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Atrazine exposure elicits copy number alterations in the zebrafish genome

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ABSTRACT

Atrazine is an agricultural herbicide used throughout the Midwestern United States that frequently contaminates potable water supplies resulting in human exposure. Using the zebrafish model system, an embryonic atrazine exposure was previously reported to decrease spawning rates with an increase in progesterone and ovarian follicular atresia in adult females. In addition, alterations in genes associated with distinct molecular pathways of the endocrine system were observed in brain and gonad tissue of the adult females and males. Current hypotheses for mechanistic changes in the developmental origins of health and disease include genetic (e.g., copy number alterations) or epigenetic (e.g., DNA methylation) mechanisms. As such, in the current study we investigated whether an atrazine exposure would generate copy number alterations (CNAs) in the zebrafish genome. A zebrafish fibroblast cell line was used to limit detection to CNAs caused by the chemical exposure. First, cells were exposed to a range of atrazine concentrations and a crystal violet assay was completed, showing confluency decreased by ~60% at 46.3 μM. Cells were then exposed to 0, 0.463, 4.63, or 46.3 μM atrazine and array comparative genomic hybridization completed. Results showed 34, 21, and 44 CNAs in the 0.463, 4.63, and 46.3 μM treatments, respectively. Furthermore, CNAs were associated with previously reported gene expression alterations in adult male and female zebrafish. This study demonstrates that atrazine exposure can generate CNAs that are linked to gene expression alterations observed in adult zebrafish exposed to atrazine during embryogenesis providing a mechanism of the developmental origins of atrazine endocrine disruption.

1. Introduction

Structural genomic variation is common within the human genome and consists of multiple components such as single nucleotide polymorphisms (SNPs), tandem repeats, transposable elements, and structural alterations (deletions, duplications, and inversions) (Freeman et al., 2006; Stankiewics and Lupski, 2010). Of these, differences in genomic copy number are widely present and are the largest known genomic variation. Copy number changes can be either amplifications or deletions that can range in size from 50 base pairs (bp) to greater than a megabase (one million bp) (Freeman et al., 2006; Russo et al., 2015). Genomic copy number differences occur throughout the genome of healthy individuals and are generally referred to as copy number variants or CNVs as they are thought to be non-pathogenic. Currently, over 25,000 CNVs including 1000 large CNVs (greater than 50 kb) are now identified. However, non-recurrent CNVs can also contribute to various disorders and diseases including obesity, cancer, and neurological disorders including autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD), and schizophrenia, Alzheimer's disease and Parkinson's disease (Sebat et al., 2007; Stankiewics and Lupski, 2010; Valbonesi et al., 2015; Walsh et al., 2008; Zhang et al., 2013). Despite recent advances in genomic technologies, limited knowledge surrounds the generation of the disease associated CNVs, which are then referred to as copy number alterations or CNAs, and the risk factors involved. Furthermore, it is hypothesized that in addition to genetic factors, exposure to environmental contaminants can lead to CNA development (Adewoye et al., 2015; Arlt et al., 2014; Peterson and Freeman, 2014).

Endocrine disrupting chemicals (EDCs) are exogenous agents that disrupt endogenous hormone signaling (Swedenborg et al., 2009). EDCs are diverse in structure and are found in numerous products such as plasticizers, pharmaceuticals, and pesticides, making human exposure to these chemicals a likely event (Birnbaum and Fenton, 2003; Ma et al., 2010; Prins et al., 2007). Numerous challenges are identified and need to be overcome when aiming to understand the mechanisms of action of EDCs. Two primary challenges of EDCs are their characteristic non-monotonic dose-response and latency period between exposure and observable effects (Vandenberg, 2012). Furthermore, studies implicate that a developmental exposure to EDCs can alter genetic and

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epigenetic processes which can result in adverse health consequences later in life and in multigenerational and/or transgenerational effects (Anway and Skinner, 2006; Casati et al., 2012, 2013, 2015; Dolinoy et al., 2007; Martinez-Arguelles and Papadopoulos, 2015).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a pre-emergent herbicide that is applied throughout the Midwestern United States and other parts of the globe on a variety of agricultural crops including corn, sorghum grass, sugar cane, and wheat (Barr et al., 2007; Eldridge et al., 2008; Solomon et al., 2008). Due to atrazine's water solubility, mobility in soil, and long half-life, atrazine frequently contaminates potable water supplies and reaches levels above the maximum contaminant level (MCL) as set by the U. S. Environmental Protection Agency (EPA) of 3 parts per billion (ppb; μg/L) (U.S. EPA, 2002; Fraites et al., 2009; Rohr and McCoy, 2010). As such the European Union banned the use of atrazine in 2003 (European Commission, 2003; Sass and Colangelo, 2006). Numerous laboratory studies have shown that atrazine alters the neuroendocrine system primarily through the hypothalamus-pituitary-gonadal (HPG) axis (Cooper et al., 2000; Foradori et al., 2009, 2013; Weber et al., 2013; Wirbisky et al., 2016a). In addition, genetic, epigenetic, and cellular mechanisms altered by atrazine exposure are under investigation (Karmaus and Zacharewski, 2015; Kucka et al., 2012; Pogrmic et al., 2009, Pogrmic-Majkic et al., 2010, 2014; Wirbisky et al., 2016a,b).

The genotoxicity of atrazine has been under investigation since the early 1990s and has provided contradictory evidence. The majority of in vitro studies have been conducted in Chinese hamster ovary (CHO) and human lymphocyte cells. Positive studies indicate that atrazine elicits genotoxicity through chromosomal aberrations (CA), sister chromatid exchanges (SCEs), and increases in the coefficient of variation (CV) at a variety of concentrations and exposure periods (Biradar and Rayburn, 1995; Lioi et al., 1998; Rayburn et al., 2001; Taets et al., 1998). While contrasting studies do not demonstrate any significant genotoxicity (Dunkelberg et al., 1994; Kligerman et al., 2000a,b; Roloff et al., 1992; Surrallés et al., 1995; Zeljezic et al., 2006). In vivo studies conducted on a variety of animal models including fish, anuran, and rodent have also shown results indicating no evidence of genotoxicity (Adeyemi et al., 2015; Cavas, 2011; Freeman and Rayburn, 2004). While other studies reported increases in micronuclei, nuclear abnormalities, and DNA damage (Clements et al., 1997; Gebel et al., 1997; Tennant et al., 2001; de Campos Ventura et al., 2008).

While various in vitro and in vivo models have been utilized to investigate the genotoxicity of atrazine, this study utilized a zebrafish fibroblast cell line derived from AB wild-type embryos. This cell line is well characterized, routinely monitored for cytogenetic changes, and has been used in previous zebrafish cytogenetic studies (Freeman et al., 2007; Peterson and Freeman, 2014). In addition, the use of this zebrafish cell line will provide ease in moving into in vivo studies utilizing zebrafish embryos as the zebrafish is a strong complementary vertebrate model used throughout many biological disciplines. A finished genome sequence and a highly conserved genetic homology to humans permits translation of molecular mechanisms observed in the zebrafish to humans (de Esch et al., 2012; Howe et al., 2013). The development of array comparative genomic hybridization (aCGH) and next-generation sequencing has allowed for the detection of CNVs and CNAs throughout the genome (Russo et al., 2015). Due to an established CNV map for the zebrafish, studies have been completed investigating CNAs in cancer models and following chemical exposure (Brown et al., 2012; Chen et al., 2013; Freeman et al., 2009; Peterson and Freeman, 2014; Zhang et al., 2013).

In this study, zebrafish fibroblast cells were used to test the hypothesis that an atrazine exposure will cause genotoxicity through the generation of CNAs detectable with the use of aCGH technology. Although genotoxicity of atrazine has been investigated in past studies through the various endpoints previously mentioned, this is the first study to assess if atrazine exposure will result in genotoxicity by evaluating the generation of CNAs. Furthermore, in order to assess potential genetic mechanisms behind the developmental origins of health and disease hypothesis, which states that developmental chemical exposure can contribute to disease onset during adulthood, links between CNAs and previously identified gene expression alterations in adult male and female zebrafish exposed to atrazine during embryogenesis was assessed (Wirbisky et al., 2015, 2016a,b).

2. Materials and methods

2.1. Zebrafish fibroblast cell line

A zebrafish fibroblast cell line established from approximately 100 embryos of the AB wildtype zebrafish strain was used in this study and is described in Freeman et al. (2007).

2.2. Cytotoxicity assay

Atrazine (CAS #1912-24-9, Chem Service, West Chester, PA, 98% purity) stock (500,000 ppb or 2315 μM) was prepared using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). A cytotoxicity assay was completed following similar protocols as previously described (Freeman and Rayburn, 2006; Peterson and Freeman, 2014; Plewa et al., 2002). Briefly, cells were harvested from cell culture flasks following a standard trypsin protocol and cell concentration was determined. The cytotoxicity assay was set up in 96 well plates with 14,000 cells per well with appropriate amount of media and chemical stock to make up 1–1125 μM. Plate set-up included a first column blank and a second column negative control. Each plate contained four subsample wells per chemical concentration. Plates were placed in an incubator at 28 °C and 5% carbon dioxide for 72 h (the equivalent of 1.5 cell cycle lengths). After 72 h, cells were fixed in 50% methanol and stained with 1% crystal violet in 50% methanol, and excess crystal violet solution was washed from the plate. Absorbance was read on a microplate reader at 595 nm (SpectraMax M2, Molecular Devices). Readings from the four subsample wells of each test concentration was averaged. Four replicate plates (containing four subsample wells per chemical concentration) were completed and the average percent negative control values for each test concentration was plotted and fit to a sigmoidal curve using SigmaPlot (SigmaPlot 10.0) to determine appropriate test concentrations for copy number alteration (CNA) analysis. Test concentrations for aCGH were at the 60% negative control value and at concentrations where no impacts on cell confluency were observed.

2.3. aCGH analysis of copy number alterations

Zebrafish cells were exposed to 0, 0.463, 4.63, or 46.3 μM atrazine (100, 1000, or 10,000 ppb, respectively) or a control treatment (media with no additional chemical treatment). 7.5×10^5 cells were initially seeded into each petri dish ($n = 4$). After set-up, petri dishes were placed in an incubator at 28 °C and 5% CO_2 for 72 h (the equivalent of 1.5 cell cycle lengths). After 72 h, cells were harvested and genomic DNA was isolated following a standard phenol:chloroform isolation method as previously described (Freeman et al., 2009; Peterson and Freeman, 2014). A zebrafish specific oligonucleotide platform was designed based upon the Zv9 zebrafish genome build. A 2×400 K array containing 418,551 unique probes approximately 60 nucleotides in length with a median spacing of 2.942 kbp was constructed using SureDesign by Agilent Technologies (Agilent Technologies, Santa Clara, CA) with avoidance of standard masked regions and restriction sites (Table 1). Array CGH analysis was performed following manufacturer's protocol (Version 7.3) using a two color hybridization strategy. Briefly, 1 μg of test DNA and 1 μg of reference DNA were fluorescently labeled with Cy5 and Cy3, respectively. Dye incorporation was assessed using a NanoDrop ND 1000 spectrophotometer. Cy5 test DNA and Cy3 reference DNA were combined and hybridized to the array for 40 h at 67 °C. Following hybridization, arrays were washed according to