Developmental Neurotoxicity Testing in *C. elegans*

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Abstract: *Caenorhabditis elegans* is a small nematode with a 3-day lifecycle that can be easily maintained using standard laboratory techniques. Many pathways involved in organismal development and neurotransmission are conserved from worms to people. The *C. elegans* digestive tract has several features that are analogous to the mammalian digestive system, making this simple model organism an attractive candidate for predictive oral toxicity testing. *C. elegans* studies have demonstrated concordance for developmental toxicity or altered motor activity when exposed to mammalian developmental toxins or neurotoxins. We have designed a novel worm Development and Activity Test (wDAT) that maps the timing of *C. elegans* developmental milestone acquisition as well as stage-specific activity levels. The wDAT was able to detect both developmental delay and hyperactivity for arsenic, lead, and mercury, developmental neurotoxins that have been associated with hyperactivity in children. Binary mixtures of arsenic, lead, and mercury produced at most additive effects on developmental delay and/or hyperactivity; no synergistic effects were detected. The wDAT can be completed by a single technician in 4 days using a relatively inexpensive activity tracker, features that would make it a cost-effective addition to integrated approaches to toxicity testing. A planned 20-compound, blinded qualification study will help determine how the wDAT might provide "fit-for-use" data to support developmental neurotoxicity testing strategies.

Introduction: The FDA's Predictive Toxicology Roadmap supports the integration of emerging methods and new technologies into regulatory safety and risk assessments. New toxicological tools that better predict human response with reduced time and expense ratios will allow rapid evaluation of many more individual compounds of concern as well as increased toxicity testing of mixtures. Traditional methods for assessing developmental neurotoxicity (DNT) are especially costly in terms of time, resources, and laboratory mammals required. As a result, a total of only about 200 such studies have been conducted, creating a vast knowledge gap that urgently needs to be addressed. New assays using alternative models for DNT assessment have the potential to fill this gap in a timely and cost-effective manner.

There is significant conservation between *C. elegans* and humans for pathways involved in organismal and neuronal development. *C. elegans* larval growth is nearly as accurate at predicting developmental toxicity in rats and rabbits as those two species are at predicting each other, and many mammalian neurotoxins have been shown to alter *C. elegans* adult locomotor activity. The activity of populations of small animals in multi-well plates can be monitored using an infrared beam interruption detection device. The wDAT utilizes the changes in population activity levels to track *C. elegans* larval development over time (Figures 1 and 2). Each wDAT peak in activity corresponds to a specific developmental stage. Delayed timing of these wDAT activity peaks in dosed populations would indicate developmental delay. Changes in stage-specific peak heights relative to controls would indicate altered activity and possible DNT effects.

Arsenic, lead, and mercury are developmental neurotoxins, and exposure to low levels of these toxic elements is associated with hyperactivity in children and developing rodents. Lithium is a mammalian developmental toxin, but it is not considered a neurotoxin and it is not associated with hyperactivity in mammals. Developmental delay in *C. elegans* may correspond to delayed acquisition of developmental milestones and/or reduced weight gain in rodents. Reduced weight gain has been found in rat pups exposed to arsenic, lead, mercury, or lithium.

The effects of exposure to individual toxic elements are generally well understood. The effects of mixtures are less well studied, and mixtures have the potential to produce additive, synergistic, or unanticipated outcomes. If the wDAT demonstrates concordance between worms and mammals for DNT effects of individual toxic elements, then the wDAT may prove useful in predicting the toxicity of mixtures.

Figure 1: *C. elegans* Development

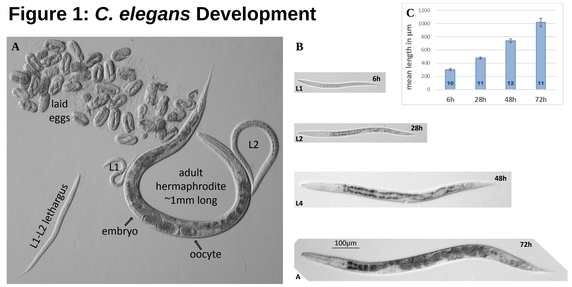

Figure 1: *C. elegans* larval development progresses through four distinct stages which can be identified by size and by morphology. Between each of these stages, the developing worm enters a short period of lethargus during which the cuticle is shed and locomotion and feeding slow or stop. A. Microscopy image showing *C. elegans* first larval stage (L1), second larval stage (L2), the lethargus between L1 and L2, an adult, and eggs in various stages of maturity. B. Representative images were taken at 6, 28, 48, and 72 h after hatching. C. Bar graph shows mean worm lengths in µm at evaluated timepoints, with inset numbers indicating the number of worms measured.

Figure 2: The worm Development and Activity Test (wDAT)

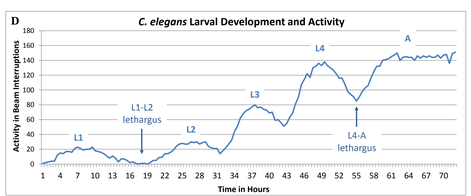

Figure 2: Approximately 900 synchronized, newly hatched *C. elegans* per well were assessed by WMicroTracker™ (WMT, PhylumTech) for three days. The WMT measures mean population activity per infrared sensor within each well as beam interruptions (y-axis) per half hour (x-axis), presented here as the mean activity in three replicate wells.

Results: With the wDAT, we found that sodium arsenate (NaAs), lead acetate (PbOAc), and mercury acetate (HgOAc) induced both developmental delay and hyperactivity, while lithium acetate (LiOAc) only induced delay (Figures 3 and 4). These data indicate that worms and humans are concordant for these endpoints, and that the wDAT can distinguish between a developmental toxin and developmental neurotoxins.

Figure 3: Mercury is a *C. elegans* Developmental Neurotoxin

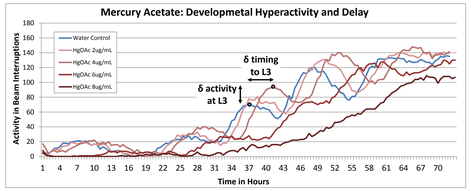

Figure 3: Dots indicate *C. elegans* third larval stage (L3) activity peak heights for the water control (blue) and 4µg/mL mercury acetate (HgOAc, pink). Increases in peak height relative to the control indicate hyperactivity while peak right-shifts indicate developmental delay.

Figure 4: Worm to Human Concordant DNT Effects

Figure 4: Bar graphs summarize data for stage-specific activity peak times and heights relative to water controls in the same plate. Control (Cont.) data shows the difference between water controls in side-by-side WMT instruments indicating experimental variability under similar conditions. A-D. *C. elegans* growth rates to the third (L3) and fourth (L4) larval stages. E-H. Activity differences between the controls and test article exposed populations at L3 and L4. Students' T-test p values: * for <0.05, ** for <0.005.

Binary mixtures of arsenic, lead, and mercury produced similar or additive effects on developmental delay and/or hyperactivity with the wDAT. No synergistic effects were detected. Mixtures with mercury produced the most significant additive effects. Assessment of cadmium mixtures is still in progress.

Mixture	Synergistic Effects	Additive Delay from NOELs	Additive Delay from LOELs	Additive Activity Effects
Arsenic + Lead	no	maybe	maybe	no
Mercury + Lead	no	maybe	yes	no
Arsenic + Cadmium	no	no	yes	maybe
Cadmium + Mercury	no	no	yes	yes
Arsenic + Mercury	no	likely	yes	yes
Cadmium + Lead	Data Soon			

Table 1: Binary combinations of NOEL and LOEL concentrations produced no synergistic effects with tested compounds. HgOAc with CdOAc or with NaAs had additive effects on hyperactivity.

CONCLUSIONS

1. **The FDA's Predictive Toxicology Roadmap ensures continued employment of cutting-edge science to assess the safety of FDA regulated products**
2. **Current methods for DNT detection are insufficient to fill critical knowledge gaps**
3. **The wDAT uses a relatively inexpensive activity tracker to simultaneously measure both developmental timing and activity levels in *C. elegans* larvae**
4. **For a limited set of test articles, worms and humans were concordant for developmental delay and hyperactivity, or just delay**
5. **A planned 20-compound wDAT qualification study will help define the wDAT's "fit-for-use" in safety assessment and regulatory decision making**

FDA Mission Relevance: New, validated toxicological tools that predict human response at reduced time and expense will allow rapid evaluation of many more compounds and mixtures of concern and thus support safety assessment and regulatory decision making. The 2016 update to the Toxic Substances Control Act mandates that U.S. Federal agencies develop leading edge predictive toxicology methods that utilize invertebrate models. The FDA supports the use of validated new approach methodologies to fill knowledge gaps and assess the safety of FDA regulated products. Evaluation of the effects of simultaneous exposure to multiple toxins is a priority for the FDA's Center for Food Safety and Applied Nutrition. The wDAT is a rapid and relatively inexpensive DNT test with the potential to contribute to each of these priorities.