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Evaluation of the developmental toxicity of lead in the *Danio rerio* body

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ABSTRACT

Lead has been utilized throughout history and is widely distributed and mobilized globally. Although lead in the environment has been somewhat mitigated, the nature of lead and its extensive uses in the past prohibit it from being completely absent from our environment and exposure to lead is still a public health concern. Most studies regarding lead toxicity have focused on the brain. However, little is found in the literature on the effects of lead in other tissues. Here, we utilize the zebrafish model system to investigate effects of lead exposure during early developmental time windows at 24, 48 and 72 h post fertilization in the body. We analyze whole body, notochord and somatic muscle changes, vascular changes of the body, as well as motor neuron alterations. We find lead exposure induces a curved body phenotype with concomitant changes in somite length, decreased notochord staining and abnormal muscle staining using live and *in situ* approaches. Furthermore, altered vasculature within the somatic regions, loss and/or alternations of motor neuron extension both dorsally and ventrally from the spinal cord, loss of Rohon-Beard sensory neurons, and increased areas of apoptosis were found. We conclude that lead is developmentally toxic to other areas of the developing embryo, not just the brain.

1. Introduction

Lead has been mined and used for over 6000 years, spiking during Roman times and the Industrial Revolution (Hernberg, 2000; Philp, 2001). During the past 100 years, lead was exclusively used in paints, canning, toy manufacturing, pesticides, lead shot, and as a gas additive (Philp, 2001; Roy et al., 2014). In the United States, use of lead in many products has been banned since the 1970s, however, lead continues to contaminate our environment and is found in dust, street dirt, soil, water and food (Tong et al., 2000). Current sources of lead exposure include past emissions of leaded gasoline accumulating in soil, abandoned industrial sites, smelting operations, older homes with leaded paint and lead pipes, as well as imported toys (Philp, 2001; Tong et al., 2000). Furthermore, a number of home hobbies can contribute to lead contamination including pottery and stained glass. Because lead was utilized globally and in such vast quantities, mitigating all the lead in our environment is impossible. Chronic exposure to low levels of lead is a common health issue and acute lead poisoning can still occur, especially among socioeconomically disadvantaged groups and in developing countries lacking policies and environmental regulations (Tong et al., 2000).

The zebrafish model has become particularly popular in the laboratory setting given its genetic and embryological similarities to higher order vertebrates including humans (Grunwald and Eisen, 2002). Zebrafish share a high degree of homology with the human genome (Dai et al., 2014; Howe et al., 2013) and thus, modeling of human diseases in zebrafish is now commonplace. From a toxicological perspective, zebrafish are particularly useful as their development is very well characterized (Hill et al., 2005; Kimmel et al., 1995) and all stages of toxicological assessment can be made *ex utero*. Of particular toxicological significance to the model, they develop organs specific to toxin conversion, like the liver, very early in their development. They also share the same liver metabolic pathways and cytochrome P450 (CYP) genes, most of which are direct orthologs of human CYPs (Goldstone et al., 2010; Padilla et al., 2012; Tao and Peng, 2009). Thus, the zebrafish can provide information that could not be gathered from other models and knowledge of the mechanisms of developmental toxicity is scarce (Teraoka et al., 2003). Since the zebrafish genome has been sequenced,

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fluorescent transgenic zebrafish are relatively easy to construct and are visualized well in the optically transparent zebrafish embryo. As zebrafish rapidly mature, transgenerational effects of toxin exposure can be assessed (Hill et al., 2005). From an eco-toxicological perspective, zebrafish have been extensively used to study heavy metals, endocrine disrupting chemicals, and persistent organic pollutants (Dai et al., 2014).

Traditional approaches to toxicology include testing on common laboratory species like rat or rabbit, but this approach is time consuming and expensive. Given the number of chemicals entering the market, the need for high throughput assays is significant. The goal of the United States Environmental Protection Agency (EPA) ToxCast™ program is to develop cost-effective approaches to rapidly screen and prioritize chemicals that would require further toxicological testing. High throughput testing has mainly involved *in vitro* assays and *in silico* modeling (Padilla et al., 2012). However, in recent years, numerous labs have correlated zebrafish developmental toxicity with mammalian developmental toxicity validating the model (Busquet et al., 2008; Padilla et al., 2012; Selderslaghs et al., 2009) and thus, the EPA has recently launched a new toxicological initiative within the umbrella of the ToxCast™ program to utilize zebrafish developmental toxicity to model human health. Two approaches can be taken to study developmental toxicity: a low dose chronic exposure or a short high dose exposure. The ToxCast™ program utilizes the latter approach and treats embryos at early developmental time points with toxic chemicals at relatively high doses to investigate overt phenotypes and other organismal toxicity (Padilla et al., 2012) for predictive modeling of human developmental toxicity. Although there is a wealth of knowledge on low dose chronic exposure to lead in children and its association with reduced IQ (Intelligence Quotient), ADHD (Attention Deficit Hyperactivity Disorder), and decreased cognitive abilities and hyperactivity (Lidsky and Schneider, 2003; Needleman, 2004; Philp, 2001; Tong et al., 2000), there are few publications in the literature on short, high dose exposures. Here, we have sought to model the EPA ToxCast™ approach to investigate a shorter high dose exposure and its effect on the developing body. A high dose, short exposure of lead in zebrafish was investigated by Dou and Zhang, (2011), who found decreased *gfap* and *huC* gene expression in the diencephalon indicating neurogenesis was significantly compromised by lead during embryonic development (Dou and Zhang, 2011). However, they noted only slight changes in two other genes required for neurogenesis, *ngn1* and *crestin*. *Neurogenin1* is expressed in the central nervous system (CNS), otic and epibranchial placodes and *crestin* in cranial and trunk neural crest cells. However, their investigation on the effects of lead did not proceed past the 24hr time point (Dou and Zhang, 2011). Springboarding off of their work and utilizing the same lead dose, we have also found structural abnormalities in the hindbrain including alterations in branchiomotor neuron development and migration. Altered neural vasculature and increased neural apoptosis were also noted (Roy et al., 2014). Here, we hypothesize that lead will also demonstrate developmental toxicity to other areas in addition to the brain and we investigate the effects of lead looking at live gross body phenotypes, notochord and muscle changes using an *in situ* and immunohistological approach and spinal neuron changes using a transgenic approach. We find lead exposure induces a curved body phenotype with concomitant changes in somite length, decreased notochord staining and altered muscle staining using live and *in situ* approaches. Additionally, altered vasculature within the somatic regions, loss and/or alterations of motor neurons extending dorsally and ventrally from the spinal cord, loss of Rohon-Beard sensory neurons, and increased areas of apoptosis were found. We conclude that lead is developmentally toxic to other areas of the developing embryo, not just the brain.

2. Materials and methods

2.1. Zebrafish breeding and embryo maintenance

Adult zebrafish were housed in the Sacred Heart University Animal Facility in a standard zebrafish module (ZMOD (zebrafish module), Aquatic Habitats, Inc). Adults were fed once daily with a combination of brine shrimp and supplemental TetraMin® Flake food. A 10% water change was performed and water quality was monitored daily in accordance with IACUC (Institutional Animal Care and Use Committees) regulations. Ammonia levels were kept below 0.5 ppm, nitrate levels below 80 ppm, nitrite levels below 1 ppm and the pH was kept between 6.5 and 7.5. The adults were maintained on a standard 14:10 h light:dark wake to sleep cycle. Two adult male and female pairs were placed in standard breeding boxes for mating purposes (Westerfield, 1993). Embryos were collected the following morning and placed in 30% Danieau Buffer (Westerfield, 1993) prior to lead treatment. Transgenic *fli-1 gfp* zebrafish were a generous gift from the Lawson Lab (University of Massachusetts Medical Center). Transgenic *neurogenin1 gfp* and *islet-1 gfp* zebrafish were generous donations from the Linney Lab (Duke University Medical Center). All embryos were staged according to Kimmel (Kimmel et al., 1995).

2.2. Lead acetate and exposure protocol

Lead acetate was obtained from Sigma–Aldrich Chemical Company. Solid lead powder was dissolved in 30% Danieau Buffer to a final concentration of 0.2 mM. Embryos were transferred to the control (30% Danieau Buffer) or lead solution (0.2 mM) at 6 h post fertilization (hpf) and treated continuously until 24, 48 and 72hpf when they were released from the lead treatment to control water. As we wished to investigate the effect of lead on body development, treatments commenced at 6hpf to coincide with the onset of gastrulation. After the treatment window ended (after 24, 48 or 72hpf), embryos were transferred to control water for safety purposes during imaging. Treatments were performed in standard Petri dishes at 28.5 °C as previously described (Roy et al., 2014). Lead solution was changed daily and a maximum of 50 embryos were placed in each dish.

2.3. Determination of dose

An LD_{50} was previously determined and the rationale for using the 0.2 mM dose was previously described (Roy et al., 2014). Embryonic survivability at the dose was also previously described (Roy et al., 2014). The 0.2 mM dose was also previously used to investigate the effect of lead on swimming patterns and to assess larval escape responses (Dou and Zhang, 2011).

2.4. Imaging and microscopy (transgenic and non-transgenic embryos)

Live images were obtained using a Leica dissection microscope attached to a Nikon Digital Sight DS-2Mv digital camera utilizing QCapture Software. Transgenic green fluorescent images were obtained with a Nikon Eclipse E400 fluorescent microscope attached to a Retiga cooled CCD (charge-coupled device) camera using QCapture Software. Embryos were sedated in tricaine methanesulfonate (MS-222) (Westerfield, 1993) to inhibit movement during microscopy. Embryos were placed in a depression slide in 3% methylcellulose for positioning purposes (Roy et al., 2014). In some cases (Fig. 5G and I and Fig. 6I), embryos were too curved to obtain in-focus whole body images and were hand manipulated into a straighter position with forceps in 1% agar for imaging.