WATER CONTAMINATED WITH THE HERBICIDE ATRAZINE:

Alteration of Cancer Associated Tumor Protein
D52-like 1 (*tpd52l1*) Gene Using the Zebrafish Model

Student Author

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chemicals such as atrazine. Devang was selected for the yearlong Cancer Prevention Internship Program (CPIP) in 2015 where he investigated the effects of chemical exposure to cancer-associated genes. He also received a grant through the Purdue Center for Cancer Research in summer 2016 where he continued his research in the Freeman laboratory. Devang plans to pursue an MD and practice medicine following his postgraduation gap year where he will be working in a clinical research setting.

Mentor

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Abstract

Atrazine is a commonly used herbicide in the Midwestern United States. Currently, Indiana uses about 26% of its land to grow corn, and atrazine is used in ample amounts, resulting in contamination of drinking water supplies. The U.S. Environmental Protection Agency (EPA) has set the Maximum Contaminant Level (MCL) at 3 parts per billion (ppb) in drinking water, but the herbicide is often found at higher concentrations. However, data suggests that atrazine can cause adverse health impacts even at 3 ppb. Previous studies from our laboratory in the zebrafish model system have shown atrazine alters gene expression associated with neuroendocrine and reproductive system function, carcinogenesis, and cell cycle regulation and an increase in head size following an embryonic atrazine exposure at 0.3, 3, or 30 ppb. From these studies, it was shown that at 72 hours postfertilization (hpf; the end of embryogenesis), atrazine elicits alterations of the gene, tumor protein D52-like 1 (*tpd52l1*). Previous studies have identified *Tpd52l1* in cell proliferation and calcium signaling, along with regulating expression at the G2-M transition in breast cancer cells. The goal of this project was to characterize the expression of *tpd52l1* during development of the zebrafish, as well as determine developmental time-point specific gene alterations caused by the embryonic atrazine exposure. To characterize the expression of *tpd52l1* throughout embryogenesis, zebrafish embryos were bred and embryos were collected at 24, 36, 48, 60, and 72 hpf for RNA isolation. In addition, alteration in *tpd52l1* gene expression following atrazine exposure was assessed at the same developmental time points. Analysis by quantitative polymerase chain reaction (qPCR) showed consistent levels of gene expression throughout embryonic development ($p > 0.05$), but a significant increase at 36 hpf in the 0.3 and 3 ppb treatments and a significant decrease at 60 hpf was observed in all three atrazine treatments ($p < 0.05$). These findings indicate that the expression of *tpd52l1* is consistent throughout embryonic development but is altered by atrazine exposure at specific developmental time points, supporting the role of this gene in the observed adverse health outcomes.

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Keywords

atrazine, developmental exposure, embryogenesis, environmental contaminant, qPCR, toxicology, *tpd52l1*, zebrafish

INTRODUCTION

Atrazine is a commonly used herbicide in the Midwestern United States; its purpose is to prevent the growth of pre and postemergent broadleaf weeds in crops such as maize and on lawns. The benzene derivative structure allows for atrazine to remain in soil for up to 4 years, allowing atrazine to readily migrate into groundwater and contaminate our drinking supply (Crawford et al., 1998). In 2004, atrazine was banned in the European Union because of its persistent contamination into groundwater and has been rereviewed for health concerns by the U.S. Environmental Protection Agency (EPA) several times (Bethsass & Colangelo, 2006). Due to the abundant use of atrazine, there has been extensive research on this compound suggesting that it may have adverse effects on the endocrine system and throughout sexual development (Freeman, Beccue, & Rayburn, 2005). Due to these controversial effects of atrazine, the EPA has set a limit, the Maximum Contaminant Level (MCL), to 3 parts per billion (ppb; $\mu\text{g/L}$) in drinking water; however, traces higher than the MCL have also been detected. It has been suspected that even very low traces of atrazine can have harmful health effects (Hayes et al., 2002).

The most understood effect of atrazine is its activity as an endocrine disrupter. Chemicals, such as atrazine, that are classified as endocrine disruptors are known to interfere with the endocrine system in mammals, often times altering various hormone levels. These adverse effects can go beyond the organism itself and have effects on the progeny and subpopulations of the organism. Many of the well understood consequences of endocrine disruptors include: birth defects, transformed tissue function, metabolism alterations, cancerous tumors, and other developmental disorders (National Institute of Environmental Health Sciences, 2013). Functions of endocrine disruptors go beyond what has been researched since they can

alter anything throughout the body that is regulated by hormones. The most commonly studied effects of endocrine disruptors are their role in decreased reproductive potential, altered growth parameters, and cancer. The primary method of operation that affects the endocrine system is through nuclear hormone receptors, which are responsible for binding to lipid soluble steroid and thyroid hormones. Once bound, these receptors are able to bind directly to DNA and regulate the expression of a multitude of genes, causing a change in expression, through up or downregulation. Endocrine disruptors can mimic the hormones these receptors are responsible for binding, either as an inhibitor or activating agent, and, as a result, elicit a change in expression. While studies have shown that atrazine disrupts the endocrine system, mechanistic studies with atrazine have shown that this chemical does not have intrinsic estrogenic activity and does not bind to the estrogen receptor (Tennant et al., 1994). The mechanisms of atrazine toxicity are not well understood at this time, and Dr. Jennifer Freeman's laboratory at Purdue University has been investigating some of these modes of action.

A previous study in the Freeman laboratory provided insight into atrazine exposure during embryogenesis of the zebrafish (Weber et al., 2013). The study encompassed the developmental time points of 1 to 72 hours postfertilization (hpf; the end of embryogenesis) and exposure of atrazine at various concentrations (0, 0.3, 3, or 30 ppb) to understand the effects of atrazine during development. The transcriptomic study revealed alterations in expression in a couple hundred genes, some of which have known roles in carcinogenesis. Of these genes, expression studies through (quantitative polymerase chain reaction) qPCR and western blot were conducted and *cyp17a1*, *glol*, *sik2*, and *tpd52ll* displayed significant upregulation in gene and protein expression at 72 hpf (Weber et al., 2013). The role of these genes goes beyond carcinogenesis; they have shown connections to cell cycle regulation, cell signaling, and reproduction. These genes have homologs in humans (*CYP17A1*, *GLO1*, *SIK2*, and *TPD52LL*, respectively), making them especially important when attempting to understand their roles during development.

This follow-up study was built off previous studies in the Freeman laboratory, and we chose to specifically focus on further investigation of the gene, tumor protein D52-like 1 (*tpd52ll*). For this study, we utilized the zebrafish vertebrate model system. Zebrafish have grown in popularity since the 1990s in their use for developmental biology and toxicology (Kalueff et al., 2012). Due to their predictive model for human development, the zebrafish make for a highly effective

model organism through their *ex utero* development. Zebrafish are advantageous since they are small in size and have rapid development, which make them easy to dose with chemicals and provide a means to attain large sample sizes. Additionally, zebrafish have near-transparent embryos that provide visualization and manipulation during developmental stages so they may be exposed to atrazine and may be used for gene expression studies (Rasooly et al., 2003). Furthermore, it has been understood that certain aspects of zebrafish development occur at known time points throughout embryogenesis. The developmental time points chosen in this study occurred throughout embryogenesis and were 24, 36, 48, 60, and 72 hpf. At these time points, what occurs developmentally for zebrafish is well understood, and this can give insight on what altered gene expression may mean and what structures are involved (see Figure 1). For example, at 24 hpf it is known that the central nervous system structures are discernable and a heartbeat is present. At 36 hpf, the touch reflexes are present and there is blood circulation in the zebrafish embryo. By 48 hpf, organ morphogenesis is complete. By 72 hpf, the blood-brain barrier has formed and embryogenesis is complete. Knowing the key developmental activities at these time points provides a great understanding for using the zebrafish as a model organism to relate developmental events with observed molecular changes associated with chemical exposure. In addition, approximately 70% of the zebrafish genes have similar functions to human genes allowing for translation of gene expression changes observed in the zebrafish associated with chemical exposure to human development (Howe et al., 2013).

Figure 1. The zebrafish early developmental time course. The developmental changes that occur in zebrafish during embryogenesis (0-72 hpf) and early larval development (72-120 hpf).

METHODS

Zebrafish were maintained and dosed with atrazine following previously established laboratory protocols (see Figure 2; Wirbisky et al., 2016). Adult zebrafish (*Danio rerio*) of the wild-type AB strain were housed on a 14-hour light–10-hour dark cycle in a Z-Mod

System (Aquatic Habitats, Apopka, FL). The environment for the zebrafish was maintained at 28°C, with a pH range of 7.0–7.2, and conductivity range of 470–550 µS. Adult fish were fed brine shrimp and bred in cages; embryos were obtained and staged following established protocols (Wirbisky et al., 2015). Treatment groups of zebrafish were exposed to various concentrations of atrazine at 0, 0.3, 3, or 30 ppb (µg/L). These test concentrations represent 0.1 to 10 times the current regulated concentration in drinking water in the US. These concentrations were prepared using a stock solution of technical grade atrazine (98.1% purity) (CAS 1912-24-9; ChemService, West Chester, PA) and were prepared as described in prior studies (Weber et al., 2013; Wirbisky et al., 2015). Following exposure to the atrazine treatment, the larvae were rinsed with system water as previously described for experimental use (Weber et al., 2013). All protocols were approved by Purdue University's Institutional Animal Care and Use Committee.

Figure 2. The methodology for the experiments that were conducted. Zebrafish were bred. Fertilized embryos were collected and allowed to develop under control conditions (fish water) in Experiment 1 or treated with atrazine (0, 0.3, 3, or 30 ppb) in Experiment 2. During the experiments, embryos were placed in petri dishes with 50 embryos per dish per each biological replicate. A total of six biological replicates were included in each experiment for each condition at each developmental time point (that is, six different plates for each). Embryos were collected in each experiment at the appropriate developmental time points. Embryos from each petri dish were pooled together in a tube and homogenized in Trizol. RNA was extracted from each sample of pooled embryos and then converted to complementary DNA (cDNA). Finally, quantification of gene expression was carried out using qPCR and data statistically analyzed to determine differences.

This research consisted of multiple parts. First, information was gathered and collectively recorded to include any roles the genes, identified with altered expression from Weber et al. (2013), play in carcinogenesis. From this analysis, *tpd52l1* was chosen as the target gene for this study. The expression of *tpd52l1* was quantified using qPCR to attain (1) a developmental expression profile and (2) identify alterations associated with an embryonic atrazine exposure. The developmental gene expression profile was first investigated for *tpd52l1* with the goal of this analysis to determine how the expression of *tpd52l1* changed throughout embryonic development of the zebrafish (through 72 hpf). The developmental time course analysis was performed in a control condition, using only fish water. This analysis allowed for the establishment of the normal expression profile of this gene. Next, the expression of *tpd52l1* was determined following atrazine exposure by measuring gene expression using qPCR from embryos collected at 24, 36, 48, and 60 hpf. Since a previous study evaluated the expression of this gene at 72 hpf and identified an increase in expression for both the gene and protein, this time point was not included in this experiment. At each of the time points listed, atrazine concentrations of 0, 0.3, 3, or 30 ppb were included with the MCL in drinking water in the US being 3 ppb.

Quantification of gene expression was conducted using established protocols to obtain developmental time point data for *tpd52l1* (Weber et al., 2013). To do this transcript analysis for all biological repetitions, 50 embryos were collected into each petri dish, comprising a single replicate. Each developmental time point (24, 36, 48, 60, and 72 hpf) included six biological replicates, requiring six plates, each with 50 embryos, for a total of 1,500 embryos. Similarly, in the atrazine experiment, following exposure to atrazine at 0, 0.3, 3, or 30 ppb, the embryos were collected from six replicate plates for a total of 1,200 embryos per time point (24, 36, 48, and 60 hpf). The atrazine treatment experiment involved the use of 4,800 total embryos. During collection, each replicate of embryos was transferred to a 1.5 mL tube and homogenized in Trizol (Life Technologies, Carlsbad, CA). The homogenized samples were then flash frozen in liquid nitrogen and stored at -80°C until RNA isolation. RNA isolation was done using the RNeasy kit (Qiagen), and cDNA was synthesized using the First Strand Synthesis Kit (Life Technologies) which follows established protocols (Peterson and Freeman, 2009). Gene expression was then quantified using qPCR for the target gene: *tpd52l1*. Similar to previous studies from our laboratory, several genes were first tested to determine a reference gene that had consistent expression during the developmental time course

Primer	Primer sequence
<i>tpd52l1</i> Forward Sequence	GCTAATATGGAGCCCAGACAAC
<i>tpd52l1</i> Reverse Sequence	ACTCATTCTCCATTTCCTCTCG
β -actin Forward Sequence	CTAAAACTGGAACGGTGAAGG
β -actin Reverse Sequence	AGGCAAATAAGTTTCGGAACAA

Table 1. Primer sequences for *tpd52l1* and β -actin.

Figure 3. *tpd52l1* expression during the embryonic developmental time course. Statistical analysis revealed no differences in expression throughout the embryonic developmental time course ($n = 6$, $p = 0.0714$). Error bars indicate standard deviation.

and following atrazine exposure. β -actin was found to maintain a stable level of expression throughout embryogenesis in zebrafish and was not changed following atrazine exposure. Thus, β -actin was used as the reference gene in this study to compare relative changes of expression. Forward and reverse primers for the target genes were designed using the Primer3 website (see Table 1). qPCR was conducted following similar methods as previously done in our laboratory (Peterson et al., 2010). The qPCR analysis used SSoAdvanced SYBR Green Supermix (Biorad) to determine gene expression levels (gene of interest/ β -actin). The cycling parameters for the qPCR were: 3 minute incubation phase at 95°C, 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s and are further explained in a previous study (Wirbisky et al., 2015; Peterson et al., 2010).

Finally, all statistical analysis was performed using SAS software with significance being established at $\alpha < 0.05$ for an analysis of variance (ANOVA) test. A least significant difference (LSD) post hoc test was performed if significance was met for the ANOVA test to establish when sets of data were different from each other, similar to other studies in our laboratory (Peterson et al., 2010; Wirbisky et al., 2015; Wirbisky et al., 2016).

Figure 4. *tpd52l1* expression following atrazine exposure. (A) Statistical analysis revealed no change in expression following atrazine exposure at 24 hpf ($n = 6$, $p = 0.2360$). (B) Statistical analysis revealed changes in expression at 0.3 ppb and 3 ppb following atrazine exposure at 36 hpf ($n = 6$, $p = 0.0253$). (C) Statistical analysis revealed no change in expression following atrazine exposure at 48 hpf ($n = 6$, $p = 0.3040$). (D) Statistical analysis revealed changes in expression at 0.3, 3, and 30 ppb following atrazine exposure at 60 hpf ($n = 6$, $p = 0.0246$). Error bars indicate standard deviation. * $p < 0.05$.

RESULTS

Gene analysis using qPCR for the zebrafish target gene *tpd52l1* was first evaluated through the embryonic developmental time course at 24, 36, 48, 60, and 72 hpf. The developmental time course for *tpd52l1* did not exhibit any significant changes throughout embryogenesis ($p = 0.0714$; see Figure 3). Second, embryos were exposed immediately after fertilization to atrazine concentrations of 0, 0.3, 3, or 30 ppb. Embryos were collected 24, 36, 48 and 60 hpf for qPCR analysis. Following atrazine exposure at 24 hpf, *tpd52l1* did not have any significant changes in gene expression for all concentrations ($p = 0.2360$; see Figure 4A). Alternatively at 36 hpf, a significant increase in expression at 0.3 and 3 ppb atrazine was observed ($p = 0.0253$; see Figure 4B). However, at 48 hpf there was yet again no significant change in

expression ($p = 0.3040$; see Figure 4C). Finally, at 60 hpf, there was a significant decrease in expression at all three concentrations of 0.3, 3, and 30 ppb ($p = 0.0246$; see Figure 4D).

DISCUSSION

As mentioned previously, atrazine is one of the most commonly used herbicides in the US, particularly the Midwest. This makes understanding toxicity of this agrochemical very relevant to where we are living as it is frequently applied in crops such as corn to prevent weeds and improve yields. Since atrazine has a stable chemical structure, it can stay in the soil for up to 4 years. Combining this chemical characteristic with atrazine's solubility in water, atrazine is commonly reported to contaminate drinking water sources in regions where this agrochemical is applied. Although the EPA has set the MCL of atrazine to 3 ppb in drinking water, there is evidence that higher concentrations are detected, and that even the set MCL concentrations may have adverse health effects. Atrazine exposure through daily drinking water is suggested to be linked to increased incidences of some cancers, altered development of reproductive organs, compromised immune development, and other adverse health effects in humans, but much more work is needed to confirm these links (Cragin et al., 2011; Freeman et al., 2011). Although we know there is a link to atrazine and various adverse health effects, it is still not understood if atrazine is confidently a carcinogen nor how it mechanistically plays a toxic role during human development.

One possible mode of its adverse health effects is through its actions on altering gene expression. In this study, we observed that during the developmental time course in control conditions the expression of *tpd52ll* does not change. However, when exposed to atrazine there are significant changes in expression of *tpd52ll* at 36 hpf for embryos exposed to 0.3 and 3 ppb. In addition, at 60 hpf, a significant change in expression of *tpd52ll* occurred for embryos exposed to 0.3, 3, or 30 ppb atrazine. It is interesting that there is no change in expression at 48 hpf and indicates expression changes are specific to developmental stages. Also, why there is an increase in expression at 36 hpf followed by a decrease in expression at 60 hpf is not well understood. One hypothesis is that the decrease at 60 hpf following the increase at 36 hpf could be a method to compensate for changes in gene expression levels that occurred earlier in development. This hypothesis is further supported considering a significant increase in expression in *tpd52ll* was also observed in a previous study in all three atrazine treatments at 72 hpf (Weber et al.,

2013). There could also be a relationship between the developmental stages of embryogenesis that could be altered due to *tpd52ll* expression alterations. Consequently, this could play a role in fluctuating the developmental progression in embryogenesis for time points of 36, 60, and 72 hpf. Moreover, the expression changes in *tpd52ll* could impact how touch reflexes or blood circulation are affected at 36 hpf or impact the completion of morphogenesis for the 60 hpf alterations. In addition, from previous and ongoing studies, we know that atrazine also changes expression of other genes too and the interactions and relationships between the expression of *tpd52ll* with these other gene targets needs further study (Weber et al., 2013; Wirbisky et al., 2015; Wirbisky et al., 2016). Overall, additional experiments need to be performed to better understand how exactly atrazine plays a mechanistic role in changing expression of the transcriptome and how these gene expression changes translate to adverse health effects.

By understanding how atrazine affects zebrafish at the genomic level, an organism with a fully sequenced genome, we may be able to better understand how atrazine alters genes in humans. Due to the relationship between endocrine disrupting chemicals and increased risk of cancer later in life, it is important to study the potential role of carcinogenesis in atrazine exposed specimens. The observation of altered expression in specific genes that have been shown to play a role in multiple adverse health effects may be the start to understanding atrazine toxicity

With approximately 41% of the US being used for farmland, and a much higher percentage in Indiana and the Midwest as a whole, the use of atrazine is very prominent. Among the 319 million people residing in the US, approximately 60% of the population is readily exposed to atrazine through drinking water (Adgate et al., 2001). Even with the EPA setting the MCL of atrazine to 3 ppb, laboratory studies indicate there may be adverse health effects associated with this exposure concentration. The research is currently limited to confidently conclude what concentration would prevent any harmful health effects. Many studies have attempted to correlate atrazine chemical exposure in adults to cancer, especially in the agricultural community (Freeman et al., 2011). These studies indicate a potential association with atrazine exposure and a variety of different cancers, which include thyroid, breast, lung, bladder, ovarian, and other cancers; however, none of these were shown to have statistical significance in their association to atrazine (Freeman et al., 2011). However, not much research has been focused on developmental chemical exposures and the association with cancer.

Knowing that atrazine is an endocrine disruptor, this chemical should be studied closely as there has been research indicating that endocrine disrupting chemicals influence the developmental origin of numerous diseases, one of which being cancer (Birnbaum & Fenton, 2003). The Developmental Origins of Health and Disease (DOHaD) paradigm has recognized the importance of understanding how developmental exposures may have an enduring adverse later-in-life health effect. This makes it especially important to investigate how certain chemicals that we are commonly exposed to, such as atrazine, may impact our future health (Uauy et al., 2011). Keeping this in mind, the approach of this project was to understand the developmental effects of atrazine on *tpd52ll* expression, a single gene that has exhibited altered expression at 36, 60, and 72 hpf. Additional research must be conducted to conclude anything about what atrazine may do to humans, but this study provides the grounds to better understand how atrazine mechanistically plays a role in altering gene expression and the associated adverse health outcomes.

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