



Research Article



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Creatine Supplement Effect Similar in Rotenone Induced and Transgenic PD Flies in *Drosophila Melanogaster*

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ABSTRACT

Creatine (Cr) is known nutritional supplement having the properties bioenergetics, anti exitoxic and anti-oxidant. Present study has been under taken to investigate protective effects of creatine supplement against rotenone induced and transgenic PD flies using oxidative stress, mortality and neurotoxicity in *D. melanogaster*. It was noticed that significant reduction in the levels of oxidative markers in whole body homogenates and better performance in a negative geotaxis assay of flies exposed to creatine supplement of rotenone (Rot) induces and transgenic PD (Parkinson's disease) flies. Further dopamine level also increased in creatine supplement of rotenone induced and transgenic PD flies. These studies suggests that protective role of creatine supplement is similar in both rotenone (Rot) induced as well as in transgenic PD models of *D. melanogaster*.

KEYWORDS: Creatine supplement; rotenone; dopamine; oxidative stress; *Drosophila melanogaster*; transgenic PD flies

INTRODUCTION

Parkinson disease (PD) is one of the neurodegenerative diseases that has affected 1-2 % of the world population [1-3]. Main symptoms of PD are loss of memory, muscle rigidity, sleep disturbance, bradykinesia, and depression. One of the important protein α -synuclein produced by α -synuclein gene is commonly found in the patient of PD and it is positively associated with single nucleotide polymorphism (SNPs) in PD patients [4-6]. Studies have also shown α -synuclein expression mimic several aspects of PD in transgenic animals [7-10]. In recent time

non mammalian models are being utilized to understand complex biological problems of human diseases [11]. *D. melanogaster* model emerged as the most powerful non Mendelian model to understand neurodegenerative diseases such as Alzheimer's and Parkinson further the proteomic analysis have also shown that more than 70% of the diseases related loci in human have a clear orthologs in *Drosophila* [12-14]. In addition to this *Drosophila* show high degree of conservation, having complex neurons system, easy for genetic manipulation, and selectively

short life span all these characters made this organism ideal to study PD. Further, study of PD has led to understand the gene affecting PD and mechanism insight into the disease. Exposure to environmental toxin also known to affect the neurons system and showing sporadic incidence of neurodegenerative disorders [15-17]. Rotenone is a plant species and it is a strong inhibitor of mitochondrial nicotinamide adenine dinucleotide (NADH) - dehydrogenase known to induce phenocopies PD [18-20]. Further chronic rotenone treatment being used to generate pharmacological model of PD and induce both dopaminergic cell loss and locomotor impairments [1, 2, 21, 22]. However, it is not known that whether or not rotenone induced PD is similar to that of PD model of transgenic animals. Therefore present study has been undertaken in *D. melanogaster* to understand similarity between PD of transgenic *Drosophila* with that of rotenone induced PD. Creatine is a natural substance that exists in the human body [23-24]. Creatine supplementation has many

beneficial effects such as perverting the damage of DNA and RNA [25-26], reduce oxidative stress [27-28] and anti-inflammatory [29-30]. Recently, Ravikumar [19] have shown in *D. melanogaster* that creatine supplementation reduced mortality, oxidative stress and restored the dopamine levels. Present experiment has been taken to understand whether or not creatine supplementation has similar effect on PD of transgenic *Drosophila* and rotenone induced PD of flies.

MATERIALS AND METHODS

Creatine monohydrate (C3630) and Rotenone (R 8875) were purchased from Sigma Chemical Co. St Louis, USA. Transgenic flies' model of Parkinson disease were collected from Bloomington *Drosophila* Stock Centre Dept. Biology, Indiana University 1001E. RNAi posttranscriptional gene silencing was achieved by conducting crosses between UAS- A30P lines and DdC- GAL4 as driver line for create transgenic flies of Parkinson model (Fig. 1).

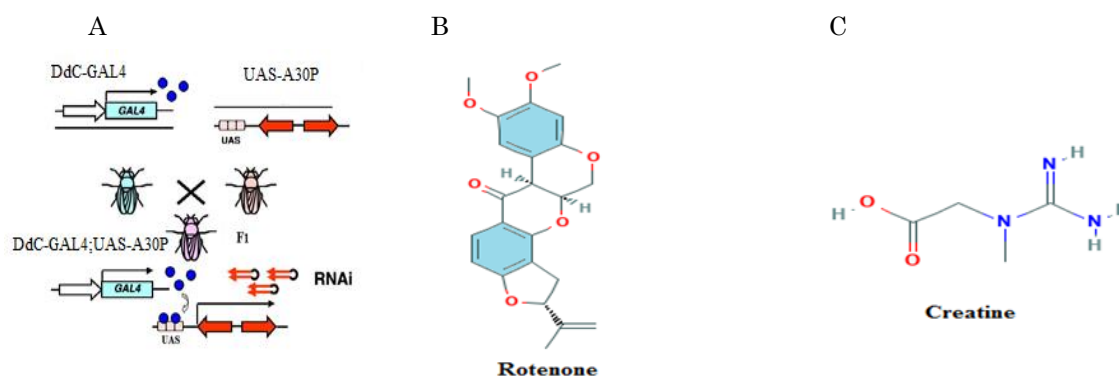


Fig. 1: (A) Transgenic flies of Parkinson model (PD). (B) Rotenone structure, (C) Creatine structure

Rotenone (Rot) Treatment

Wild type flies of *D. melanogaster* were collected from *Drosophila* stock Centre at manasagangotri, Department of studies in zoology, University of Mysore, Karnataka, India. These flies were cultured using wheat cream agar media (standard media) and maintained them at $22 \pm 1^\circ\text{C}$ and 70 – 80% relative humidity. These flies were used to treat rotenone, although in the preliminary experiment. Flies were exposed to four different concentration of rotenone (125 μM , 250 μM , 500 μM and 1000 μM) to determine lethality following the procedure of Ravikumar [19].

However 500 μM showed sub lethality therefore this concentration was used in all the experiment. Rotenone control (untreated), rotenone treated (500 μM) for 7 days, transgenic PD and control transgenic PD were used separately and co-treated them with 10 mM creatine. These flies were subjected for biochemical analysis involving Quantification of oxidative markers (Malondialdehyde (MDA), reduced glutathione (GSH) content, catalase (CAT), superoxide dismutase (SOD), Acetyl cholinesterase (AchE)), dopamine level and climbing assay.

Locomotion Assay

Fifty experimental flies were anesthetized and placed in a vertical plastic column (length, 25 cm; diameter, 1.5 cm). After 20 to 30 min recovery, flies were gently tapped from the bottom of the column. After 1 minute numbers of flies that reached the top and those of flies which remained at bottom were counted separately. Data obtained was expressed as flies escaped beyond minimum distance of 6 cm in 60 second of interval [11]. This experiment repeated for three times to obtained average value. Experimental assay was carried out separately for each of the experimental grouped flies.

Biochemical Assays

Flies were used to study oxidative stress through Quantification of oxidative markers viz., Malondialdehyde (MDA), reduced glutathione (GSH) content, and activities of antioxidant enzymes viz., catalase (CAT) and superoxide dismutase (SOD) were determined using head homogenate of the flies. Further, mitochondrial oxidative impairments were assessed in terms of reactive oxygen species (ROS) generation. In addition to this, cholinergic functions were measured in terms of the activities of acetylcholinesterase (AChE) in whole body homogenates.

Catalase (CAT)

Catalase activity of samples was determined by using Aebi method [31]. The reaction mixture consisted of sodium phosphate buffer (0.1M, pH 7.0), H₂O₂ (3%, 8.8mM). To prepare 1 ml reaction mixture, H₂O₂ was mixed with sodium phosphate buffer, then sample was added to reaction mixture. The changes in absorbance of hydrogen peroxide was recorded at 240 nm for 3 minutes. Molar extinction coefficient of hydrogen peroxide (H₂O₂) was 44.1 mM⁻¹ cm⁻¹.

Superoxide Dismutase (SOD)

Activity of superoxide dismutase (SOD) was determined according to the SOD-mediated inhibition of pyrogallol auto-oxidation by using method of Marklund and Marklund [32]. To carry out the experiment, sample, Tris HCl buffer and distilled water were mixed in a quartz cuvette. Then pyrogallol solution was added to the quartz cuvette. The OD values were recorded at 412 nm for 3 min to calculation of pyrogallol auto-oxidation. The enzyme activity

was said as units. Therefore one unit was equivalent to 50% of inhibition of pyrogallol auto-oxidation.

Reactive Oxygen Species (ROS)

The level of Reactive oxygen species (ROS) was measured by using non-fluorescent probe was called dichloro-dihydro-fluorescein diacetate (DCFH-DA) explained by Black and Brandt method [33]. Fifty head of adult male flies were decapitate, then homogenized into a micro tube containing 1 ml ice-cold Tris-HCl buffer (0.1 M; pH 7.4) and centrifuged at 13000g for 10min at 4 C°. Then supernatant was filtered. 15 µl of 5 µM DCFH-DA was poured into each well of micro plate, and then 100µl of filtered sample was added into micro plate containing DCFH-DA. After incubation at room temperature for one hour, the micro plate was read in a spectrofluorometer. The conversion of DCFH-DA to DCF was measured at 489 nm excitation and 525 nm emission wavelengths.

Lipid per Oxidation (LPO)

Lipid per oxidation was determined by using of Buege and Aust method [34]. To carry out of this experiment, 1.5 ml of 20 % acetic acid (pH 3.5) , 1.5 ml of 2-thiobarbituric acid (TBA , 0.8 % w/v) and 600 µl of distilled water were mixed . Then 200µl of sample was added to this mixture reaction, and mixed it well. The mixture reaction was incubated at 90 C° for 60 min. After incubation, 3 ml of butanol was added, the mixture was centrifuged for 3 min at 10000g. One ml of centrifuged supernatant was taken to absorbance and read at 532 nm.

Glutathione Assay (GSH)

Reduced glutathione (GSH) content was determined by using of Ellman method [35]. Fifty head collected from flies were homogenized in one solution containing (TCA, 10%) and (EDTA, 10mM) with ratio of 1:1, and centrifuged at 13000 g for 10 min at 4 C°. To prepare the reaction mixture, 50 µl of DTNB (10 mM) was mixed with 2750 µl of Tris-buffer (0.2 M; pH 8.0) into 3 ml quartz cuvette, then 200 µl of sample was added into reaction mixture and kept in room temperature for 10 min . Following this absorbance was recorded at 412nm and value was expressed as µg GSH/mg protein.

ChE Enzymes

Enzyme activity of Acetylcholinesterase (AChE) was determined by using of Ellman's method [36]. 30 μ l of sample was mixed with phosphate buffer (0.1 M, pH 8.0) and DTNB (10 mM). Then ACTI (78 mM) was added to mixture cuvette. Change in absorbance for 3 minute was monitored at 412 nm.

Determination of Dopamine

Head of the flies were homogenized in ice cold per chloric acid (PCA, 0.1 M) then centrifugation at 10000g for 10 min. 15 μ l of supernatant filtered injected directly into HPLC column (C-18 column reverse phase, Linchrospher 100RD-18, 14.5 cm, 5 μ m, E. Merck) equipped with ultraviolet detector set at 280 nm. Mobile phase consisted of 0.2% aqueous trifluoroacetic acid and methanol (70:30 v/v) and the flow rate was maintained at 1 ml/min [37].

Protein Estimation

Concentration of protein was determined by Lowry's method [38]. To carry out this experiment, 100 μ l of sample was mixed with distilled water and alkaline cooper solution (Lowry's reagent), mixture was incubated for 15 min at room temperature (dark place). Then

Folin-Ciocalteu's reagent (FC-reagent) was added to mixture and mixed well. The mixture was kept at room temperature for 30 min (dark place). The absorbance of samples was read at 660 nm, by using of bovine serum albumin (BSA) as standard curve.

Statistical Analysis

Data obtained in above experiments were subjected for one way ANOVA followed by post hoc (Tukey's post hoc test) using SPSS IBM Statistics 20 version.

RESULTS

Protective Effect of Creatine on Treated Rotenone and Transgenic Flies (PD).

Effect on Lethality of Flies

In the present study we used one concentration of rotenone (500 μ M) and two concentration of creatine. It was noticed that percentage of lethality of rotenone (500 μ M for 7 days) treated flies was 58.5% as compared its control. Interestingly when flies were co-exposure to creatine (5 mM and 10mM) significant decreased percent of mortality (Fig. 2). This shows that creatine supplement protect against rotenone induced lethality.

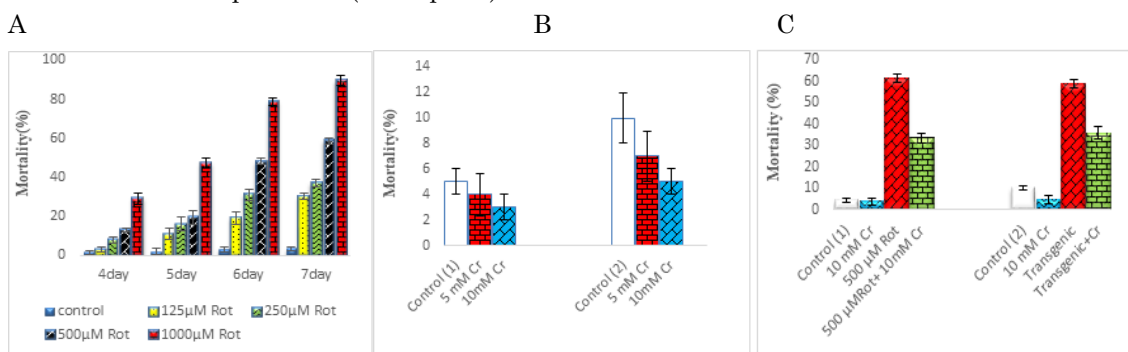


Fig. 2: Mortality of *D. melanogaster* flies. (A) Flies exposed to four different concentration of rotenone (125, 250, 500 and 1000 μ M) up to 7days. (B) Files exposed to different concentration creatine (5 mM and 10mM). (C) Flies co-exposed to rotenone + 10 mM creatine and transgenic PD flies exposed to 10 mM creatine; control (1) is wild type flies and control (2) is UAS-A30P flies (control for transgenic PD flies). (Values are mean \pm SE; three replicates, 50 flies per replicate).

Effect on Motion of Flies

Both rotenone treated flies (500 μ M) and transgenic PD flies exhibited significant decrease in climbing ability as compared to their

control. However flies co-exposure to creatine showed significant improved in locomotor ability (Fig. 3).

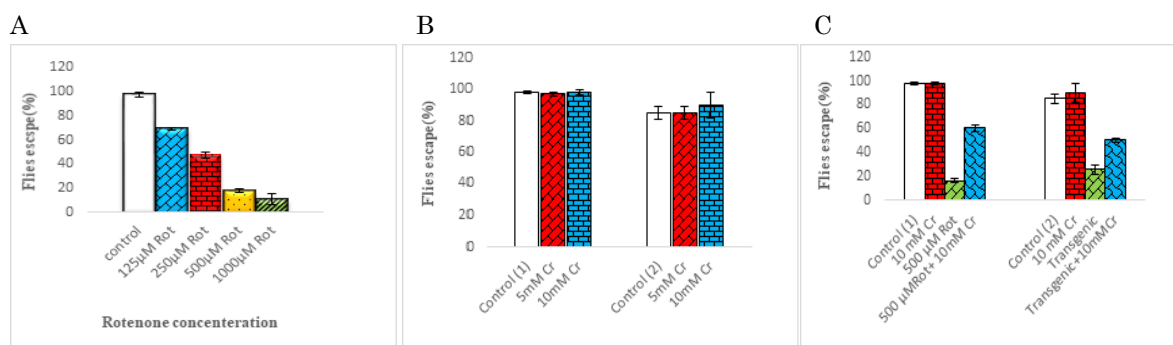


Fig. 3: Climbing assay of *D. melanogaster* flies. (A) Flies exposed to four different concentration of rotenone (125, 250, 500 and 1000 μM) up to 7 days. (B) Flies exposed to different concentration creatine (5 mM and 10 mM). (C) Flies co-exposed to rotenone + 10 mM creatine and transgenic PD flies exposed to 10 mM creatine; control (1) is wild type flies and control (2) is UAS-A30P flies (control for transgenic PD flies). (Values are mean \pm SE; three replicates, 50 flies per replicate).

Effect on Level of Dopamine

Rotenone treated (500 μM) and transgenic PD flies showed dopamine (DA) depletion. However in control (creatinine alone treated) did not

showed significant effect on dopamine level. Further it was noticed that co-exposure of rotenone and creatine supplement restored of dopamine in the head (Fig. 4).

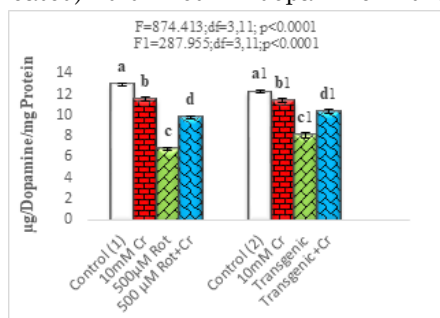


Fig. 4: Quantity of dopamine in Rotenone and transgenic PD flies in *D. melanogaster*. Values are mean \pm SE (three replicates, $n = 50$ flies per replicate). Different letter on the superscript of bar graph is superscript indicate significance at 0.05 level by Tukey's test post host test.

Effect of Creatine on Oxidative Stress and Antioxidant Markers Enzyme

Effect on Activity of CAT and SOD Enzymes

In rotenone treated (500 μM) and transgenic PD flies showed increased level of CAT and SOD activity as compared to their controls. Co-

exposure of rotenone treated flies (PD) and transgenic flies to creatine supplement had decreased the amount of enzyme CAT (29.68%) and SOD (40.84%). Thereby restoring its normal function (Fig. 5 A, B).

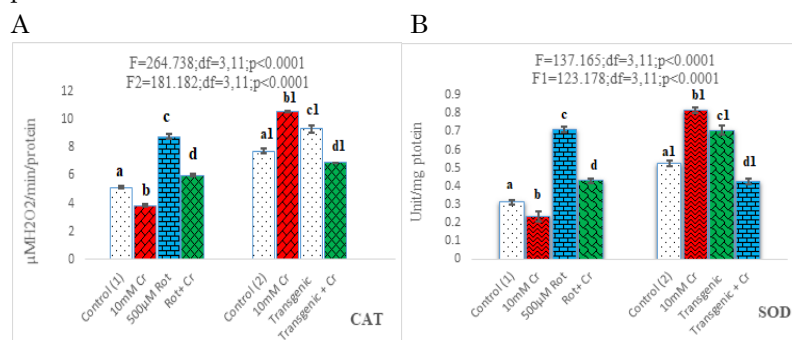


Fig. 5: Regulatory effect of creatine on CAT and SOD enzymes in rotenone induced and transgenic PD flies of *D. melanogaster*. Values are mean \pm SE (three replicates, $n = 50$ flies per replicate). Different letter on the superscript of bar graph indicate significance at 0.05 level by Tukey's post host test.

Effect on ROS Level

Figure reveals that rotenone treated (500 μ M) and transgenic PD flies showed significant

increase in level of ROS as compared to their controls. Co-exposure treatment of creatine supplement had restored the ROS level (Fig. 6).

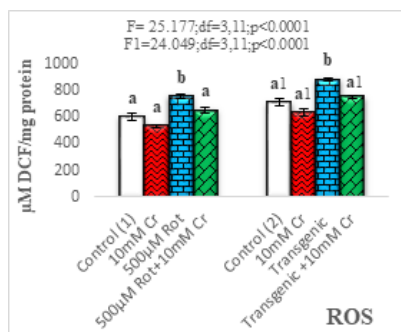


Fig. 6: Regulatory effect of creatine on ROS enzyme in rotenone induced and transgenic PD flies of *D. melanogaster*. Values are mean \pm SE (three replicates, n = 50 flies per replicate). Different letter on the superscript of bar graph indicate significance at 0.05 level by Tukey's post host test.

Effect on GSH Level

Even in GSH level rotenone treated (500 μ M) and transgenic PD flies showed decreased level as compared to their controls. When rotenone treated flies and transgenic PD flies exposed to

creatine supplement increased GSH level compared to normal. Thereby restoring its function in rotenone treated and transgenic PD flies in *D. melanogaster* (Fig. 7).

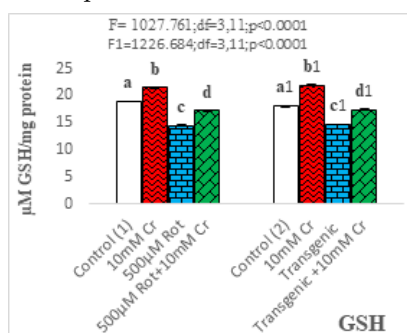


Fig. 7: Regulatory effect of creatine on GSH enzyme in rotenone induced and transgenic PD flies of *D. melanogaster*. Values are mean \pm SE (three replicates, n = 50 flies per replicate). Different letter on the superscript of bar graph indicate significance at 0.05 level by Tukey's post host test.

Effect on Lipid per oxidation Product (MDA)

Malondialdehyd is one of the products of lipid per oxidation. Rotenone treated and transgenic PD flies showed increased level of MDA as

compare to their respective control. Creatine supplement fed with treated rotenone and transgenic PD flies decreased the level of MDA to the normal (Fig. 8).

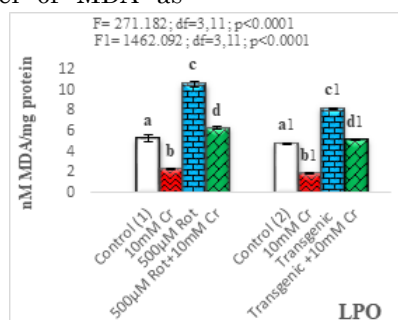


Fig. 8: Regulatory effect of creatine on LPO enzyme in rotenone induced and transgenic PD flies of *D. melanogaster*. Values are mean \pm SE (three replicates, n = 50 flies per replicate).

Different letter on the superscript of bar graph indicate significance at 0.05 level by Tukey's post host test.

Effect on Acetylcholinesterase (AChE)

The enzyme activity of Acetylcholinesterase in rotenone treated (500 μ M) and transgenic PD

flies increased as against control. But after co-exposed the flies with creatine, the activity of AChE was decreased (Fig. 9).

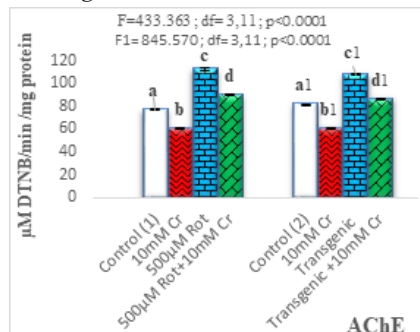


Fig. 9: Regulatory effect of creatine on AChE enzyme in rotenone induced and transgenic PD flies of *D. melanogaster*. Values are mean \pm SE (three replicates, n = 50 flies per replicate). Different letter on the superscript of bar graph indicate significance at 0.05 level by Tukey's post host test.

DISCUSSION

Most compelling studies on the pathophysiology of neurodegenerative mechanisms have shown that energy depletion, oxidative stress and mitochondrial dysfunctions are important factors associated with many of the neurodegenerative disorders [39-40]. Thus the treatment of such neurodegenerative disorders has been attempted with using anti-oxidant compounds on neuroprotective properties of neurodegenerative disorder. In recent years *Drosophila* used widely to understand mechanism involved in the pathophysiology in neurodegenerative disorders. Rotenone is a known respiratory inhibitor known to cause sporadic PD in humans [41]. Even in *Drosophila* chronic exposure to sub lethal doses of rotenone showed main symptom of PD i.e. selective loss of dopaminergic neuron including locomotor defects (F4) [42]. When such animals were subjected for oxidative stress analysis have also showed elevated level of MDA hydro peroxide levels and elevated activity of antioxidant enzymes namely CAT and SOD (F5, F6, F7, F8, F9). These studies suggests that rotenone known to induce PD in *Drosophila*. Our results also confirms studies Ravikumar [19] in *D. melanogaster*. They also found that rotenone treatment induce PD in flies. Many of the phytochemicals, has antioxidant properties i.e. Bacopa monnieri has been used to treat rotenone induced neurotoxicity [43]. Ravikumar [19] had used creatine supplement against rotenone

induced mitochondrial oxidative stress in *Drosophila*. However these studies did not studied creatine supplement on transgenic model of PD. Therefore present investigation has been undertaken to test the hypothesis that creatine supplement has similar effects on chemically induced (rotenone) and transgenic model of PD in *Drosophila*.

It is known that creatine is used as ergogenic aid to improve the performance in humans and as a treatment in the ameliorate oxidative stress mediated disorders. In the present study flies (transgenic model of PD and rotenone induced PD) provided with creatine supplement showed significant reduction in the endogenous levels of oxidative markers such as malondehyde and hydro peroxide suggesting that anti-oxidative property of creatine (F5, F6, F7, F8, F9). Ravikumar [19] have also noticed similar results with reference to creatine supplement against rotenone induced PD in *D. melanogaster*. It is clear from our study whether the PD is induced by chemical stress or transgenic model the pathophysiology involved in neurodegenerative mechanisms of PD is similar. Our results also confirms the previous report of fly model in understanding antioxidant property of creatine both in vitro and in vivo studies. Lawler [44] have also shown the creatine has the ability to scavenge ABTS⁺, superoxide anions and proxy nitrite radicals. Even in the studies of all model organism have also proved beyond doubt as anti-oxidant property of creatine [26, 45, 46].

Recently in rat Deminice [47] have shown that creatine supplementation had reduced endogenous lipid peroxidation biomarkers which suggests a protective role of creatine against oxidative damage. In the present study rotenone caused high mortality (50- 60 %) among flies during a 7 day exposure period and low incidence of mortality among creatine co-exposed flies suggests that presence of antioxidant property in creatine (F2). Further, it was also noticed that rotenone induced neurotoxicity could be evidenced in high rate of locomotor deficits as measured in the negative geotaxis assay. Further, it was also found that flies with locomotor deficits have tendency to stay at the bottom of glass column and do not appear to coordinate their legs in a normal fashion. On the other hand, flies co-expose to rotenone and creatine supplement had showed increased rate of locomotor activity (F3). Further in the present study we found significant correlation between locomotor dysfunction and dopamine deficiency (F4).

CONCLUSION

From all the biochemical parameter studied it was propose that dietary feeding of creatine supplement to *D. melanogaster* has the potentiality to reduce oxidative stress both in rotenone induced as well as transgenic PD flies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research article.

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