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Herbicide paraquat induces sex-specific variation of neuroinflammation and neurodegeneration in ***Drosophila melanogaster***

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There are several reports on herbicide paraquat (PQ)-induced Parkinsonian-like pathology in different animal models, including ***Drosophila melanogaster***. Also, the role of some inflammatory factors, such as nitric oxide is reported in PQ-induced neuroinflammation of ***Drosophila***. Although invertebrate model is valuable to study the conserved inflammatory pathway at the time of neurodegeneration, but neuroinflammation during PQ-mediated neurodegeneration has not been studied explicitly in ***Drosophila***. In this study, the inflammatory response was examined in ***Drosophila*** model during PQ-induced neurodegeneration. We found that after exposure to PQ, survivability and locomotion ability were affected in both sexes of ***Drosophila***. Behavioural symptoms indicated similar physiological features of Parkinson's disease (PD) in different animal models, as well as in humans. Our study revealed alteration in proinflamatory factor, TNF-α and ***Eiger*** (the ***Drosophila*** homologue in TNF superfamily) was changed in PQ-treated ***Drosophila*** both at protein and mRNA level during neurodegeneration. To ensure the occurrence of neurodegeneration, tyrosine hydroxylase (TH) positive neuronal cell loss was considered as a hallmark of PD in the fly brain. Thus, our result revealed the conserved inflammatory events in terms of expression of TNF-α and ***Eiger*** present during a sublethal dose of PQ-administered neurodegeneration in male and female ***Drosophila*** with significant variation in proinflamatory factor level among both the sexes.

Keywords: Paraquat, ***Drosophila***, Neurodegeneration, Pro-inflammatory factors.

Animal models are invaluable tools for studying the pathogenesis and therapeutic intrusion strategies of human disease, including Parkinson's disease (PD)[1,2]. Many neurotoxins like paraquat (PQ), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP), rotenone etc. have been used to develop Parkinsonism in various animal models[3]. For last few decades, PQ, a non-selective and non-systemic cationic bipyridylium herbicide has been widely used in the agricultural sector throughout the world. Considering the viability of experimental models, in most of the cases doses selected for the oral route are acute and in the higher range compared to the original field exposure and at these concentrations lead to mortality through neurodegeneration.

Among the animal models, ***Drosophila melanogaster*** is one of the most suitable organisms to evaluate PD and other neurological dysfunctions due to similarities between the cellular and molecular mechanisms of neurodegeneration in ***D. melanogaster*** and those of vertebrates[4,5] and similarities in motor behaviour between the Parkinsonism induced in flies and PD in humans. Also, PQ exposure in rodent animals results in loss of dopaminergic (DA) neurons in ***Substantia Nigra pars compacta*** (SNpc) and reduces the animals' general motor activity[6,7].

Flies with PQ-induced Parkinsonism can be compared with other animals, for example, a prominent symptom like bradykinesia, also present in flies can be estimated using simple locomotor assays. Several lines of evidences suggest a significant role of neuroinflammation in PD and loss of DA neurons caused by pro-inflammatory factors. Tumour necrosis factor-α (TNF-α) is a protein that has key role in inflammation and cognitive function in the brain. Inflammation mainly caused by microglial activation in the central nervous system (CNS) is also responsible for the PD pathogenesis[8,9]. The increased titer of a pro-inflammatory cytokine, such as TNF-α is proposed to be toxic to DA neurons in mammal[7]. Also, recently it is reported that excessive influx of nitric oxide levels as inflammatory response in the central complex region of the brain accelerates the DA neurodegeneration during PD in ***Drosophila***

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Abbreviations: DA; dopaminergic; DAPI, diamidino-2-phenylindole; MFI, mean fluorescence intensity; PD, Parkinson's disease; PQ, paraquat; TH, tyrosine hydroxylase; TNF-α, tumour necrosis factor-α.

model. It is also reported that ***Eiger***, a first invertebrate TNF superfamily ligand induces cell death in ***Drosophila***[10]. However, the gender-specific changes of that proinflamatory factor TNF-α in the brain of an invertebrate model like ***Drosophila*** are not clear**.**

In this study, we have examined the role of TNF-α mediated neuroinflammation during tyrosine hydroxylase (TH) specific neuronal loss in both sexes of PQ-administered ***Drosophila*** and sex-specific changes due to PQ exposure.

Materials and methods

Reagents

Paraquat (PQ) was purchased from Sigma-Aldrich (St Louis, MO, USA). Selected antibodies were used to detect various protein expression levels, included anti-TNF-α from (Cell Signaling, Danvers, MA, USA), α-tubulin and tyrosine hydroxylase (TH) (Developmental Studies Hybridoma Bank, University of Iowa), alkaline Phosphate (AP) conjugated secondary antibody (Abcam, UK) and fluorochrome-tagged secondary antibody (Life technologies), and DAPI (Vector Laboratories). All analytical grade chemicals were used for this study.

Fly stocks

Wild-type Oregon-R strain of ***Drosophila melanogaster*** was maintained on standard corn meal agar food medium. All life cycle stages were reared at 22 ± 1ºC and 80% relative humidity and 12 h light: 12 h dark cycle[11]. All experiments were performed with the same strain.

Experimental design

Fifty male and female flies, ten days (d)-old were placed into bottles containing corn meal agar food medium and with a selected concentration of PQ. Before treatment, flies were starved for 4 h in separate vials. To determine the lethal dose$_{50}$ (LD_{50}), male and female flies were transferred separately into bottles containing 5, 10, 15, 20, 25 and 30 mM of PQ with food medium for 48 h[12]. Depending on the LD_{50} value, we selected a sublethal dose for the maximum toxic effects on minimum mortality and the next parts of experiment were performed with selected dose. All the experiments were performed in triplicate.

Locomotion assay

The climbing assay of fly was used to quantify mobility as described previously[13]. After PQ exposure, treated and control flies (male and female) were placed in a vertical plastic column (length 25 cm; diameter 1.5 cm). After 1 min, flies that reached the top of the column and that remained at the bottom were counted separately. Three trials (n = 50) were performed at 1 min intervals in each experiment.

Whole mount immunostaining

Whole mounts immunostaining was performed in 12-15 finely dissected brains, according to described protocol[14]. Brains were dissected in cold phosphate buffered saline (PBS, 1x). For fixation, tissue was incubated in fresh 4% paraformaldehyde and then transferred into tubes containing PBT [PBS with Triton X (v/v)]. Brains were incubated for 2 h in PBXDG buffer (PBT, 5% normal goat serum, 1% BSA, deoxycholate and Triton X-100) and immunostained with primary antibody at 4ºC overnight. Then the samples were washed with PBXDG buffer under agitation at room temperature, than Alexa Fluor® 660-labelled secondary antibody (1:500) was used[15].

Confocal microscope (Olympus FV1200) was used to collect images with Z-stack section using laser line 405 for DAPI and 635 for Alexa Fluor® 660-conjugated antibody and the magnification was 20× as indicated. Mean fluorescence intensity of DA neurons was quantified in terms of TH-positive cells from the total area of the brain using ImageJ software.

Protein extraction and Western blotting

For Western blotting, protein was extracted from head of male and female flies (n = 40). The fly heads were then homogenized in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktails (Thermo Scientific, USA) and 1 mM phenylmethanesulfonylfluoride (PMSF) and kept for 45 min on ice. Then the homogenate was centrifuged (eppendorf Centrifuge 5810 R) at 14,000 rpm for 15 min at 4ºC. The concentration of proteins was estimated using Bradford reagent. The membranes (Merck Millipore, India) were incubated in primary antibody (anti-TNF-α, 1:1000) for overnight at 4ºC and then anti-rabbit AP-conjugated secondary antibodies (1:1500) were used and antibody complexes were visualized by calorimetric detection with NBT/ BCIP (Himedia, India).

RNA isolation and gene expression by reverse transcription-PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's

recommended protocol. Gene-specific primer sequences were designed with Integrated DNA Technology (IDT) and custom-made by Excelris Genomics (Ahmedabad, India). In semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis, cDNA was subjected to PCR amplification with a primer set of target genes (Table 1) and PCR premix Taq V2.0 (Excelris Genomics, Ahmedabad, India). All target genes expression was calculated in relation with loading control ***Actin***. All RT-PCR quantification experiments were performed in triplicate.

Table 1—Primer sequences of targeted genes

Drosophila gene	Product size	Primer sequences
Actin5C	108	Forward: CGAAGAAGTTGCTGCTCTGGTTGT
		Reverse: GGACGTCCCACAATCGATGGGAAG
Eiger	81	Forward: GCTGTCTGTGAGGTTGTTT
		Reverse: CGAGCTTTGTCGCACTTTATATG

Statistical analysis

Results were representative of three independent experiments performed in triplicate for each group (n = 50), male and female, respectively and represented as mean ± SD. Data were analysed and the significance of the differences between the mean values of control and treated flies. A one-way analysis of variance (ANOVA, Bonferroni test, Fisher test, Tukey test) was performed, where significant level stands for *p<0.05, ** p<0.005*** p<0.0005.

Results

Determination of LD_{50}

Figure 1A shows the survival rates of male and female flies as a function of PQ doses. PQ decreased the survivability of ***Drosophila*** in a dose-dependent manner. At 5 mM dose, survival of male and female flies was 94% (± 4.3) and 97% (± 2.5), at 10 mM 92% (± 6.1) and 95% (± 3.5) and at 15 mM dose 88% (± 5.5) and 90% (± 5.4), respectively. But, the survival rate decreased to 49.3% (± 4.1) and 51.7% (± 4.6) at 20 mM, 32% (± 2.9) and 37% (± 2.5) at 25 mM for male and female flies, respectively.

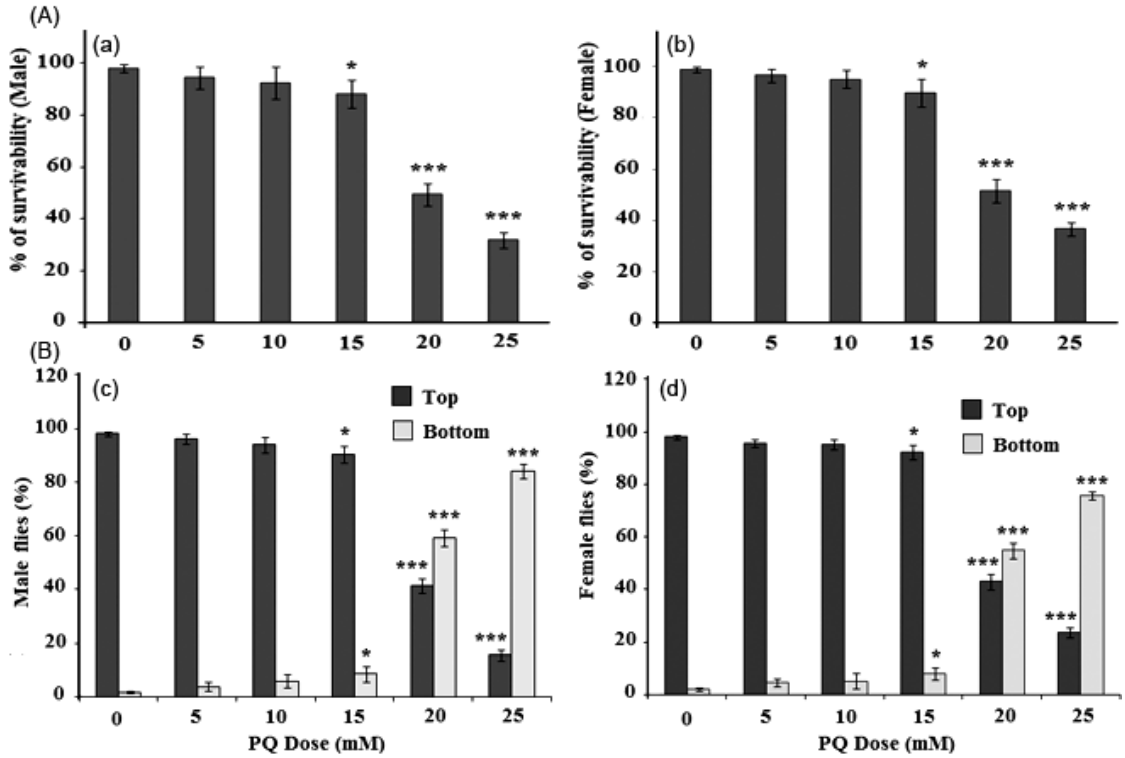

Fig. 1—Effect of different concentrations of paraquat (PQ) exposure on survivability (A) and locomotor activity (B) of both sexes of ***D. melanogaster*** after 48 h [Survivability of male (a) and female (b) flies, locomotor activity of male (c) and locomotor activity of female (d) flies. Results are representative of three independent experiments performed in triplicate for each group (n = 50), male and female, respectively and represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni test) was performed, where significant level stands for *p<0.05, ** p<0.005*** p<0.0005]

PQ exposure causes locomotor deficits

In mammalian studies, PQ exposure is found to induce DA neurodegeneration, although the neuronal loss is not sufficient enough to emulate PD movement characteristics[2,16-18]. Therefore, we tried to observe the effects of PQ on the mobility of adult flies. Locomotion activity was gradually decreased with increasing concentrations of PQ after 48 h of treatment. At the time of climbing on the vial wall, sometimes flies were frozen and often fell down in the bottom of the vial. Male flies exhibited symptoms earlier than the females and both were robustly affected at 20 mM PQ concentration and so on.

To determine the change in locomotion activity, we placed flies in contact with PQ-supplemented feeding medium having different doses of PQ (i.e. 5, 10, 15, 20, 25 and 30 mM) (Fig. 1B). At 5 mM PQ dose, 96% (± 1.5) of both males and females were found on top of the bottle, at 10 mM, 94% (± 2.8) of male and 95% (± 1.9) of female flies and at 15 mM dose, 91% (± 3.1) of males and 92% (± 2.7) of females were found at the top with uninterrupted locomotion. However, at 20 mM PQ, locomotion activity of the male and female flies was found to be sporadic, with only 41% (± 2.7) of males and 43% (± 2.9) of females were able to reach the top of the bottle, while at 25 mM, 16% (± 2.1) of male and 23% (± 1.9) of female flies were able to climb to the top of the bottle. Finally, at 30 mM dose no fly was left for examination.

Thus, considering the survivability and locomotion activity, we selected 15 mM PQ as sublethal dose, which was insufficient to cause maximum death, but seemed to have profound effects on neural study. Therefore, we continued our further experiments with 15 mM dose of PQ.

Increased level of TNF-α in PQ-treated *D. melanogaster*

Immunoblotting was performed to evaluate the expression of proinflamatory factor TNF-α (Fig. 2). The expression of TNF-α was significantly increased after PQ exposure in both sexes. Interestingly, the expression was higher in treated male and female, when compared with their respective controls. Among the controls, the female flies expressed higher TNF-α than males. The increase level of TNF-α expression in treated flies than their respective gender-wise control was found to be significantly higher in males than in females.

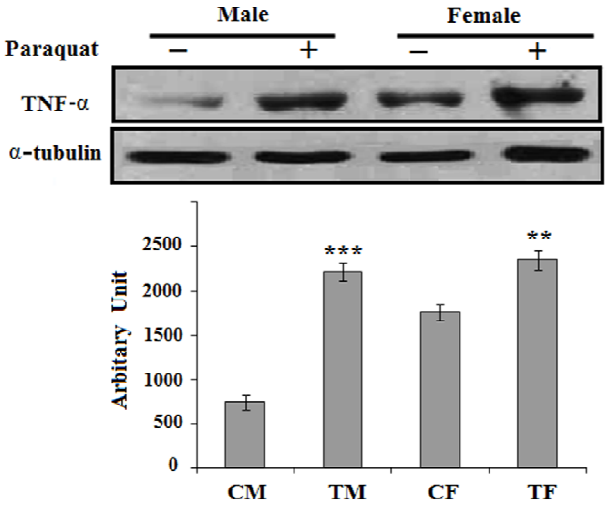

Fig. 2—Paraquat (PQ) induces expression of pro-inflammatory factor TNF-α in both sexes of ***D. melanogaster*** [Flies were treated with PQ for 48 h to each group. Protein was isolated from PQ-treated and untreated fly heads. Results are representative of three independent experiments performed in triplicate for each group (n = 100), male and female, respectively and represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni test) was performed, where significant level stands for *p<0.05, ** p<0.005*** p<0.0005. α-Tubulin was used as loading control. CM signifies control male, TM PQ treated male and CF control female and TF treated female.

Elevated expression of *Eiger* in PQ-treated *D. melanogaster*

We also investigated whether sublethal dose of PQ concentration affected the mRNA expression of ***Eiger*** (TNF-α homologue in ***Drosophila***). RT-PCR analysis revealed that the expression of ***Eiger*** gene was amplified in PQ-administered male and female ***Drosophila*** (Fig. 3). Interestingly, the expression was found to be significantly higher in control females than respective control males. In addition, it was observed that elevated level of ***Eiger*** expression was higher in males than in females, than their respective controls.

PQ induces dopaminergic neurodegeneration

To assess inflammation-mediated DA neurodegeneration in brain of both sexes of ***Drosophila***, we used a marker TH, a rate-limiting enzyme involved in DA production[1]. Immunofluorescence analysis of the brain of both male and female showed that those with two days of exposure of PQ had decreased immunoreactivity of TH expression (Fig. 4a and b). Interestingly, after PQ treatment, the reduction of mean fluorescence intensity was relatively less in female flies compared to male.

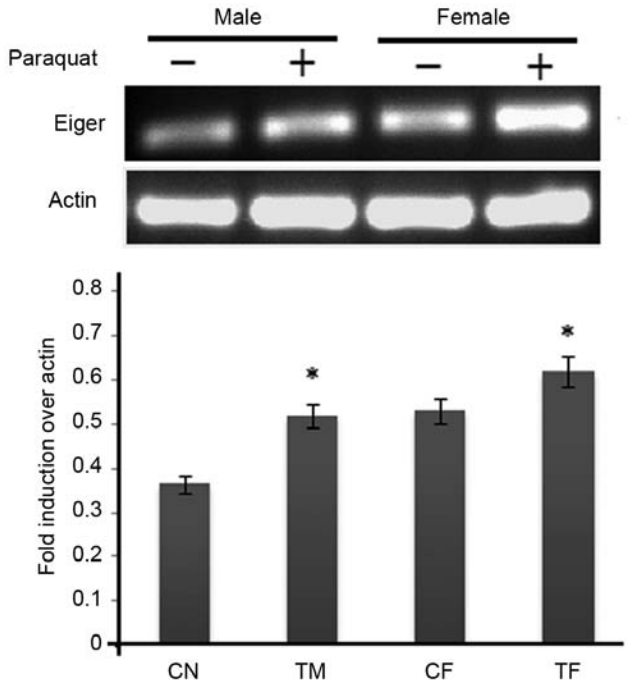

Fig. 3—PQ exposure (48 h) upregulated the expression of ***Eiger*** (TNF-α homologue in ***Drosophila***) in both sexes of ***D. melanogaster*** [RNA was isolated from treated and untreated fly heads. ***Actin*** was used as the loading control. CM signifies control male, TM PQ treated male and CF control female and TF treated female. Results are representative of three independent experiments performed in triplicate for each group (n = 100), male and female, respectively and represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni test) was performed, where significant level stands for *p<0.05, ** p<0.005, *** p<0.0005]

Discussion

PQ exposure in human may occur either via contamination through food indirectly or during cultivation directly. Even at low concentration while consuming processed foods, PQ causes developmental and neurological disorder in human. To elucidate the basic molecular events in the neuroinflammation during PQ-induced Parkinsonism, we selected relevant ***in vivo Drosophila*** model, as this system has been widely used in the area of neurobiology due to similarities in terms of human disease gene, protein sequences and behavioural features of PD, including resting tremors, bradykinesia and postural instability when Parkinsonism is induced in flies[2,19].

In this study, we found that PQ also caused the motor disorder, similar to that of PD; the survival rate was more detrimental with increasing PQ concentrations and different expressions of neuro-inflammatory factor in sex-specific response against PQ-administered ***D. melanogaster***. We observed that at 5 and 10 mM PQ, both male and female flies were hyperactive, but interestingly, at 15 mM, males were hypoactive, whereas females were still hyperactive. The 50% lethality observed against both male and female flies at 20 mM concentration of PQ and was also similar to other studies[14,20].

Although insect models, such as ***Drosophila*** is very much useful to study of evolutionary interlinking pathways, still the conserved inflammatory pathway are not well-defined in flies

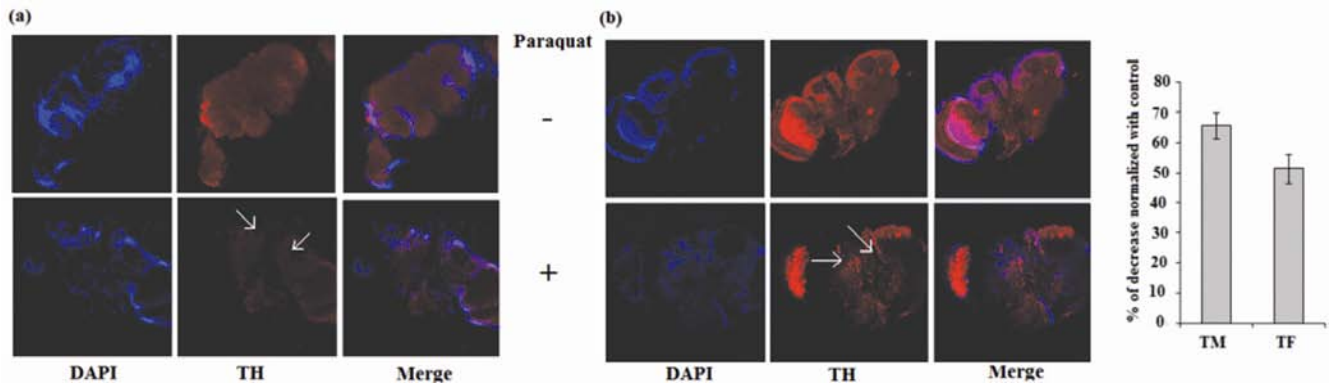

Fig. 4—PQ exposure (48 h) exposure reduced the expression of tyrosine hydroxylase (TH) [Immunofluorescence study of treated and untreated fly brain for both sexes of ***D. melanogaster*** was done using anti-TH primary antibody and Alexa Fluor® 660-labelled secondary antibody (1:500) was used[15]. DAPI was used for nuclear staining. Confocal microscope was used to capture images with Z-stack section using laser line 405 for DAPI and 635 for Alexa Fluor® 660- conjugated antibody and the magnification was 20× as indicated. Mean fluorescence intensity (MFI) of dopaminergic neurons was quantified in terms of TH-positive cells from the total area of the brain using ImageJ software. (a) TH expression in male brain, and (b) in female brain. The MFI of TH expression is represented by bar graph. Arrow heads indicate neurons altered by exposure to PQ]

and this event has already been studied in mammals[21]. Continuous exposure to toxic agents intensifies chronic inflammation, followed by neurodegeneration. The degenerative process, in turn, further promotes inflammation as well as more degeneration in the feedback loop. In neurodegenerative diseases, inflammation may be triggered by the accumulation of abnormal changes in protein conformation and/or signals coming forth from injured neuron[8]. ***Drosophila*** could also be used to study the direct effects of cytokines on neurons and glial cells[22,23]. In contrast, the neurotoxin-mediated inflammatory response in ***Drosophila*** has been less studied.

In this study, we observed for the first time the gender-specific variations in expression level of neuroinflammation during DA neurodegeneration. For this purpose, a pro-inflammatory cytokine TNF-α was chosen, since it is involved in the response to neural lesion in mammals[7,24]. So, we investigated the correlation between neuroinflammation and PQ-exposed Parkinsonism in ***Drosophila***. The PQ promoted the upregulation of TNF-α more in males rather than in females. To confirm whether the TNF-α is an invertebrate TNF family ligand ***Eiger***, we examined the expression of ***Eiger*** at mRNA level[10]. RT-PCR data revealed the elevated expression of the ***Eiger*** in PQ-treated ***Drosophila*** of both sexes than their respective control. The results also indicated that ***Eiger*** expression was higher in treated males, as compared to the treated females.

Earlier, it is shown that expression of TNF-α and TNFR1 is upregulated due to the presence of estradiol in anterior pituitary cells[26]. In our study, we also found that the basal level expression of TNF-α was higher in the untreated female flies than the untreated males, although the neurotoxic effect of PQ promoted the upregulation of TNF-α more in males rather than in females. Thus, the data from mRNA and protein level suggested that PQ exposure might induce neuroinflammation in ***Drosophila***. Earlier report has shown that the significant exposure of neurotoxin in rodent aggravates chronic inflammation, followed by neurodegeneration which can be determined by the expression of TH, a potent marker of DA neuron[7]. During immunofluorescence assay, we observed a significant reduction in the post-treatment fluorescence intensity of TH in both sexes of ***Drosophila***, particularly in males.

In conclusion, the exposure of sublethal doses of PQ in both sexes of ***D. melanogaster*** caused low survival rate, locomotor impairment and DA neurodegeration. The PQ-induced neurodegeneration was initiated with the pro-inflammatory factor, such as TNF-α. Thus, our study confirmed that during neurodegeneration, a sublethal exposure of PQ induced neuroinflammation in both male and female ***Drosophila*** with significant variation in the level of pro-inflammatory cytokine TNF-α.

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