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Glyphosate induces neurotoxicity in zebrafish

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ABSTRACT

Glyphosate based herbicides (GBH) like Roundup® are used extensively in agriculture as well as in urban and rural settings as a broad spectrum herbicide. Its mechanism of action was thought to be specific only to plants and thus considered safe and non-toxic. However, mounting evidence suggests that GBHs may not be as safe as once thought as initial studies in frogs suggest that GBHs may be teratogenic. Here we utilize the zebrafish vertebrate model system to study early effects of glyphosate exposure using technical grade glyphosate and the Roundup® Classic formulation. We find morphological abnormalities including cephalic and eye reductions and a loss of delineated brain ventricles. Concomitant with structural changes in the developing brain, using *in situ* hybridization analysis, we detect decreases in genes expressed in the eye, fore and midbrain regions of the brain including *pax2*, *pax6*, *otx2* and *ephA4*. However, we do not detect changes in hindbrain expression domains of *ephA4* nor exclusive hindbrain markers *krox-20* and *hoxb1a*. Additionally, using a Retinoic Acid (RA) mediated reporter transgenic, we detect no alterations in the RA expression domains in the hindbrain and spinal cord, but do detect a loss of expression in the retina. We conclude that glyphosate and the Roundup® formulation is developmentally toxic to the forebrain and midbrain but does not affect the hindbrain after 24 h exposure.

1. Introduction

Glyphosate based herbicides (GBHs) are utilized globally and are used both in agricultural and non-agricultural (domestic and urban) areas for weed control and acts as a broad-spectrum, post-emergent herbicide (EPA; Uren Webster et al., 2014; WHO). Glyphosate is the main ingredient in formulations including Roundup®, Rodeo® and Touchdown®, each varying slightly in chemical composition and surfactant composition (Howe et al., 2004). Glyphosate strongly absorbs to soil, but it is susceptible to microbial degradation (Uren Webster et al., 2014). Due to glyphosate's low persistence, repeated applications become necessary for weed control (Ayoola, 2008). Glyphosate is also water soluble and contamination is noted during heavy rainfall. Increased river sediment loads are also noted during turbulent flooding events (Botta et al., 2009; Giesy et al., 2000; Uren Webster et al., 2014). High levels of glyphosate have also been noted in rivers near urban runoff and wastewater treatment effluent (Botta et al., 2009; Uren Webster et al., 2014). In faster moving, more diluting bodies of water, glyphosate concentrations are generally lower averaging around 10–15 μg/L (Byer et al., 2008; Struger et al., 2008; Uren Webster et al., 2014). However, in stagnant bodies of water, like isolated ponds or wetlands, higher levels of glyphosate have been noted. The lack of water flow leads to less dilution and dispersion of the glyphosate (Giesy et al., 2000). Given glyphosate's high water solubility and its extensive use in the environment, exposure to non-target organisms is inevitable (Tsui and Chu, 2003). Interestingly, glyphosate nor its various formulations (with surfactants) are tested or regularly monitored in surface waters (Uren Webster et al., 2014). Glyphosate's specific mechanism of action is inhibition of the enzyme 5-*enol*pyruvoylshikimate-3-phosphate synthase (EPSPS). This plant enzyme is required in the shikimate pathway, part of the biosynthetic steps leading to formation of aromatic amino acids, but is not required in vertebrates (Schonbrunn et al., 2001; Steinrucken and Amrhein, 1980). Thus, glyphosate was not thought to have a common molecular target in animal species (Sandrini et al., 2013). However, mounting evidence suggests non-target species may also be affected (Giesy et al., 2000).

Acute toxicity and teratogenicity in response to glyphosate was first noted in amphibian species as the nature of their reproduction and early developmental stages depends on aquatic areas making them particularly susceptible to glyphosate (Howe et al., 2004; Mann and Bidwell, 1999; Perkins et al., 2000). More recent studies focused on exposures during sensitive stages of amphibian development. Howe et al. have shown glyphosate exposure led to smaller

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animals than the controls as determined by decreased lengths from the snout to the vent (Howe et al., 2004). Additionally they noted delayed metamorphosis compared to controls, as well as defects in the tail regions including necrosis and blistering and abnormal gonads including intersex gonads (Howe et al., 2004). A more comprehensive study by Paganelli et al. detailed that glyphosate based herbicides induced alterations in *Xenopus* body, brain and eye development (Paganelli et al., 2010). Specifically, the authors noted alterations in neural crest development, primary neuron differentiation and loss of hindbrain rhombomere patterning using *in situ* hybridization approaches. Additionally, craniofacial and cephalic defects including reduction of the optic vesicles and microcephaly were noted that were attributable to glyphosate induced misregulation of the Retinoic Acid pathway (Paganelli et al., 2010).

There is also growing evidence that glyphosate based herbicide (GBH) toxicity is not limited to aquatic life. In rural areas, particularly in farm heavy regions of South America and Paraguay where GBHs are extensively used, an alarming trend of birth defects is starting to appear including microcrocephaly, anencephaly, cleft palates and a variety of other facial defects (Benitez Leite et al., 2009; Campana et al., 2010). Additionally, glyphosate is used in Colombia to eradicate coca plantations. Epidemiological studies between 2004 and 2008 found increased rates of cyclopia at endemic levels (Lopez et al., 2012; Saldarriaga, 2010). Glyphosate has been shown to permeate the human placenta (Poulsen et al., 2009) and thus the risk of glyphosate induced teratogenesis in human development is evident.

In situ hybridization using neural specific markers is a key tool in investigating gene expression changes in response to chemical challenge. The unique patterns by which each gene is expressed allows one to investigate changes in specific areas or in multiple areas of the developing brain. For example, *krox-20* is a zinc-finger transcription factor expressed uniquely in rhombomere stripes 3 and 5 and is directly activated by *hox* genes (Giudicelli et al., 2001). *hoxb1a* is a regulatory transcription factor expressed as a single stripe in rhombomere 4 (Rohrschneider et al., 2007). Thus, the unique pattern of these genes provide information on proper hindbrain patterning. Alterations in the stripes would indicate defects in hindbrain development. *ephA4* can provide information on the developing hindbrain, forebrain and midbrain as it is expressed in multiple regions (Jessell and Sanes, 2000). Thus, *ephA4* is a good marker to investigate changes in multiple areas of the developing brain. *otx2* is a key regulator specifically in developing forebrain structures (Mori et al., 1994; Pannese et al., 1995). *pax* genes are essential transcription factors in development. Specifically, *pax6* is necessary for mammalian eye and nervous system development and acts as a master control gene which controls the development of a single eye field in the anterior neural plate into two eye fields which form the left and right optic vesicles and optic cups (Graw, 2010). Any chemical induced alterations to *pax6* expression could lead to detrimental defects in the anterior cephalic regions and the eyes. Mutations in *pax6* are known to induce eye disorders (Bhatia et al., 2013). Likewise *pax2* plays an important role in eye development (Pfeffer et al., 1998).

Zebrafish is commonly used as a vertebrate model in developmental neurotoxicity studies given their genetic and embryological similarities to higher order vertebrate species (Dai et al., 2014; de Esch et al., 2012; Grunwald and Eisen, 2002; Hill et al., 2005; Parng et al., 2007; Teraoka et al., 2003). Zebrafish embryos are especially suited for neurotoxicological studies as fluorescent neural transgenic yield real-time phenotypes, neurons and axons are easily visualized and behavioral protocols have become well established (Linney et al., 2004; Ton et al., 2006). The zebrafish model has been used extensively to model environmental toxins including heavy metals, persistent organic pollutants and endocrine disrupting chemicals (Dai et al., 2014).

Currently, there is limited data regarding exposure to glyphosate during the windows of embryonic development. Most data in the literature is general and involves death as an end-point. Here we seek to investigate the effects of glyphosate-based herbicide exposure using technical grade glyphosate and the Roundup® Classic formulation on the developing brain using the zebrafish vertebrate toxicity model system. We investigate structural changes to the fore, mid and hindbrain by examining gross structural morphology and further investigate morphological abnormalities by investigating gene expression changes *via in situ* hybridization, immunohistological and transgenic approaches. We conclude that glyphosate and the GHB herbicide Roundup® Classic are neurotoxic to the fore and midbrain, but does not induce hindbrain changes as seen in other species.

2. Methods

2.1. Adult and embryo handling

Wild-type AB strain and transgenic adult zebrafish were housed in a ZMOD (zebrafish module) System (Aquatic Habitats Inc.) on a 14:10 h light:dark cycle. 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. Ammonia levels were kept below 0.5 ppm, nitrate levels below 80 ppm, nitrite levels below 1 ppm and pH was kept between 6.5 and 7.5 values. Transgenic fish *RGYn* (Retinoic Acid Responsive Element – yellow fluorescent protein) were attained from the Linney Lab (Duke University Medical Center) (Perz-Edwards et al., 2001). Embryos were generated by natural pair-wise mating in zebrafish mating boxes (Westerfield, 1993). Embryos were placed in Petri dishes in 30% Danieau Buffer (50X Danieau's Solution [169.475 g NaCl, 2.61 g KCl, 4.93 g $MgSO_4{\cdot}7H_2O$, 7.085 g $Ca(NO_3)2{\cdot}4H_2O$, 0.5 M Hepes at a pH of 7.6, autoclaved]). A solution of 30% Danieau's buffer was prepared by mixing 6 ml of the 50X concentrated solution into 1 L of distilled H_2O at 28 °C for 5 h (h) before moving into treatment. Zebrafish were staged in accordance with standard staging series (Kimmel et al., 1995). All treatments were approved and met ethical standards by the Sacred Heart University Institutional Animal Care and Use Committee (IACUC).

2.2. Solutions and exposure protocols

Embryos were collected after pair-wise male/female mating and transferred to control (30% Danieau Buffer) or 50 μg/ml glyphosate concentration by diluting Roundup® (commercially purchased) or pure glyphosate (Sigma-Aldrich) in 30% Danieau Buffer at 5 h post fertilization (hpf) (just before gastrulation) and treated continuously until 24 h in development when the major brain ventricles and structures have formed and are clearly delineated visually (Fig. 1). We chose the 5–24 h time window to initiate treatments at the onset of gastrulation and cover major neural developmental stages including segmentation, somitogenesis and neurulation. Embryos were raised at 28.5 °C in standard glass petri dishes. For each type of experiment (live gross morphology, *in situ* hybridization (per gene), immunohistochemistry, transgenics) embryos were placed in the control, Roundup® dilution or pure glyphosate dilution and treated until the 24 h time point (Fig. 1). To ensure the data was not skewed by slowly developing embryos, embryos were examined for 24 h hallmarks including presence of the otic vesicle, development of the lens and retina and pericardial cavity.

2.3. Live gross morphology

A total of ten embryos for control, Roundup® treated and glyphosate treated were tested. Thus, one experimental replicate