Expression of rnf14 and ttc3 are Consistent During Zebrafish Embryonic Development with Expression Only Altered for ttc3 at 60 hpf with Atrazine Exposure

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Abstract

Atrazine is a herbicide commonly applied to crops in the Midwest part of the United States. The chemical moves into drinking water sources after rainfall events. The United States Environmental Protection Agency (EPA) set the Maximum Contaminant Level (MCL) at 3 parts per billion (ppb) in drinking water, but even these levels are suspected to cause adverse health effects. Developmental exposure to atrazine is reported to increase birth defects and result in endocrine disruption. Previous studies in our laboratory have shown that genes have altered expression in the zebrafish after an embryonic exposure to atrazine. Two of these genes, rnf14 and ttc3, were further tested in this study. rnf14 is a gene that interacts with the androgen receptor. When expression of rnf14 is increased, it is expected to cause abnormal cell growth and lead to carcinogenesis. ttc3 is expected to be involved with neuronal proliferation and differentiation when expression is increased. Overexpression of ttc3 leads to strong inhibition of neurite extension. The purpose of this experiment was to first determine how gene expression changed over the zebrafish embryonic developmental time course at 24, 36, 48, 60, and 72 hpf. Then, gene expression was assessed following atrazine exposure at 0, 0.3, 3, or 30 ppb at developmental time points. The data showed that the rnf14 and ttc3 expression is steady throughout embryogenesis with no significant change (p=0.05) and that changes caused by atrazine exposure only occurs at the time points of 60 (p=0.0099; n=6) and 72 hpf for ttc3 and only at 72 hpf for rnf14.

Introduction

Atrazine

Atrazine is a herbicide used to control the growth of broadleaf weeds (1). It is the second most common herbicide used on crops in the United States with the annual use being approximately 76 million pounds (2). Atrazine gets into water sources, like groundwater and streams, when it is washed from the fields with rainfall. Atrazine has a slow chemical breakdown in water sources at a half-life of greater than three months, and it has been found in 20 Superfund sites in the United States. Studies done have shown that there may be a link between atrazine and some types of cancer. It has also been shown to cause other adverse health effects since it is an endocrine disrupting chemical. Due to all of these factors, atrazine is commonly found as a contaminant in drinking water. The European Union banned the use of atrazine in 2004 to keep it out of their drinking water. The Environmental Protection Agency (EPA) of the United States has set the Maximum Contaminant Level (MCL) at 3 parts per billion (ppb, µg/L) in drinking water. However, it is suspected that atrazine still causes adverse health effects at lower contaminant levels (1).

rnf14

rnf14 was chosen as the gene to analyze because previous studies have shown that expression changes from atrazine exposure at 72 hpf (3). This gene is expected to cause cell growth and carcinogenesis when expression is increased. rnf14 is a coactivator that stimulates androgen receptor stimulated gene expression in the prostate. The growth in the prostate can help promote prostate cancer. When there is a dominant negative mutant of rnf14 it inhibit the growth brought on by an androgen receptor; therefore inhibiting prostate growth (4).

ttc3

ttc3 also showed gene expression changes from atrazine exposure at 72 hpf, which is why it was analyzed (3). This gene is expected to be involved with neuronal proliferation and differentiation when expression is increased. Overexpression of the gene leads to strong inhibition of neurite extension. The dosage imbalance alters neuronal differentiation, which may contribute to the mental retardation in Down syndrome (5). Due to this, ttc3 may be involved in carcinogenesis, particularly cancers that people with Down syndrome are more at risk of getting, like leukemia (5).

Zebrafish Model System

Zebrafish are a vastly used model organism in studies for the fields of developmental biology, genetics, and toxicology. The advantages of using this species are their rapid ex utero embryonic development, near transparency of their embryo that permits easy visualization and manipulation through early developmental stages, prolific breeding, and similar genetic structure to humans. The rapid ex utero embryonic development allows zebrafish embryos to be dosed during development with ease making them ideal candidates for studying the effects of atrazine exposure on gene expression.

Materials and Methods

Profiling rnf14 and ttc3 Expression Throughout Development

Table 1. rnf14 and ttc3 expression analysis with quantitative PCR using the zebrafish model. For the developmental time course, 50 zebrafish embryos were collected at each time point of 24, 36, 48, 60, and 72 hpf. In each Petri dish the embryos were pooled to obtain a single replicate and six total plates were collected at each developmental time point to obtain six biological replicates. When collecting for the atrazine experiments, we used 6 replicate plates for each treatment of 0, 0.3, 3, and 30 ppb atrazine. For collection, embryos were transferred and homogenized in Trizol. After homogenization, samples were frozen in liquid nitrogen and stored at -80°C prior to RNA isolation. Total RNA was isolated using Trizol and RNeasy kit (Qiagen). cDNA was synthesized using the First Strand Synthesis Kit (Life Technologies). qPCR was then done using the SYBR Green Express (BioRad) to determine gene expression levels of rnf14 and ttc3. The expression levels were compared to the relative expression of the reference gene, β-actin, for analysis and an ANOVA was completed to statistically test the variance (p=0.05).

Table 2. rnf14 and ttc3 expression analysis with quantitative PCR using the zebrafish model. The developmental time course, 50 zebrafish embryos were collected at each time point of 24, 36, 48, 60, and 72 hpf. In each Petri dish the embryos were pooled to obtain a single replicate and six total plates were collected at each developmental time point to obtain six biological replicates. When collecting for the atrazine experiments, we used 6 replicate plates for each treatment of 0, 0.3, 3, and 30 ppb atrazine. For collection, embryos were transferred and homogenized in Trizol. After homogenization, samples were frozen in liquid nitrogen and stored at -80°C prior to RNA isolation. Total RNA was isolated using Trizol and RNeasy kit (Qiagen). cDNA was synthesized using the First Strand Synthesis Kit (Life Technologies). qPCR was then done using the SYBR Green Express (BioRad) to determine gene expression levels of rnf14 and ttc3. The expression levels were compared to the relative expression of the reference gene, β-actin, for analysis and an ANOVA was completed to statistically test the variance (p=0.05).

Conclusions

Overall, there was a significant change for expression of ttc3 following atrazine exposure at 60 hpf, but there was no significant differences observed for its expression during the developmental time course or following the atrazine exposure at 24, 36, or 48 hpf. There was also no significant differences observed for expression of rnf14 during the developmental time course or following atrazine exposure at 24, 36, 48, or 60 hpf. This data indicates expression alterations in ttc3 and rnf14 associated with an embryonic atrazine exposure at the time points analyzed were specific to the end of embryogenesis at 60 and 72 hpf for ttc3 and at 72 hpf only for rnf14 as shown in a previous study (3).

References

4. RNF14 Finger Protein 14 [Homo Sapiens (Human)]. (November 2015). National Center for Biotechnology Information.