Characterizing the Effects of Diazinon on Zebrafish Growth and Survival

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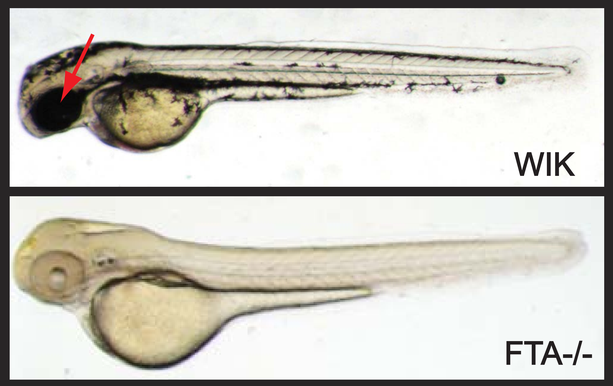
Abstract

Developmental toxicology is an emerging field that investigates the effects of pesticides, insecticides, and other toxic substances on embryonic development. The zebrafish (Danio rerio) is a widely-studied developmental and genetic model system whose fecundity, rapid development, and optically clear embryos allow researchers to investigate most aspects of vertebrate development on a cellular level. This model system has been very useful in testing toxicity of potentially harmful substances and is gaining popularity in the field of ecotoxicology. We have examined the effect of an organophosphate, diazinon, on early embryonic development. We first determined that using a triple pigment mutant for these studies is useful for viewing organ systems and for future experiments. We found that a 5 mg/L diazinon concentration is the concentration that produces 50% survival and total body length, spinal cord diameter and eye diameter are significantly reduced in the presence of diazinon. These data suggest that diazinon has teratogenic effects on the zebrafish embryos.

Developmental mecahnisms in zebrafish are akin to those in more complex vertebrates, including humans. This research will expand our knowledge of the cellular and molecular impact of organophosphates on vertebrate development and help us to understand if irreversible developmental defects in the nervous system are present.

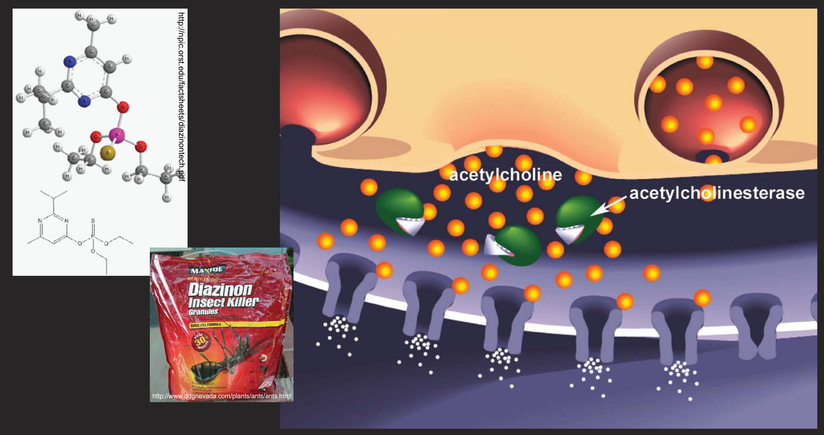
1. Comparison of WIK and FTA zebrafish embryos at 48 hpf



Shown are images of a wild-type (WIK) and triple pigment mutant (FTA) zebrafish at 48 hpf. The head is facing to the left and tail to the right. There is dark pigmentation throughout the head to tail axis, including the eye (arrow) of the WIK embryo compared to complete absence of pigment in the FTA embryos. For our studies, we utilized the transparent zebrafish allowing for simple visualization of many organ systems in vivo, including the nervous system. The use of the FTA strain is advantageous because (1) we are interested in characterizing diazinon effects after pigmentation has taken place and (2) future studies using adult zebrafish will be more easily conducted in the transparent background.

We performed the survival and body measurement experiments on WIK embryos and compared those data to our FTA results. There was no statistical difference between WIK and FTA (data not shown); therefore, we feel confident that diazinon does not affect embryonic development differently in the absence of pigment.

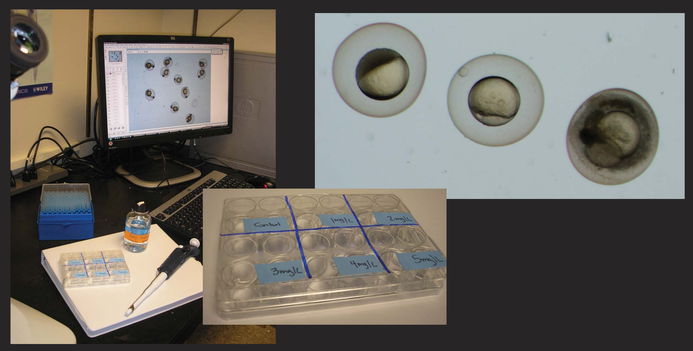
2. Diazinon chemical structure and its target at the neuromuscular junction



In the nervous system, acetylcholine is the primary neurotransmitter at the axon terminal-skeletal muscle interface. After release from the neuron, acetylcholine binds to a receptor on the skeletal muscle; it also accumulates in the synaptic cleft until it is degraded by acetylcholinesterase. Our chemical of interest, diazinon, inhibits the function of acetylcholinesterase, causing persistence of acetylcholine in the synapses. When this happens, the skeletal muscle receives a continuous signal to contract resulting in paralysis.

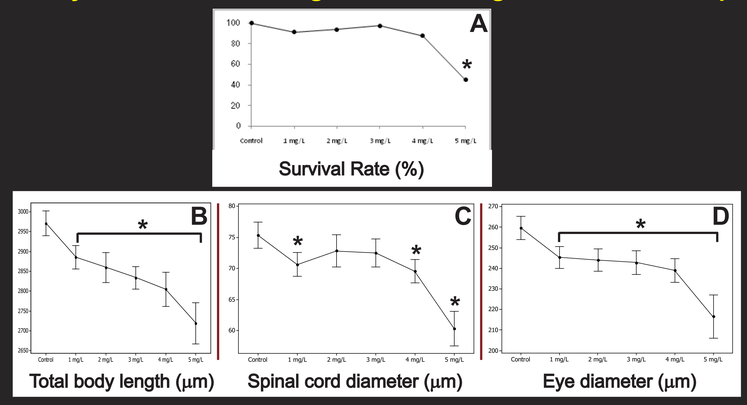
Diazinon is one of a large group of insecticides called organophosphates. While a common ingredient in household pesticides prior to 2001, diazinon is now only permitted to be used for industrial agricultural applications. Studies examining diazinon effects in zebrafish have been limited. Further, many diazinon studies in fish have focused the adult or juvenile animals, while few have examined effects on early embryonic development.

3. Diazinon treatment protocol



Adult male and female FTA zebrafish were self-crossed and fertilized embryos were identified prior to the eight-cell stage. Five embryos were added to each well of a 24-well plate. The embryo media was extracted from each well and replaced with 1mL of diazinon with concentrations ranging from 1 mg/L-5 mg/L. Embryos were placed in the climate control chamber set at 28°C to ensure optimal growth conditions. Survival rates were taken at 24 hpf and 48 hpf by observing the dish under a stereomicroscope. Dead embryos were removed from each well to reduce contamination at each time point. Surviving embryos were then dechorionated and placed in 4% paraformaldehyde at 48hpf. Post-fix, embryos were mounted on a microscope slide in 3% methylcellulose and imaged on an Olympus SZX10 equipped with a DP72 digital camera. Measurements were then taken using analysis tools in Microsuite 5.

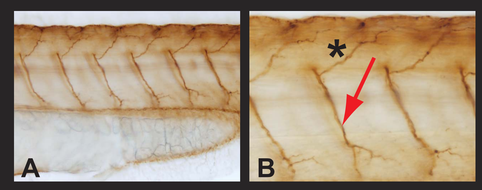
4. Embryo survival rate and growth in 0-5 mg/L diazinon at 48 hpf



When performing toxicological experiments determining the LC50, the drug concentration which is lethal to 50% of specimens, is the first parameter to measure. Survival rates are shown in figure A with control embryos having 100% survival compared to 45% survival at 5 mg/L at 48 hpf. From these results, we were interested in determining the effect of diazinon on basic body plan such as total body length (B), spinal cord diameter (C), and eye diameter (D). All measurements showed a significant decrease correlated with increasing diazinon concentration. Specifically, compared to control embryos, all concentrations of diazinon produced a significant decrease in total body length and eye diameter. Spinal cord diameter was significantly smaller in diazinon concentrations of 1, 4, and 5 mg/L compared to control. These data indicate that diazinon has terotogenic effects on zebrafish embryos.

For each parameter 10 embryos/concentration were analyzed and a Tukey test was used to determine significance. S.E.M shown in B-D.

5. Motor neuron axon detection with anti-acetylated tubulin at 48 hpf



Future studies will examine the structure of motor neuron axons in control versus diazinon treated embryos. Activity dependent mechanisms are critical for proper nervous system development, therefore, we hypothesize that the presence of diazinon during axon outgrowth may result in shorter, misdirected, or less branched motor neuron axons. Shown are two images of 48 hpf embryos that have been stained for anti-acetylated tubulin, a protein that is present in mature axons (brown staining). The image on the left was captured at 20X magnification, while the image on the right is at 40X. Two classes of motor neuron axons are present (arrow and asterisk).

6. Summary and future directions

1. There is no significant difference in survival or body measurements between WIK and FTA embryos.
2. As the concentration of Diazinon increases, survival rate and growth measurements are significantly decreased, especially at 5 mg/L.
3. Analysis of motor neuron axon outgrowth, shape and length is currently underway using anti-acetylated tubulin to detect mature axons.

7. Acknowledgements

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