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Effect of Brown Rice on PINK1/Parkin-Mediated Mitophagy in a Drosophila Model of Iron-Induced Parkinson's Disease

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Abstract

Background: Parkinson's disease (PD) involves the progressive loss of dopaminergic neurons and is linked to brain iron accumulation and free radicals. Brown rice (BR) possesses antioxidant properties that may help mitigate oxidative stress-related damage. This study aimed to compare the effects of BR against WR on iron-induced PD in a fruit fly model.

Materials and Methods: Adult fruit flies were divided into five groups and fed an iron-supplemented diet for 10 days to induce oxidative stress. The groups were then given different diets for 5 days: normal, iron-only, BR + iron, white rice (WR) + iron, and L-dopa + iron diets. Behavioural tests (motor function, memory, anxiety), iron, dopamine, malondialdehyde (MDA), and antioxidant enzyme levels were measured, along with the expression of antioxidant genes (CAT, GPx and NRF2), iron metabolism-related genes (ferritin and DMT-1) and autosomal activity-related genes (PINK1/PARKIN).

Results: BR improved motor and cognitive functions, reduced MDA and iron levels, and increased antioxidant enzyme levels compared to the iron-only and WR groups. BR also upregulated the expression of antioxidant and iron metabolism genes, and positively affected the expression of the PINK1/PARKIN genes. These findings suggest BR could be developed as a complementary dietary supplement in combination with standard PD treatments.

Conclusion: BR may be beneficial as a therapeutic dietary intervention for PD by reducing motor/non-motor symptoms and regulating PINK1/Parkin-mediated mitophagy.

Keywords: Parkinson's disease, Ferric iron, White rice, Brown rice, L-Dopa.

Introduction:

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised by debilitating motor deficits resulting from the loss of dopaminergic (DA) neurons in the midbrain's substantia nigra (1). The neuropathology of PD is characterised by selective neuronal loss associated with accumulation and aggregation of alpha-synuclein in Lewy bodies (LBs), cytoplasmic inclusions in neurons of specific brain regions, including the brainstem and cortical regions (2). While the precise pathogenesis of PD remains elusive, mounting evidence suggests that free radicals produced locally within the basal ganglia play a role in damaging nigral neurons, leading to the progressive demise of substantia nigra neurons (3). Deletions or compound heterozygous mutations in the Parkin gene are the most common cause of recessive forms of PD, indicating a role for impaired proteasomal activity in PD pathogenesis. Mutations in key protective genes PINK1 and DJ-1 (PARK7), which generally help cells withstand oxidative stress, emphasise how cellular damage contributes to PD development (4).

Furthermore, when the amount of iron exceeds the detoxification systems of the cell, increased levels of iron, especially Fe2+, enhance the conversion of H2O2 to ●OH through the Fenton reaction. This process favours greater turnover in the Haber-Weiss cycle, resulting in amplified oxidative stress. This may induce a vicious circle, as oxidative stress may increase the levels of free iron (5). Similarly, Fe accumulation in the substantia nigra correlates with disease severity in PD patients (6).

As the population ages, the number of patients with neurodegenerative diseases also rapidly increases, along with an increased socioeconomic burden (7). Globally, morbidity and mortality due to PD are increasing rapidly, second only to Alzheimer's disease among degenerative diseases. By 2030, the number of people affected is expected to double, emphasising the urgent need for more effective treatments, cures, or preventive measures (8). In West Africa, including Nigeria, where PD prevalence varies, the disease poses a significant public health challenge (9,10).

The central role of dopamine in PD means that treatment options are directed at mimicking dopamine activity in the central nervous system. L-3,4-dihydroxyphenylalanine (L-dopa) is used as a gold standard to treat PD, although it does not inhibit the progression of the disease and side effects have been associated with this and other drugs used (11).

Brown rice (BR), obtained by de-husking harvested paddy rice, retains beneficial bioactive compounds lost in white rice (WR) during polishing (12). With the highest annual consumption in sub-Saharan Africa, the genetic properties, cultivation conditions, and postharvest treatments of rice contribute to this compositional variation (13).

Drosophila melanogaster is increasingly being used as a model for studying metal toxicity, responses, and metal-related diseases (14-16). With its simpler nervous system compared to humans (17), D. melanogaster allows for a clearer understanding of metal homeostasis and metal-protein interactions in the brain. In this model organism, iron toxicity occurs either through the direct introduction of iron into the diet (iron-induced toxicity) or through the dysregulation of iron-regulating proteins, which leads to overload, oxidative damage, and toxicity (18). The conserved genes involved in dopamine dynamics and signal transduction

between fruit flies and humans make Drosophila a valuable model for researching Parkinson's disease (19).

While there is currently no cure for neurodegenerative diseases, effective management strategies are available. Recent studies have identified bioactive molecules with antioxidant properties and amino acids essential for dopamine synthesis in BR (20–23). We hypothesised that BR supplementation into the diet would reduce iron-induced oxidative stress and improve PD symptoms through PINK1/Parkin-mediated mitophagy.

Materials and Methods

Reagents and Chemicals

Ethanol was purchased from Merck, Sigma Aldrich (Germany). Baker's Yeast was bought from STK Royal Ltd (Lagos, Nigeria). Ferrous sulphate, FeSO4·7H2O (MW-278.02 g/mol) was from Kermel (China), while L-Dopa (Carbidopa/Levodopa) was from Organon & Co. (Jersey City, NJ, USA). Powdered milk (peak milk) was purchased from FrieslandCampina WAMCO Nigeria Plc. (Lagos, Nigeria). Agar-Agar powder (Bacteriological grade-B1CA1HV01) was purchased from Titan Biotech Ltd (Rajasthan, India), while Nipagin (Methyl Paraben) was from Molychem (Mumbai, India). Similarly, sucrose was purchased from LOBA Chemie Pvt Ltd. (Mumbai, India), while Fe, Dopamine, Catalase, Glutathion peroxidase and Malondialdehyde assay kits were obtained from Solarbio Life Science (Beijing, China). Nucleic acid extraction kit was purchased from Liferiver®, Shanghai ZJ Bio-Tech Co., Ltd (China), while SYBR-Green One-Step qRT-PCR Master Mix kit was purchased from TransGen Biotech Co., Ltd. (Beijing, China).

Sample Preparation

Damboto rice, one of the popularly consumed rice cultivars in North West Nigeria, which was shown to be rich in antioxidants (Kalman, 2014; Rappold et al., 2011; Saka et al., 2023; Sibian et al., 2017), was used in this study. The rice cultivar was obtained from farmers after harvest and stored in the laboratory at room temperature before the commencement of the research. The paddy rice was de-husked to generate BR, and a subset of the cultivar was polished to produce WR. The BR and WR were then ground into a fine powder, sifted and placed in polythene bags at ambient temperature for future investigations.

Fly stock and Husbandry

Harwich strain of *Drosophila melanogaster* was acquired from the fly lab of the Centre for Advanced Medical Research and Training (CAMRET). Usmanu Danfodiyo University, Sokoto. The flies were cultured in vials and maintained at the fly optimum temperature (22-25°C) and humidity (60-65%). They were provided the standard cornmeal diet (Table 1) and subjected to a natural light-dark cycle. The fly media was regularly replaced every three days to avoid contamination.

Table 1: Nutritional Composition of Experimental groups

Component	Fly Culture (per 100 mL)	Iron-Diet (per 100 mL)	BR +Fe Diet (per 100 mL)	WR+Fe Diet (per 100 mL)	L-Dopa+Fe Diet (per 100 mL)
Corn flour	10 g	-	-	-	-
Sucrose	-	2% w/v	1% w/v	1% w/v	1% w/v
Agar-agar	1 g	1% w/v	1% w/v	1% w/v	1% w/v
Baker's yeast	1 g	1% w/v	1% w/v	1% w/v	1% w/v
Distilled Water	0.15 L	0.15 L	0.15 L	0.15 L	0.15 L
Methyl paraben	80 mg	0.08% w/v	0.08% w/v	0.08% w/v	0.08% w/v
Powdered milk	-	1% w/v	1% w/v	1% w/v	1% w/v
Brown rice	-	-	1% w/v	-	-
White rice	-	-	-	1% w/v	-
Iron (Fe)	-	1 Mm	1 mM	1 mM	1 mM
L-Dopa	-	-	-	-	1 mM

Experimental Design

Three-day-old adult flies were blindly randomised into five groups, with 20 flies per vial in triplicate. Some of the flies were fed Fe diets for 10 days (Figure 1) and then treated with their respective diets for 5 days (Fe+BR, Fe+WR, Fe+L-Dopa groups). The control group (CT) was raised on a normal diet for 15 days (Table 1). The behavioural assessments (climbing assay, grooming, aggressiveness and aversive Phototaxis suppression) were carried out while the fly heads were used for biochemical (iron, Dopamine, Catalase, GPx and MDA) and gene expression (Catalase, GPx, NRF2, DMT-1, ferritin and PARK-7) analyses.

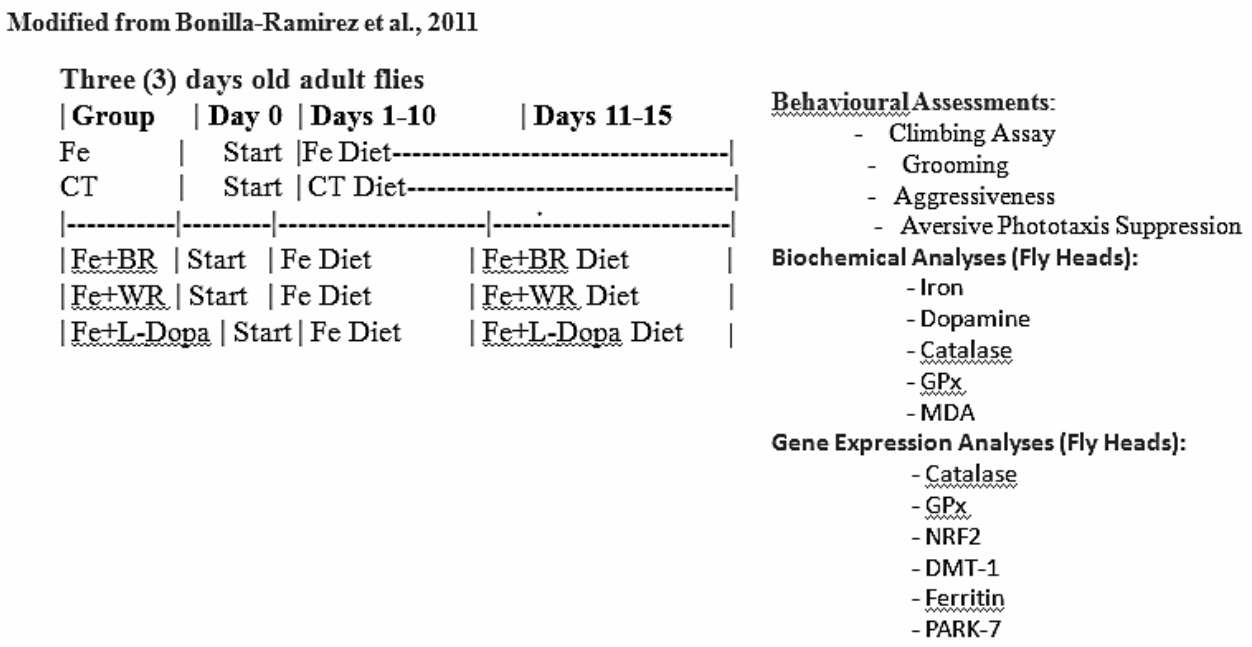

Figure 1: Experimental design showing treatment groups and timeline. Flies of the following groups (Fe+BR, Fe+WR, Fe+L-Dopa) were raised on iron diets for 10 days, then treated with their respective diets, brown rice (BR), white rice (WR) and L-dopa for 5 days. While the flies of the BR, WR and L-dopa groups were raised on a normal diet for 10 days, and fed BR, WR and L-dopa diets respectively for 5 days. Control group (CT) flies were raised on a normal diet for 15 days. WR- White rice; BR- Brown rice; L-dopa- levo dopa, Fe- iron.

Behavioral Assessment

Climbing Assay

Twenty flies per interventional diet were anaesthetised on ice and placed in empty 50 mL measuring cylinders to assess locomotor activity (23). The cylinder was marked at 6 cm from the bottom. Flies were allowed to acclimatise at room temperature for 10 min. The cylinder was gently tapped such that all the flies were at the bottom of the tube, and the number of flies that crossed the 6 cm mark in six seconds was recorded. This was repeated three times on the 16th day of exposure to interventional diets for each group, with a 2-minute resting time, to determine the average pass rate per group.

Memory Test

The flies' memory was evaluated using an aversive phototaxis test for quinine sensitivity (24), with some modifications. Before the tests, we checked whether the flies exhibited phototaxis response (i.e., if they were attracted to light), which is an important factor for the test. Firstly, a test was performed to assess the locomotor mobility of the flies using the negative geotaxis test. After the flies did not exhibit a locomotor deficit and phototaxis sensitivity, they were considered able to perform the test. The flies were then individually placed in a dark cylindrical tube. After one minute, the dark tube with the fly inside was positioned horizontally on the device, alongside another tube that was fully lit (16 W lamp). The device has free passage between one tube and another. If the fly left the dark tube and went towards the light (in 10 s), it was considered valid to carry out the next test steps. In the second moment, the flies were trained; a filter paper moistened with 120 µL 1 M chloroquine solution was then added to the end of the illuminated tube. The fly was placed again in the dark tube and coupled to the illuminated tube containing chloroquine. When going to the illuminated tube (i.e., the light), the fly was repelled by the chloroquine. This procedure was repeated five times; the training was performed at zero time (PC0), and this test corresponded to short-term memory. Then, 6 h after the initial training session (PC6), the flies performed the same procedure; however, the chloroquine was removed from the illuminated tube. Therefore, if flies remained on the dark side of the tube, it meant that they remembered the presence of a repellent at the illuminated end of the tube, which repels them. Each trained fly was evaluated six hours after training, following the same protocol as at time zero. The number of times the fly avoided entering the lighted tube was recorded. The last stage of the test, PC6 (6 hours after conditioning), is related to long-term memory. In total, 15 flies were used per group, corresponding to three independent experiments (n = 3).

Aggressiveness Test

The aggressiveness test was evaluated as reported previously (25) with some modifications. After the experimental period, the flies were placed in a Petri dish for 90 min without food before the test began. Five of the male flies were placed in a circular arena (45-mm radius and 12-mm height) with a 5% drop of sucrose in the center of the arena. Behavioural tests were performed at 22 ºC. After the acclimatisation period, the males were placed in the arena for 5 min to observe any aggressive events. Aggressive events were considered when flies extended their legs to the other fly, resulting in physical contact, chasing, quickly approaching the other fly (leading to disorientation), fast wing flapping in response to proximity/approach from the other fly, and the impact involving the front legs of both flies. A digital camera was used in recording all the assays. The score corresponding to the number of aggressive events among the five pairs of male flies per group was used for the evaluation. A total of 20 pairs per treatment group was assessed (n = 3).

Grooming Test

Through the repetitive behaviour test, flies' grooming behaviour is related to the dopaminergic and octopaminergic systems. Here, we evaluated the number of grooming events as a parameter related to

stress. The performance evaluation of repetitive hygiene behaviors was performed as previously described (26), with some modifications. The number of times the fly performed grooming movements was observed and recorded over 2 minutes. Six independent experiments were conducted (n = 6), with five flies per group used for the tests. The mean time of the five flies in each group was used for statistical analysis.

Biochemical Assays

Haemolymph extraction

Upon cold anaesthesia, a pooled population of thirty flies per group was transferred into 1.5mL Eppendorf tubes filled with 200 µL phosphate-buffered saline (PBS). The flies were then homogenised using a handheld homogeniser (Hangzhou Miou Instrument Co., Ltd., Hangzhou, China) and centrifuged at 8,000 × g for 10 min at 4°C (TOMY MX-301 High Speed Refrigerated Microcentrifuge). The supernatant was gently collected, dispensed into a sterile 1.5mL tube and kept at -20°C for downstream biochemical analyses.

Determination of Fe concentration

The Fe concentration of the whole fly and fly head were quantified separately using Iron biochemical detection assay kit (Beijing Solarbio Science & Technology Co., Ltd, China; Cat No BC1735), in accordance with manufacturer instructions. The absorbance was measured at 520 nm using a microplate reader (Infitek Microplate Reader, MPR-H200BC, Shandong, China).

Determination of dopamine concentration

The dopamine concentration of the whole fly and fly head were quantified using dopamine ELISA detection assay kit (Elabscience, China) in accordance with manufacturer instructions. The absorbance was measured using a microplate reader (Infitek Microplate Reader, MPR-H200BC, Shandong, China) at 450 nm, and a four-parameter (4PL) logistics curve (y = 0.8925x + 2.221; R2 = 0.997) was used to calculate the dopamine concentrations.

Oxidative stress and antioxidant markers assay

Malondialdehyde (MDA) level and activities of Glutathione peroxidase (Gpx) and catalase (CAT) in the whole fly haemolymph were determined using colorimetric assay kits (Solarbio Life Science, Beijing, China) according to the manufacturer's instructions. Absorbance readings were measured using microplate reader (Infitek Microplate Reader, MPR-H200BC, Shandong, China) at the appropriate wavelengths (240 nm for CAT; 412 nm for Gpx; 450 nm, 532 nm, and 600 nm for MDA)

Primer Design

The gene-specific primers were designed using Primer Quest. The primer sequences are provided in Table 2.

Table 2: Primary gene sequences

Gene Products (mRNA)	Strand	Primer (5`-3`)
GPx	Forward	CGAATATCTGTGCGAGGATGG
	Reverse	CTGCTCGTTGGAGATGTAGC
Fer1HCH	Forward	CTGGCTGTTCCTGAGATTACC
	Reverse	CATGGCCAAGTACTGGTAGG
Rpl32	Forward	GGATCGATTCCTGTGAGAGTTC
	Reverse	TGGGCAGTATCCATTGAGTTT
Nrf2	Forward	GGAAGAAGAAGCAGCGAAGA
	Reverse	GATGATGAGACCCGACTACAAC
DJ-1	Forward	CTCGTATCCCTCAATGAAGCC
	Reverse	CCTCGACTGGTGATCAGATTG
Mvl	Forward	CGGTCAGTTCTCAATGGAAGG
	Reverse	GGCAGAAGGTGGGAATAATGG
Cat	Forward	CTGCCCTACTGTAGTTAATTCCG
	Reverse	GTATATCGAGTGACCACTGTGC

PCR conditions: Reverse transcription at 45oC for 5 mins; pre-denaturation at 94oC for 30 sec (pre-denaturation); 40 cycles of denaturation at 94oC for 5 sec, annealing at 60oC for 15 sec and extension at 72oC for 10 sec. cat: catalase; Nrf2: Nuclear factor erythroid 2-related factor 2; GPx: Glutathione peroxidase; DJ-1: Protein deglycase; Fer1HCH: ferritin heavy chain; mvl: Malvolio.

Gene Expression Studies

Total RNA extraction

Total RNA was isolated from whole flies using Liferiver® isolation kit (Shanghai ZJ Bio-Tech Co., Ltd., Shanghai, China) following the manufacturer's guidelines. Ten flies were used per group for each extraction. RNA was quantified using Nanodrop spectrophotometer (Bioevopeak Nuclei acid analyser, SP-MUV200F, Shandong, China) and A260/280 readingsof 1.8 to 2.0 and A260/230 values of 2.0 to 2.2 were considered good.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

For mRNA expression, one-step reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed according to TransScript® Green one-step qRT-PCR Supermix kit protocol (AQ211-01, TransGen Biotech, China) on a Rotor-Gene Q 5plex HRM qPCR machine (QIAGEN, Germany). All the reactions were carried out in triplicate. Following melting curve analysis, target mRNA expression was normalised to RPL-32 mRNA reference gene according to the 2-ΔΔct formula.

Statistical Analysis

Data generated were subjected to normality test using Shapiro-Wilk's normality test. The normally distributed data were recorded in mean ± SD. One-way ANOVA was used where necessary and the Tukey *post-hoc* test was used to compare significance across groups. GraphPad Prism v7.0 (GraphPad Software Inc., San Diego, California) was used for statistical analyses.

Results

Behavioural Analysis

BR improve climbing assay (locomotive performance)

Male flies exposed to Fe and WR+Fe groups exhibited locomotor behavior deficit, evidenced by reduced climbing rate compared to the control group. This adverse impact, however, was mitigated by treatment with both BR and L-dopa. Flies in these groups demonstrated improved climbing abilities in BR + Fe and BR+L-dopa significantly ($p < 0.05$). (Figure 2).

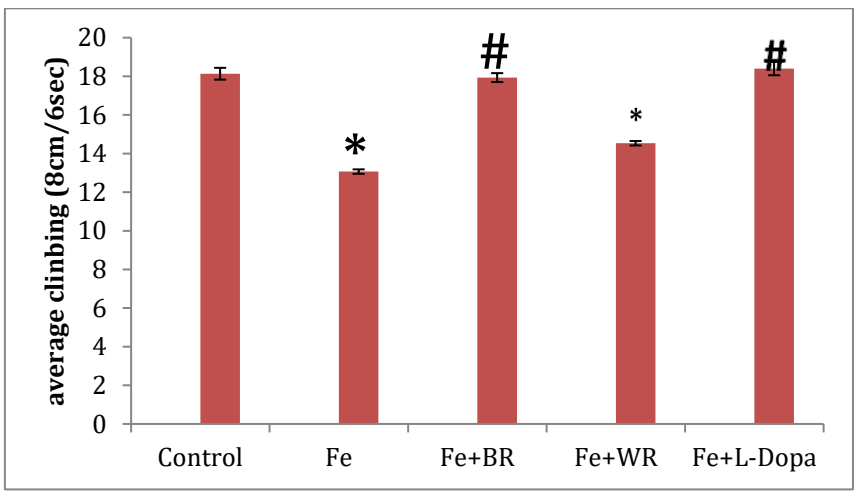

Figure 2: Climbing assay (locomotion) of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 20 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P<0 .05

BR reduced autogrooming observed in iron induced Parkinson's disease

In the grooming test, a sequence of grooming movements was observed (Figure 3). Flies in to Fe and Fe+WR exhibited a higher frequency of grooming episodes compared to the control group ($p < 0.05$). In contrast, the groups treated with BR (BR+Fe) demonstrated fewer grooming episodes compared to the Fe-exposed group, with statistically significant differences from both the Fe-exposed and Fe+WR groups. Remarkably, a similar effect was observed in the groups treated with L-dopa (L-dopa + Fe).

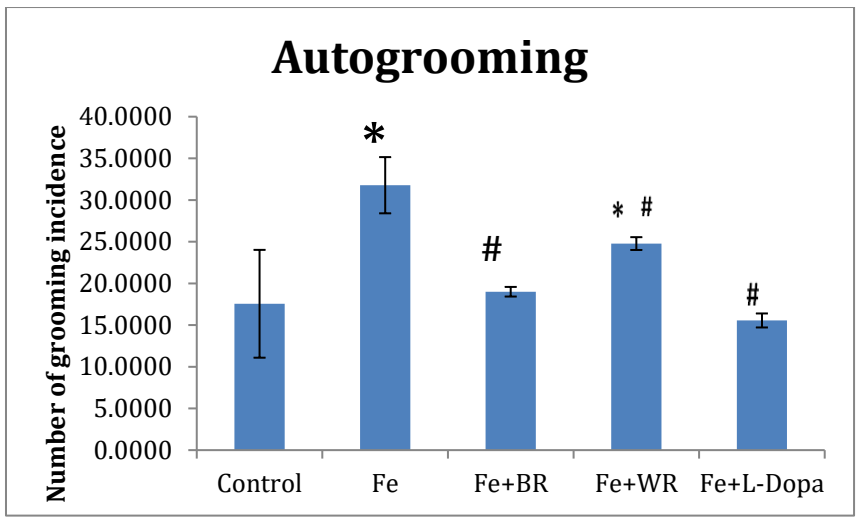

Figure 3: Autogrooming behaviour of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD. (n=3; 5 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P<0.05

BR reduced aggressiveness observed in iron-induced parkinson's disease in fly

The aggressiveness of the flies was assessed based on the number of observed aggressive encounters (Figure 4). Flies in Fe and Fe, Fe+WR groups displayed an increased level of aggressiveness compared to the control group ($p<0.05$). In contrast, groups treated with BR and L-Dopa exhibited reduced aggressiveness compared to the Fe group ($p < 0.05$). Moreover, the behaviour of these groups was comparable to that of the control group.

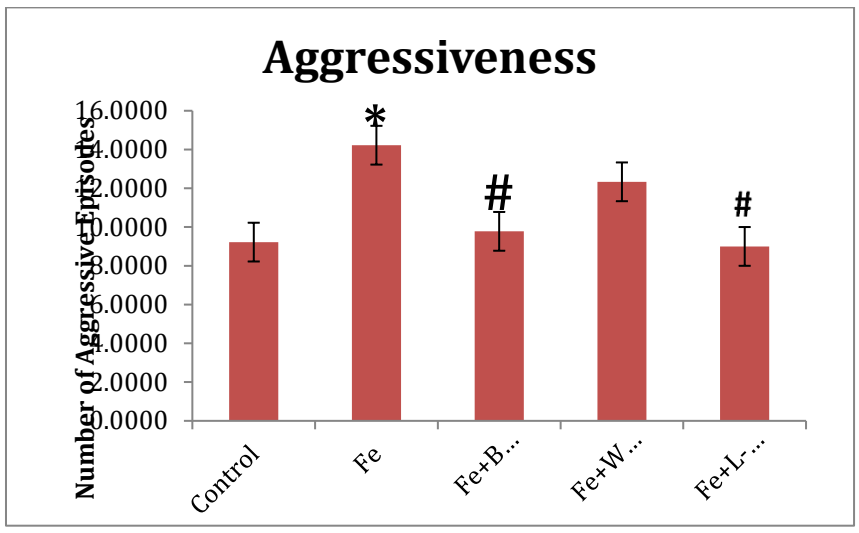

Figure 4: Aggressive episode of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 2 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< .05

BR restored reduced learning and short-term memory in iron-induced Parkinson's disease

We investigated the impact of BR on Parkinson-like non-motor changes, specifically learning and short-term memory, induced by Fe exposure in Drosophila melanogaster. The aversive phototaxis test was employed for both assessments, with learning evaluated through the "0-hour" post-conditioning test (PC0) (Figure 5a) and short-term memory assessed through the 6-hour post-conditioning test (PC6) (Figure 5b). Statistical significance was determined using Kruskal-Wallis non-parametric analysis. In the Fe and WR+Fe groups, the flies' learning capacity (short-term memory; PC0, Figure5a) was diminished, and their long-term memory (PC6, Figure 5b) was compromised ($p < 0.05$). Conversely, the Fe + BR and Fe + L-dopa groups exhibited preserved short- and long-term learning and memory capabilities.

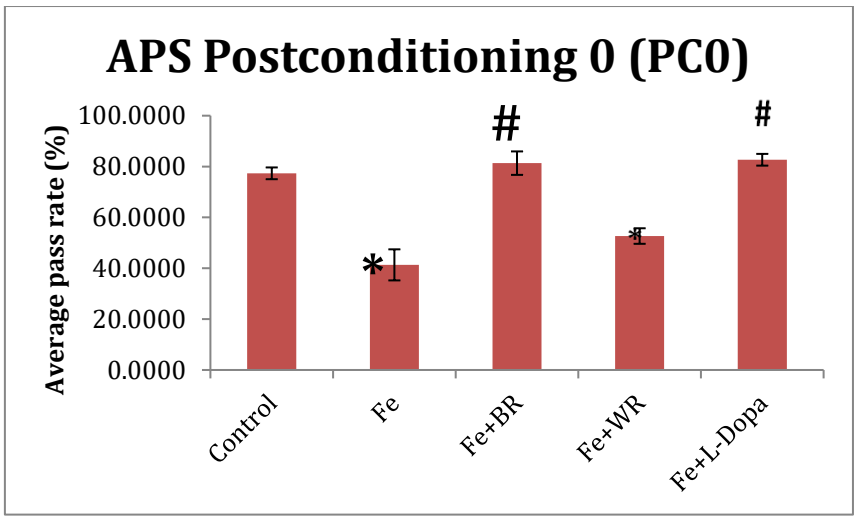

Figure 5a: Aggressive Phototaxis suppression 0 (PC0) of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and Levo dopa treatments. Values represent mean ± SD. (n=3; 10 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P<0 .05

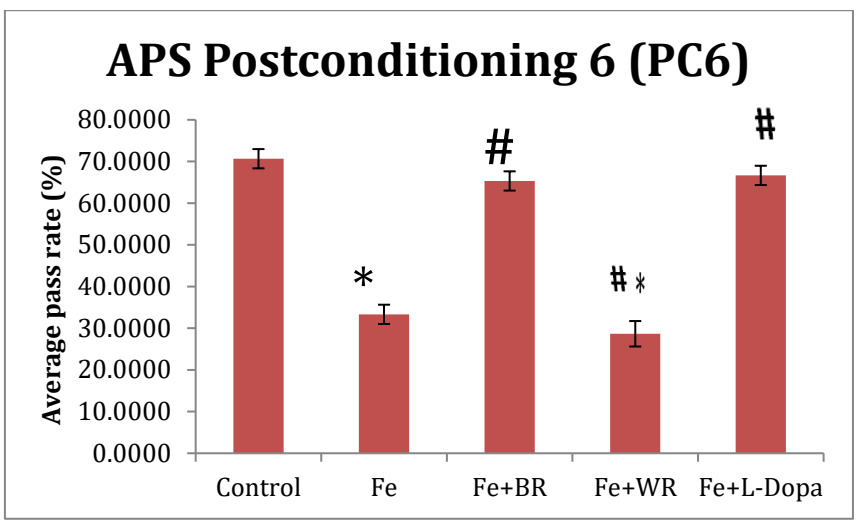

Figure 5b: Aggressive Phototaxis suppression 6 (PC6) of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and Levo dopa treatments. Values represent mean ± SD. (n=3; 10 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P<0 .05

BR reversed iron overload of the whole fly and fly head Fe levels of iron induced Parkinson's disease

Fe levels in the fly head (Figure 6a) and whole fly (Figure 6b) of *Drosophila melanogaster* were quantified following a 15-days exposure. Each treatment group consisted of 25 heads and 20 bodies per sample (n = 3). The results revealed that the Fe, WR+Fe groups exhibited significantly higher concentrations of Fe in both the head and body in line with the control group ($p < 0.05$). Notably, flies treated with BR (BR + Fe, groups) and L-dopa (L-dopa + Fe groups) demonstrated lower Fe levels in the head ($p < 0.05$).

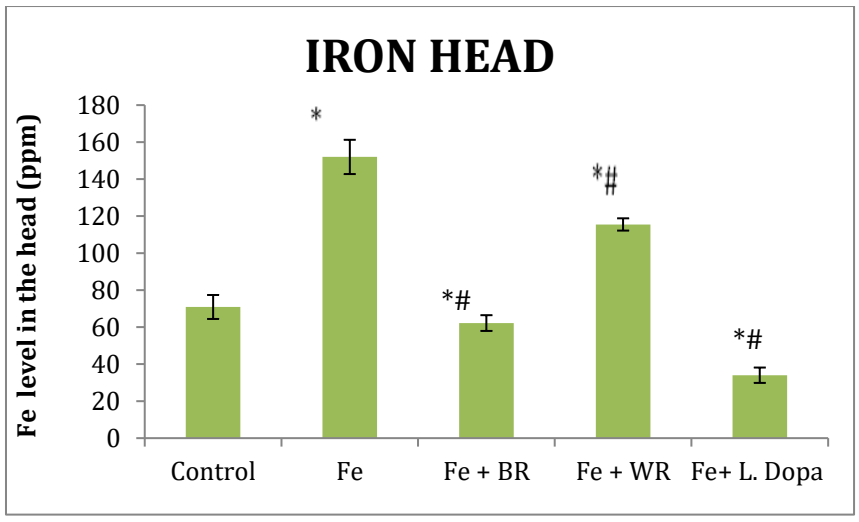

Figure 6a: Iron level in the head of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 25 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< .05.

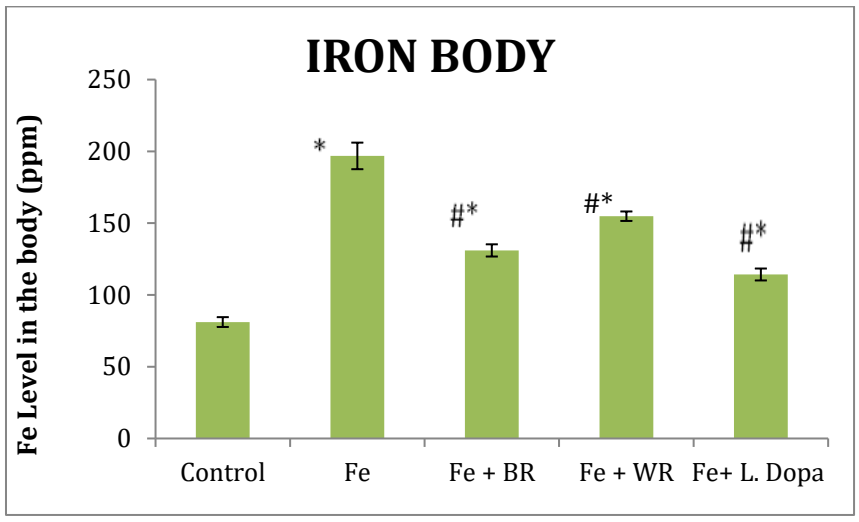

Figure 6b: Iron (Fe) level in the body of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 25 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< .05

BR ameliorated reduced level of dopamine displayed in the fly head of iron-induced Parkinson's disease

Figure 7 shows the results of dopamine levels in the fly heads. It is observed that flies exposed to iron (Fe group) had significant decrease in dopamine levels in their heads ($p < 0.05$). However, treatment with Brown rice (BR+Fe) and L-dopa (Fe+L-dopa groups) resulted in increased dopamine levels, in line with the control group ($p < 0.05$).

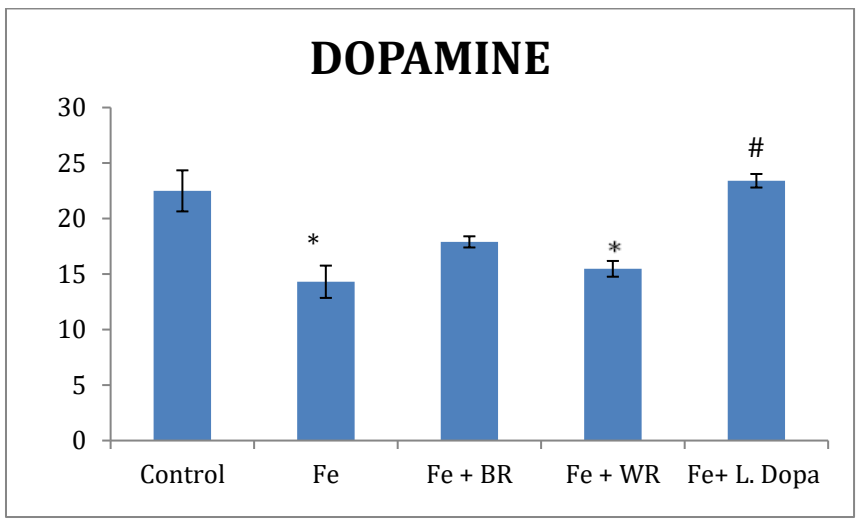

Figure 7: Dopamine (DA) level of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 25 flies per replicate) * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05

BR attenuated oxidative stress and improve antioxidant markers in the fly head of iron-induced Parkinson's disease

Oxidative stress indicators assessed included CAT, GPx and MDA (Figure 8 a, b and c, respectively). The results revealed that flies exposed to Fe exhibited a significant reduction in CAT and GPx activities compared to the control group ($P < 0.05$). Treatment with Brown rice and L-dopa effectively reversed this effect, showing a significant increase in CAT and GPx activities compared to treatment with WR.

The analysis of lipid peroxidation, as indicated by MDA concentration, demonstrated an increase in oxidative stress in flies exposed to Fe compared to the control. Interestingly, treatment with BR significantly attenuated this effect ($P < 0.05$). However, the MDA levels in flies treated with L-dopa and WR were lower than those treated with Fe alone, although these were not statistically significant.

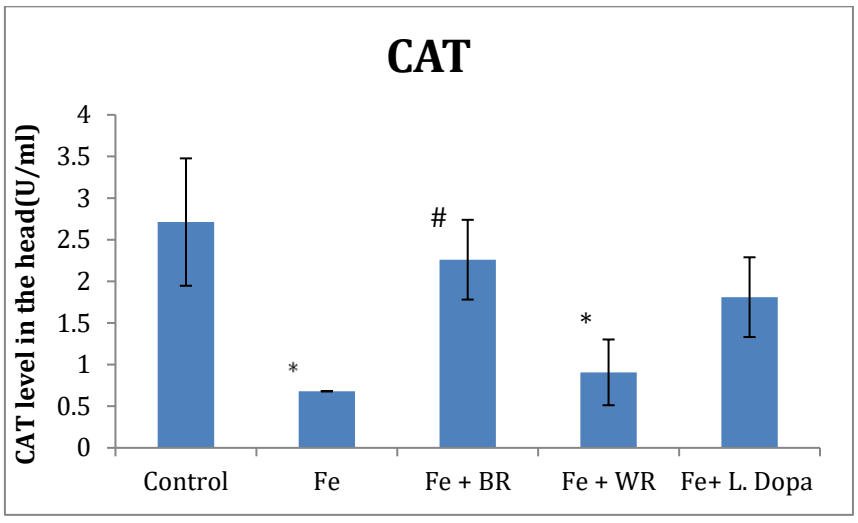

Figure 8a: Catalase activities of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 25 flies per replicate) * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< .05.

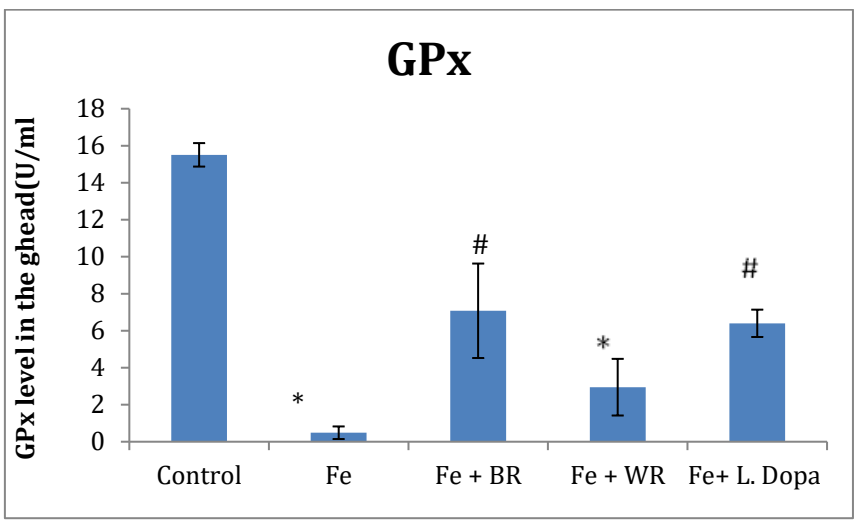

Figure 8b: Glutathione peroxidase activity of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 25 flies per replicate) * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< .05.

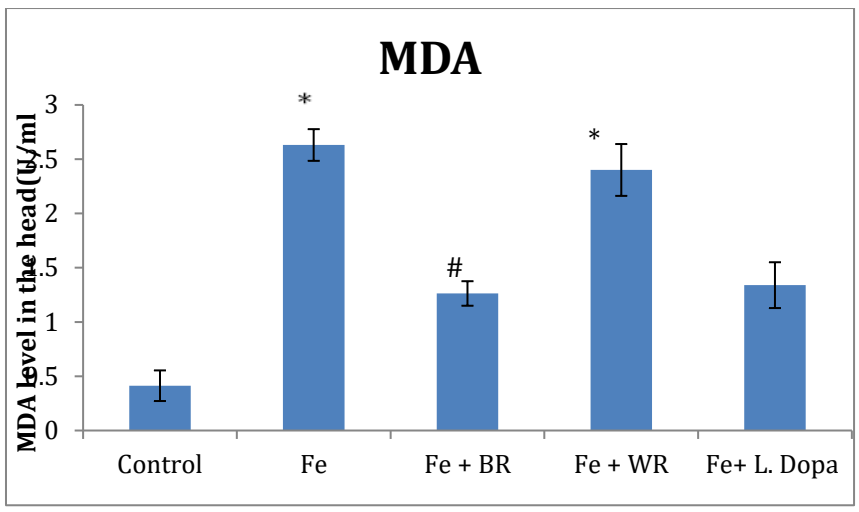

Figure 8c: Malonyldehyde activity of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 25 flies per replicate) * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< .05

BR prevented the downregulation of antixiodant enzymes and modulated Fe-related mRNA levels in fly head of iron induced Parkinson's disease

Figure 9 shows the effectsof BR, WR and L-dopa on the expression of Catalase, GPx, NRF2 and DJ-1 genes in the head of *Drosophila melanogaster* compared to non-treatment (Fe group) and control groups. The results indicated that the Fe group had downregulation of the mRNA levels of CAT, GPx, and Nrf2 genes compared to the control group ($P<0.05$). However, treatment with BR (BR+Fe) and L-dopa led to a significant upregulation of these genes ($P<0.05$). Similarly, the expression of ferritin and MVL genes in the head of Drosophila melanogaster was evaluated (Figures 10, 11, and 12). The results indicated that the Fe group had significant upregulation of ferritin mRNA levels and significant downregulation of MVL in the nontreated iron group. However, treatment with brown and L-dopa maintains it at a normal control level ($P<0.05$).

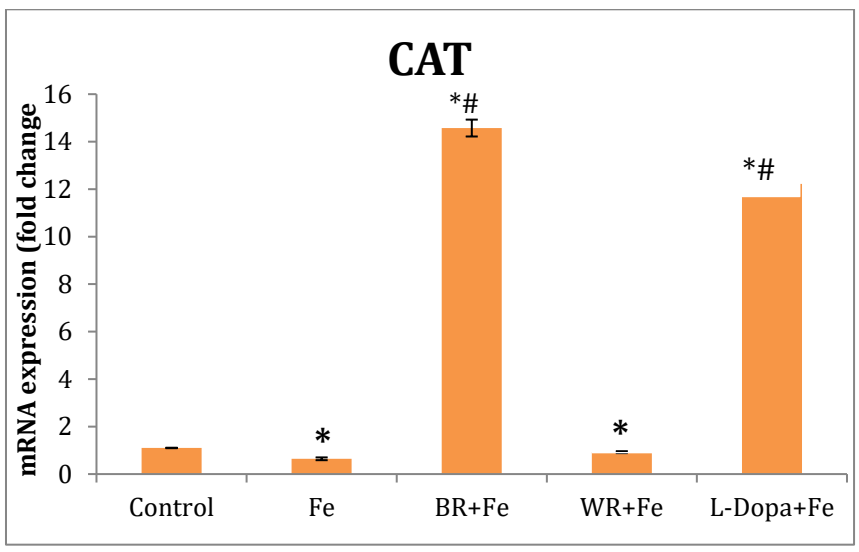

Figure 9a: Catalase mRNA expression activity of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

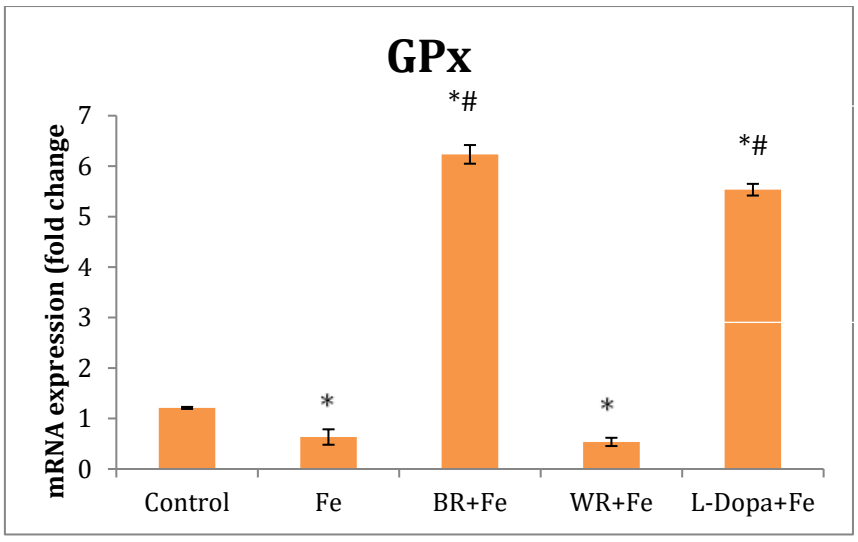

Figure 9b: Glutathion peroxidase mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

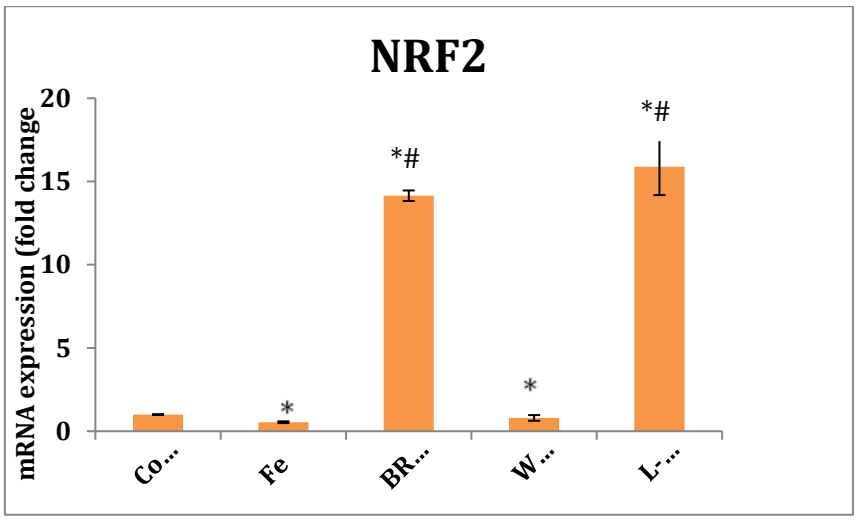

Figure 9c: Nuclear factor erythroid 2-related factor 2 (NRF2) mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

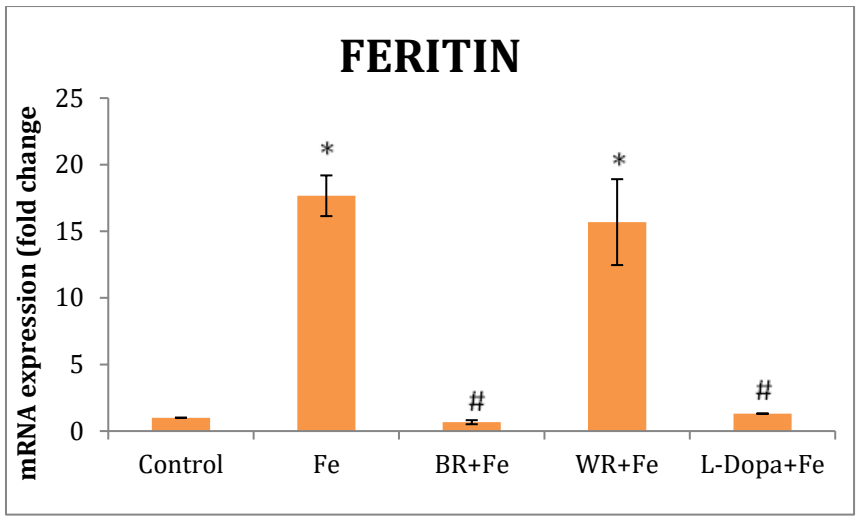

Figure 10a: Feritin mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

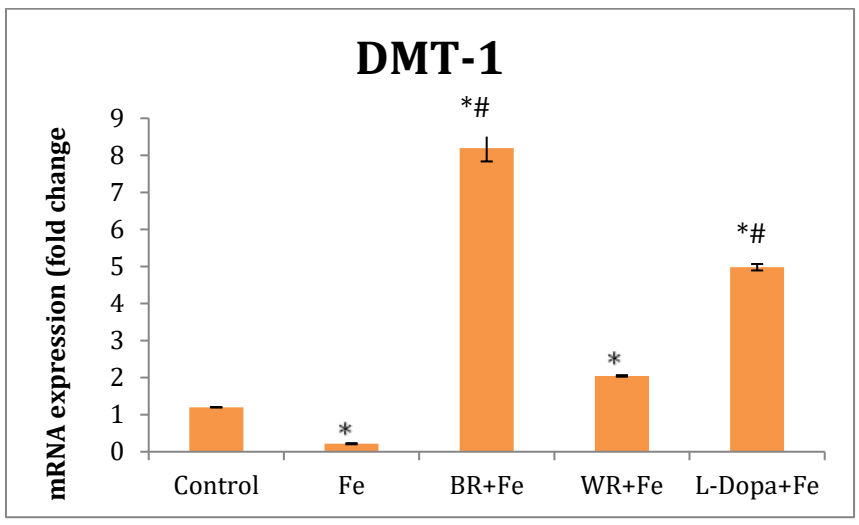

Figure 10b: Malvolio (Mvl) mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and Levo dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

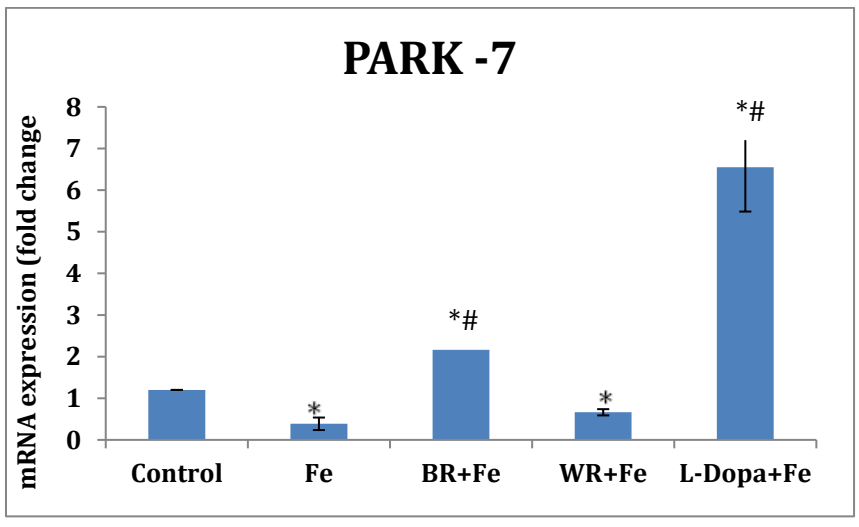

Figure 10c: PARK7 mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

BR modulate PINK1/PARKIN mRNA expression in iron-induced Parkinson's disease

Figure 11 shows the effects of BR, WR and L-dopa on expression of PINK1/PARKIN genes in the head of *Drosophila melanogaster* compared to non-treatment (Fe group) and control groups. The results indicated that the Fe group had downregulation of the mRNA levels of PINK1/PARKIN genes compared to the

control group (P<0.05). However, treatment with BR (BR+Fe) and L-dopa resulted in a significant upregulation of these genes (P < 0.05).

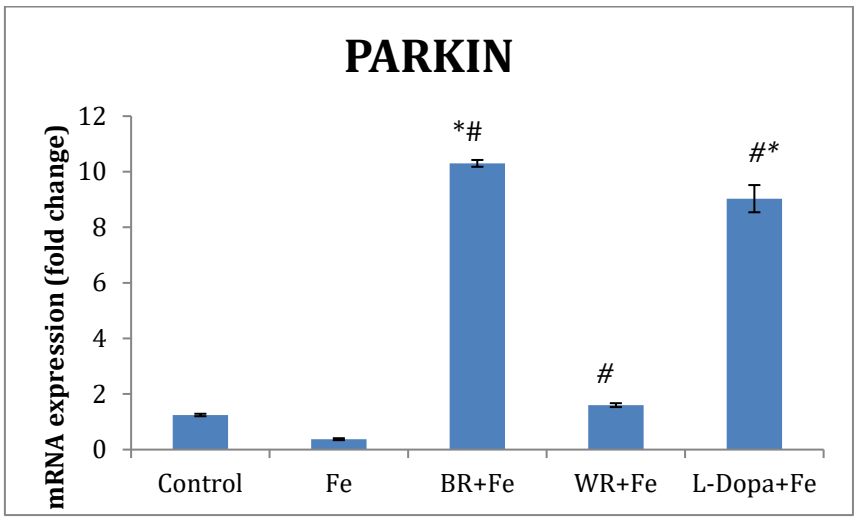

Figure 11a: PARKIN mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and Levo dopa treatments. Values represent mean ± SD (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

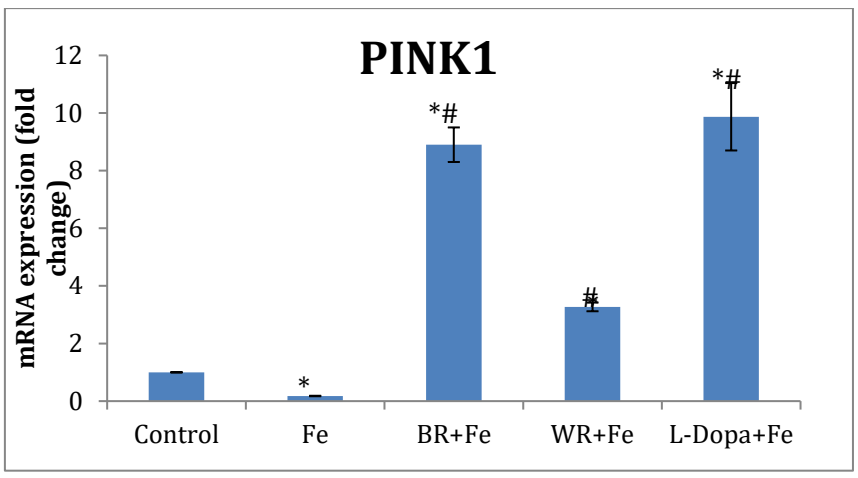

Figure 11b: PINK1 mRNA expression of male *Drosophila melanogaster* exposed to Fe, Brown rice, White rice and L-dopa treatments. Values represent mean ± SD, (n=3; 30 flies per replicate). * = statistically different from control; # = statistically different from the Fe-diet group. Statistically significant at P< 0.05.

Discussion

Iron plays a crucial role in various cellular processes in the brain, including mitochondrial respiration, myelin and DNA synthesis, oxygen transport, neurotransmitter synthesis, and cellular metabolism (27,28). However, iron dyshomeostasis can cause neuronal damage in iron-sensitive brain regions. Neurodegeneration with brain iron accumulation reflects disorders caused by iron overload in the basal ganglia. High iron levels and iron-related pathogenic triggers have also been implicated in sporadic neurodegenerative diseases, particularly, Parkinson's disease (PD) (29).

This study investigates iron metabolism dysregulation as a key factor in disease progression. In flies, iron is rapidly metabolised and stored in ferritin after consumption (30). Higher iron levels in fly heads indicate metabolised and circulating iron, supporting existing research (31). Excess non-ferritin iron can cause harmful effects, including cell death (32). Our study revealed that flies treated with brown rice (BR + Fe) significantly reduced iron levels in their heads, similar to the control group, suggesting effective iron chelation. This effect may be due to the rich antioxidant content of BR, mainly phenolic compounds, compared to white rice (WR) (12,23). Phenolic compounds can chelate iron with their functional groups (33,34). Thus, BR may act as an effective iron chelator, potentially influencing the course of Fe-induced Parkinson's disease in flies. Similarly, L-dopa also preserved iron levels in the flies' heads, supporting its Fe chelating properties (25,35).

Our study identified significant correlations between iron levels in fly heads, dopamine levels, antioxidant enzymes, and locomotion. Elevated iron levels increased susceptibility to neurotoxicity, emphasising the relationship between iron levels and neurodegenerative processes (35,36). Flies subjected to chronic iron treatment showed significant locomotor deficits, particularly in locomotive+ tests. This observation is consistent with previous studies indicating that elevated iron concentrations can induce Parkinsonism-like effects in Drosophila melanogaster, potentially due to disruptions in iron metabolism or homeostasis (36). BR treatment alleviated neuromotor deficits and locomotor impairments, likely due to its polyphenol-rich antioxidant activity, which helps maintain redox balance (37).

Non-motor changes in the early stages of PD are less well-studied than motor alterations (1). Recognising the link between elevated brain iron levels and PD development, we employed a Drosophila melanogaster model induced by chronic iron exposure to simulate Parkinson-like disease. Identifying non-motor alterations could guide research toward novel pharmacological interventions to enhance quality of life and longevity for individuals with PD. BR administration positively impacted dopaminergic functionality, reducing iron levels in the head and impeding non-motor alterations progression. Improvements included memory deficits and anxiety-like behaviors. BR-treated flies performed better than L-dopa-treated flies, although the difference was not statistically significant.

Recognising that non-motor alterations, such as anxiety and memory deficits, precede motor alterations in PD, our investigation focused on these aspects to explore BR as a potential therapeutic alternative. The aversive Phototaxis Suppression tests (APS) evaluated the impact of BR on the flies' memory capacity. Flies exposed to iron showed a decline in learning capacity. Interestingly, BR and L-dopa preserved cognitive ability. The PC6 test, conducted 6 hours after training, indicated short-term memory based on task recollection (24,35). Cognitive changes correlate with elevated iron levels and neurodegenerative diseases (35). These memory deficits often coincide with increased oxidative stress and apoptotic markers (38).

Auto-grooming behavior, used to assess anxiety levels, showed that BR-treated flies had grooming episodes comparable to the control group, indicating behavioral stability. In contrast, iron and WR exposure increased grooming episodes, suggesting behavioral disturbances. The aggressiveness test revealed that BR and L-dopa had a protective effect against aggressive behaviour, maintaining levels similar to the control group. BR's anxiolytic action may result from its high Gamma-aminobutyric acid (GABA) content, a neurotransmitter known for its calming effect (39).

Dopamine is essential for controlling various functions, including locomotion, learning, memory, cognition, and emotion (40) . Loss of dopaminergic neurons in the substantia nigra leads to a significant reduction in dopamine, crucial for PD's clinical characteristics (41). In our study, iron-exposed flies exhibited decreased dopamine levels, whereas BR maintained dopamine levels comparable to those of the control and L-dopa-treated flies. Similar results were observed by Poetini et al. (35), who found that excess iron oxidises dopamine, forming neurotoxic products. Long-term L-dopa use can exacerbate neurodegeneration through toxic conjugates formed in dopamine auto-oxidation (42). BR's high tyrosine, a precursor of dopamine synthesis and antioxidant bioactives may modulate PD progression without long-term side effects by reducing oxidative stress (43,44).

Elevated lipid peroxidation (MDA) and decreased enzymatic activities of CAT and GPx following iron exposure indicated oxidative stress (45). BR and L-dopa treatments restored

antioxidant defenses and prevented oxidative damage. Ferulic acid from BR may favor anti-apoptotic mechanisms in the substantia nigra pars compacta (SNpc) region, underscoring its neuroprotective action (46). This supports the protective effects of brown rice against oxidative stress-induced damage (47).

Our study provides insights into the mechanistic effects of iron, BR, WR, and L-dopa in the context of Parkinson's disease. Upregulation of ferritin mRNA in iron-exposed flies indicates iron overload and homeostasis disruption, leading to dysregulated iron metabolism in neuronal cells. BR and L-dopa treatments normalised ferritin gene expression, suggesting their potential in mitigating iron-induced disturbances in neuronal iron metabolism. The upregulation of the Mvl gene in flies treated with BR and L-dopa suggests a regulatory mechanism contributing to iron homeostasis normalisation. Ferritin expression increases under iron accumulation, while DMT1 mRNA levels decrease (48). This inhibits iron absorption and enhances storage.

Our investigation also revealed the downregulation of antioxidant defense and cellular protection genes, namely Nrf2, Catalase, GPx, and PARK7, in iron-exposed flies. BR and L-dopa treatments upregulated these genes, suggesting improved cellular resilience and antioxidant defence. PARK7's role in inhibiting Nrf2 degradation further connects the dots, as upregulated Nrf2 in treated flies may enhance stress response enzymes, particularly NQO1 (49). Nrf2, the master regulator of cellular responses against oxidative stress, is typically downregulated under iron overload, reflecting impaired stress response, while its activators protect against oxidative stress (50,51). GPx and CAT are also involved in the breakdown of hydrogen peroxide (52). Their mRNA downregulation in iron-exposed flies suggests a compromised ability to detoxify reactive oxygen species, while their upregulation in BR and L-dopa-treated flies hints at a restored antioxidant capacity.

Mitophagy is linked to neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease, and Huntington's disease (53,54). The PINK1/Parkin pathway is vital for mitochondrial quality control, and defects in this pathway are thought to cause PD (55). PINK1 detects changes in mitochondrial membrane potential and acts as a receptor for damaged mitochondria (56). PINK1, a 63 kD protein, consists of a mitochondrial target sequence (MTS), a transmembrane segment (TM), and a kinase domain(57,58). Under normal conditions, PINK1's MTS is cleaved by mitochondrial processing proteases, and the TM segment is cut by PARL, resulting in a 52 kD form that is transported to the cytoplasm (59,60). When mitochondria are damaged, the membrane potential decreases, stabilising the full-length PINK1 (63 kD) on the outer membrane. This form of PINK1 phosphorylates ubiquitin and recruits Parkin to the mitochondria, initiating PINK1/Parkin-mediated mitophagy (61).

In this study, brown rice (BR) was found to positively modulate the PINK1/Parkin genes by upregulating them, thereby enhancing PINK1/Parkin-mediated mitophagy. The upregulation of these genes is also associated with the upregulation of NRF2, a master transcription factor that induces genes involved in cytoprotection and detoxification. This finding aligns with Murata et al. (2015), who indicated that NRF2 positively regulates PINK1 expression.

Conclusion

The study found that excessive iron caused Parkinson's disease (PD)-like symptoms in fruit flies, affecting both their movement and other functions. This damage might be linked to changes in the activity of genes related to antioxidants and iron management. Importantly, two substances, BR and L-dopa, were shown to counteract these negative effects by restoring antioxidant defences and enhancing the process of clearing damaged mitochondria via PINK1 and PARKIN pathways. These results suggest that BR and L-dopa may be promising treatments for PD, particularly in managing symptoms associated with iron-induced oxidative stress. The study offers new insights into developing therapies to improve the quality of life for PD patients, particularly by addressing their non-motor symptoms.

Limitations of the Study

The current study did not examine neuroinflammation and protein aggregation, which are equally important processes in PD. Similarly, it did not assess mitochondrial dysfunction, a key factor in iron-induced PD. These limitations may impact the overall scope of the findings, suggesting that further research is necessary to fully explore the neuroprotective effects of brown rice in PD models.

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