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Paraquat affects mitochondrial bioenergetics, dopamine system expression, and locomotor activity in zebrafish (*Danio rerio*)

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HIGHLIGHTS

- Paraquat is an aquatic contaminant with multiple modes of action.
- Exposure to 1, 10, 100 μM paraquat induced premature hatching in zebrafish.
- Paraquat at 100 μM increased activity of zebrafish larvae.
- Maximal respiration of 24 hpf larvae was affected by 100 μM paraquat.
- Genes related to oxidative stress and dopamine signaling were altered by paraquat.

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ABSTRACT

The dipyridyl herbicide paraquat induces oxidative stress in cells and is implicated in adult neurodegenerative diseases. However, less is known about paraquat toxicity in early stages of vertebrate development. To address this gap, zebrafish (*Danio rerio*) embryos were exposed to 1, 10 and 100 μM paraquat for 96 h. Paraquat did not induce significant mortality nor deformity in embryos and larvae, but it did accelerate time to hatch. To evaluate whether mitochondrial respiration was related to earlier hatch times, oxygen consumption rate was measured in whole embryos. Maximal respiration of embryos exposed to 100 μM paraquat for 24 h was reduced by more than 70%, suggesting that paraquat negatively impacts mitochondrial bioenergetics in early development. Based upon this evidence for mitochondrial dysfunction, transcriptional responses of oxidative stress- and apoptosis-related genes were measured. Fish exposed to 1 μM paraquat showed higher expression levels of *superoxide dismutase 2, heat shock protein 70, Bcl-2-associated X protein*, and *B-cell CLL/lymphoma 2a* compared to control fish. No differences among groups were detected in larvae exposed to 10 and 100 μM paraquat, suggesting a non-monotonic response. We also measured endpoints related to larval behavior and dopaminergic signaling as paraquat is associated with degeneration of dopamine neurons. Locomotor activity was stimulated with 100 μM paraquat and *dopamine transporter* and *dopamine receptor 3* mRNA levels were increased in larvae exposed to 1 μM paraquat, interpreted to be a compensatory response at lower concentrations. This study improves mechanistic understanding into the toxic actions of paraquat on early developmental stages.

1. Introduction

Paraquat is a widely used dipyridyl herbicide that induces oxidative stress in cells. The high propensity of this herbicide to propagate reactive oxygen species (ROS) is a predominant mechanism underlying paraquat-induced neurotoxicity in different

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animal models (Czerniczyniec et al., 2011; Hosamani and Muralidhara, 2013). Paraquat is proposed to impair mitochondrial complex I, leading to an aberrant electron transport chain system and the overproduction of ROS (Castello et al., 2007; Cochemé and Murphy, 2008; Jackson-Lewis et al., 2012; Nunes et al., 2017). Hence, the resulting mitochondrial dysfunction caused by paraquat is a central component of its toxicity. Oxidative stress arising from the redox cycling of paraquat has been documented in multiple animal models, such as rodents, fish and *Drosophila* (Czerniczyniec et al., 2011; Jahromi et al., 2015; Wang et al., 2016). Thus, this herbicide appears to act through a conserved mechanism of mitochondrial dysfunction, which is independent of the animal model used. Several studies demonstrate that the oxidative stress caused by paraquat can lead to an increased antioxidant response, interpreted to be a compensatory response to the increase in ROS (Cochemé and Murphy, 2008; Wang et al., 2016; Nunes et al., 2017). Therefore, paraquat-induced neurotoxicity likely arises from an imbalance in the redox state of cells.

Paraquat is associated with increased risk for neurodegenerative diseases including Parkinson's disease (PD) (Bortolotto et al., 2014; Nellore and Nandita, 2015; Nunes et al., 2017) and has been labelled an environmental neurotoxin. Neurodegenerative diseases are typically associated with oxidative stress and mitochondrial dysfunction (Reddy et al., 2012; Schmitt et al., 2012; Grimm et al., 2014; Nunes et al., 2017). There is also growing evidence that paraquat disrupts dopaminergic signaling through increased ROS production (Zhou et al., 2011; Hosamani and Muralidhara, 2013), thus leading to Parkinsonian-like syndromes and behavioral impairments. Altered dopaminergic signaling and locomotor deficits caused by paraquat exposure have also been reported in rodents, fish, *Drosophila* and *Caenorhabditis elegans* (Litteljohn et al., 2009; Bortolotto et al., 2014; Lima et al., 2014; Jahromi et al., 2015). Using zebrafish as a model, Nellore and Nandita (2015) observed significant reductions in dopamine levels and locomotion in larval zebrafish following paraquat exposure. Conversely, in adult zebrafish, studies reported increased dopamine levels and decreased locomotion with long-term exposure to paraquat using an injection route of exposure (Bortolotto et al., 2014). These recent studies support the hypothesis that paraquat disrupts dopaminergic signaling in zebrafish, which may lead to impaired locomotion and behavior.

Dopamine (DA) is a catecholamine and a major neurotransmitter in the vertebrate central nervous system (CNS). DA mediates several important brain functions including motor movement, cognition, emotion, reward, feeding behavior and social behavior (Davie, 2008; Schultz, 2010). Characterization of the effects of paraquat exposure on dopaminergic signaling during development requires the analysis of genes involving DA synthesis, transport and signal transduction. Paraquat exposure generally diminishes DA level with a concomitant reduction of TH level in rodents (Reeves et al., 2003; Somayajulu-Niţu et al., 2009), but this may not involve concomitant alteration of *th* expression in adult zebrafish (Bortolotto et al., 2014). It has also been reported that a significant decrease in *dat* expression can occur due to paraquat exposure both in rodents and adult zebrafish (Ossowska et al., 2005; Ren et al., 2009; Bortolotto et al., 2014). Moreover, DA receptors have been shown to be associated with paraquat-induced neurotoxicity in *Drosophila* (Cassar et al., 2015). Taken together, data show that the dopamine system is a significant target for paraquat neurotoxicity in a variety of animal models.

Zebrafish are an excellent model for neurotoxicity and neurobehavioral studies, due to its well-characterized neurotransmitter systems and repertoire of well described behavioral endpoints (Panula et al., 2010; Flinn et al., 2008; Tierney, 2011). During early development, zebrafish are highly sensitive to many environmental pollutants, and toxicology screens using this species can generate insight into the wide range of adverse impacts on physical health and swimming behavior. Dopaminergic signaling and behavior, as well as mitochondrial bioenergetics, serve as important endpoints to better understand molecular mechanisms underlying paraquat-induced toxicity. Additionally, there is evidence that exposure to paraquat early in development affects organisms, resulting in behavioral deficits in zebrafish and loss of dopamine neurons in mice (Nellore and Nandita, 2015; Zhou et al., 2011). Therefore, the aims of this study were to (1) determine how paraquat effects early development, (2) test the hypothesis that paraquat induced mitochondrial dysfunction in fish embryos (3) and to determine if any impairments to mitochondrial bioenergetics and dopamine signal transduction were associated with higher level endpoints at a later time, such as swimming activity.

2. Materials and methods

2.1. Fish husbandry

Adult wildtype zebrafish (*Danio rerio*, AB strain) were obtained from the Animal Care Services at the University of Florida and maintained at controlled temperature ~28 ± 1 °C with a light/dark cycle of 14/10 h according to the method of Westerfield (2000). Embryos were collected and staged using the criteria of Kimmel et al. (1995). Embryo rearing medium (ERM) was prepared by adding 8 g NaCl, 0.4 g KCl, 0.035 g Na_2HPO_4, 0.6 g KH_2PO_4, 0.14 g $CaCl_2$, 0.12 g $MgSO_4$, 0.35 g $NaHCO_3$ into 1 L double distilled water (ddH_2O) with a pH of 7.2. The experimental protocols were approved by the University of Florida Animal Care Committee and were carried out at the Center for Environmental and Human Toxicology at the University of Florida.

2.2. Animal exposures

Paraquat dichloride ($C_{12}H_{14}Cl_2N_2$, purity ≥ 95%, CAS Number: 1910-42-5, Sigma, USA) stock solution (100 mM) was prepared in distilled water. The exposure solutions were prepared by diluting the stock solution into ERM to achieve final nominal concentrations of 1, 10 and 100 μM paraquat. Concentrations of paraquat within this range (10–200 μM) have been used in previous studies to assess oxidative stress responses and DNA damage in zebrafish embryos (Imamura et al., 2011; Ling et al., 2017) and we decided to bracket this range in the current study as 200 μM paraquat increased the oxidative stress response in zebrafish embryos (<24 h) by more than 6-fold (Ling et al., 2017). Control embryos were treated with ERM alone. Toxicity of paraquat was first assessed in embryos over the 4 days. Ninety-six (96) embryos at 6 h post fertilized (hpf) (around the shield stage) were transferred to each well of a 96-well plate (n = 24 embryos/treatment). The exposure was initiated at 6 hpf because at this stage, non-fertilized and dead eggs are readily discernable and are removed from the experiment prior to the initiation of treatments. The embryos are also less susceptible at this stage to mechanical damage from any manipulations into treatments. The 96-well plate was pre-coated with ddH_2O or paraquat at the desired concertation 24 h prior to exposure. Development, survival rate and hatch time of embryos were assessed by EVOS FL Auto Imaging System (Life Technologies) to obtain bright field images every hour over a 96 h exposure. Zebrafish that were assessed for mitochondrial respiration (24 h exposure) and gene expression analysis (96 h exposure) were incubated in glass beaker replicates with 10 replicates for control and 8 replicates for each treatment. Zebrafish were also exposed in two 96-well plates separately for 5 and 7 days post-fertilization