Effects of Exposure to Atrazine on Retinoid Signaling in Zebrafish

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ABSTRACT

Atrazine is a widely used herbicide developed for use in range and pastureland. It is present in many surface waters, contaminating nontarget organisms due to its persistence. In this study, the effects of acute exposure to atrazine on retinoid signaling were investigated in zebrafish. Zebrafish embryos were exposed to atrazine from 6 hours post-fertilization (hpf) to 120 hpf. The contents of retinal and retinoic acid were decreased significantly. The mRNA expression levels of retinal dehydrogenase (*raldh2*), retinol dehydrogenase (*rdh1*), retinol binding protein (*rbp1a*), retinoic acid receptor subunit (*raraa*), and cellular retinoic acid binding protein (*crabp1a* and *crabp2a*) were significantly reduced, which indicated that retinoid signaling was interrupted. However, the transcriptional levels of five opsin genes (*zfrho*, *zfuv*, *zfred*, *zfblue*, and *zfgr1*) were increased. These results indicated that exposure to atrazine could inhibit retinoid signaling and impair the eye development of zebrafish larvae.

INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamine-s-triazine) has been widely used to controlling broadleaf weeds and grasses in agriculture and submerged vegetation in stagnant or slow-running waters (Solomon, Giesy et al. 2013). Atrazine is a selective systemic herbicide, acts as a photosynthesis inhibitor, which can be absorbed by leaves and roots. It is translocated acropetally in the xylem and accumulated in the apical meristems (Görge and Nagel 1990, Cole 1999). Atrazine belongs to the most significant water pollutants

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in rain and limnic, marine, and ground water (Tasli, Patty et al. 1996), where aquicolous organisms are affected. Due to the persistence of atrazine, especially in soils under anaerobic or denitrifying conditions, and in some aquatic systems (Topp, Gutzman et al. 1995), chronic exposure are nearly lifelong time (Hussein, El-Nasser et al. 1996). Adverse effects have been observed in ecosystems affected by atrazine contaminated run-off, with biological effective levels persisting for several weeks (Pratt, Melendez et al. 1997).

Atrazine caused damage to the fish in physiological disturbances, including disarrangements of osmoregulation, increased respiration, decreased re# exes, and inhibition of the acetylcholinesterase in blood serum and brain (Gluth and Hanke 1985, Hussein, El-Nasser et al. 1996); increased renal excretion of sodium, potassium, chloride, and proteins (Santa Maria, Vilas et al. 1986, Fischer-Scherl, Veeser et al. 1991); the gill epithelium (Andrews 1996); necrosis of kidney endothelial cells and renal hematopoietic tissue (Wiegand, Krause et al. 2001, Plhalova, Blahova et al. 2012, Glisic, Hrubik et al. 2014). The behavior of zebrafish is changed at an environmentally relevant concentration of 5 μg/L atrazine (Steinberg, Lorenz et al. 1995). A bioaccumulation factors of 19 is found in zebrafish embryos, due to the lipophilicity of atrazine, the chorinons providing no protection against the the herbicide (Wiegand, Pflugmacher et al. 2000, Wiegand, Krause et al. 2001).

Retinoid is related to a wide range of physiological processes, including vision, immunity, cell growth, differentiation, embryogenesis and reproduction (Ross and Zolfaghari 2004, Napoli 2012, Gu and Cui 2015). The processes of

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mobilization of the retinyl ester into retinol and further delivered to target tissues are highly regulated (Napoli 1996, Quadro, Blaner et al. 2004, André, Ruivo et al. 2014, Gu and Cui 2015). During fish embryonic development, retinol binds to the plasma retinol binding protein monomer (Palace and Werner 2006, André, Ruivo et al. 2014), which then been transported from the yolk to various target organs including eyes (Wei 2003, Bastien and Rochette-Egly 2004). Retinol is transported by cellular retinol binding protein type I (CRBP I) in the eyes. Retinol is converted to retinoic acid (RA) by retinol dehydrogenase and retinal dehydrogenase (Dobbs-McAuliffe, Zhao et al. 2004). RA is an important signaling molecule for photoreceptor development in the visual systems of vertebrates (Prabhudesai, Cameron et al. 2005).

The objectives of this research were to investigate the effects of atrazine exposure on retinoid signaling and the potential impacts on the eye development. Zebrafish embryos were treated with various concentrations of atrazine, and retinoid profiles in the larvae were measured. The transcriptions of genes that were involved in retinoid transport and metabolism were also determined.

MATERIALS AND METHODS

Chemicals

Atrazine, DMSO, standards for retinol, retinal and retinoic acid were purchased from Sigma Aldrich (St Louis, MO, USA). Chemicals used for retinoid measurement were of HPLC grade.

Zebrafish maintenance and atrazine treatment

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Wild-type (AB) zebrafish (*Danio rerio*) were maintained and raised under standard conditions (Gu, Yang et al. 2014, Zhai, Gu et al. 2014). Developmental stages of zebrafish embryos were characterized as described previously (Kimmel, Ballard et al. 1995).

One hundred zebrafish embryos were incubated in 10-cm dishes containing 10-ml atrazine exposure solutionn (0, 4, 10 and 20 mg/L) from 12 hours post-fertilization (hpf) under standard condition. The selected exposure concentrations were based on a previous study (Wiegand, Krause et al. 2001). Control and treated embryos received 0.01% (v/v) DMSO. The embryos were collected, immediately frozen in liquid nitrogen and stored at -80 °C for subsequent gene, protein and retinoid analysis at 120 hpf. Each experimental and control group contained three replicates.

The animal protocol for this research was approved by the Animal Care and Use Committee of Hubei Province in China and by the Institutional Animal Care and Use Committee of Hubei University of Technology (Approval ID: Keshuizhuan 0800).

RT-PCR

Total RNA was extracted from 20 homogenized zebrafish embryos exposed to each treatment using TRIzol (Invitrogen) according to the manufacturer's instruction, and single-stranded cDNA was systhesized as described previously (Gu, Yang et al. 2013, Zhong, Yuan et al. 2014).

Real-time qPCR

Real-time quantitative PCR (qPCR) was performed as described

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previously (Song, Li et al. 2012, Yang, Gu et al. 2015). SYBR green master mix (Toyobo) was used for PCR in a real-time detection system (Bio-Rad). The primer sequences of target genes were designed using the Primer 5 program. Relative gene transcription levels were determined after normalizing to the mRNA content of reference gene β-actin. The primer sequences for the target genes were listed in Table S1.

Atrazine extraction and analysis

The atrazine in water samples were extracted with solid-phase extraction columns as described previously (Muldoon and Stanker 1997). Briefly, water samples were passed through glass-fiber filters to get rid of the particulates and sonicated in 40% methanol (v/v) for 30 min. The water samples were then purified by the preconditioned SPE columns. The collected extracts were further cleaned.

Retinoid measurement

Retinoid profiles were extracted and determined by HPLC as described previously (Karpińska, Mikołuć et al. 2006). Briefly, 500 zebrafish larvae were collected and sonicated. An external standard was added to measure retinoid recovery. The suspension was vortexed vigorously for 30 min and the supernatant was transferred to a new tube, dried under nitrogen and resuspended in methanol for subsequent analysis by HPLC.

Statistical analyses

All data were analyzed with SignaPlot 12.5 for statistical significance and were reported as mean ± standard error (SEM). Differences in gene expression

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were analyzed with the unpaired Student's t test. Significance levels of $P < 0.05$ and $P < 0.01$ are denoted in graphs by a single asterisk (*) or double asterisks (**), respectively. Representative results from at least three independent biological replicates are shown unless stated otherwise.

RESULTS

Concentration of atrazine in water

The detected actual atrazine concentrations in the water samples were 4 ± 0.2, 10 ± 0.6, 20 ± 1.1 μg/L in the nominated (4, 10 and 20 μg/L) exposure groups, respectively. No atrazine was detected in the control water samples.

Developmental toxicity of atrazine treatment

The survival rates of embryos exposed to 0, 4, 10 and 20 μg/L of atrazine were 92 ± 1.2 %, 87 ± 4.2 %, 80 ± 3.2 % and 78 ± 5.6 %, respectively. These results showed a significant decrease after exposure to 5 μg/L atrazine, however, the atrazine exposure had no overt affect on the malformation rates. The embryos appeared developmental delay before 72 hpf, but there was no obviously significant difference in hatching after 96 hpf.

Retinoid profiles after treated with atrazine

Compared to the controls, no significant changes were observed in the atrazine treated groups. However, retinal contents were significantly reduced in the 5 μg/L atrazine exposure group (Table 1). RA contents were lower in the atrazine-exposed group compared to the controls (Table 1). These reductions in RA contents occurred in a dose-dependent manner (Table 1).

Transcriptional changes in atrazine-exposed zebrafish embryos

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The mRNA level of *crbp1a*, which is involved in intracellular retinol and retinal transport, was significant decreased (Figure 1). The retinol dehydrogenase, *rdh1*, that transforms retinol to retinal, was also reduced, while *raldh2*, which encodes the retinal dehydrogenase that is responsible for converting retinal to RA, was significantly increased (Figure 1). The mRNA expression levels of the two isoforms of the cellular retinoic acid binding proteins (*crabp1a* and *crabp2a*), were down-regulated in atrazine-treated embryos (Figure 1). Gene transcriptions of *raraa* was decreased in a dose-dependent manner by atrazine exposure (Figure 1). The transcription of *zfrho, zfuv, zfred, zfblue* and *zfgr1*, that encode rhodopsin and ultraviolet, red, blue and green opsins, were up-regulated in a dose-dependent manner (Figure 2).

DISCUSSION

The disruptive effects of exposure to atrazine on retinoid contents and the potential negative effects of atrazine on eye development in fish largely remain unknown. In this study, zebrafish larvae were treated with various concentration of atrazine. In the laboratory research, high concentrations of toxicants usually have been used to investigate the potential mechanisms of toxicity (council 2010). This study showed that acute exposure to atrazine disrupted retinoid signaling and may affect eye development in zebrafish larvae.

The total retinol level did not change in the zebrafish. A previous study shows that retinol concentrations in the skin, an important target tissue, remain constant, which likely reflects compensatory transfer from fat tissues to maintain

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physiological functions (Herkenne, Alberti et al. 2008). Different responses to toxins may be explained by exposure time. Therefore, exposure to atrazine does not appear to interfere with retinol content in the larvae during this short period of exposure. In zebrafish, retinol is present in high quantities in eggs (Solomon, Carr et al. 2008), suggesting that the retinol content in eggs is sufficient for embryonic development. When exogenous stress in introduced, the storage form of retinol can be mobilized and excreted into the plasma to restore homeostasis (Nieman, Romero et al. 2013).

The mRNA expression levels of the retinol binding protein (*rbp4*) did not change after atrazine exposure, indicating normal delivery of plasma retinol to target organs. However, the concentration of retinal was reduces, may due to the inhibition of the *CRBP I* and *RDH* genes, which demonstrated that the retinol cellular transport pathway was interrupted and resulted in few retinol being converted to retinal. (Lara-Ramírez, Zieger et al. 2013). The expression pattern of *crbp1a* mRNA could serve to regulate retinal metabolism and RA production in retina development and the maintenance of retina function (Greiling and Clark 2012).

A previous study reports that RA has a negative feedback mechanism through repression of raldh2 to regulate its production (Greiling and Clark 2012). In this study, the mRNA level of *raldh2* was increased, which can be explained as a compensatory response to lower levels of RA in order to produce more RA. The transcription of *cyp26a* is independent of endogenous RA production via *raldh2* in zebrafish larvae (Dobbs-McAuliffe, Zhao et al. 2004). However, *cyp26a*

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mRNA levels remained unchanged in this study, which suggested larval zebrafish possessed a constant capability for RA degradation.

RA acts as an embryogenesis signal by controlling the expression of genes involved in several systems, including the central nervous system, limb and eye, and embryos deficient in RA demonstrate defects in these organs (Dobbs-McAuliffe, Zhao et al. 2004). The RA levels were down-regulated in a atrazine dose-dependetn manner, indicating that the transcription of RA responsive genes may have been changed. Considering the importance of RA to early eye development, decreases in RA levels might have adverse implications for early stage photoreceptor development in zebrafish.

CRABPs regulates the access of RA to its nuclear receptor RAR, and hence play an important role in transcription initiation of target genes during RA targeting and metabolism (Sharma, Saxena et al. 2005). In developing zebrafish, *raraa* is expressed across the hindbrain, tailbud and eye, and this overlaps with *raldh2* and *crabp2a* expression (Hale, Tallafuss et al. 2006). Therefor, the decrease of raraa gene in this study suggested down-regulation of RA tissue concentrations.

RA is an important signaling molecule for photoreceptor development in the visual systems of vertebrates (Cvekl and Wang 2009). RA can modulate gene transcription required for eye morphogenesis by binding and activating RARs and RXRs nuclear receptors (Novák, Beníšek et al. 2008). Various animal and cell culture models have also indicated an important role for RA in regulating the development of retina photoreceptors. In zebrafish, application of exogenous

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RA during optic primordial development causes duplication of the retina (Hyatt, Schmitt et al. 1992). Therefor, the decreased RA levels ovserved in the present study might impact the photoreceptor development. In the present study, five zebrafish opsin genes (*zfrho, zfuv, zfred, zfblue*, and *zfgr1*) that encode rhodopsin, ultraviolet, red, blue and green opsins, respectively, were all significantly up-regulated. These results may suggest a compensatory response to the reduced supply of chromophore retinal and the disturbance of RA signaling in eye photoreceptors in order to increase visual acuity to light. The acute atrazine exposure concentrations used in the zebrafish were higher than those measured in surface water. Therefore, further studies are warranted to examine the effects of longer-term exposures to atrazine at environmentally relevant concentrations on retinoid homeostasis and visual function in fish.

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Table 1 Retinoid profiles (ng/mg protein) in zebrafish embryos after exposure to various concentration of atrazine untile 120 hpf

	0 μg/L	4 μg/L	10 μg/L	20 μg/L
Retinol[a]	150.3 ± 12.5	189.4 ± 15.6	145.2 ± 3.7	180.8 ± 5.8
Retinal[a]	98.3 ± 2.6	102.5 ± 6.9	113.8 ± 15.2	59.3 ± 11.7
Retinoic acid[a]	17.9 ± 2.4	14.5 ± 1.7	4.7 ± 0.9*	N.D. **

N.D., not detected.

[a] Values are mean ± SEM of three replicates.

* Significant differences compared to the control group (Two-way ANOVA, followed by Student's t test: $p < 0.05$).

** Significant differences compared to the control group (Two-way ANOVA, followed by Student's t test: $p < 0.01$).

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Figure 1 Gene expression patterns of *crbp1a, rdh1, raldh2, crabp1a*, *crabp2a* and *raraa* in zebrafish embryos treated with various concentrations of atrazine until 120 hpf. Values are mean ± SEM of three replicates. Significant differences compared to the control group are indicated by asterisks. *$p < 0.05$, ** $p < 0.01$.

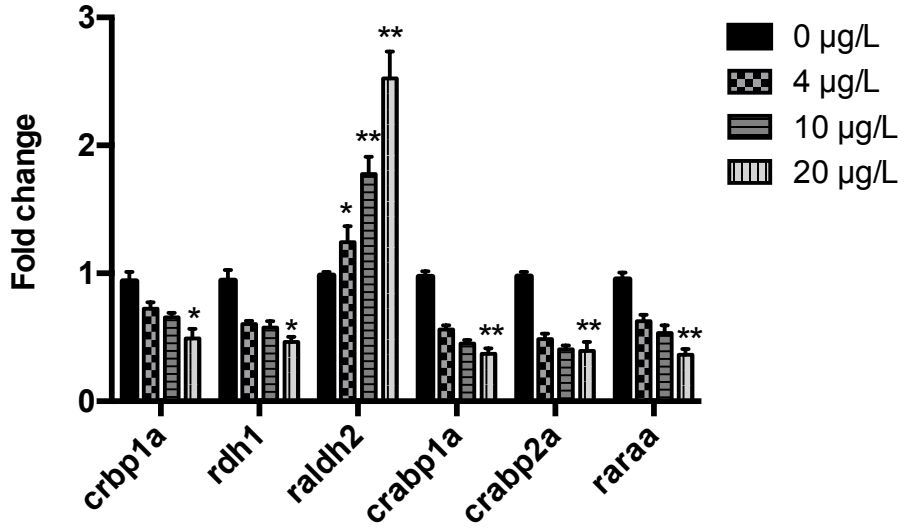

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Figure 2 Opsin gene transcriptions of *zfrho, zfuv, zfred, zfblue* and *zfgr1* in zebrafish embryos treated with various concentrations of atrazine untile 120 hpf. Values are mean ± SEM of three replicates. Significant differences compared to the control group are indicated by asterisks. *$p < 0.05$, ** $p < 0.01$.

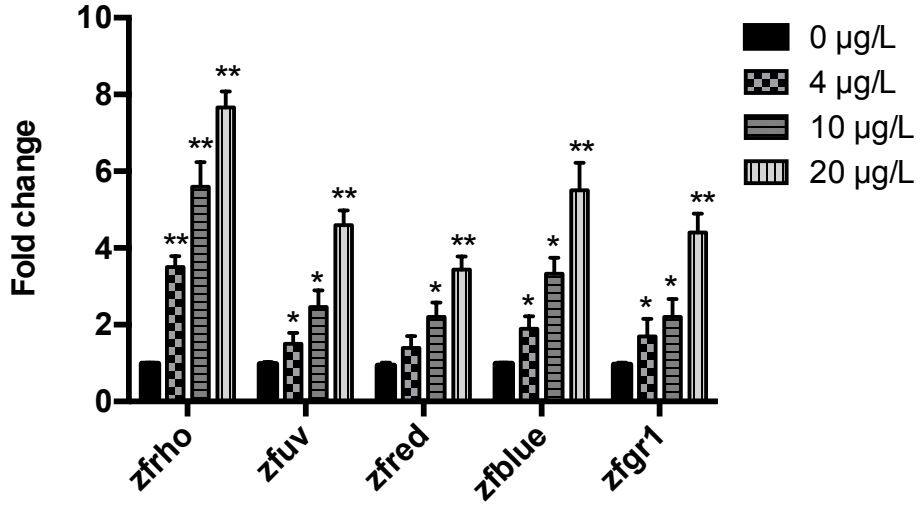

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Table S1 Primers used in this study.

Gene name	Sequences (5'-3')
rbp4	F-GGCCATAGTGACACAGAAGAA
	R-GCTCCTTGATGCAATGGTTTAG
crbp1a	F-TCTCTCTCTCTCTCTCTCTCTCT
	R-GGTCCATCCAGTGACATTCTT
rdh1	F-GGAGGATACTGCCTGTCCAA
	R-CTCCAGCGTTTCTTCAGGTC
raldh2	F-CATTTTTGCAGATGCTGATTTTG
	R-CAAAGATACGGGAACCAGCAGT
cyp26a	F-AGTGGCCAGCATCAGTGAGAA
	R-GAACGCCCTCATAATGGCCT
crabp1a	F-TACGAGAATGAGGGCGAATC
	R-CAGCAATGGCTGAGAATTGA
crabp2a	F-CGACAGAACGGAAGATGGAT
	R-TCACTGAGGTGGACGTCTTG
raraa	F-GAGAACTACACGCTGAGCCC
	R-CCATAAATCCACATCCAGGG
zfrho	F-AGCCATGAACGGTACAGAGG
	R-CTTCTTGTGCTCGATGGTGA
zfuv	F-CCTAGCAGGCTTCATTTTCG
	R-AAGGGTTTGCAGATGACCAC
zfred	F-CTGCACTGTGGTCGTTGACT
	R-GAGGCCAGTATCTGCTCCAG
zfblue	F-CAGCTTACAGCCCTTTCCTG
	R-CAAGTTGGAAATGGCAAGGT
zfgr1	F-AGGCTGAGAGGGAAGTGACA
	R-TGTTAAGCATGCAGCTACGG
β-actin	F-ATGAAGATCCTGACCGAGAGA
	R-TCAAAGTCAAGGGCCACATAG

AUTHOR CONTRIBUTIONS

WZ conceived and designed this work. YF, HL designed and performed the experiments. YF, HL and PX analyzed data and contributed manuscript writing. WZ wrote and revised the manuscript.

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