Rotenone Neurotoxicity Causes Dopamine Neuron Loss in Zebrafish

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ABSTRACT

Objectives: We sought to determine whether rotenone, a commonly used pesticide, exhibits neurotoxicity in zebrafish by causing dopamine neuron loss through rotenone-induced oxidative damage. **Methods**: We exposed transgenic zebrafish embryos expressing green fluorescent protein under the control of the *cis*-regulatory elements of *dopamine transporter* (*dat*) to rotenone to determine the neurotoxic effects of rotenone on dopamine neuron abundance and pattern distribution, as well as the presence of apoptotic markers. The oxidative stress potential of rotenone on embryos was assessed using a live MitoSOX Red assay, and behavioural testing on adult zebrafish was assessed using video recordings of midline crossing events. **Results**: Zebrafish embryos treated with rotenone displayed a 50% reduction in dopamine neurons in the ventral diencephalon when exposed to 30µM rotenone (n=6, $p<0.001$), and rotenone-exposed zebrafish raised to adulthood demonstrate an anxiety-like behaviour (n=5, $p<0.01$). Furthermore embryos exposed to rotenone also demonstrated a logarithmic increase in markers of oxidative damage (n=3, $p<0.001$) and apoptotic activity in their diencephalic neurons. **Conclusions**: These results show that rotenone can induce dopamine neuron loss in zebrafish, providing a useful model for studying the environmental causes of Parkinson's disease.

RÉSUMÉ

Objectif: Nous cherchons à déterminer si la roténone, un élément commun dans les pesticides, démontre de la neurotoxicité dans les poissons-zèbres en causant une perte de dopamine dans leurs neurones à travers le dommage oxydatif induit par la roténone. **Méthode:** Nous avons exposé des embryons de poissons-zèbres transgéniques qui expriment la protéine fluorescente verte sous le contrôle d'éléments *cis*-régulateurs des *transporteurs sélectifs de dopamine* (*dat*) à la roténone pour déterminer les effets neurotoxiques de ce dernier sur les niveaux dopaminergiques dans leurs neurones. De plus, nous avons évalué la présence de marqueurs apoptotiques. Le stress oxydatif potentiel de la roténone sur les embryons a été analysé par le « live MitoSOX Red assay » et les tests comportementaux sur les poissons-zèbres adultes furent analysés en utilisant des enregistrements vidéo. **Résultats:** Les embryons de poissons-zèbres qui ont été traités avec la roténone ont démontré une réduction de dopamine de 50% dans les neurones localisés dans le diencéphale ventral, quand exposés à 30µM de roténone (n=6, $p<0.001$). Ils ont également illustré une augmentation logarithmique dans les marqueurs de dommage oxydatif (n=3, $p<0.001$) et une activité apoptotique dans les neurones du diencéphale. Les poissons-zèbres exposés à de la roténone qui ont atteint l'âge adulte ont démontré des comportements d'anxiété (n=5, $p<0.01$). **Conclusion:** Les résultats démontrent que la roténone peut induire une perte dopaminergique dans les neurones des poissons-zèbres. Ces résultats s'avèrent utiles pour étudier davantage les causes environnementales reliées à la maladie de Parkinson.

INTRODUCTION

Parkinson's disease is a neurodegenerative disease that presents as a chronic progressive movement disorder. In the majority of cases, Parkinson's disease (PD) is idiopathic, and more than 85% of patients have no known genetic association or other primary causes [1]. Parkinson's disease is now the second most common neurodegenerative disorder after Alzheimer's disease [2] and the incidence of idiopathic Parkinson's disease greatly increases with age. It is estimated that the disease affects over 1% of the population above the age of 65 [3]. This disease is caused by the progressive death of dopamine neurons in the substantia nigra, a deep midbrain region of the brain responsible for the modulation of motor signals via the basal ganglia. While many neurotoxic chemicals such as paraquat and oxidopamine (6-OHDA) [4] are suspected to contribute to the development of Parkinson's disease, very few have shown to cause dopamine neuron degeneration in live animal models. Longitudinal studies of exposure to chronic pesticide-use have been associated with the development of Parkinson's disease later in life [5], and further investigation of this phenomenon predicates itself on the development of a biologically representative animal model of PD.

In recent years, the zebrafish has become a prominent live animal model in many fields of medical and neurological research [6]. With a short generation time, high fecundity and transparent tis-

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sue during embryonic development, zebrafish present a unique and novel platform for studying PD. Developing zebrafish larvae can be exposed to environmental neurotoxins simply by administering the neurotoxin to the fish water [7, 8]. Furthermore, the neurologic effects of these neurotoxins are readily apparent on the developing zebrafish, leading to behavioral abnormalities in the larvae and a series of morphological changes in the larval brain. Lastly, there are a number of genetic models of neurodegeneration in zebrafish due to the apparent ease with which we can genetically manipulate the well-annotated zebrafish genome [9]. The catecholaminergic system in zebrafish is very similar to that of other vertebrates, containing regions within the brain corresponding to homologous structures in more complex vertebrates. The posterior tuberculum neurons of the ventral diencephalon send ascending neural projections to the ventral telencephalon and appear functionally equivalent to the meso-striatal and meso-limbic systems in mammals [10]. Serotonergic neural clusters, such as the caudal hypothalamic neural cluster of the embryonic zebrafish, exhibit a strong susceptibility to the toxic effects of certain catecholaminergic neurotoxins (oxidopamine and MPTP). This leads to a decrease in serotonin within the fish brain, consistent with the supposed cause of behavioural and psychiatric deficits observed in patients with Parkinson's disease [10, 11]. Olfactory loss is very strongly linked to the development of PD and its rapid progression [12, 13], and changes within the olfactory bulb in zebrafish can be easily visualized.

Rotenone is one of the pesticides that have been associated with the development of PD [5], and displays a strong cytotoxic oxidative effect through mitochondrial complex I inhibition [14]. The only vertebrate model of rotenone-induced PD was until recently limited to the Lewis rat [15] with milder phenotypes seen in mouse models [16, 17, 18]. Although small scale neurotoxin screens (including rotenone) using behavioural analysis have been performed on zebrafish to identify potential PD-inducing agents, MPTP was the only toxin to yield conclusive results [19]. Here we show that rotenone affects the dopaminergic system of the zebrafish through a dose-dependent ablation of dopaminergic neurons in the diencephalon by oxidative stress–induced apoptotic activity.

METHODS

FISH CARE AND HUSBANDRY

Zebrafish embryos were obtained by natural spawning of adults maintained on a 14-hour light/10-hour dark cycle and fed a diet of fish pellets and *Artemia*. After cleaning and sorting, embryos were raised at 28.5°C from birth to 15 days post-fertilization (dpf) in embryo media (13mM NaCl, 0.5mM KCl, 0.02mM Na_2HPO_4, 0.04mM KH_2PO_4, 1.3mM $CaCl_2$, 1.0mM $MgSO_4$, and 4.2mM $NaHCO_3$). To prevent formation of pigmentation, phenylthiourea (Sigma-Aldrich) was added to the embryo media at 24 hours post-fertilization (hpf) to a final concentration of 0.2mM. A transgenic zebrafish line, Tg(*dat:EGFP*), in which the green fluorescent protein (GFP) is expressed under the control of *cis*-regulatory elements of the *dopamine transporter* (*dat*) gene, was used in this experiment [20]. The University of Ottawa Animal Care Committee approved all animal care procedures, and all animals used were governed by protocols in strict accordance with the recommendations of the Canadian Council for Animal Care.

ROTENONE PREPARATION AND ADMINISTRATION

Rotenone powder (Sigma-Aldrich) was freshly dissolved in DMSO (Fisher Scientific) at a concentration of 1mM, then serially diluted up to 1,000,000-fold in double distilled water to half-log working concentrations between 1nM and 1000nM of rotenone. Rotenone solutions older than 4 h were never used. Embryonic zebrafish were collected and raised in 6-well, 12-well or 24-well plates (Corning) with no more than 20 embryos per well. Embryos were exposed to a single dose of rotenone at 24 hpf without water changes until 7 dpf to ensure that embryos were exposed to the maximum bioavailable dose of rotenone. Controls were embryonic zebrafish exposed to a solution of 0.1% DMSO in embryo media, corresponding to the DMSO concentration used in the maximum concentration of rotenone exposure. A petri dish containing a bed of 2% agarose gel was prepared with 1 mm-deep troughs cut into the agarose using a standard glass microscopy slide. Embryos were then anesthetized with a 0.168 mg/ml solution of MS-222 (Sigma-Aldrich), gently placed into a trough in the agarose bed in a dorsal-ventral orientation, then imaged on a Leica MZ6 epifluorescence stereomicroscope to examine for changes in green fluorescence patterning in a single-blind fashion. Images were then assembled into panels using Adobe Photoshop CS5. Rotenone from a different source (Fisher Scientific) was also assessed, and demonstrated reproducible changes in neuronal patterning (data not shown). After exposure, all rotenone-containing solutions were bleached and subsequently disposed as hazardous wastes.

IMMUNOHISTOCHEMISTRY ON ZEBRAFISH EMBRYO CRYOSECTIONS

Embryos were fixed in 4% paraformaldehyde dissolved in phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, PBS) overnight at 4°C, rinsed thrice in PBS and equilibrated in a 30% sucrose/PBS solution overnight at 4°C. Embryos were then flash frozen in Tissue-Tek ® O.C.T™ media (VWR Canada) and sectioned with a Leica CM 1850 cryostat (Leica Microsystems, Weltzar, Germany) at a thickness of 14µm on to Superfrost glass slides and stored at -20°C until use. After a short rehydration period for 10 minutes in PBS containing 0.1% Tween 20 (PBT), sections were blocked in 10% goat serum in PBT

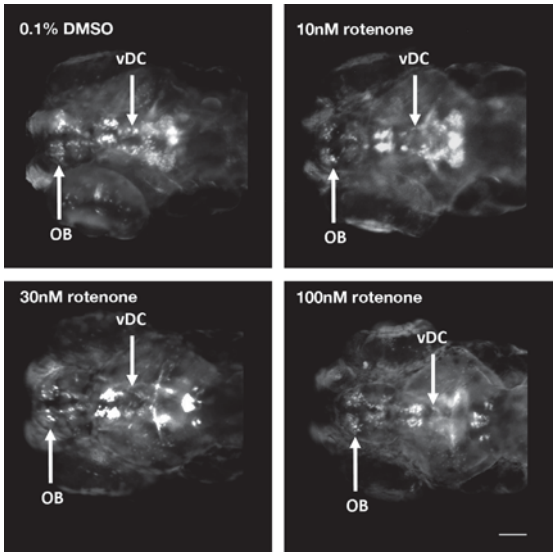

Figure 1. Morphology and distribution of dopaminergic neuron clusters in 7 dpf zebrafish embryos exposed to rotenone. Zebrafish embryos were exposed at 24 hours post-fertilization to either a 0.1% DMSO solution, or 10nM, 30nM, or 100nM rotenone solutions then imaged at 7 dpf. GFP expression was examined in the olfactory bulb (Ob), and ventral diencephalon (vDC). All animals are shown in a dorsal view, anterior to the left. Scale bar = 50µm.

at room temperature for 3 hours. Sections were then incubated overnight at 4°C with a 1:1000 dilution of monoclonal mouse anti-GFP antibody (AS-55887, Anaspec) and 1:1000 dilution of polyclonal rabbit anti-caspase 3 (AS-55372, Anaspec). After three washes in PBT, sections were incubated for three hours at room temperature with a 1:1000 dilution of goat anti-mouse antibody Alexa 488 conjugate (A-11001, ThermoFisher Scientific) and a 1:1000 dilution of goat anti-rabbit antibody Alexa 594 conjugate (A-11012, ThermoFisher Scientific), then washed three times in PBT. Sections were then incubated in NucBlue® Fixed Cell ReadyProbes® Reagent (R37606, ThermoFisher Scientific) for 10 minutes before being rinsed thrice and mounted in Aqua-Poly/Mount (18606, Polysciences Inc.) and viewed on a Zeiss Axiophot upright epifluorescence microscope. Images were assembled using ImagePro, FIJI and Adobe Photoshop CS5.

MITOSOX RED OXIDATIVE STRESS ASSAY

To assess for oxidative stress, we used a whole animal reactive oxygen species detection method as outlined in [21]. Embryos exposed to rotenone were grown to 3 dpf, anesthetized with a 0.168 mg/ml solution of MS-222, collected and rinsed thrice in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich). Embryos were then incubated in the dark for 15 minutes at 28ºC in a 5µM solution of MitoSOX Red (ThermoFisher Scientific) dissolved in HBSS. Following exposure to MitoSOX Red, embryos were then washed twice in HBSS then gently placed into a trough in an agarose bedded plate in a dorsal-ventral orientation, and imaged as described previously.

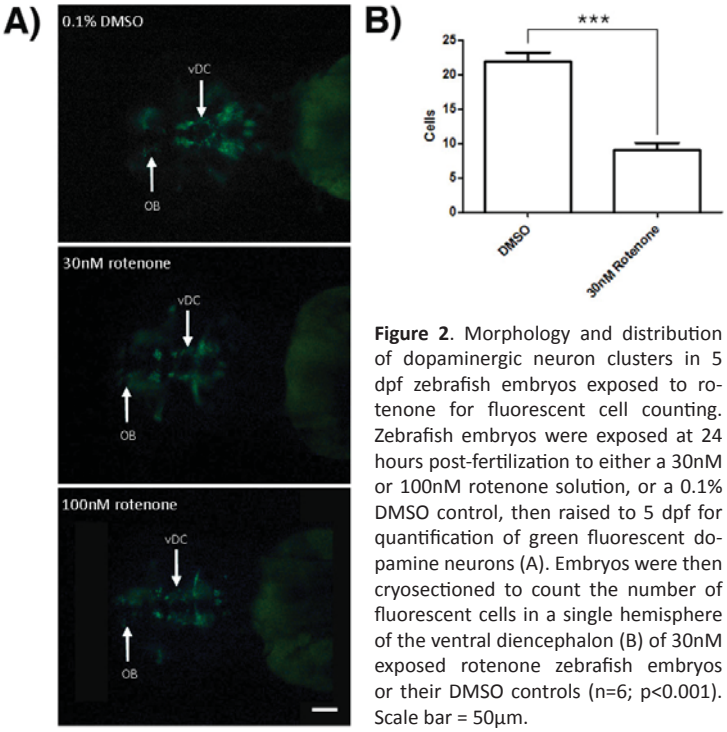

Figure 2. Morphology and distribution of dopaminergic neuron clusters in 5 dpf zebrafish embryos exposed to rotenone for fluorescent cell counting. Zebrafish embryos were exposed at 24 hours post-fertilization to either a 30nM or 100nM rotenone solution, or a 0.1% DMSO control, then raised to 5 dpf for quantification of green fluorescent dopamine neurons (A). Embryos were then cryosectioned to count the number of fluorescent cells in a single hemisphere of the ventral diencephalon (B) of 30nM exposed rotenone zebrafish embryos or their DMSO controls (n=6; p<0.001). Scale bar = 50µm.

ADULT BEHAVIOUR ASSESSMENT

Zebrafish embryos that were exposed to either 0.1% DMSO alone, or 10nM or 100nM rotenone were raised for 12 months to adulthood in accordance with animal care protocols. These adult zebrafish were then placed into individual tanks in an isolated environment and allowed to acclimatize to their new environment for 30 minutes before being recorded for 5 minutes using a Nikon Coolpix A camera. Each tank had the midline depth demarcated with a rubber band, and manual analysis of the video recordings allowed us to count the number of times each fish crossed the midline depth of the tank. All behaviour experiments were performed in biological quintuplicates.

RESULTS

Rotenone induces a dose-dependent chemical ablation of the dopaminergic neurons in the ventral diencephalon (vDC) and olfactory bulb (OB) of zebrafish embryos

Transgenic zebrafish embryos expressing eGFP under the regulation of dopamine transporter *cis*-regulatory elements [11] were exposed to various concentrations (10nM, 30nM and 100nM) of rotenone and to a 0.1% DMSO vector at 24 hours post-fertilization. The embryos were imaged at 7 dpf (Figure 1) to examine changes in fluorescence patterning. Labelling in the ventral diencephalon is markedly reduced at 10nM in comparison to the 0.1% DMSO control, and reductions become more severe with a 30nM exposure. Neurons were judged to be either severely mispatterened or almost absent at a 100nM exposure, following

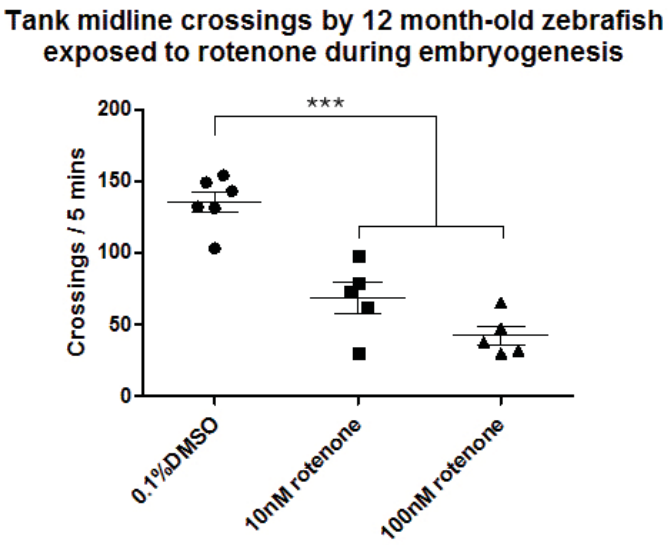

Figure 3. Tank midline crossings by 12 month-old zebrafish exposed to rotenone during embryogenesis. Adult zebrafish exposed to either 10nM rotenone, 100nM rotenone or 0.1% DMSO as embryos were placed in a standalone tank and monitored for five minutes for the number of times they crossed the midline depth of the tank (n=5, p<0.01).

the same criteria as [22]. A less severe dose-dependent reduction in fluorescence in the olfactory bulb became apparent, with the amount of labelled neurons decreasing with increasing concentrations of rotenone. Embryos exposed to a concentration of 300nM did not survive past the second day of treatment. The numbers of eGFP labelled neurons were also manually quantified in single hemispheres of 5 dpf embryos exposed to 30nM rotenone or DMSO alone (Figures 2A and 2B), and confirmed with immunohistochemistry (IHC) on frozen sections. At 5 dpf, the number of labelled neurons in the ventral diencephalon were reduced by more than 50–60% following exposure to a 30nM concentration of rotenone.

Neurodegenerative effects of rotenone cause behavioural changes in treated embryos

Having observed that rotenone had an effect at the cellular level, we wanted to investigate whether the neurodegenerative effects of rotenone translated into observable behavioural changes in treated fish. The tank midline-crossing test allowed us to assess motor function and behaviour patterns of adult fish. Reduced tank midline crossing indicates either hypomotility or anxiety-like behaviour [23]. Zebrafish that were exposed to rotenone showed significantly less midline crossing than the unexposed fish (Figure 3). Fish exposed to 10nM rotenone crossed the midline of the tank nearly 50% less than the controls, and the fish exposed to 100nM rotenone crossing the midline nearly 70% less often. This observed behaviour seemed to suggest a pattern of anxiety-like behaviour rather than overall hypomotility since we observed similar velocities and distance traveled between rotenone-exposed fish and their unexposed controls (data not shown).

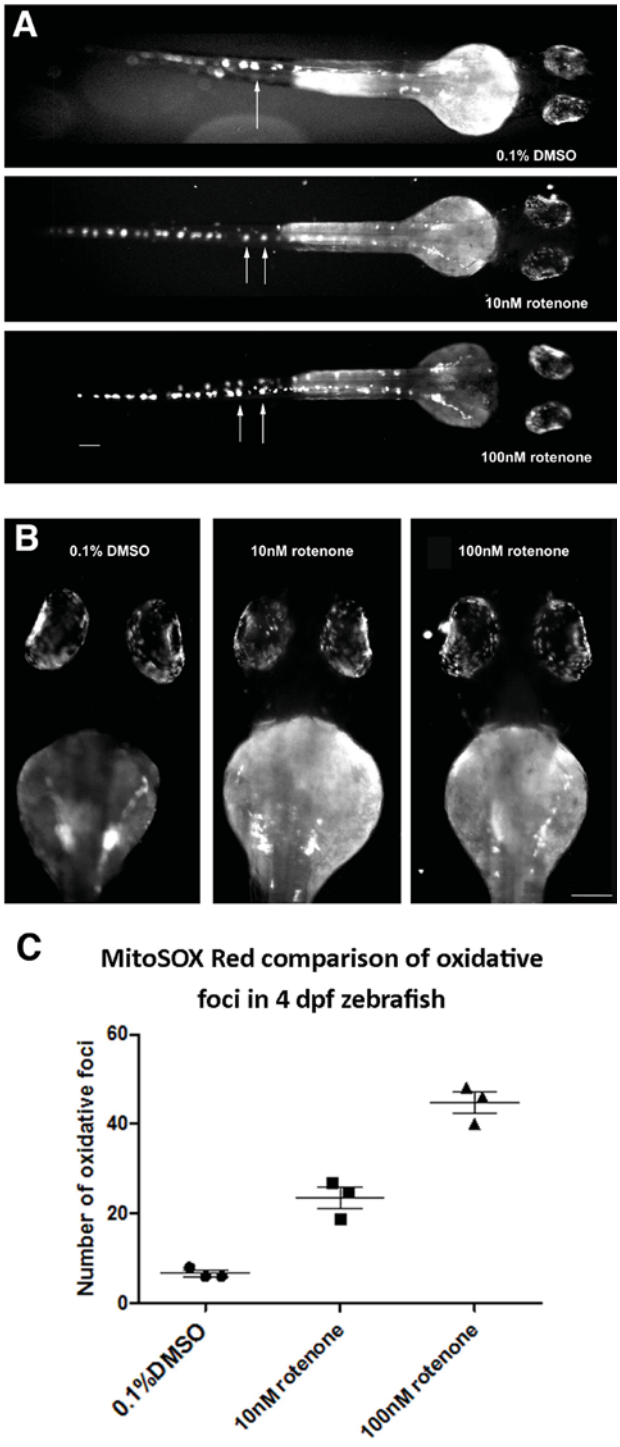

Figure 4. Assessment of reactive oxygen species–mediated damage in embryos exposed to rotenone by MitoSOX Red. Embryos exposed to either 10nM rotenone, 100nM rotenone or 0.1% DMSO raised to 4 dpf were assessed for foci of oxidative stress along the trunk (A, arrows) and head (B). The number of MitoSOX Red foci in the trunk of zebrafish embryos was manually quantified for each treatment group (C) as a marker of oxidative stress (n=3, p<0.001). All animals are shown in a dorsal view; embryos were oriented anterior to the right in panel A, and anterior to the top in panel B. Scale bars = 100µm.

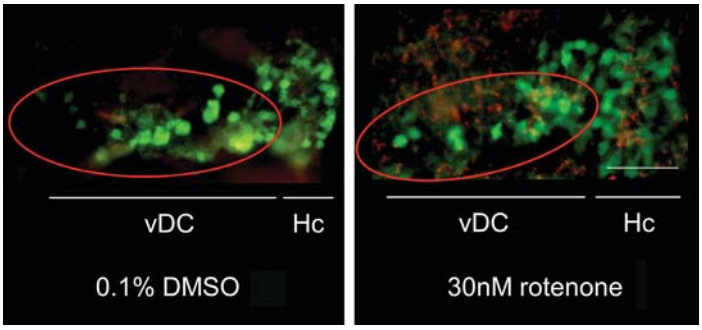

Figure 5. Immunohistochemistry for apoptosis markers in deep brain structures. Frozen sections (14µm thickness) of 5 dpf embryos exposed to 30nM rotenone (right) or 0.1% DMSO (left) were immunostained with anti-GFP (green) and anti-caspase 3 (red) in the ventral diencephalic cluster (vDC) and hypothalamic clusters (Hc) of a single hemisphere. The red ellipse marks comparable regions of neuronal mispatterning between the vDC of rotenone-exposed embryos to vehicle controls. Both panels are shown in a dorsal view, anterior to the left. Scale bar = 50µm.

Chronic rotenone exposure induces dose-dependent oxidative stress in zebrafish embryos

In order to explore the mechanism of action of rotenone on treated zebrafish, we used the mitochondrial superoxide indicator MitoSOX Red for live detection of reactive oxygen species damage. Since rotenone is believed to induce cell death by causing high levels of oxidative stress, this assay allows the determination of the levels of oxidative stress experienced by embryos treated at increasing concentrations of rotenone. Rotenone-exposed embryos raised to 4dpf were incubated with MitoSOX Red for 15 minutes, and subsequently imaged (Figures 4A and 4B). However, this incubation time was insufficient to yield reagent penetration and fluorescent foci formation in deep brain structures within the head of the embryos. Longer incubation times yielded embryonic toxicity and nonspecific superficial staining (data not shown). Although no fluorescent foci were observable in the cephalic regions of both the control and the exposed fish (Figure 4B), there appeared to be a correlation between the total number of red fluorescent foci along the trunk of the zebrafish embryo (Figure 4A, arrows) and concentration of exposed rotenone. Embryos exposed to higher concentrations of rotenone showed more red fluorescent foci than the control embryos (Figure 4C), demonstrating that rotenone is associated with dose-dependent oxidative stress.

Oxidative stress induces apoptosis in dopamine neurons

In order to improve cellular resolution and determine the cellular consequences of rotenone toxicity, immunohistochemistry was performed on frozen sections of 5dpf embryos. We sought to characterize the distribution of green fluorescent neurons, and investigate their co-localization with markers of apoptosis, a common cellular consequence of oxidative stress. We were able to resolve and co-localize green eGFP distribution (as a marker of dopamine neurons; green) with active caspase 3 (red) in double fluorescence images (Figure 5). Rotenone-exposed zebrafish showed a significant reduction in green fluorescent-labelled neurons in the ventral diencephalon (red circled area) when compared to unexposed embryo, showing an increase in apoptotic activity in zebrafish exposed to rotenone.

DISCUSSION

The zebrafish is a promising model for the study of environmentally induced Parkinson's disease. Our study aims to expand current knowledge in regards to the association between pesticides and an increased risk of developing PD. Additionally, since reliable live vertebrate models of Parkinson's disease are limited, this study may provide the foundations for a new model of environmentally induced neurodegeneration. While previous studies in the zebrafish focused on behavioural changes [19], our approach consists of studying changes at the cellular level to identify neurodegeneration-inducing compounds.

Embryos from the *dat*:eGFP transgenic zebrafish line express green fluorescent protein under the control of *dat* regulatory elements, a key protein in dopamine neurons [24]. This provides a powerful *in vivo* fluorescent marker for dopamine neuron health and distribution. In an example of leveraging the transparency of zebrafish embryos, we were able to visualize dopamine neurons *in vivo* of *dat*:eGFP embryos exposed to rotenone. Our results show that rotenone administered in a manner to mimic a single neurotoxic exposure during an embryonically plastic stage appears to cause pharmacological disruption of the diencephalic dopaminergic neurons in a dose-dependent manner. Treated embryos demonstrate a significantly reduced amount of dopaminergic neurons and severe mispatterning within the ventral diencephalon of the midbrain and the olfactory bulb in comparison to the untreated embryos. Dopaminergic neuron disruption in the ventral diencephalon of the treated zebrafish is particularly interesting since these posterior tuberculum neural populations are homologous to the human nigro-striatal pathways affected in PD [10, 25]. These data suggest that rotenone exposure may cause dopamine neuron death in a pathophysiologically similar pattern to human patients with PD. Unfortunately, only neurons expressing the dopamine receptor can be assessed using this protocol, and future work should aim to assess for rotenone's neurotoxic effects on other neuronal populations.

To corroborate our previous observations, we proceeded to verify if changes at the cellular level affected the behaviour and mobility of the zebrafish. Since zebrafish are active swimmers, reduced midline crossing has been associated with hypomotility and general anxiety during movement, two symptoms also observed in human PD patients [23]. Adult zebrafish exposed to rotenone during embryogenesis appeared more hesitant to explore the different tank regions (as evidenced by the impaired midline crossing), demonstrating that a loss of dopaminergic neurons may also affect the fish by inducing anxiety-like behav-

iour. Although this does not appear to correlate with a motor phenotype of PD, anxiety and other psychiatric symptoms manifest in the later stages of patients suffering from PD [26]. It is interesting to note that the 30nM concentration of rotenone used to treat the embryos for cellular counts and behavioural tests resulted in a 50–60% decrease in ventral diencephalic dopaminergic neuron populations, similar to a loss of at least 50%–70% of dopaminergic nigral neurons in human PD patients at symptom onset [27].

In order to investigate the mechanism of action by which rotenone induces neurodegeneration, we used MitoSOX red, an oxidative stress marker for detecting mitochondrial damage from superoxide formation in an attempt to quantify the levels of oxidative stress experienced by rotenone-exposed zebrafish embryos. While it is known that short exposures (under 15 minutes) to high concentrations of rotenone induce rapid and severe oxidative stress in zebrafish [22], few studies have examined the lasting neuronal effects and embryonic survival in response to much lower doses of the rotenone. The fish treated with sub-lethal concentrations of rotenone exhibited an increased number of red fluorescent foci on their bodies, indicating acute oxidative stress. In comparison to the DMSO control group, the number of oxidative foci nearly tripled in fish treated with 10nM of rotenone, and increased by seven-fold when the fish were exposed to 100nM of rotenone. This demonstrates that rotenone induces large amounts of mitochondrial oxidative stress. Oxidative stresses from rotenone toxicity may also disproportionately affect dopaminergic neurons due to the increased oxidative stress of synthesizing dopamine *de novo* within these neurons [28]. The inability to address the increased oxidative stress would typically lead to an accumulation of reactive oxygen species (ROS), which may overwhelm endogenous anti-oxidant capability. This can cause subsequent cellular damage, followed by programmed cell death by apoptosis. Our data demonstrated an increase in caspase signalling land other markers of cell death suggesting that apoptosis from ROS damage may be the mechanism of rotenone neurotoxicity. These results yield insight on the mechanism of action of rotenone, while linking oxidative stress to a decrease in dopaminergic neuron populations.

CONCLUSION

This study presents an interesting perspective on the established correlation between pesticide use and the increased risks of developing Parkinson's disease. In addition, these data provide a foundation for the study of environmentally induced Parkinson's disease using the zebrafish as a live animal model.

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