



# Paraquat exposure induces behavioral deficits in larval zebrafish during the window of dopamine neurogenesis



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## ARTICLE INFO

### Article history:

Received 6 April 2015

Received in revised form 29 May 2015

Accepted 11 June 2015

Available online 16 June 2015

### Keywords:

Cholinergic system

Motor defects

Paraquat

Early life

## ABSTRACT

Exposure to environmental risk factors such as herbicides in early life has been proposed to play important roles in the development of neurodegenerative disorders in adult life. To test this hypothesis, we used a zebrafish model to link the herbicide paraquat (PQ) to disease etiology. Strikingly, treatment of 18 hpf embryonic zebrafish with low-dose PQ treatment (0.04 ppm, lower than the accepted human daily exposure) resulted in 50% display of neurodegenerative phenotypes and motor deficits at various developmental stages (segmentation to larval stage). Wide arrays of biomarkers have been employed to delineate the toxic responses which include lipid peroxidation, glutathione (GSH) and apoptosis studies. A decrease in the GSH levels, increase in lipid peroxidation and apoptosis, respectively, were observed at various developmental stages. Unexpectedly, we show that the exposure to paraquat during the window of dopamine neurogenesis causes Parkinsonian like motor defects in later life by perturbing cholinergic system due to oxidative stress.

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## 1. Introduction

Neurotransmitter systems work in concert in the vertebrate nervous system to regulate signal transduction pathways that govern processes like behavior [1]. Dysfunction in the dopaminergic nervous system can lead to conditions that feature motor abnormalities. Parkinson's disease (PD) is a common neurodegenerative disease characterized by the loss of dopamine neurons in the substantia nigra and patients present with extensive motor abnormalities [2]. Neurodevelopmental motor disorders can also result from dopaminergic dysfunction, the most well documented of these being attention deficient hyperactivity disorder (ADHD) [3]. In addition to altered dopaminergic signaling, effects on serotonergic pathways have been identified in PD and ADHD patients [4,5]. The etiology of sporadic dopamine related movement disorders is largely unknown. Epidemiological studies, however suggest environmental factors may contribute. One such risk factor is exposure to chemicals like pesticides. Paraquat has been linked to selec-

tive dopaminergic degeneration, which has been shown to produce parkinsonian syndrome, and thus, are also used in animal models of PD [6]. Interestingly, evidence has shown that neurodegenerative diseases may result from insults during development [7]. More studies need to be conducted in order to evaluate environmental exposures as a causal factor in the onset of movement disorders. Also, it is not clear whether developmental insults on the dopaminergic nervous system are the underlying cause for disorders that occur later in life.

Zebrafish may be a useful model in addressing these qualms. In zebrafish, no dopaminergic cells lie in the midbrain; therefore, the nigrostriatal pathway, which extends from the substantia nigra (SN) to the striatum (Str) in mammals, is homologous to the dopaminergic ascending pathway in zebrafish, extending from cells in the posterior tuberculum (PT) to the subpallium (Vd), both of which are present in the forebrain [8]. As determined by tyrosine hydroxylase and dopamine transporter staining, dopamine-containing neurons can first be detected in the posterior tuberculum in the midbrain as early as 18 hpf [9]. Other components of the dopaminergic nervous system such as the spinal cord and ventral diencephalon contain functioning neurons by 24 hpf. Also at 48 hpf, dopaminergic neurons are present in the olfactory bulb, preoptic region, telencephalon, paraventricular organ, and hypothalamus. By 72 hpf, dopaminergic neurons are present in the retina, pretectum, and ventral thalamus, with the tectum gaining

**Abbreviations:** PQ, paraquat; hpf, hours post fertilization; LPO, lipid peroxidation; GSH, glutathione; ADHD, attention deficient hyperactivity disorder; PD, Parkinson's disease; DTNB, 5 5'-dithiobis-(2- nitrobenzoic acid); EM, embryo medium; ppm, parts per million.

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<http://dx.doi.org/10.1016/j.toxrep.2015.06.007>

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dopaminergic neuronal activity by 96 hpf [10]. By the end of 4 days (96 hpf), the full complement of dopaminergic neuronal groups is present in the zebrafish [11].

Relatively few studies have assessed paraquat induced effects on locomotor activity during zebra fish development in small testing environment (e.g., a multi-well microtiter plate) that would be required for large-scale chemical screening in toxicology. Hence, we began investigating the locomotion of zebrafish during development, as well as the factors that affect this activity following paraquat exposure during the window of dopamine neurogenesis i.e., 18 hpf.

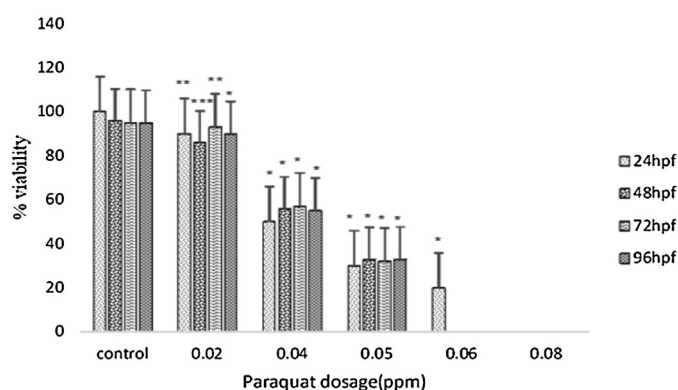
## 2. Materials and methods

### 2.1. Chemicals

DTNB (5,5'- dithiobis-(2-nitrobenzoic acid)), 2 -thiobarbituric acid, methanol, chloroform were purchased from Southern Scientific Corporation, Chennai. Other chemicals that were used were of analytical grade. Paraquat was obtained from Microbial Technology Laboratory, Sathyabama University, Chennai.

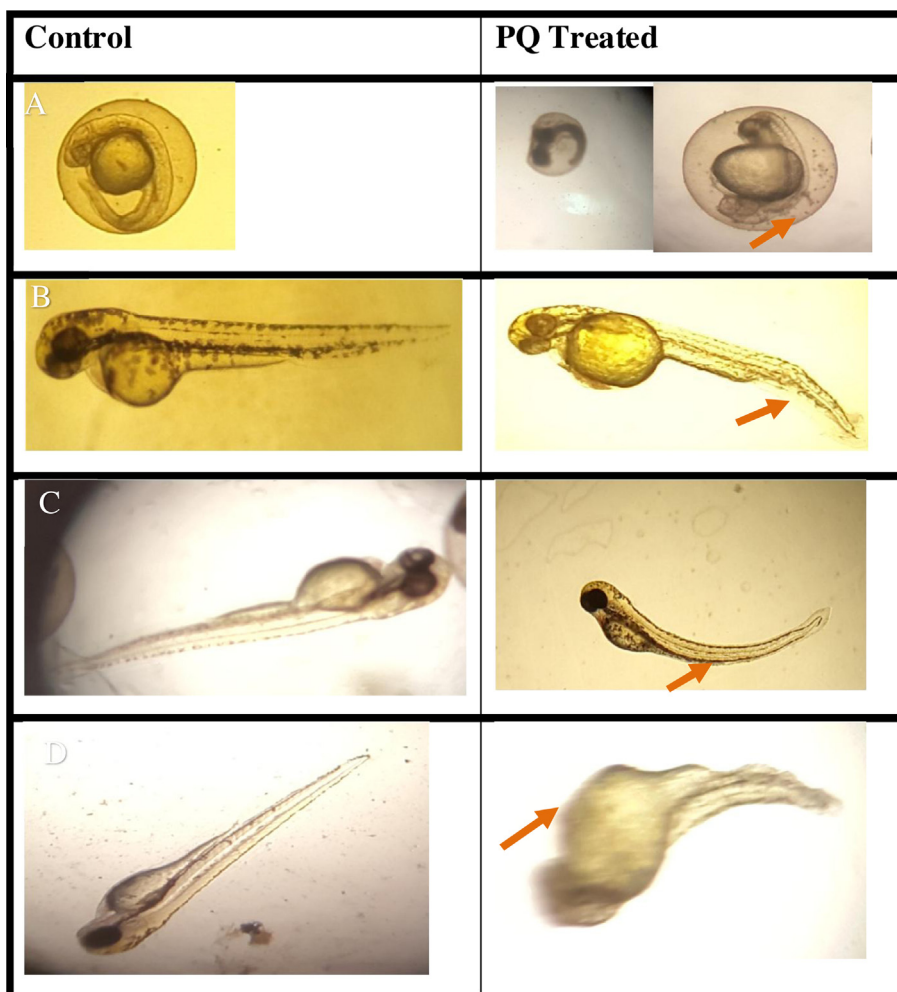
### 2.2. Zebrafish

Wild-type adult (<8 months old) zebrafish were reared and maintained at the aquatic research laboratory at Sathyabama Uni-

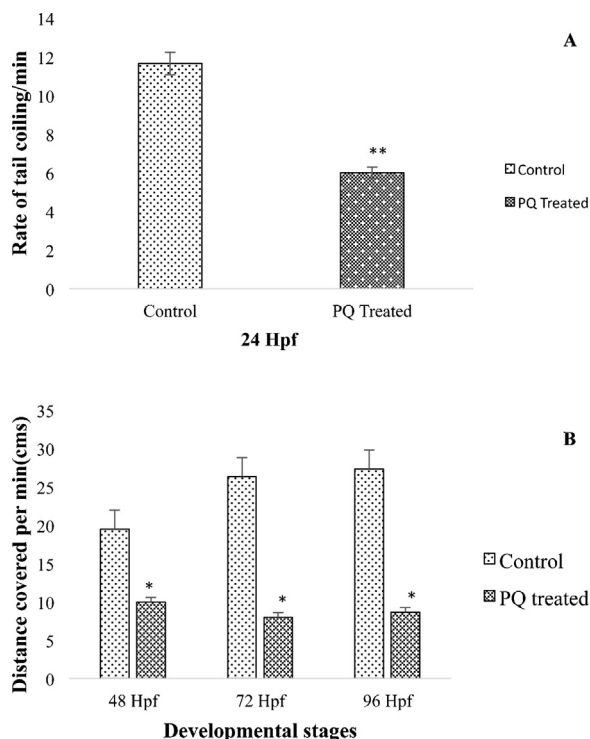


**Fig. 1.** Effect of various concentrations of paraquat on the viability of embryos at various developmental stages following exposure during the window of dopamine neurogenesis. Statistical significance was determined by multivariate analysis for each developmental stage (\* $p < 0.0001$ , \*\* $p < 0.02$  and \*\*\* $p < 0.03$ ). Error bars depict SEM.

versity, Chennai. The temperature was maintained at 28 °C and the lighting was maintained in a cycle of 14 h of light and 10 h of dark for the fish to breed. The next day morning, the embryos were collected and staged according to the method of [12] and used for analysis. The embryos were maintained in embryo medium (EM)



**Fig. 2.** Display of morphological alterations including a flat head, small eyes, pinched midbrain–hindbrain boundary, thin yolk extension, and curved-up body in zebrafish embryos exposed to 0.04 ppm of paraquat when photographed in lateral orientation through a stereomicroscope at 24 hpf (A) 48 hpf (B) 72 hpf (C) and 96 hpf (D).



**Fig. 3.** (A) Effect of paraquat on spontaneous tail coiling at 24 hpf (B) Effect of paraquat on the distance covered by the larvae at 48, 72 and 96 hpf following exposure at 18 hpf. Statistical significance determined by Student's *t*-test for each developmental stage (\* $p < 0.0001$ ). Error bars depict SEM.

at a temperature of 28 °C in an incubator till 96 hpf. The medium was replaced with fresh medium on a daily basis.

### 2.3. Experimental groups

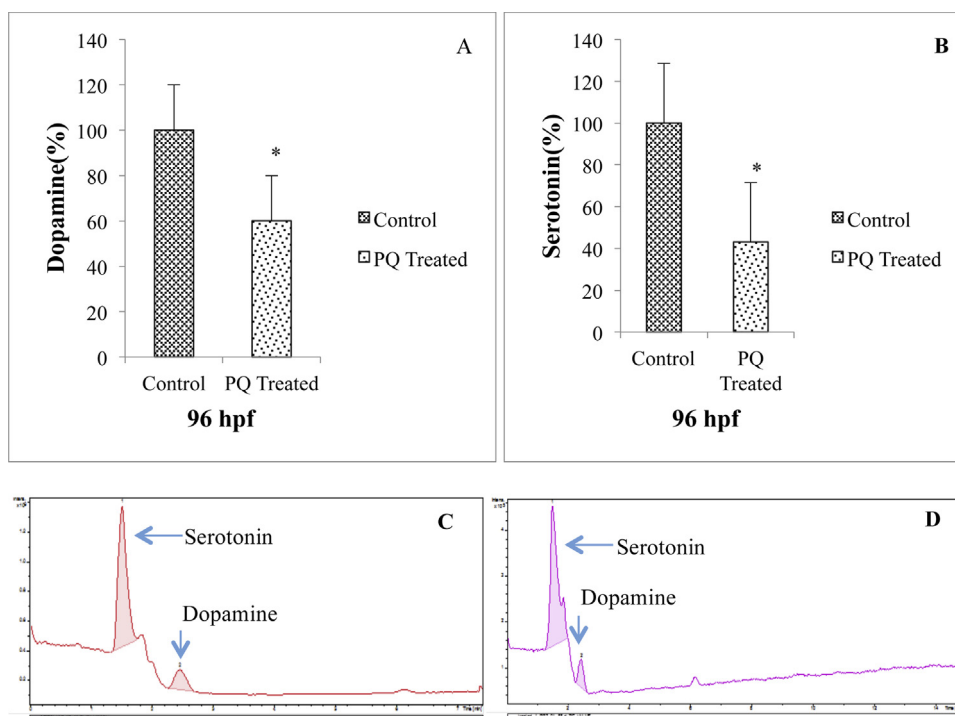
The experimental groups used for the tests comprise the following (i) control group (untreated zebrafish embryos) (ii) zebrafish embryos treated with paraquat. The embryos were incubated with various concentrations of paraquat ranging from 0.02 ppm to 0.08 ppm. The 50% lethal concentration (LC<sub>50</sub>) of paraquat was determined based on the concentration-% survival and concentration-% effect neurodegenerative phenotypes reported at all the end points.

### 2.4. Preparation of embryo homogenates

Embryos were collected at different developmental stages viz., 24, 48, 72 and 96 hpf, respectively, for analysis. The whole embryo homogenates were prepared in 0.1 M phosphate buffer and centrifuged at 3000 × *g* for 30 min. The supernatants were used for further analysis.

### 2.5. Analysis of lipid peroxidation (MDA) and glutathione (GSH) levels

The embryo supernatants were used for the measurement of MDA and GSH levels. GSH was measured according to the method of [13], which is based on the reaction with 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) to give a yellow colored compound that absorbs at 412 nm. The Thiobarbituric acid reactive substances was measured to analyze the amount of malondialdehyde (MDA) produced by measuring the optical density of the supernatant at 532 nm [14].



**Fig. 4.** Quantification of dopamine and serotonin in zebrafish embryos at 96 hpf following exposure to 0.04 ppm paraquat at 18 hpf. Post paraquat administration during dopamine neurogenesis (A) 60% reduction in the levels of dopamine and (B) 43% reduction in the levels of serotonin was observed. Each point is an average of fifteen embryos. Calculations of the concentrations of each developmental stage were determined by HPLC. (C) Typical HPLC chromatogram of control zebrafish at 96 hpf. (D) Typical HPLC chromatogram of 0.04 ppm paraquat exposed zebrafish at 96 hpf. Statistical significance was determined by Student's *t*-test for each neurotransmitter (\* $p < 0.0001$ ). Error bars depict SEM.

## 2.6. Apoptosis assay

In zebrafish, live embryos can be used to detect and quantify cell death in the whole animal with the utilization of acridine orange. The control and PQ exposed embryos at various developmental stages were manually dechorionated and rinsed twice with EM and incubated with 5 µg/ml acridine orange dissolved in EM for 45 min at room temperature in the dark [15]. The embryos were then washed with EM three times for 5 min each. All embryos were examined with a fluorescent microscope (Nikon, Japan) and representative pictures were shown.

## 2.7. Locomotor analysis

Locomotor activity was monitored at every stage of embryogenesis of control and PQ treated zebrafish embryos. At 24 h, the tail movement of the embryos was observed under a stereozoom microscope. At 48–96 h, the locomotor activity of the embryos was studied in a petriplate marked with 1 cm grid lines, containing embryo medium. The movement of the embryos across the grid lines was observed for a period of 1 min, from which the distance covered by the embryo was calculated.

## 2.8. Neurochemical measurements

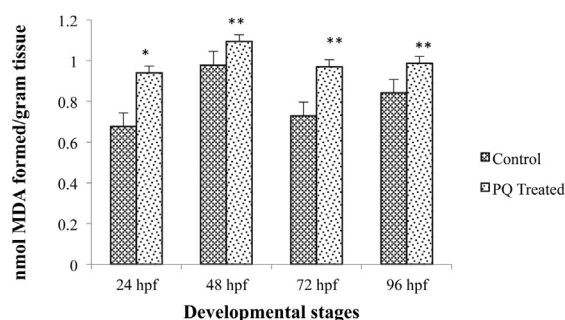
Neurochemical measurements were determined according to the method of [16]. Briefly, 15 larvae were homogenised in 0.5 mL of cold perchloric acid (0.4 M). Subsequently, the sample was centrifuged at 20,000 × g for 10 min at 4°C, and the supernatant was transferred to a clean tube and measured for volume. One-half volume of a solution containing 0.02 M potassium citrate, 0.3 M potassium dihydrogen phosphate, and 0.002 M disodium ethylenediaminetetraacetate (Na<sub>2</sub>EDTA) was added and mixed thoroughly to deposit perchloric acid. After incubation in an ice bath for 60 min, the mix was centrifuged at 15,000 × g, for 20 min at 4°C. Supernatants were analyzed for neurotransmitters by HPLC (125 mm × 3 mm I.D. column, packed with nucleosil 100 C 18; 3 µm particle size) and electrochemical detection (INTRO, ANTEC Leyden, The Netherlands; cell potential = 800 mV). The mobile phase consisted of 5% acetonitrile, 10 g/L citric acid, 4 g/L Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), 0.1 g/L ethylenediaminetetraacetic acid (EDTA), and 0.175 g/L octanesulfonic acid; pH 3.0. Neurotransmitters were quantified as using a standard curve generated from injection of high purity standards.

## 2.9. Statistical analysis

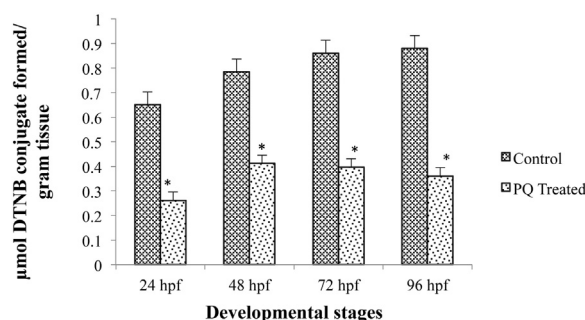
Quantitative data was expressed as mean ± SEM, depending on the distribution of the data. The Student's *t*-test was used to test for differences between the paraquat exposed and the control zebrafish embryos for locomotor activity, dopamine and serotonin levels at various developmental stages. A multivariate analysis (the developmental stage and treatment as covariates; the lipid peroxidation and GSH levels as dependent variable, respectively) was performed using the SPSS software version 16.0 to evaluate the differences in the mean value of viability, GSH level and Lipid peroxidation at various developmental stages in paraquat exposed and control. (\* = *p* < 0.0001, \*\* = *p* < 0.002).

## 3. Results and discussion

In the survival rate test, during the window of dopamine neurogenesis i.e., at 18 hours post fertilization (hpf) zebrafish embryos were treated with embryo medium alone for control and paraquat within a range of concentrations 0.02–0.08 ppm for up to various developmental stages of zebrafish viz., 24 hpf, 48 hpf, 72 hpf and



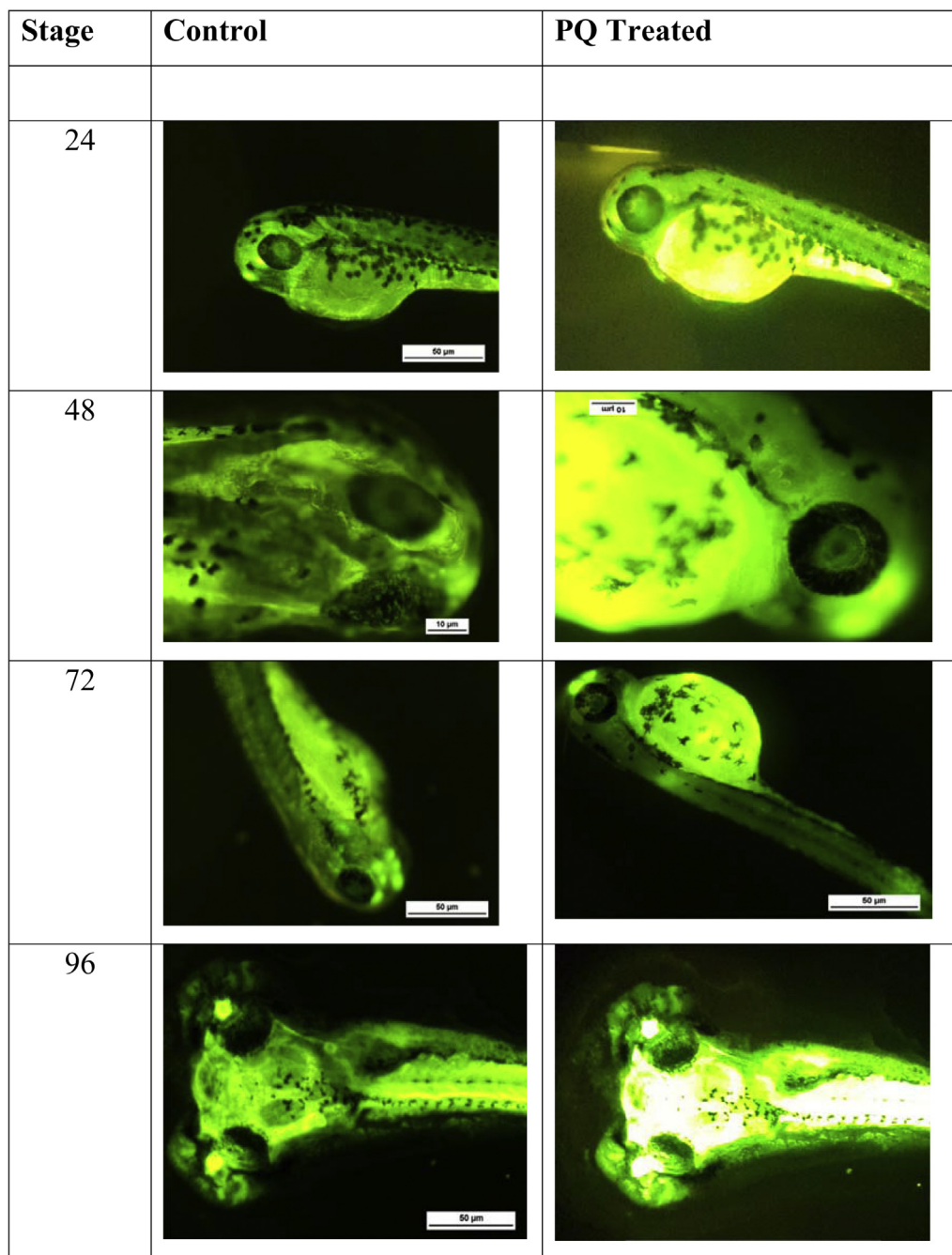
**Fig. 5.** Effect of 0.04 ppm paraquat on lipid peroxidation at various developmental stages of zebrafish following exposure during the window of dopamine neurogenesis i.e., at 18 hpf. Statistical significance determined by a Multivariate analysis (the developmental stage and treatment as covariates; the MDA levels as dependent variable, respectively) was performed using the SPSS software version 16.0 to evaluate the differences in the mean value of the MDA levels at various developmental stages in paraquat exposed and control. (\* = *p* < 0.0001, \*\* = *p* < 0.002). Error bars depict SEM.



**Fig. 6.** Effect of 0.04 ppm paraquat on GSH levels at various developmental stages of zebrafish following exposure during the window of dopamine neurogenesis i.e., at 18 hpf. Statistical significance was determined by Multivariate analysis (the developmental stage and treatment as covariates; the GSH levels as dependent variable, respectively) which was performed using the SPSS software version 16.0 to evaluate the differences in the mean value of the GSH levels at various developmental stages in paraquat exposed and control embryos. (\* = *p* < 0.0001). Error bars depict SEM.

96 hpf, respectively. At 0.02 ppm, no significant effect was recorded on the hatching rate and mortality at various developmental stages. Subsequent experiments showed that 0.04 ppm was required to cause 50% mortality and the remaining surviving embryos displayed significant neurodegenerative phenotypes like bent tail structure, curved spine structure and distorted yolk sac respectively at 24 hpf, 48 hpf, 72 hpf and 96 hpf respectively as shown in Fig. 1. Focusing on the 0.05, 0.06 and 0.08 ppm treated groups showed reduced survival are unhatched with necrosis, reduced heart beads and edemas were detected at 24 hpf, 48 hpf, 72 hpf and 96 hpf, respectively following exposure at 18 hpf as shown in Fig. 2. Based on the concentration-% survival and concentration-% effect neurodegenerative phenotypes reported at all the end points, 0.04 ppm was determined as the LC<sub>50</sub> value.

The most noticeable consistency was a distinct curved body axis most apparent in our study in embryos exposed to 0.04 ppm. Previous studies have demonstrated that this curved tail down phenotype has been associated with central nervous system (CNS) development, supporting the idea that CNS is the target organ system underlying behavioral abnormalities [17]. To directly correlate the paraquat exposure during the window of dopamine neurogenesis to locomotor activity, 0.04 ppm exposed embryos at 24 hpf, 48 hpf, 72 hpf and 96 hpf, respectively, were selected for testing locomotor activity. 48.27% reduction in spontaneous tail coiling was recorded at 24 hpf as shown in Fig. 3(A). At 48 hpf, 72 hpf and 96 hpf the locomotor activity was significantly reduced by 48.66%,



**Fig. 7.** Treatment with Paraquat during dopamine neurogenesis induced apoptosis throughout the central nervous system (CNS) and development of neurodegenerative phenotypes. Image shows cell death in CNS by acridine orange staining at 24 hpf, 48 hpf, 72 hpf and 96 hpf, respectively, following exposure to 0.04 ppm paraquat during the window of dopamine neurogenesis i.e., 18 hpf.

69.61% and 68.53% respectively in response to 0.04 ppm paraquat as shown in Fig. 3(B).

A second key component in our study was to elucidate the effect of paraquat on locomotor activity through measuring the levels of modulatory neurotransmitters dopamine and serotonin. As shown in Fig. 4, a significant reduction in dopamine and serotonin levels was reported in larval zebrafish which could be a manifestation of reduced locomotor activity following paraquat exposure during dopaminergic neuron development. A recent study emphasized that dopaminergic dysfunction mediates the behavioral effect observed in zebrafish following deltamethrin exposure during development. It is nevertheless intriguing that paraquat disturbs the dopamine homeostasis through increased generation of reac-

tive oxygen species and reduced locomotor activity in rodent models [18].

Of the many biological targets of oxidative stress, lipids are the most involved class of biomolecules. Lipid oxidation gives rise to a number of secondary products. Malondialdehyde (MDA) is the principal and most studied product of polyunsaturated fatty acid peroxidation. This aldehyde is a highly toxic molecule and should be considered as more than just a marker of lipid peroxidation. There is overwhelming evidence that PQ toxicity is caused by oxidative stress, a process that plays an important role in dopaminergic neuronal degeneration [19].

Oxidative stress damages key cellular pathogenetic proteins and disrupts lipid membranes that in turn cause more ROS production.

This observation emphasized the need to monitor the levels of MDA at various developmental stages of zebrafish following paraquat exposure at 18 hpf. As shown in Fig. 5, a 38% increase in MDA levels were demonstrated at 24 hpf compared to control. While so, 11%, 33% and 17.47% increase in MDA levels were reported at 48 hpf, 72 hpf and 96 hpf, respectively.

Several studies suggest that the reduced levels of endogenous antioxidant molecules such as glutathione (GSH), may lead more vulnerable to oxidative stress leading to selective degeneration of dopaminergic neurons [20]. It was also reported that the neurotoxic mechanism of paraquat specifically involve enhancement of the oxidative pathway of dopamine metabolism through coupling with the antioxidant GSH system of the substantia nigra [21]. In concurrence to this, our results also demonstrated a decrease in GSH levels by 59.2% at 24 hpf, 47.5% at 48 hpf, 53.89% at 72 hpf and 59.02% at 96 hpf following exposure to paraquat during development as shown in Fig. 6. It is shown that a decrease in GSH triggers the activation of neuronal 12-lipoxygenase (12-LOX), which leads to the production of peroxides, the influx of  $Ca^{2+}$ , and ultimately to apoptosis [22].

In the same way, paraquat was associated with increased risk of dopaminergic neuron degeneration, by stimulating glutamate efflux from neurons and thereby increasing calcium influx ultimately leading to apoptosis [23]. The spatial and temporal patterns of apoptosis in vertebrate embryonic development are tightly regulated events [24]. Disruption of the regulated occurrence of apoptosis as a result of genetic mutations or exposure to toxicants leads to developmental abnormalities. In this study, our first focus was to obtain a quantifiable measurement of the occurrence of apoptosis at the brain and spinal region following exposure to paraquat during development. Following acridine orange staining the apoptotic cells significantly increased in the brain region with increase in duration of exposure to paraquat as shown in Fig. 7.

In our studies, we observed that, early assaults can lead to a broad range of lifelong problems in both physical and mental health that can be devastating. During early development, the brain is highly sensitive to many chemicals. When certain substances reach dangerous levels at particularly sensitive points in time, they can disrupt the developmental process through toxic effects on the general health of brain cells and their capacity to perform specialized functions. These toxic functions can weaken the foundation structure of the brain and result in permanent impairment, thereby leading to a wide range of lifelong adverse impacts on learning, behavior and health.

There is considerable evidence that paraquat may cause the onset, or accelerate the development, of Parkinson's disease; that the longer the exposure the greater the risk; that there may be a lag time between exposure and development of symptoms; and that early exposures are the most deleterious. The unborn fetus and children are most at risk. Pregnant women and children should not be exposed to this chemical as it can cross the placenta and cause acute poisoning including death of the fetus or chronic effects that can persist for the lifetime.

In light of our findings and additional data from literature we emphasize that the biochemical investigations in our research project, will be a supportive marker for prognosis and diagnosis of PD. This could help physicians and neurologists to find out risk of initiation and progression of PD. It also might allow them to find treatments that will halt the disease process in the early stages.

#### 4. Conclusion

In conclusion, by analyzing the lethal and sub lethal categorical endpoints, the zebrafish embryos were found to be more sensitive to even low concentration of paraquat (0.04 ppm) and the toxic

effect of paraquat in zebrafish embryo increased with increase in concentration and exposure time. In this study, the toxicity of paraquat was investigated by analyzing the behavioral functions during dopamine neurogenesis. We showed that continuous exposure to paraquat at every developmental stage caused accumulative damage to dopaminergic neurons in zebrafish. The levels of modulatory neurotransmitters namely dopamine and serotonin were reduced significantly post paraquat exposure, thus hampering the locomotor activity.

Immature brain is vulnerable to neurotoxins, and exposure to environmental toxicants early in the life may play an important role in PD pathogenesis. Although the mechanisms involved in the pathogenesis and progression of PD are not fully understood, there is overwhelming evidence that oxidative stress and neuronal apoptosis play an important role in dopaminergic neuronal degeneration, in coherence with which, our results demonstrated a drop in the levels of the glutathione and marked increase in the malondialdehyde, protein and apoptosis after PQ exposure.

#### Conflict of interest

We declare that we have no conflict of interest.

#### Acknowledgement

Gratefully thanks to the management of Sathyabama University for providing facilities to the author.

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