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Research Article

Biochemical and Neurobehavioral Changes in the Rat Model of Parkinson's Disease with Suprathreshold Exercise and Usnic Acid

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Abstract: Finding a solution for Parkinson's disease has been a challenge to researchers despite advances in medicinal research. None of the options available so far has been able to reverse dopaminergic damage or increase endogenous dopamine production from existing dopaminergic cells in Parkinson's disease. Research in Parkinson's disease has not so far resulted in finding better solutions for reversing dopaminergic damage or increasing dopamine production. Above-the-threshold (suprathreshold) exercises producing improvement have been documented with supporting clinical and imaging findings but without any biochemical evidence. Above-the-threshold (suprathreshold) exercises producing improvement in Parkinson's disease have been documented with supporting clinical and imaging findings. To the best of our knowledge, the biochemical basis of this improvement has not been studied in detail. Recently, biomolecules like usnic acid have been shown to improve dopamine levels through protective and regenerative effects. This study aimed at understanding the biochemical basis of the improvement following suprathreshold exercises and usnic acid. The objectives were to assess whether the suprathreshold exercise produces improvement in PD rat models and, if so, to understand the biochemical basis of improvement and compare the mechanism with that of usnic acid.The effect of suprathreshold exercise and usnic acid on Parkinson's disease Hence, this study was conceived to understand whether the exercise produces results due to a similar mechanism that had never been reported before. Parkinson's disease was compared with rotenone alone, rotenone and high-fat diet models, and control. Induced with rotenone and a high-fat diet, the effect of suprathreshold exercise was compared to that of usnic acid, the rotenone alone model, and control. Locomotor behaviour was assessed with an actophotometer and rotarod. Western blotting assessed the blood biochemical parameters, antioxidant and dopamine levels in addition to α-synuclein, tyrosine hydroxylase, Nurr1, and HSP70 levels. Usnic acid decreased oxidative stress markers like MDA, peroxynitrite and nitric oxide, indicating lesser oxidative stress, increased dopamine and serotonin levels and decreased α-synuclein expression. But the suprathreshold exercise did not significantly affect oxidative stress markers nor increased dopamine levels. However, α-synuclein expression was decreased. Compared with usnic acid, lower Nurr1 levels in the suprathreshold group demonstrate lesser protective and regenerative effects on dopaminergic neurons. HSP70 levels indicate that improvement in suprathreshold exercise resulted from preventing misfolding of α-synuclein or through a noncanonical interaction, and comparable locomotor behaviour was observed in both groups. Biomolecules like brain-derived neurotropic factors produced by suprathreshold exercises acting centrally and in muscles might have also contributed to improvement in this group. When usnic acid increased dopamine levels in the brain through protective effects on dopaminergic neurons, antioxidant effects, and reduced expression of α-synuclein, suprathreshold exercise did not increase dopamine, decreased α-synuclein and had less antioxidant effects. This study biochemically proves that suprathreshold exercise alleviates Parkinson's disease symptoms by preventing α-synuclein misfolding and not by mechanisms other than increasing dopamine, neuroprotection or regeneration of dopaminergic neurons, which require further studies in humans.

Keywords: Parkinson's disease, above-the-threshold exercise, dopamine, Nurr1, HSP70, α-synuclein, antioxidant damage.

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1. INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder that manifests as generalised slowness of movements and at least one other symptom of resting tremor or rigidity in later life, affecting more than 6 million people worldwide. The symptoms are caused by the loss of dopaminergic (DA) neurons in the basal ganglia, which is the primary pathology.[1] Both motor and non-motor symptoms of PD exist, and the symptoms vary depending on the stage of the disease, although they usually appear after 80% DA neurodegeneration. Bradykinesia, tremors, an inability to pass over obstacles, balance issues, a forward-leaning (festinant) gait, dementia, mood swings, apathy, anxiety, and impulsiveness are linked to PD. [2] Since the loss of olfactory capacity is linked to the development of PD, olfactory tests could be an early, sensitive clinical indication. [3] PD is mostly idiopathic and is seen as a hereditary condition in only 15% of cases. [4] Though treatment options like pharmacotherapy, including L-dopa, and surgical options like deep brain stimulation are available, the disease symptoms are difficult to control due to their permanent and progressive nature. [5, 6] Hence, there is a need for finding new molecules and alternative methods with better therapeutic effects. Certain foods' bioactive compounds can be an antidote to some of the risk factors in normal and injured neurons. [7] In this context, the utility of usnic acid (UA), a naturally occurring compound, as a treatment option was considered and proved to be useful. UA has been proven to cause ROS scavenging in DAergic neurons of the CNS, reduce the inflammatory effects in oligodendrocytes, and cause regeneration of damaged DAergic neurons. [8] The better utility of UA over L-Dopa has been proven in the above study at biochemical, molecular, and histological levels.

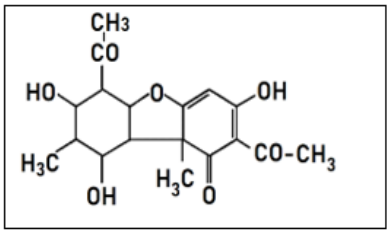

Fig I: Chemical structures and IUPAC (International Union of Pure and Applied Chemistry) names of Usnic Acid. 2,6-Diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzo [b, d] furan-1,3(2H,9bH)-dione. The chemical formula is $C_{18}H_{16}O_7$.

As a treatment option for PD, deep brain stimulation [9, 10] may not be preferred by most patients. Pallidotomy, thalamotomy, and gamma knife surgery have emerged as alternative interventions for advanced PD patients who have completely utilised standard treatments. [11] Recent studies have denoted the importance of exercise in the management of PD.[12, 13] Currently, exercise is increasingly being recognised as a key component in the treatment strategy for PD and has received significant attention due to its wide accessibility, low cost, and lack of complex equipment needs. [14, 15] Through aerobic exercises in PD, postural stabilisation and functional gains connected with mobility are targeted during training to improve balance and gait. On the other hand, aerobic training has no discernible effect on temporal or spatial performance. This can be achieved by skilled exercises that improve dexterity, resulting in improvements in the above parameters. [16] Despite this improvement, neuro-behavioural and biochemical analysis of changes in the brain after skilled exercise in Parkinson's disease models has yet to be proven conclusively. Further, this human improvement's biochemical, histological, or neurobehavioral basis has yet to be proven. Another study on PD patients demonstrated that forced training improved by 41% in stiffness, 38% in tremors, and 28% in bradykinesia. But, functional magnetic resonance imaging (fMRI) with aerobic exercise showed increased activity in cortical and subcortical areas, and responses were like those observed with anti-parkinsonian medication. [17] A meta-analysis of fMRI findings in PD patients reported that improvements following exercise result from coordinated changes in multiple brain areas. Increased activation was noted mainly in the cerebellum, occipital lobe, parietal lobe, and frontal lobe but did not activate a single brain area. [18] Few animal studies provide a possible explanation for the improvements, possibly not directly related to DAergic neurons. A study examined the effects of treadmill exercise on PD rats. The exercise-induced neuronal and behavioural recovery in PD rats was associated with an improvement in mitochondrial function and an increase in brain-derived neurotropic factors (BDNF) in the nigrostriatal region of the brain. [19] Another study demonstrated the influence of physical exercise on brain plasticity, resulting in neuroregenerative, neuroadaptive, and neuroprotective responses due to the release of neurotrophic factors, the reduction of oxidative stress and the decrease in the production of reactive oxygen species (ROS). These trophic factors release signalling biomolecules, such as an increase in β-CaMKII (calmodulin-dependent protein kinase II) activation induced by aerobic exercise, resulting in increased brain-derived neurotropic factors (BDNF) expression in the hippocampus. [20, 21] It has been found that many neurotropic factors, like BDNF, act on multiple systems, including the brain, following exercise. Increased neurotropic factors like BDNF lead to increased dendritic arborisation and volumes in cortical areas and mitochondrial biogenesis and play a role in co-regulating the serotoninergic system. As a result, it is hypothesised that these factors have broad plasticity on PD symptoms and can be modified with adequate physical activity. [13] However, biochemical evidence, including levels of dopamine in the nigrostriatal region following above-the-threshold exercises and suprathreshold exercises, and its comparison with other interventions, especially with hyperlipidemia as a comorbidity, has not been well documented. Hence, this study was conceived to understand the biochemical basis of improvement following suprathreshold exercise (SE) and compare the same with UA for managing PD in Wistar rats.

2. MATERIALS AND METHODS

2.1. Animals and Ethical Approval Materials

2.1.1. Animals and Ethical approval

Male Wistar rats from Biogen Laboratory Animal Facility, Bengaluru (250-300gm) maintained in polypropylene cages at 23 ± 2 °C with a relative humidity of 40–60% under a 12:12 hour light: dark cycle with a commercial solid diet and water *adlibitum* was used in the study. This study was conducted as per the guidelines of the Committee for 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). In addition, the study and manuscript drafting was done following the ARRIVE guidelines for animal research.

2.2. METHODS

2.2.1. Chemicals

The main chemicals were procured from the following sources: Rot from KEP7C, TCI China; Tonact10 (atorvastatin) from Sikkim (India); Syndopa110 (L-dopa) from Sun Pharma (India); and UA from Sigma-Aldrich (India). All mandatory laboratory health and safety procedures have complied within the course of conducting experimental work reported in this work.

2.2.2. Drug Treatment & Experimental Design

2.2.3. Animal grouping and experimental protocol

PD was induced with rotenone and a high-fat diet, which aggravated the PD pathology. [8] The rats were randomly assigned into five groups (n = 5 per group) as follows:

Group-1-Vehicle (Control): Dimethyl sulfoxide (DMSO) (0.3µl) and olive oil [2 mL/kg, intraperitoneally (i.p)] once daily.
Group-2 (Rot): Once daily, Rotenone (2.5 mL/kg, i.p.).
Group-3 (Rot+HFD): Rotenone (2.5 mL/kg, i.p.) and corn oil (10 ml/kg p.o.) once daily.
Group-4 (Rot+HFD+SE): rotenone (2.5 mL/kg, i.p) and corn oil (10 ml/kg, p.o.), and UA (25 mg/kg) once daily, followed by supra-threshold exercise.
Group-5 (Rot+HFD+UA): Rotenone (2.5 mL/kg, i.p), corn oil (10 ml/kg, p.o) and UA (25 mg/kg) once daily.

Animal experimentation and grouping were performed as per approved institutional guidelines. [22, 23] The development of PD symptoms such as bradykinesia, postural instability, gait disturbances, and rigidity was carefully monitored twice daily. The animals were anaesthetised with isoflurane when they developed debilitating behavioural changes, i.e., limiting mobility, feeding, or grooming (Raman and Weil, Mumbai). Groups 1, 2, 3, 4, and 5 were to receive the respective drugs for 14 days or until they developed full behavioural changes, whichever was earlier. Group 4 received above-threshold exercises using a rotarod following the development of the clinical endpoint for PD symptoms. Groups 1, 2, 3, and 5 were sacrificed after measuring the values on the rotarod, whereas group 4 was subjected to an above-threshold exercise and then sacrificed for assessing biochemical characteristics.

2.2.4. Rotarod test

Rats were observed for their ability to run on a 3 cm diameter rotarod at speed adjusted to 4 rpm. Three days before PD induction, the animals were subjected to pretraining. As previously described, pretraining was accomplished by exposing the rotarod to 4 rpm for 3 minutes, with an acceleration rate of 20 rpm/min and a maximum speed of 40 rpm. [24] After manifesting PD, they were again subjected to above-the-threshold/suprathreshold exercise (SE) using a rotarod. It was decided to test for five days, as one rat day is equivalent to 4 human weeks. [25] The rats were subjected to increasing pre-set speeds of 6, 12, 18, 24, 30, and 36 rpm for 5 minutes each with a 5-minute rest in between trials for normalisation of heart rate. Group 4 animals were tested above the SE, each receiving three such trials in a day with a minimum of 15 minutes between the sets of trials. The time rats spent on rotarod without falling was recorded for each trial. In addition, the average performance at 6, 12, 18, 24, 30, and 36 rpm for five rats in the SE group was calculated.

2.2.5. Actophotometer

Animal locomotor behaviour was assessed with an actophotometer. The provided actophotometer included a digital counter, a photocell, and a light source to measure horizontal locomotor activity in rats. Each rat from groups 1–5 was kept for 5 minutes after the exercise, and its activity score was recorded.

2.2.6. Blood sample collection

Blood samples were collected by retro-orbital puncture into vacutainer tubes with potassium ethylene diamine tetraacetic acid (K3 EDTA). The blood was allowed to clot for 15–30 minutes at room temperature and centrifuged at 3,500 rpm for 10 minutes in a cooling centrifuge (REMI CPR-24PLUS, India) to obtain serum. After isoflurane anaesthesia, animals were euthanised by cervical dislocation, organs were harvested, and brains were rapidly removed and bisected mid-sagittaly

2.2.7. Cardiac Perfusion and brain homogenate preparation

After anaesthetising the animal, cardiac perfusion was done using normal saline (0.9% NaCl) to eliminate the blood clots, and the skull was slit open centrally at the dorsal aspect. The midbrain was carefully separated and washed with ice-cold phosphate-buffered saline (PBS) at 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 4°C for 10 min using a Remi refrigerated centrifuge, after which the supernatant was analysed. [26]

2.2.8. Estimation of Glucose

For biochemical analysis, standard commercial kits were utilised according to the manufacturer's instructions. The glucose level was determined by the glucose oxidase-peroxidase method using the kit from Randox Laboratories Ltd., UK.

2.2.9. Assay of Serum Aspartate Aminotransferase (AST) and Serum Alanine Transaminase (ALT)

The AST and ALT were assayed according to Mohun's method. [27] To 0.1 mL of serum, buffered substrate (1 mL) was added and incubated for 1 hour at 37°C. The DNPH reagent (1 mL) was added to arrest the reaction. The DNPH reagent

and serum (0.1 mL) were added to the blank tubes. After allowing the vials to sit for 10 to 15 minutes, 15.0 mL of NaOH was mixed and measured at 510 nm in a Shimadzu UV spectrometer. The reagents and method used for ALT were the same as those used for the analysis of AST, except for the substrate solution, which was incubated for 30–40 minutes. In a buffer, 1800 mg DL-alanine and 0.037 g 2-oxoglutarate were combined. 1 ml of NaOH was added, and the volume was adjusted to 0.1 L, so the identical process as with AST was followed.

2.2.10. Estimation of Creatinine

Creatinine was estimated by the method of Slot, as followed by Malathi. [28] To 3 ml of deproteinised supernatant (0.1 ml of serum + 3.9 ml 10% TCA), added 2 ml of alkaline picrate solution. Blanks containing 3 mL of water and aliquots of a standard containing 4 mL of water were allowed to react in the same form. Forty minutes later, the colour was observed at 520 nm in contrast to the reagent blank. The values were expressed as mg/dl.

2.2.11. Estimation of Blood urea

By using the enzymatic colourimetric test of Berthelot, serum urea was determined. In water and urease, urea gets hydrolysed and produces ammonia and carbon dioxide. In an altered Berthelot reaction, a green dye has been made through the reaction of ammonium ions 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 the standard reaction, and one as a sample. A blank reaction was performed initially: 0.02 ml of distilled water and 2 ml of reagent were pipetted into the cuvette. 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. The sample (0.02 ml) was pipetted into the cuvette, and the reagent (2 ml) and the absorbance were measured with a spectrophotometer (Elico, B-200, Hyderabad, India). The concentration of urea was calculated.[29] The normal value of blood urea in Wistar rats is 62.1±8.4 mg/dl. [30]

2.2.12. Estimation of total cholesterol

Cholesterol estimation was done according to the procedure of Parekh and Jung, as noted by Santhosh. [31] Ferric chloride-uranyl acetate reagent (2.9 ml) was added to the sample (0.1 ml). After centrifugation, sulfuric acid-ferrous sulphate reagent (2.0 ml) was added gradually 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. The optical density was measured using a Shimadzu UV spectrophotometer (530 nm after 20 minutes). The levels of cholesterol were measured in mg/dl.

2.2.13. Estimation of triglycerides

Triacylglycerol estimation was done according to the Rice procedure, as noted by Santhosh. [31] To the 0.1 ml sample, activated alumina (50 mg) and isopropanol (3.9 ml) were added mixed well. This was 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 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 analyser (ROBONIK) using a diagnostic reagent kit (Aspen Laboratories, Delhi). The following formulas are used to calculate VLDL and LDL:

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

2.2.14. Estimation of MDA

The levels of malondialdehyde (MDA) were quantified to measure the oxidative damage using the thiobarbituric acid $C_4H_4N_2O_2S$ reaction method. To measure the MDA level, a working solution consisting of thiobarbituric acid, trichloroacetic acid, and 0.20 N HCl was made. After adding 250 L of tissue homogenate and 500 L of working solution, the mixture was centrifuged for 10 minutes at 3000 rpm. After centrifugation, the supernatant was taken in, and the OD of samples was assayed at 540 nm, as suggested by Shin. [32] The MDA concentration, an indicator of LPO, is expressed as nmol mL^{-1}.

2.2.15. Estimation of Nitric Oxide (NO^{-2})

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-hydrolysed, and subjected to GC/MS analysis. ONOO was synthesised from 2-ethoxy ethyl 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. [33]

2.2.16. Estimation of Peroxynitrite

The peroxynitrite level was determined according to the method. In brief, 100µl of homogenate was placed in a glass test tube, to which 5 mM phenol in 5 M sodium phosphate buffer pH 7.4 was added for a final volume of 2 ml. The resulting solution was then incubated for 2 hours at room temperature, and then 15 µl of 0.1 M sodium hydroxide was added. 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). [34]

2.2.17. Estimation of Total antioxidant activity

The serum's total antioxidant capacity (TAC) was determined using the Benzie and Strain method. [35] In brief, a FRAP (ferric reducing antioxidant power) working solution was created by combining buffer acetate with a 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) solution in HCl. After that, $FeCl_3$ was added and mixed. Next, 8 µL of sample supernatant and 240 µL of the working solution were mixed and incubated for 10 min at

room temperature. The optical density of samples was measured at 532 nm.

2.2.18. Brain urea quantification

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 brain samples by HPLC coupled with MS. Brain samples were extracted in 50:50 (v/v) methanol: chloroform containing labelled urea internal standard. The methanol: chloroform internal standard solvent was prepared. After that, LC-MS grade water was added to the samples before centrifugation at 2,400 g for 15 minutes to separate the polar and non-polar phases. The methanol phase was transferred to a test tube in a centrifugal concentrator. 0.1% formic acid was added to the samples after they had dried. The resulting solution was transferred to 300µl autosampler vials, with two blanks containing only 0.1% (v/v) formic acid also prepared. Standard solutions containing an internal urea standard in 0.1% v/v formic acid were prepared. Separation was carried out on a Hypersil Gold AQ column with a diameter of 2.1 mm, length of 100 mm, and 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 300 µl/min. Urea quantification was performed using Thermo TSQ Quantum Access Max (Thermo Fisher Scientific, MA, United States).

2.2.19. Molecular analysis

HSP70 (Heat Shock protein), α-Syn (Alpha-synuclein), Nurr1 (Nuclear receptor-related protein-1) and Tyrosine hydroxylase (TH) were analysed using Western blot assay. The mid-brain tissue homogenate was prepared and lysed in lysis buffer containing 1% protease inhibitor (Himedia Laboratories) for 1 h at 4°C. Samples were then centrifuged at 10,000g at 4°C for 15 min. Supernatants were collected, and protein concentration was determined using a Bradford protein assay kit (Biorad Laboratories, USA). Equal amounts (30 µg) of protein were separated by 10% SDS-PAGE and electro-transferred onto PVDF. Membranes were blocked for 1 hour at room temperature with 5% non-fat milk before being incubated overnight at 4 °C in a solution of rabbit polyclonal β-actin (1:500) and anti-rabbit polyclonal antibodies (1:1000) (purchased from Invitrogen, USA). The next day, membranes were washed three times with tris buffered saline and incubated with HRP-labelled goat anti-rabbit (1:1000) secondary antibody (Abcam, US) for 1 hour at room temperature. Then they washed three times with tris buffered solution with 0.5% tween. Targeted proteins were visualised using enhanced chemiluminescence (ECL) reagent and exposed to a film. Densities of specific protein bands were acquired using Adobe Photoshop CS6 software (version 12.0).

3. STATISTICAL ANALYSIS

SPSS version 21 (IBM Corp., Armonk, NY, USA) was used for analysis, and PRISM-8 software for Windows was used for generating graphical representations and data analysis. The information was presented 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.

4. RESULTS AND DISCUSSION

4.1. Results

The effects of SE and UA for possible use in management were studied by monitoring biochemical parameters and the neurobehavioral patterns of PD Wistar rats. Based on rotarod and movement analysis, all the groups other than the control had bradykinesia, postural instability, and rigidity. In addition, baseline rotarod performance and actophotometric movement analysis were done. Animals fell to their maximum at 35 rpm during the pretraining.

4.1.1. Rotarod performance

After induction, the rats fell from the rotarod maximum at settings of 18 and 24 rpm for groups 3 and 4, followed by group 2 and then by group 5 when compared with control "Figure II". Following the above-threshold exercise in group 4, when rats were reassessed in rotarod, it was noted that rats in this group significantly improved their performance. (ANOVA $F=14.84$, $R^2=0.748$, $p<0.0001$. A Bonferroni multiple comparison between the groups showed no significant difference in fall rate between 18 and 24 rpm (mean12; $t=0.425$) "Figure III".

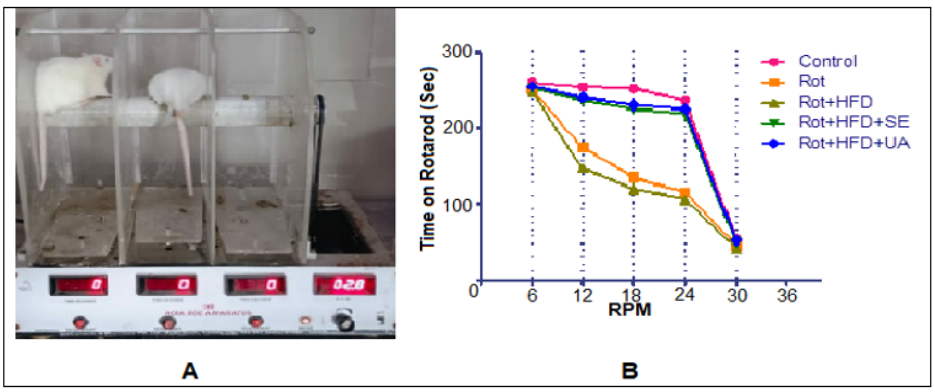

Fig II: Performance on rotarod after PD Induction. (A) Photograph of Rotarod training and (B) Time on Rotarod. Rats in Rot+HFD+SE had a curve similar to Rot/ Rot+HFD group, but after exercise, it became similar to the UA group.

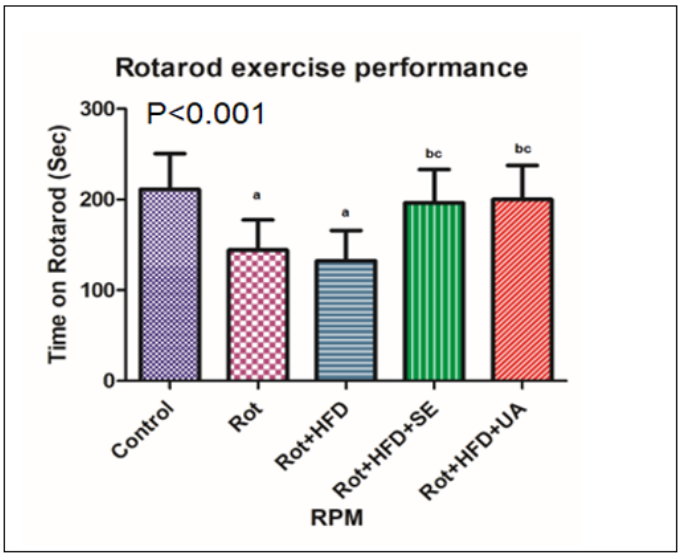

Fig III: Multi-pair comparison of time spend on rotarod by different groups. The values are mean ± standard deviation (n = 5 each). The "P" values are from a one-way analysis of variance. The letters indicate the results of the Bonferroni multiple comparison tests: a, significantly different from the control group; b, significantly different from the Rot group; c, significantly different from the Rot + HFD group; d, significantly different from Rot + HFD + SE. Abbreviations: HFD, high-fat diet; Rot, rotenone; UA, usnic acid, SE, suprathreshold(above-the-threshold) exercise.

4.1.2. Actophotometer performance

After 5 minutes of exposure inside the actophotometer, the activity score was recorded. The mean±SD activity scores of groups 1, 2, 3, 4, and 5 were 185.6±17.62, 80±7.22, 72.67±8.08, 73.6±7.68, 166.3±8.737 movements/minute. Following the rotarod exercise, the values in group 4 increased to 133.7±7.77. Oneway ANOVA analysis showed significant differences between means with p<0.0001. However, the Bonferroni test shows no significance in the difference in mean between the control and UA group (19±2.19SD). On the other hand, the difference in means is significant between the SE and Rot, Rot+HFD and UA group (p<0.05) "Figure IV".

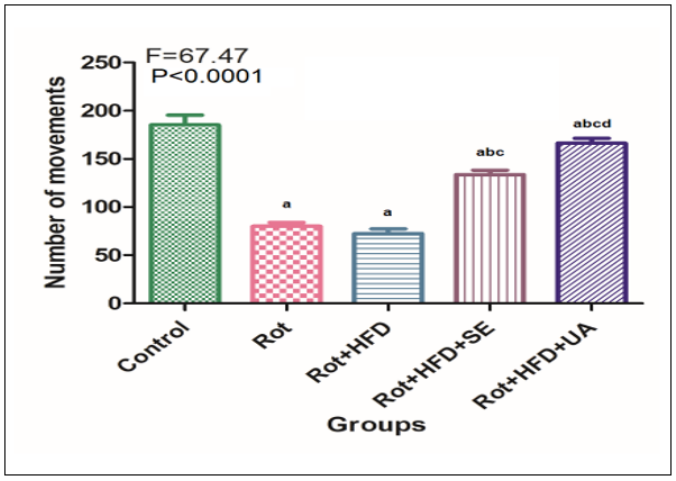

Fig IV: Comparison of experimental groups' performance on Actophotometer. The values are mean ± standard deviation (n = 5 each). The "F" and "P" values are from a one-way analysis of variance. The letters indicate the results of the Bonferroni multiple comparison tests: a, significantly different from the control group; b, significantly different from the Rot group; c, significantly different from the Rot + HFD group; d, significantly different from Rot + HFD + SE. Abbreviations: HFD, high-fat diet; Rot, rotenone; UA, usnic acid, SE, suprathreshold(above-the-threshold) exercise.

4.1.3. Changes in biochemical parameters

The biochemical results of (Glucose, AST, ALT, urea, and creatinine levels) were analysed to assess the adverse effects. The mean value of glucose in control (group-1), Rot (group-2), rotenone +HFD (group-3), Rot+HFD+SE (group-4) and Rot+HFD + UA (group5) was 87.6 ± 6.5, 118.1 ± 3.8, 130.1 ± 2.9, 121.6 ± 2.82 and 101.5±11.1 respectively. Unpaired t-test showed significance with a p-value of <0.001. Multiple pairwise comparisons showed significance for values comparing group 5 vs 1,2,3 and 4 as well as group4 vs 1,2,3 and5. The liver function test (LFT) was significantly elevated compared to the control in all the groups. Groups 3, 4 and 5 showed 2.6, 1.8 and 0.9-fold increases in values compared with the control.

Analysis of ALT values of groups showed high results corresponding to 1.5, 2.6, 2.1, and 1.2-fold increases compared with the control. Analysis of renal functions revealed blood urea values were 20.76 ± 2.965, 66.04 ± 5.038, 89.88 ± 1.532, 76.7 ± 8.76 and 40.96±9.75 respectively. A comparison of the SE and UA group showed a 3.6 Vs 1.9-fold change in urea compared to the control. Creatinine values (mg/dl) were also elevated in all groups compared to the control. Creatinine values increased 4-fold in SE compared to a 1.6-fold increase in the UA group. The lipid profile assessed showed no significant increase in TC in groups 1,2,4, and5 when compared with a significant increase in group3. Though TGL values were significantly increased in group3 and 5, groups 2 and 4 showed reductions compared to the control. LDL was significantly lower in groups 2 (40.36 ± 1.70) and 4 (44.75 ± 1.48) when compared with group 1 (57.52 ± 1.48), group 3 (84.10±1.09) and group 5 (67.540±3.16). HDL levels (mg/dl) in groups 2,3,4 and 5 were lower than in group1. This decrease of HDL in groups 2, 3 and 4 was significant on Bonferroni pairwise comparison with group 1($p < 0.001$). Total cholesterol did not show any significant fold changes between SE and UA groups. However, HDL values in group-2 vs group-3, group-3vs group-4 or group-2 vs group-4 are not significant. Similarly, pair wise comparison showed that the difference in mean of TC, TGL and LDL in group-2 Vs group-1,3 and4, group-3 Vs group-1 and 4, were all significant "Figure V".

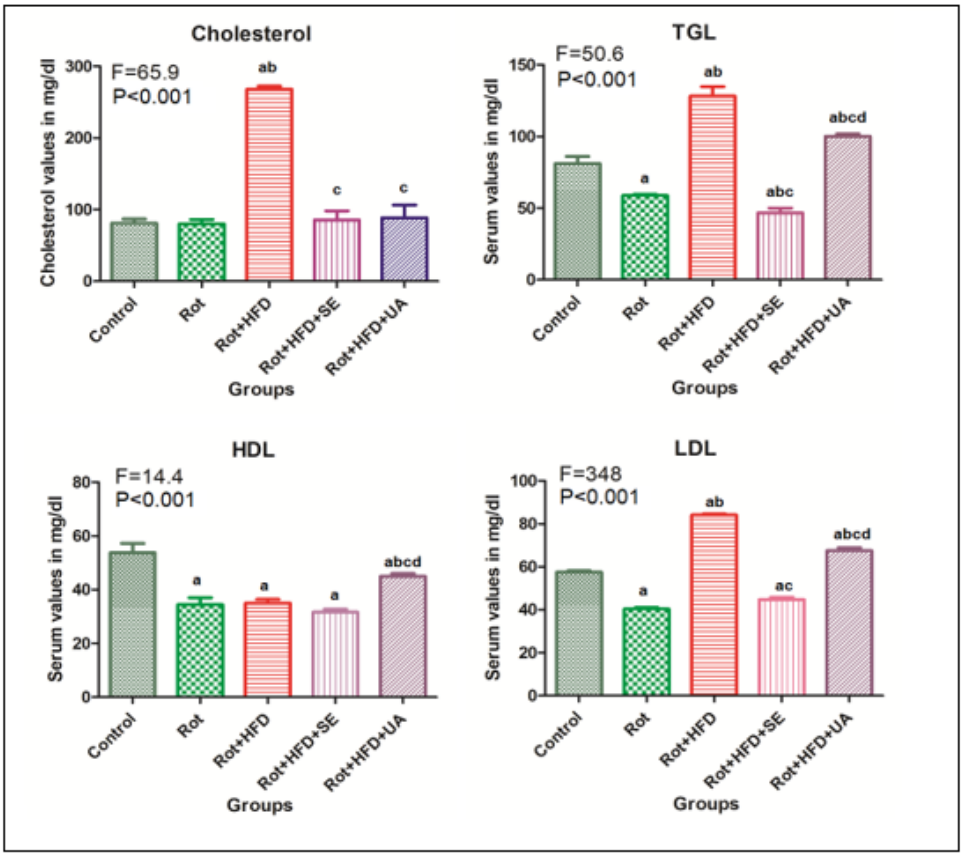

Fig V: Changes in serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in the experimental groups compared with the control group. The values are mean ± standard deviation (n = 5 each). The "F" and "P" values are from a one-way analysis of variance. The letters indicate the results of the Bonferroni multiple comparison tests: a, significantly different from the control group; b, significantly different from the Rot group; c, significantly different from the Rot + HFD group; d, significantly different from Rot + HFD + SE. Abbreviations: HFD, high-fat diet; Rot, rotenone; UA, usnic acid, SE, suprathreshold(above-the-threshold) exercise.

4.1.4. Changes in oxidative stress markers

Assessment of oxidative stress markers like MDA, total antioxidant capacity (TAC), showed that MDA increased in all the groups when groups compared with control. The Mean±SD values of MDA in group-1, 2, 3, 4 and 5 were 1.846 ± 0.369, 5.126 ± 0.091, 5.678 ± 0.387, 5.64±0.13, and3.48±0.29. SE(group-4) did not have a change in MDA levels when compared with group2 and 3, but UA (group5) had a 1.9-fold reduction. The TAC value in group-1, 2, 3, 4 and 5 was 698.08 ± 27.653, 388.94 ± 54.897, 324.12 ± 17.145, 350.95±59.75, 526.0±42.54 respectively. TAC values of SE and groups2 and3 were similar, whereas UA increased compared to them. Analysis of markers of induction of oxidative damage like nitric oxide and its toxic product showed increased values. Peroxynitrite was increased in all the groups- compared to the control. There was no significant difference between peroxynitrite or nitric oxide levels in groups 3 and 4; group 5 had significantly lower levels compared to both "Figure VI".

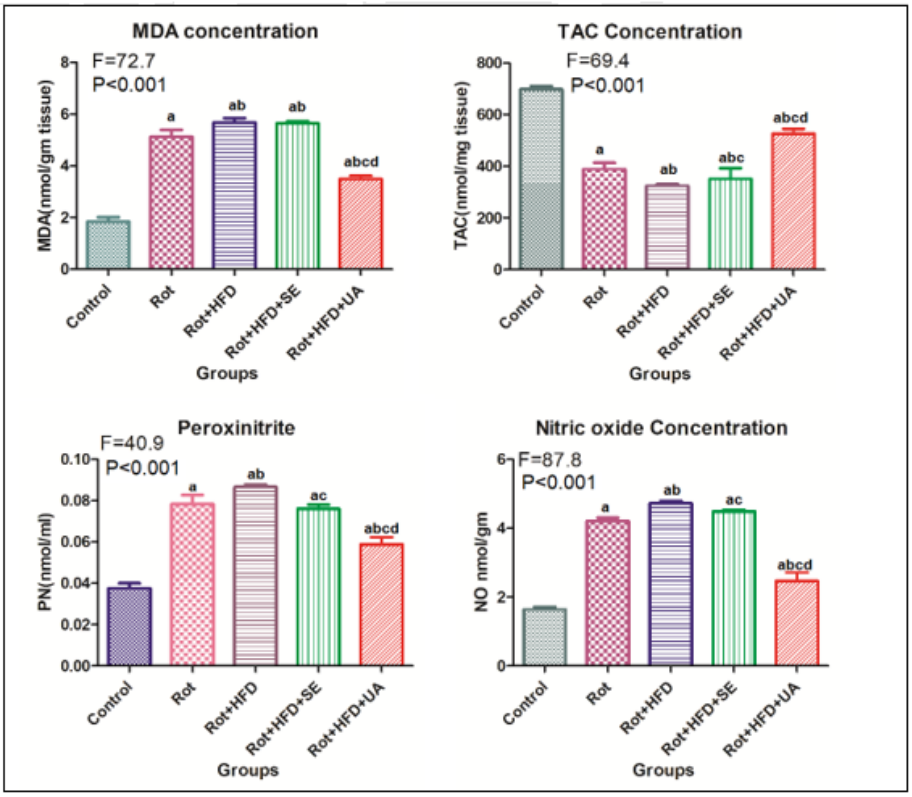

Fig VI: Changes in serum oxidative stress markers in the experimental group compared with the control group. The values are mean ± standard deviation (n = 5 each). The "F" and "P" values are from a one-way analysis of variance. The letters indicate the results of the Bonferroni multiple comparison tests: a, significantly different from the control group; b, significantly different from the Rot group; c, significantly different from the Rot + HFD group; d, significantly different from Rot + HFD + SE. Abbreviations: HFD, high-fat diet; Rot, rotenone; UA, usnic acid, SE, suprathreshold (above-the-threshold) exercise.

4.1.5. Analysis of neurotransmitters and brain urea

The brain's levels of serotonin and dopamine were assessed to understand the effect of improvement seen following both SE and treatment with UA. Mean dopamine of group1,2,3,4 and 5 were 0.878±0.04, 0.474±0.03, 0.496±0.05, 0.5±0.04, and 0.808±0.05. One-way ANOVA analysis gave significance at <0.001. Bonferroni multiple comparisons gave significance (<0.05) between group4 and 5, group5 vs 1,2 and 3. Comparison between group4 vs2 and3 returned as insignificant. Mean serotonin levels of group 1,2,3,4 and 5 were 0.476±0.023, 0.21±0.026, 0.252±0.02, 0.246±0.02, and 0.394±0.02. One-way ANOVA showed significance between groups at p<0.001. Bonferroni test showed a significant difference between group 4 and 5 serotonin levels but no significant difference(p<0.05) in levels between group 4 vs2 and3 (Figure VII). Levels of Brain urea were also analysed in all the groups. The mean±SD values of Group-1,2,3,4 and 5 were 36.7 ± 3.883, 73.2 ± 1.43, 69.86 ± 5.638, 73.65±0.6364 and 50.46±1.69SD respectively. Bonferroni multiple Pairwise comparison of brain urea levels between group-4 and 5 showed a significant difference in mean (M=23.8; t=11.3; p-value < 0.05). Comparison between group4 and groups2,3 did not return any significance, whereas group 5 vs groups 2 and 3 returned significant(p<0.05) "Figure VII".

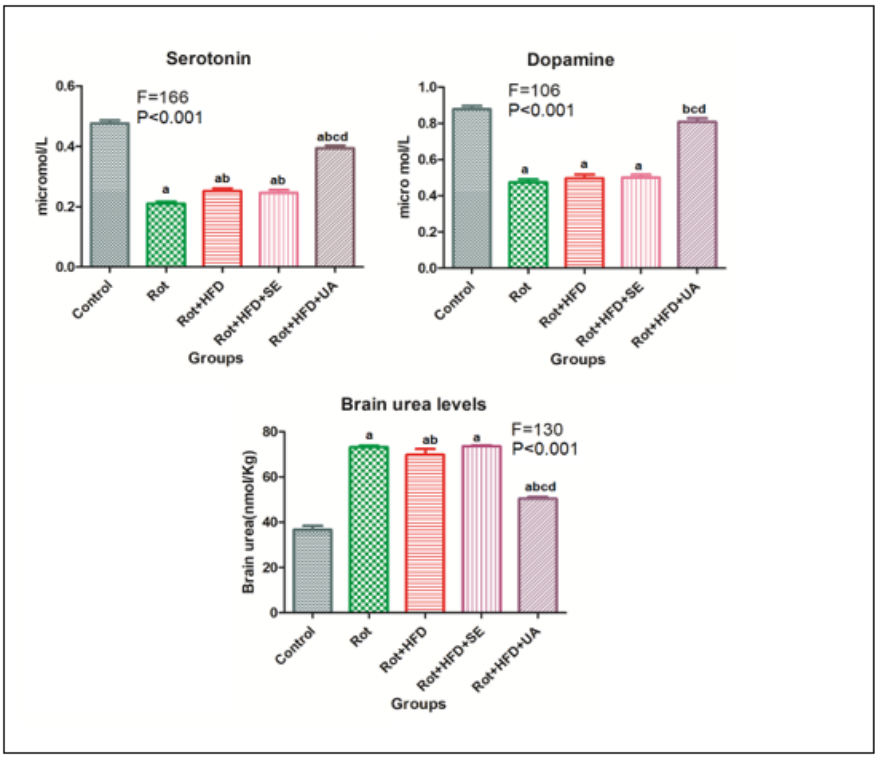

Fig VII: Changes in brain serotonin, dopamine and urea levels in the experimental groups compared with the control group. The values are mean ± standard deviation (n = 5 each). The "F" and "P" values are from a one-way analysis of variance. The letters indicate the results of the Bonferroni multiple comparison tests: a, significantly different from the control group; b, significantly different from the Rot group; c, significantly different from the Rot + HFD group; d, significantly different from Rot+HFD+SE. Abbreviations: HFD, high-fat diet; Rot, rotenone; UA, usnic acid, SE, suprathreshold (above-the-threshold) exercise.

4.1.6. Indicators of PD damage and regeneration- Western Blot

"Figure VIII" shows representative α-syn, TH, Nurr1 and HSP70 western blotting, and "Table I" shows the densitometry. Compared with the control group, the Rot-HFD group showed the most marked changes: a significant increase in α-syn and a significant decrease in TH, Nurr1 and HSP70. Each tested treatment reversed these changes, and the UA group returned the protein levels to those seen in the control group. UA had the most pronounced effects: in addition to decreasing α-syn, it also normalised the levels of TH, Nurr1 and HSP70, with no significant differences compared with the control group. SE group showed a decrease in α-syn.

Table 1: Alpha-synuclein, TH, HSP-70 and Nurr-1values by Western blotting.

Relative expression of	C	Rot	Rot+HFD	Rot+HFD+SE	UA-H
α -syn Mean	2.20	5.83	6.43	5.07	2.50
SD	1.05	0.78	0.42	0.15	0.87
TH Mean	5.83	2.66	1.56	3.43	5.4
SD	0.20	0.81	0.47	0.64	0.6
HSP-70 Mean	5.93	1.53	2.46	4.26	5.36
SD	0.15	0.58	0.45	0.20	0.51
Nurr1 Mean	5.76	2.1	1.7	3.1	5.3
SD	0.49	0.2	0.26	0.15	0.52

Quantitative data are expressed in intensity units.

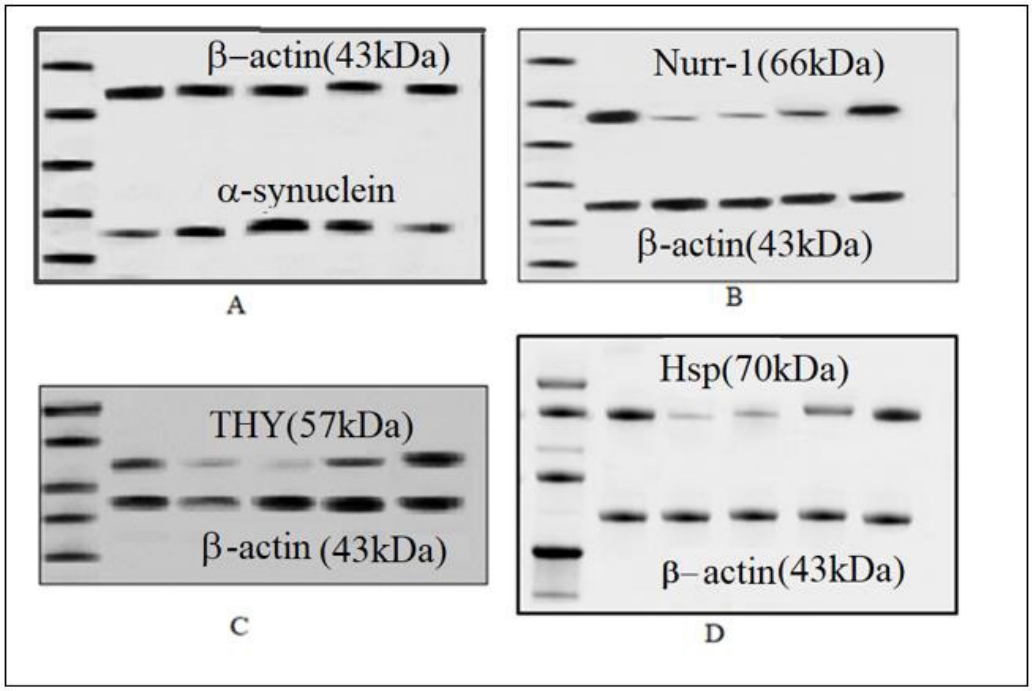

Fig VIII. WESTERN BLOT ANALYSIS. Representative western blots of (A) α -synuclein, (B) Nurr1, (C) tyrosine hydroxylase and (D) HSP70.

5. DISCUSSION

Biochemical analysis of the blood showed that blood sugar levels were increased than in control but remained within the normal range for Wistar rats-i.e, 95th centile for FBS/PBS-6.2mmol/L; 7.9 mmol/L. [36] This may be attributed to the dysautonomia associated with PD leading to impaired insulin response. Since the blood glucose did not increase beyond the reference ranges, it can be inferred that though there was a tendency for glucose to increase when induced with Rot+HFD, the levels were still within the normal range, as noted in an earlier study. [37] Post-exercise, the values were within normal limits. This is in line with the post-exercise glucose values of rats described elsewhere. [38] The significant elevation seen in AST, ALT, blood urea, and creatinine levels, though never beyond the upper limit of the reference range of rats, can be attributed to the rotenone and HFD-induced alterations due to excessive ROS generation in the renal and hepatic systems, as reported elsewhere. [39] The present study shows significant protective effects of UA at high doses on the liver and renal functions in the Rot+HFD PD model. These protective effects were also significantly better than the earlier Rotenone alone induction model. [8] Significant reductions were seen in both AST and ALT in the SE group compared with Rot+HFD. However, such reduction in AST and ALT was not seen in pre and post-exercise normal rats. [38] Marginal increase in renal parameters in the SE group may be due to muscle damage induced by rotenone and strenuous exercise. Normalisation of the lipid profile, as seen in the present study, with better control of total cholesterol seen in the SE and UA group, points to the lipid-lowering action of both. [8] Such reduction in TC following strenuous exercise was not seen in a similar study on normal rats. [38] This is probably due to the lipid-lowering effect in PD augmented by both interventions. Similarly, exercise-induced reduction seen in TGL, HDL and LDL in the present study did not correlate with a similar study done with normal rats. [38] However, TGL, HDL, and LDL in the SE group did not significantly change in values compared with Rot and Rot+HFD. This may be attributed to cholesterol-lowering effects due to PD, as seen in a recent meta-analysis which concludes that cholesterol biosynthesis may be impaired in PD, especially if associated with hyperlipidaemia. [40] Few human researchers have found that high-intensity exercise for a short time significantly declines blood triglycerides. [41, 42]

5.1. A comparison of UA and SE effects on oxidative markers

Oxidative stress parameters like MDA, TAC, peroxynitrite, and nitric oxide, were following deranged exercise. MDA values showed a significant increase when compared with control and UA. TAC value in the SE group was comparable with Rot+HFD but significantly lower than both UA and control. Peroxynitrite and nitric oxide showed higher values when compared with both UA and control. The comparable results of increased nitric oxide levels (but higher than controls) in UA and SE show that exercise might have stimulated endothelial NO synthetase production. These findings are in line with a clinical trial conducted on PD patients. [43] In a similar experiment, it was found that UA reversed the alterations in oxidative stress markers (including ROS generation, glutathione system, and lipid peroxidation levels) in neurons. [44] A similar study done with normal rats showed normalisation of MDA levels following strenuous exercise. [45]

5.2. Neurotransmitter and intervention

Analysis of the effect on neurotransmitters showed that UA had raised the serotonin and dopamine values more than SE or other groups. The decrease in serotonin and dopamine in both Rot+HFD and Rot alone is comparable to the SE group. [46] The oxidant damage induced by rotenone and inflammatory damage caused by ω6 rich corn oil in the extrapyramidal system is evident from the decreased dopamine levels showing manifestations of PD in both group2 and 3. A similar study also shows increased damage in the extrapyramidal system, confirmed by histopathology and biochemical findings. [47, 8]

5.3. α-Syn expression & treatment options

α-Syn expression was significantly increased in group-2,3, and 4. However, the reduction in the expression of α-Syn was significant in the UA group in which α-Syn levels were comparable to the control, indicating the possibility of a mechanism independent of anti-inflammatory and antioxidant effects. This may be due to the attenuation of α-Syn by UA and the regeneration of the residual dopaminergic cells. Though exercise produced a slight reduction in α-Syn levels, it was much lower when compared with UA, indicating continued damage and less regeneration of DAergic cells. Hence the improvement obtained through suprathreshold exercise could be due to mechanisms other than the regeneration of DAgic neurons or a protective effect. Interaction of soluble TH with α-Syn leads to a reduction of TH activity. [48, 49] The effect of UA on TH was found to be significant in group 5, indicating that enhancement of the enzymatic activity of TH might have resulted in a higher conversion of Tyrosine in the central nervous system leading to better dopamine levels and better clinical manifestations. However, exercise could produce only marginal benefits compared to the UA group.

5.4. Effect of treatment options on neuronal protective mechanisms

Nurr1 is necessary for developing and maintaining a dopaminergic phenotype in the nigrostriatal dopaminergic system. Reduced levels of Nurr-1 are seen as a major risk factor for PD. [50] A recent experimental study with rats demonstrated that α-Syn dependent PD-related pathophysiology is mediated partly by Nurr1 down-regulation. [51] The pathogenesis of PD may be influenced by neuroinflammation linked to activated microglia and higher levels of proinflammatory mediators. In addition to the roles in DA neurons, Nurr1 is supposed to be part of anti-inflammatory pathways in microglia and astrocytes, which protects the DA neurons from inflammatory damage. This dual role of Nurr-1 protection from anti-inflammatory cell death, especially with microglia and the development and maintenance of midbrain DA neurons may explain the results obtained in the present study. [52] The present study shows significantly higher levels of Nurr1 values in the UA group comparable to controls showing remarkable protection to DAergic cells. In contrast, the SE group showed a marginal increase in expression. Reflecting on a related study, the UA group may have promoted the cell cycle by modulating cell cycle-related molecules. [53] Nurr1 and its transcriptional targets are downgraded in midbrain DA neurons of PD patients that express high α-Syn levels. [54] Therefore, higher levels of Nurr1 in the UA group compared with Rot+HFD shows that UA probably annuls the down-grading and stimulates higher Nurr1 resulting in the activation of the dopaminergic pathway as noted by better clinical-biochemical parameters in group-5. Whether UA directly binds to the ligand binding domain of Nurr1 and acts as a Nurr1 agonist to facilitate the dual role of dopaminergic neuroprotection and anti-inflammatory effects in Parkinson's disease, as discovered in a recent study, remains to be studied. [52] In another study, it was found that Nurr1 agonists may offer protection from PD through neuroprotective and anti-inflammatory effects. [55] This protective action in the UA group needs to be studied further to understand the mechanism behind the protective effect of Nurr1 on PD. However, it is evident from Nurr-1 results that the mechanism by which SE-induced recovery from PD symptoms is biochemically different from mere protection or recovery of DAergic neurons. HSP70, a chaperone elevated in stress-induced conditions, has been shown in cell and animal models to protect against α-Syn misfolding and α-Syn driven neurodegeneration. HSP70 chaperones block α-Syn oligomer formation, an early event in α-Syn misfolding. A recent study using truncations, mutations, and inhibitors, confirmed that HSP70 interacts with α-Syn through canonical and unidentified noncanonical interaction sites in the C-terminal domain. [56] The noncanonical alternative binding site for α-Syn on HSP70 blocks α-Syn oligomerisation, conferring protection against α-Syn pathogenicity. HSP70 increased in the present study indicates that it either directly through preventing misfolding of α-Syn or through the recently identified noncanonical interaction might have resulted in a near normalisation of HSP70 levels in the UA group, comparable to the control. However, the SE group also seems to be acting through this mechanism to give protection from PD with only minor differences in mean±SD values compared to UA or controls. HSP70 is crucial for immunological control, cell protection during exercise, and the effectiveness of the regeneration and repair processes. [57] Leem [58] provided evidence for neuroprotective effects and improved neuroplasticity in PD through motor skill learning training through their experiment using rota rod walking, suggesting that neurogenesis and restoration of walking performance were by modulating the AMPK/BDNF pathway. Another experiment in a Hemi-Parkinson model proved that exercises ameliorate the motor symptoms of PD and improve dopaminergic functions. [59] Running wheel exercises which also improve the skill level of motor ability including dexterity had been proven to reduce α-Syn levels and improve motor symptoms and cognition in transgenic PD rats. [60] In the present study, it is proven that the improvement through skill level exercises like rotarod and UA happened through 2 different mechanisms. UA group showed improvement through regeneration, and the neuroprotective effects of UA and exercise did not create any difference in dopamine or Nurr-1, suggesting that the mechanism differs from the regeneration of DAergic neurons**.** BDNF produced due to SE, acting both centrally on neurons and peripherally on muscles, might have increased both the performance of skilled exercise which improves the dexterity on rotarod and the endurance in muscles, resulting in improvement in scores as well as clinical improvement.

6. CONCLUSION

This study analysed the biochemical basis of neurobehavioural improvement in PD following above the threshold/suprathreshold exercise and newer biomolecule-Usnic acid, which was never reported in PD management. SE did not produce an increase in dopamine levels nor a decrease in antioxidant levels, implying mechanisms other than dopaminergic neurons in producing clinical improvement. But UA produces antioxidant, protective and regenerative effects on dopaminergic neurons, increasing dopamine and corresponding improvement in locomotor behaviour. So far, no drugs have been proven to be useful in regenerating DAergic neurons in PD. Further studies are needed to ascertain the receptor-level interactions of UA and SE. Hence SE can be used complementarily with current modalities of treatment of PD, as the mechanism of action is independent of DAergic neurons, till newer drugs that facilitated regeneration/protection of DAergic neurons like UA are made available after further studies.

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8. AUTHORS CONTRIBUTION STATEMENT

Each author has made substantial contributions, read and approved this version for submission in this journal, and agrees to be personally accountable for their own contribution. Conceptualisation, Methodology and Validation: R Vijayaraghavan, S Senthil Kumar and Devika S Kumar; Formal analysis and Investigation: Devika S Kumar; Software and Data curation: R Vijayaraghavan, Devika S Kumar and Lal D V Nair; Writing-original draft preparation: Devika S Kumar, Lal D V Nair; Editing and Supervision: R Vijayaraghavan and S Senthil Kumar.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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