 died during the course of the experiment, the deficiency was covered up by adding 3 more rats into the groups. To prepare serum, the blood was allowed to clot for 15–30 min at room temperature and centrifuged at 3,500 rpm for 10 min in a cooling centrifuge (REMI CPR-24PLUS, India). Animals were euthanized after isoflurane anaesthesia by cervical dislocation, to harvest the organs for study. Brains were rapidly removed and bisected mid-sagittaly.

Cardiac Perfusion and Brain Homogenate Preparation: After anesthetizing the animal, cardiac perfusion was done using normal saline (0.9% NaCl) to eliminate the blood clots. The skull of the rats was slit open centrally at the dorsal aspect, and the midbrain was carefully separated and washed with ice-cold phosphate-buffered saline (PBS), pH 7.4. The whole midbrain tissue isolated from each rat was kept on ice and homogenised with 0.1 M PBS (pH 7.0) using a Potter–Elvehjem PTFE-coated Teflon pestle and glass tube (PRO Scientific Inc., Oxford, CT, USA), run at 600 rpm for 3 minutes. It was then centrifuged at 3000 rpm at 4ºC for 10 min using a Remi refrigerated centrifuge, and then the supernatant was taken for analyzing of the parameters [25].

Estimation of Glucose: Standard commercial kits were utilised according to the manufacturer's instructions for biochemical analysis. The glucose level was determined by the glucose oxidase-peroxidase method using the RANDOX Laboratories Ltd., UK kit.

Assay of Serum Aspartate Aminotransaminase (AST): The aspartate aminotransaminase activity was assayed according to Mohur's method. To 0.1 mL of serum, buffered substrate (1 mL) was added and incubated for 1 hour at 37ºC. To arrest the reaction, DNPH reagent (1 mL) was added. To the blank tubes, DNPH reagent, as well as serum (0.1 mL) were added. After letting the vials sit for 10 to 15 minutes, 15.0 mL of NaOH was mixed and measured in a Shimadauz UV spectrometer at 510 nm.

Assay of Serum Alanine Transaminase (ALT): The activity of Alanine transaminase (ALT) was analyzed according to Mohur's method. The reagents and method used were the same as those used for the analysis of aspartate transaminase, except for the substrate solution and incubated for 30-40 minutes. 1800 mg of DL-alanine and 0.037 g of 2-oxoglutarate were combined in a buffer. Then 1 ml of NaOH was added, and the volume was adjusted to 0.1 L, so the identical process as with AST was followed.

Estimation of Creatinine: Creatinine was estimated by the method of Slot as followed by Malathi [26]. To 3 ml of deproteinized supernatant (0.1 ml of serum + 3.9 ml of 10% TCA), added 2 ml of alkaline picrate solution. The blank, having 3 ml of water and aliquots of standard in 4 ml of water, was allowed to react in the same form. 40

minutes later, the colour was observed at 520 nm in contrast to the reagent blank. The values were expressed as mg/dl.

Estimation of Blood Urea: Using Berthelot's enzymatic colorimetric test, serum urea was determined. In the presence of water and urease, urea gets hydrolyzed and produces ammonia and carbon dioxide. In an altered Berthelot reaction, a green dye has been made through the reaction of ammonium ions when reacted with hypochlorite and salicylate. At 578 nm, the absorbance increases and is proportional to the urea concentration in the sample. There were three cuvettes for the reaction; one as a blank, one as a standard, and one as a sample. Initially, a blank reaction was performed: 0.02 ml of distilled water was pipetted into the cuvette and reagent (2 ml). For proper mixing, the solution inside the cuvette was pipetted again and transferred to the heated photometer. Initial absorbance was measured accurately 30 seconds after pipetting the working solution, and the subsequent absorbance was measured after a minute. To the cuvette and reagent (2 ml), a sample (0.02 ml) was pipetted out, and absorbance was taken using a spectrophotometer (Elico, B-200, Hyderabad, India). The concentration of urea was calculated. The normal blood urea level in Wistar rats is 62.18.4 mg/dl [28].

Estimation of Total Cholesterol: Cholesterol estimation was done according to the procedure of Parekh and Jung, as noted by Santhosh [29]. Ferric chloride-uranyl acetate reagent (2.9 ml) was added to the sample (0.1 ml). After centrifugation, the sulphuric acid-ferrous sulphate reagent (2.0 ml) was added and mixed well. A blank containing ferric chloride-uranyl acetate reagent (3.0 ml) and sulphuric acid-ferrous sulphate reagent (2.0 ml) was used. A calibration graph was made with standard cholesterol. In a Shimadzu UV spectrophotometer, the optical density was taken (530 nm after 20 minutes). The levels of cholesterol were measured in mg/dl.

Estimation of Triglycerides: Triacylglycerol estimation was done according to the procedure of Rice, as noted by Santhosh [29]. To a 0.1 ml sample, activated alumina (50 mg) and isopropanol (3.9 ml) were added and mixed well. This was then kept for 15 minutes. Centrifugation was done, and the supernatant (2.0 ml) was taken for examination. Alkaline potassium hydroxide (0.6 ml) was allowed to react with the canisters and was kept for 10 minutes at 60°C. The tubes were chilled, and sodium meta periodate (1.0 ml) reagent was added to the tubes, followed by the addition of acetylacetone reagent (0.5 ml). Finally, the reading was measured with a Shimadzu UV spectrophotometer at 420 nm against a blank.

HDL was estimated by a semi-auto analyzer (ROBONIK) using a diagnostic reagent kit (Aspen Laboratories, Delhi). The calculation of VLDL and LDL by using the formulae:

LDL-C (mg/dl) = sample x calibrator concentration (mg/dl);
VLDL = Triglycerides (mg/dl) / 5

MDA Estimation: The levels of malondialdehyde (MDA) were quantified using the thiobarbituric acid C4H4N2O2S reaction method to measure oxidative damage. A working solution of thiobarbituric acid, trichloroacetic acid, and 0.20 N HCl was prepared to measure the level of MDA. 250 µL tissue homogenate and 500 µL working solution were added and centrifuged for 10 min at 3000 rpm. After centrifugation, the supernatant was then taken in, and the OD of the samples was assayed at 540 nm. The MDA concentration, an indicator of LPO, is expressed as nmol mL^{-1}.

Nitric Oxide (NO-2) Estimation: *In-vitro* oxidation reactions (1 mg brain protein/ml) were performed at 37 °C in buffer B (50 mM sodium phosphate, pH 7.4).When indicated, buffer B was supplemented with 25 mM $NaHCO_3$. Reactions were terminated by adding 0.2 mM DTPA (pH 7.4), 300 nM catalase, and 0.1 mM BHT. Proteins were precipitated with ice-cold trichloroacetic acid (10% final concentration), acid-hydrolyzed, and subjected to GC/MS analysis. ONOO−was synthesized from 2-ethoxyethyl nitrite and H_2O_2 and stored at −80 °C. ONOO− was thawed immediately before use, and its concentration was determined spectrophotometrically at 540 nm[30].

Estimation of Peroxynitrite: The peroxynitrite level was determined according to the method described by Beckman and referred by Kruk [31]. In brief, 100µl of homogenate was placed in a glass test tube, to which 5mM phenol in 5M sodium phosphate buffer pH 7.4 was added to a final

volume of 2 ml. After mixing, the resulting solution was incubated for 2 hours at room temperature and then added 15 µl of 0.1 M sodium hydroxide. The method is based on the oxidation of o-phenylenediamine, a colourless substance, by peroxynitrite to give a coloured product, and the absorbance is read at 412 nm. The absorbance increase seen from the reaction is proportional to the concentration of peroxynitrite in the range of 4.4×10^{-7} to 8.0×10^{-6} mol L^{-1} with a detection limit of 1.7×10^{-7} mol L^{-1} (3σ); described in the procedure of Jablonska, noted by Rodriguez Garcia [32].

Total Antioxidant Activity Estimation: Total antioxidant capacity (TAC) of serum was measured according to the method of Benzie and Strain, as suggested by Munteanu [33]. By combining buffer acetate with 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) solution in HCl, a working solution of FRAP (ferric reducing antioxidant power) was obtained. After that, $FeCl_3$ was added and mixed. 8 µL of sample supernatant and 240 µL of the mentioned working solution were mixed and incubated for 10 min at room temperature. The optical density of samples was measured at 532 nm.

Measuring Brain Urea: Brain samples were cut into small sections of 50 mg each for urea quantification and stored at −80°C before extraction. Urea was quantified in the brain sample by HPLC coupled with MS. Brain samples were extracted in 50:50 (v/v) methanol: chloroform containing labeled urea as an internal standard. The methanol: chloroform internal standard solvent was prepared. LC-MS grade water was then added to samples before separating polar and non-polar phases by centrifugation at 2,400 × g for 15 min. The methanol phase was transferred to a test tube in a centrifugal concentrator. After drying, 0.1% formic acid was added to the samples. The resulting solution was transferred to 300-µl autosampler vials, with two blanks containing only 0.1% (v/v) formic acid also prepared. Standard solutions in 0.1% v/v formic acid were prepared using an internal urea standard. Separation was carried out on a Hypersil Gold AQ column with a diameter of 2.1 mm, a length of 100 mm, and a particle size of 1.9 µm (Thermo Fisher Scientific) maintained at 25°C with a 0.5µm pre-column filter (Thermo Fisher Scientific). Gradient elution was performed using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at 300µl/min. Urea quantification was performed on the Thermo TSQ Quantum Access Max (Thermo Fisher Scientific, MA, United States).

Estimation of Serotonin: Biochemical analysis of serotonin neurotransmitters was performed using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) (HPLC-ECD). The samples were centrifuged at 25,000 g for 10 minutes at 4°C, and supernatants were collected. Serotonin (5-HT) content was assayed by HPLC-ECD, equipped with a column of 5µm spherical C18 particles. The mobile phase consisted of 0.1 M phosphate buffer (pH 2.6) containing 0.2 mM octane sulfonic acid, 2.5% methanol, and 4.5% acetonitrile. The content of serotonin is expressed as ng/mg.

Estimation of Dopamine: The biogenic amine, dopamine, was estimated by the HPLC method using an electrochemical detector as described by Church in the study done by Pathania[34]. Waters standard system consisting of a high-pressure isocratic pump, a 20 µl sample injector valve, C18 reverse phase column, and an electrochemical detector was used. Data were recorded and analyzed with the help of Empower software. The mobile phase consisting of 2% citric acid, 2% $KHPO_4$, 1mMEDTA, 1.2% MeOH, and 70 mg/ml of sodium octylsulphate, pH of the mobile phase was adjusted to 3 with the help of HCl (6N). Separation was carried out at a flow rate of 1 ml/min. Samples (20 µl) were injected manually. Electrochemical conditions for the experiment were +0.800 V, and sensitivity ranged from 5~50 nA. The content of dopamine is expressed as ng/mg.

Histopathology of Brain Tissue: For histopathological analysis using light microscopythe brain tissues were administered for paraffin sectioning. Then the tissues were hydrated and dehydrated in various grades of alcohol series. It was then cleaned with xylene and chloroform before being preserved in paraffin wax using a rotary microtome. Tissue sections were then cut and stored at room temperature overnight. It was then deparaffinized and moistened with descending alcohol concentrations, followed by distilled water. Using haematoxylin and eosin stain, the sections

were stained and then subjected to ascending grades of alcohol. The permanent slide was prepared using a DPX mount. The slides were observed under a light microscope (Olympus microscope), and photomicrographs were taken using a Sony digital camera [35].

Statistical Analysis: The data were expressed as the mean ± standard deviation. The statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by a Bonferroni t-test for multiple comparisons. The statistical analysis was performed using the following software programs: immunohistochemistry quantification, SPSS version 22.0 (IBM Corp., Armonk, NY, USA); and urea measurements, Sigma Plot 14.5 (Systat Software, San Jose, CA, USA).

RESULTS:

Duration of Induction: Group 1 (control) received DMSO+O live oil for 21 days, while Group 2 received HFD (Corn oil) alone; neither developed any PD manifestation. Group 3 (20.21 ± 0.3742 SD) received rot only for 21 days, and group 4 (14.00 ± 0.7071 SD) received Rot+HFD for 14 days, at which they developed a full behavioural phenotype. The unpaired t-test showed significance with a p-value of < 0.0001. Based on rotarod and movement analysis, all the group-4 and group-3 (Rot) animals (n=5 each) had bradykinesia, postural instability, and rigidity.

Changes in Biochemical Parameters: The biochemical results (glucose, AST, ALT, urea, and creatinine levels) shown in **Fig. 1** were analyzed to assess the adverse effects caused by HFD and rotenone + HFD induction. The mean value of glucose in control (group-1), HFD (group-2), rotenone (group-3) and HFD + rot (group-4) was 87.6 ± 6.5 SD, 126.7 ± 6, 118.1 ± 3.8 and 130.1 ± 2.9 respectively.

In the liver function test (LFT) compared to control, in all the groups, both AST and ALT (IU/L) were significantly elevated when compared with control. The Mean±SD AST value of group-1,2,3 and 4 was 72.06 ± 5.623, 169.26 ± 3.62, 116.58 ± 9.812 and 188.1 ± 5.135. Analysis of ALT values showed similarly high results with groups-1,2, and 4 with mean± SD values of 53.28 ± 13.772, 102.38 ± 8.642, 83.9 ±9.746 and 140.82 ± 2.142 respectively.

Analysis of renal function tests (RFT) showed higher values when compared with control. The mean±SD values of blood urea (mg/dl) in group-1,2, 3 and 4 were 20.76 ± 2.965; 39.98 ± 4.977, 66.040 ± 5.038 and 89.88 ± 1.532 respectively. Creatinine values (mg/dl) were also elevated in all groups when compared with control. Mean±SD values in group-1, 2, 3 and 4 were 0.420 ± 0.0837, 1.242 ± 0.188, 1.54 ± 0.270 and 2.38 ± 0.130 respectively.

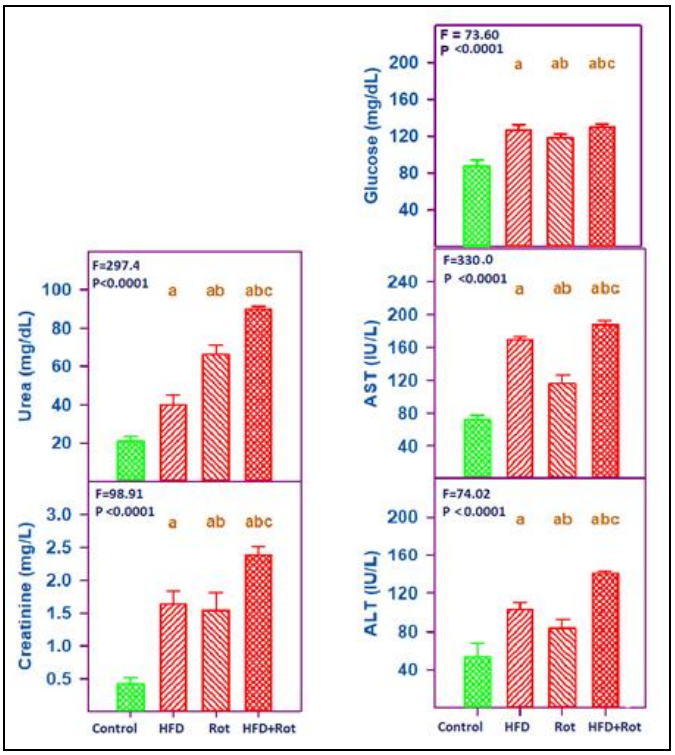

FIG. 1: COMPARATIVE EFFECT OF HIGH-FAT DIET (HFD), ROTENONE (ROT) AND HFD+ROT INDUCED CHANGES ON SERUM GLUCOSE, ASPARTATE AMINOTRANSAMINASE (AST), ALANINE AMINOTRANSAMINASE (ALT), BLOOD UREA AND SERUM CREATININE. Values are mean ± SD (n = 5 each). The 'F' and 'P' values are by one-way ANOVA with Bonferroni't' test. A-Significantly different from the control group. B-Significantly different from the HFD group. C-Significantly different from PD group.

Lipid profile was assessed with total cholesterol (TC), triglyceride (TGL), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). There was a significant increase in TC, TGL, and LDL in groups 2, 3 and 4 compared with group 1 expressed as mean±SD values.

HDL levels (mg/dl) in group-2 (32.08 ± 1.301), group-3 (34.46 ± 5.826) and group-4 (35.04 ± 3.078) were lower than in group 1(53.78 ± 7.702). This decrease in HDL in groups 2, 3, and 4 was significant on Bonferroni pairwise comparison with group 1($p<0.05$). However, HDL values in group 2 vs. group 3, group 3 vs. group 4, or group 2 vs. group 4 are not significant. Similarly, group-2 vs. group-1, group-2 vs. group-4, group-3 vs. group-4, group-3 vs. group-1, and group-2 vs. group-3 all had significant differences in mean TC, TGL, and LDL **Fig. 2.**

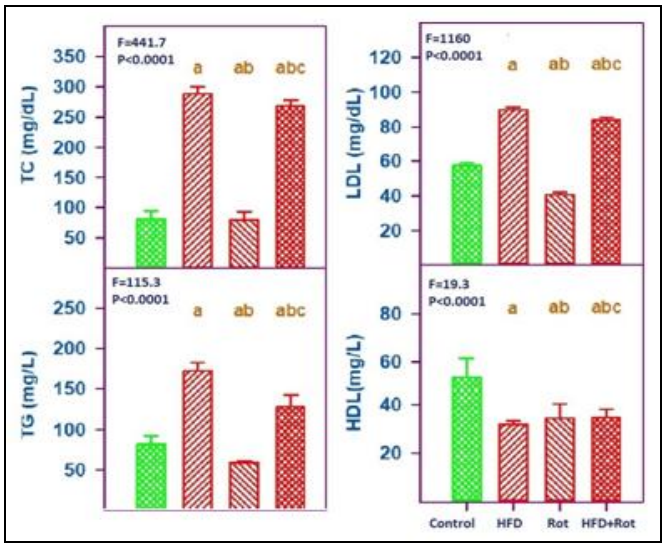

FIG. 2: COMPARATIVE EFFECT OF HIGH FAT DIET(HFD), ROTENONE(ROT), AND HFD+ROT INDUCED CHANGES ON SERUM TOTAL CHOLESTEROL (TC), HIGH-DENSITY LIPOPROTEIN (HDL), LOW-DENSITY LIPOPROTEIN (LDL), TRIGLYCERIDE (TGL). Values are mean ± SD (n = 5 each). The 'F' and 'P' values are by one way ANOVA with Bonferroni't' test. A-Significantly different from control group. B-Significantly different from HFD group. C-Significantly different from PD group.

Alterations in Oxidative Stress Markers: Assessment of oxidative stress markers like MDA and total antioxidant capacity (TAC) shows that MDA increased in all the groups when compared with control. The Mean±SD values of MDA in group-1, group-2, group-3 and group-4 were1.846 ± 0.369, 5.412 ± 0.502, 5.126 ± 0.091, 5.678 ± 0.387. The TAC value in group-1, group-2, group-3, and group-4 was 698.08 ± 27.653, 435.82 ± 21.831, 388.94 ± 54.897, and 324.12 ± 17.145. Analysis of markers of induction of oxidative damage like nitric oxide and its toxic products showed increased values.

Peroxynitrite was increased in all the groups-compared to the control. Group-1, group-2, group-3 and group-4 was 0.0376 ±0.00541, 0.0706 ± 0.00607, 0.0784 ± 0.00961 and 0.0866 ± 0.00288 expressed as Mean±SD values. Nitric oxide values in group 1 were low (1.642 ± 0.154 SD) when compared with group 2 (4.216 ± 0.0859 SD), group 3 (4.206 ± 0.218 SD), and group 4 (4.724 ± 0.168 SD) **Fig. 3.**

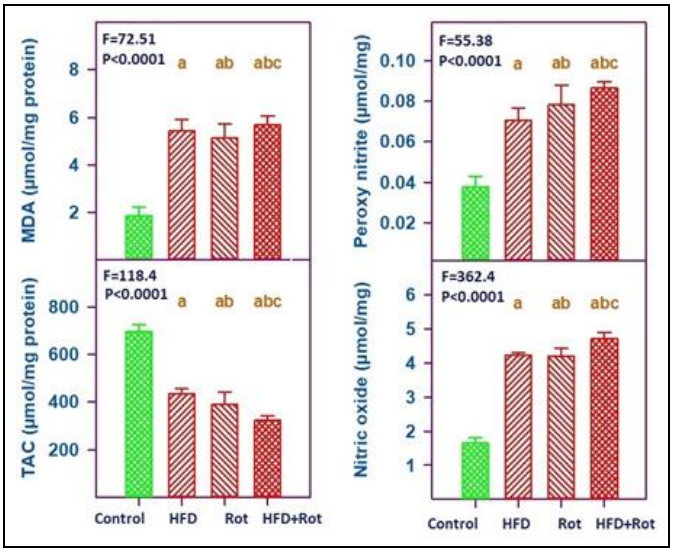

FIG. 3: COMPARATIVE EFFECT OF HIGH FAT DIET(HFD), ROTENONE(ROT) AND HFD+ROT INDUCED CHANGES ON SERUM TOTAL CHOLESTEROL (TC), HIGH-DENSITY LIPOPROTEIN(HDL), LOW-DENSITY LIPOPROTEIN (LDL), TRIGLYCERIDE (TGL) ON MDA, TAC, PEROXYNITRITE AND NITRIC OXIDE. Values are mean ± SD (n = 5 each). The 'F' and 'P' values are by one way ANOVA with Bonferroni't' test.

A pairwise comparison of TAC and MDA values from groups 3 vs. 4 and 2 vs. 4 revealed no statistical significance. However, peroxinitrite and nitric oxide values showed significant differences in mean in group-2 vs. group-4, though no significant difference in mean was observed between group-3 vs. group-4 or group-2 vs. group-3 on the Bonferroni test.

TABLE 1: PAIRWISE MULTIPLE COMPARISON OF OXIDATIVE STRESS MARKERS

Comparison of MDA values	Diff of Means	t	P
HFD+Rot vs. Control	3.832	16.770	<0.001
HFD+Rot vs. Rot 0.5522.416	0.610		
HFD+Rot vs. HFD0.2661.164	1.000		

HFD vs. Control 3.566	15.605<0.001		
HFD vs. Rot0.2861.252	1.000		
Rot vs. Control 3.280	14.354	<0.001	
Comparison of TAC values	**Diff of Means**	**t**	**P**
Control vs. HFD+Rot373.960	19.677	<0.001	
Control vs. Rot 309.140	16.267<0.001		
Control vs. HFD 262.260	13.800<0.001		
HFD vs. HFD+Rot	111.700	5.878	<0.001
HFD vs. Rot 46.880	2.4670.542		
Rot vs. HFD+Rot64.820	3.411	0.051	
Comparison of Peroxinitrite value	**Diff of Means**	**t**	**P**
HFD+Rot vs. Control	0.0490	12.662<0.001	
HFD+Rot vs. HFD	0.0160	4.135	0.007
HFD+Rot vs. Rot	0.0082	2.119	1.000
Rot vs. Control	0.0408	10.543<0.001	
Rot vs. HFD	0.0078	2.016	1.000
HFD vs. Control	0.0330	8.528<0.001	
Comparison of Nitric Oxide Value	**Diff of Means**	**t**	**P**
HFD+Rot vs. Rot	0.518	2.988	0.153
HFD+Rot vs. HFD	0.508	2.930	0.177
HFD vs. Control	2.574	14.848	<0.001
HFD+Rot vs. Control	3.082	17.778	<0.001
HFD vs. Rot0.01000	0.0577	1.000	
Rot vs. Control	2.564	14.790	<0.001

Analysis of Neurotransmitters in Brain Tissues: Serotonin (ng/mg) and dopamine (ng/mg) showed a significant decrease when compared with the control. Group 1 showed Mean±SD values of 0.476 ± 0.023, group 2 (0.318 ± 0.013), group 3 (0.210 ± 0.0158), and group 4 (0.252 ± 0.0192) for serotonin. Dopamine levels in brain tissue showed a mean value of 0.878 ± 0.0715 SD, group-2 (0.840 ± 0.0515 SD). However, groups 3 (0.474 ± 0.0358 SD) and 4 (0.496 ± 0.0483 SD) had significantly lower values **Fig. 4.**

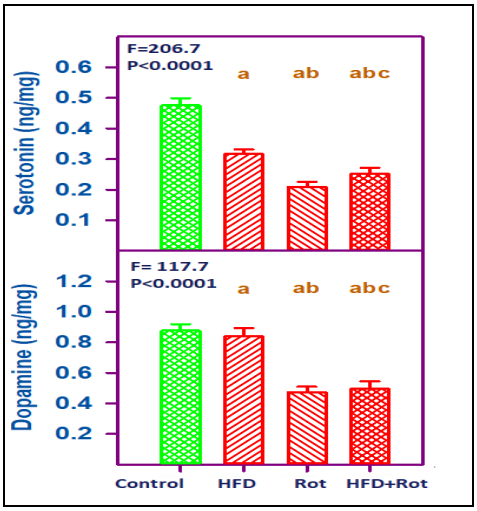

FIG. 4: COMPARATIVE EFFECT OF HIGH-FAT DIET (HFD), ROTENONE (ROT) AND HFD+ROT INDUCED CHANGES ON BRAIN SEROTONIN AND DOPAMINE. Values are mean ± SD (n = 5 each). The 'F' and 'P' values are by one way ANOVA with Bonferroni't' test.

Urea Changes in the Brain: Levels of brain urea were also analyzed in all the groups. The Mean±SD values of group-1(36.7 ± 3.883) and group-2 (39.72±0.779) did not show a statistically significant difference. But, the mean values were significantly elevated in groups 3 (73.2 ± 1.43) and 4 (69.86 ± 5.638). Bonferroni, multiple Pairwise comparison of brain urea levels between groups 3 and 4, showed a significant difference in mean (M=23.8; t=7.425; p-value < 0.001) **Fig. 5.**

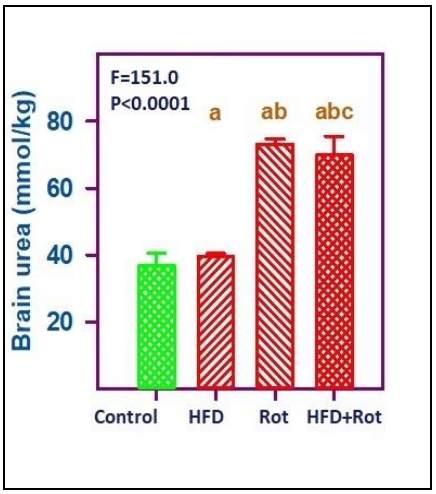

FIG. 5: COMPARATIVE EFFECT OF HIGH-FAT DIET (HFD), ROTENONE (ROT) AND HFD+ROT INDUCED CHANGES ON BRAIN UREA. Values are mean ± SD (n = 5 each). The 'F' and 'P' values are by one way ANOVA with Bonferroni't' test.

Comparison of α-Syn in Different Groups: The evidence for cell damage due to the oxidative stress induced in different models assessed with levels of expression of α-Syn by Western Blotting showed a significant increase in values in both groups-3 and 4 when compared with group-1 and group-2; but there was no significant difference in means between group-3 and group-4 **Table 2, Fig. 6.**

TABLE 2: QUANTITATIVE DATA OF A-SYN EXPRESSION BY WESTERN BLOT

	Group 1	Group 2	Group 3	Group 4
Mean (intensity Units)	2.20	5.07	5.83	6.43
SD	1.05	0.15	0.78	0.42

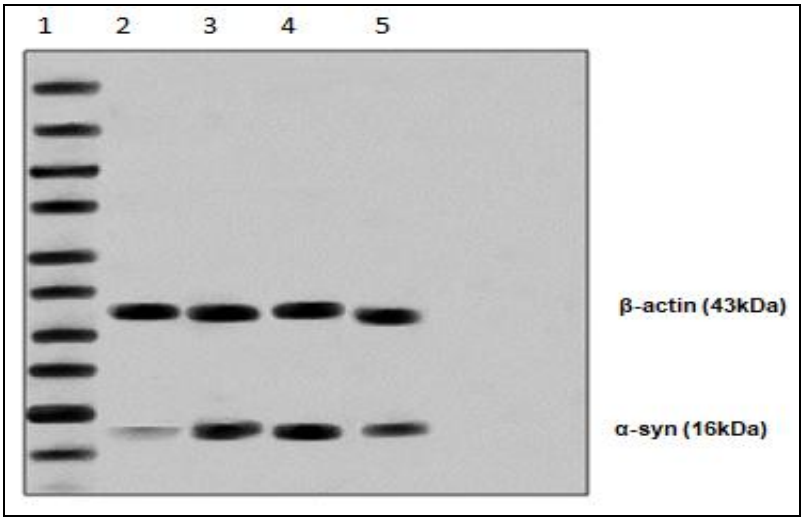

FIG. 6: A-SYN EXPRESSION- WESTERN BLOT. LANE 1- MARKER LANE; LANE 2 – CON; LANE 3 – ROT; LANE 4 – ROT+HFD; LANE 5 – HFD

Histopathological Analysis: Histopathological examinations using Hematoxylin and Eosin staining and viewed at 40x magnification showed that the control group had normal cells with no pathological alterations, whereas the rotenone group had darkly stained nuclei, indicating neuronal damage, as well as Lewy bodies (dark arrow). The HFD group showed significant inflammatory cell response and vacuolation, whereas the HFD+Rotenone group showed vacuolation and pericellular haloes (red arrow) in neurons and significant neuronal loss.

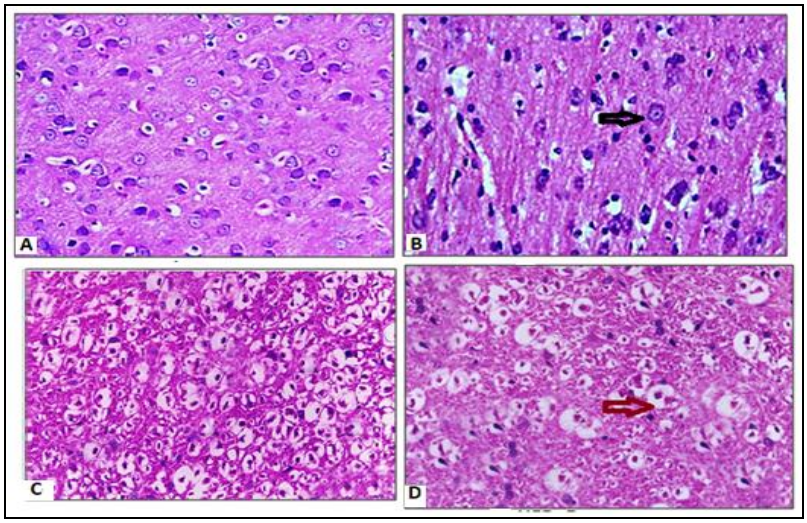

FIG. 7 HISTOPATHOLOGY OF BRAIN: HISTOPATHOLOGY OF BRAIN TISSUE WITH HAND E AND MAGNIFIED AT 40X IS SHOWN AS – A- CONTROL; B- ROTENONE; C- HFD; D- HFD+ROTENONE

DISCUSSION: Rotenone-induced PD models have been extensively used for the study of the pathogenesis of PD [36]. Current evidence suggests that intraperitoneally administered rotenone

induces PD much earlier and efficiently with less mortality than orally administered rotenone does [37]. In the present study, Parkinson's disease induced by rotenone alone and rotenone and corn oil combination did not show a significant difference in findings. The death rate of animals was low (n=3; 10%), but mostly due to trauma. The main limitations of the rotenone model have been variability (i) in the percentage of animals that develop a nigrostriatal dopaminergic lesion, (ii) lesion magnitude, and (iii) mortality. Group-4 (Rot + HFD) showed less mortality and duration of induction with rotenone, with much quicker and better development of PD symptoms and less weight loss when compared with the PD alone model ($p<0.05$).

Blood sample analysis revealed increased glucose levels when compared to controls, implying glucose dysregulation, most likely due to PD-associated dysautonomia, leading to impaired insulin response, as reported in a recent study [38]. According to an earlier study, rotenone treatment did not cause an increase in blood glucose [39]. But, treatment with rotenone increased blood glucose in the present study; though the increase was never beyond the 95th centile value for FBS/PBS of Wistar rats (95% upper limit was 6.2 mmol/L and 7.9 mmol/L, respectively) [40].

Significant elevation seen in AST, ALT, blood urea, and creatinine levels in the present study, though never beyond the upper limit of the reference range of rats, can be attributed to the rotenone and HFD-induced alterations. The altered renal function, elevation in AST and ALT levels following rotenone were attributed to excessive ROS generation in the hepatic and renal systems [41, 42]. This shows that the combination of Rotenone and HFD may cause rise in creatinine levels, but not to the extent of causing increased mortality or morbidity as the duration of induction is reduced, especially if we follow standard protocols of ensuring optimal dose, fresh preparation of drug each time, and ensuring full dilution before giving I/P as done in the present study.

Many studies have reported changes in lipid profiles, including an increase in total cholesterol, TGL, and LDL in the HFD and Rot + HFD groups as reported in the present study. But the above parameters were decreased in group 3 when compared with the control. The lipid profile in the present study is in line with a recent meta-analysis concluding cholesterol biosynthesis seems to be impaired in PD. Still, the direction of the change differs among PD aetiologies. A strong correlation exists between PD and lipid metabolism, especially between PD and fatty acids (PUFA, SFA etc.) and lipoproteins (LDL, HDL and VLDL [43 ,44]. PUFA strongly interacts with the N-terminal region of α-Syn [45], increasing its *in-vitro* and *in-vivo* oligomerization. Loss of striatal dopamine terminals was evident in rotenone animals treated at 3.0 mg/kg/day. But animals treated with a lower dose of rotenone, 2.75 mg/kg/day for 21 days, also achieved a debilitating PD phenotype, without overt striatal lesions [46].

Many other studies have also reported a variable period for induction ranging from 60 days to a minimum time frame of 21-25 days [47, 48]. However, in the present study, it was possible to establish the same PD phenotype along with biochemical evidence of brain damage as early as 14 days after administering rotenone at 2.5 mg/kg/day and 10 mL/kg/day of corn oil. This was possible by utilising the ROS-generating property of ω-6 PUFA in corn oil. Corn oil has a ω6:3 ratio of 46:1, which is well above the ideal ratio for human use at 4:1 [22].

Though ω-3 PUFA should be obtained through diet, other fatty acids can be synthesized in the body; therefore, the effects of self-synthesized fatty acids on our findings cannot be ruled out. Abdel–salem *et al.* proved that rotenone administration resulted in a significantly increased MDA level by 113.8% compared with control. There was also a significant increase in nitric oxide content by 80.7% (41.5±2.78 vs 22.96±1.66 μmol/g tissue) and decreased TAC by 38.4%(1.86±0.05 vs. 3.02±0.11 μmol/g tissue) in the rotenone only group [49]. These observations were in line with findings in the present study, with an increase in MDA and a corresponding decrease in TAC suggesting increased redox reaction.

The insignificant difference between group-3 and group-4 in the above parameters and in peroxinitrite and nitric oxide proves that the group-4 model is equivalent to conventional PD models in inducing oxidative damage. Markers of induction

of oxidative damage like nitric oxide and its toxic product, peroxinitrite, showed altered in values [50]. Increased ROS will reduce the amount of bioactive NO by chemical inactivation to form toxic peroxynitrite. Peroxynitrite can "uncouple" NO synthase to become a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress [51]. Peroxynitrite was increased in all the groups compared to the control. Nitric oxide values in group 1 were low when compared with groups 2, 3 and 4 [52].

Brain urea levels are elevated in many neurodegenerative diseases like Alzheimer's, Huntington's, and PD [53]. Urea being a nitrogenous base of protein metabolism, its increase in the brains of PD patients has been subjected to much debate, especially in conditions where blood urea remained normal. The blood-brain barrier (BBB) is impermeable to urea; hence, increased brain urea levels in the present study indicate either increased synthesis of brain urea inside the central nervous system or breakage of BBB[54]. A similar experimental study on PD also suggests increased brain urea in Wistar rats induced with rotenone [37]. In the present study, the brain urea levels in group 4, elevated compared to group 1, were almost similar to those in group-3, indicating increased urea due to neuronal destruction.

The serotonin and dopamine decrease in groups 3 and 4 are also comparable. The oxidant damage induced by rotenone and inflammatory damage induced by ω6 rich corn oil in the extrapyramidal system is evident from the decreased dopamine levels showing manifestations of PD. This is also confirmed in the present study by histopathology, which shows a decreased number of neurons and inflammatory damage in dopaminergic neurons in the HFD+Rot group compared with the rot lone or HFD alone group [55].

The interaction of α-Syn with cholesterol seems to be associated with α-Syn accumulation [56, 57, 58] and aggregation, a key determining factor in α-Syn's ability to form pores [58, 59]. Hence, high levels of cholesterol aggravate α-Syn-associated pathology. In the present study, the expression of α-Syn was highest in group 4 (Rot + HFD), followed by group 3 (Rot alone) and group 2 (HFD alone). In group 3, α-Syn was increased as expected. The increase in α-Syn in group 2 can be explained by the α-Syn accumulation stimulated by increased cholesterol as reported by Eriksson [19]. In group 3, serum cholesterol levels were found to be reduced due to a possible decrease in endogenous production [59, 60].

α-Syn levels were elevated due to ROS and inflammatory damage caused by rotenone. However, in groups 4 and 2, a significant increase in the expression of α-Syn was found; the increase was highest in group 4, indicating a synergistic effect.

Erickson reported that accumulation of lysosomal cholesterol impairs lysosomal function, reducing autophagic flux and consequently increasing α-Syn levels. This study suggests a dual role of cholesterol in PD-acting as both a protector against lysosomal membrane protein-induced cell death and a stimulator of α-Syn accumulation [19].

The present study also showed a significant increase in α-Syn accumulation in group-4 (Rot + HFD) when compared with group-3 (Rot), suggesting the role of HFD in accumulation. Taken altogether, the study findings indicate that group-4 (Rot + HFD) can serve as a better PD model than group-3 (Rot) taking into consideration the quicker induction (less time point), minimal distress to rats and alterations in biochemical parameters associated with PD pathogenesis.

CONCLUSION: The present study concludes that the Rot + HFD model is better than the Rotenone alone model of Parkinson's disease with respect to clinical manifestation, biochemical evidence of dopaminergic neuronal destruction, and oxidative and inflammatory damage to neurons in a shorter time frame. The better animal outcome, less suffering for animals, and a shorter duration of induction are seen when compared with Rotenone alone models.

It also brings out the role of ω 6:3 ratio of PUFA, with a higher inflammatory role of ω6 in dopaminergic neurons. Hence, contrary to the current argument that saturated fatty acids increase PD, PUFA with higher ω6 also contributes to the damage of dopaminergic neurons, resulting in aggravation of PD. This new animal model can be used for PD research.

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