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Long-term exposure to the non-steroidal anti-inflammatory drug (NSAID) naproxen causes thyroid disruption in zebrafish at environmentally relevant concentrations

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ABSTRACT

The presence of trace levels of pharmaceuticals is an emerging issue impacting the aquatic ecosystem. Naproxen (NPX) is a nonsteroidal anti-inflammatory drug (NSAID) that has been frequently detected in aquatic environments worldwide. Recently, concerns regarding endocrine disruption by NSAIDs have increased; however, their effects on the thyroid system have yet to be understood. In this study, zebrafish were utilized to evaluate the thyroid-disrupting effects of NPX. After a 60-day exposure to various concentrations of NPX (0.1, 1, 10 and 100 μg/L), the body length and weight of the zebrafish were significantly decreased. The decrease of cytochrome P450 gene expression and enzyme activity might inhibit the metabolism of NPX, which might result in the significant bioconcentration in zebrafish. Thyroid hormone (TH) analysis showed that both triiodothyronine (T3) and thyroxine (T4) levels were substantially decreased. Gene transcription expressions along the hypothalamic-pituitary-thyroid (HPT) axis were also markedly affected. Significant down-regulation of *dio1*, *dio2*, *nis*, *nkx2.1*, *pax8*, *tg*, *tpo*, *trβ* and *ttr* levels, along with the stimulation of the *tshβ* gene, were also observed in exposed fish compared to controls. Western blot analysis indicated that expression of the TTR protein was significantly decreased, which coincides with the results of the gene expression analysis. Collectively, our observations show that NPX increases the risk of bioconcentration and thyroid disruption in zebrafish. Given the continued increasing consumption and emission of pharmaceuticals, thyroid disruption should be considered when assessing the aquatic risk of long-term exposure to environmentally relevant concentrations of pharmaceuticals.

1. Introduction

Over the past few years, pharmaceuticals have become a unique contaminant group of emerging concern (Brausch et al., 2012; Li, 2014; Wu et al., 2014; Comber et al., 2018). The increasing consumption plus incomplete elimination in wastewater treatment plants leads to continuous emission of pharmaceuticals into the surface water (Kolpin et al., 2002; Li, 2014; Comber et al., 2018). Although pharmaceuticals are usually detected at trace levels, chronic exposure can cause numerous detrimental effects on aquatic organisms, due to their inherent bioactivities and environmental pseudo-persistence (Zenker et al., 2014; Fabbri and Franzellitti, 2016; Ebele et al., 2017). One major concern is their ability to interfere with the endocrine system and disrupt homeostasis (Ebele et al., 2017). However, the endocrine-disrupting pharmaceuticals are not limited to hormonal drugs, such as steroids. Many nonsteroidal anti-inflammatory drugs (NSAIDs) predominating in the analyses of environmental samples (Kosjek et al., 2005; Mezzelani et al., 2016), have been reported to cause endocrine disruption of vertebrates in recent studies (Ebele et al., 2017; Ji et al., 2013)

The thyroid is an important component of the endocrine system that exerts significant control over the development and growth of fish, particularly in their early life stages (Nelson and Habibi, 2009). Although numerous xenobiotics have been shown to have a thyroid-disrupting effect on fish, the current understanding of the effects of NSAIDs on aquatic organisms is limited. However, clinical studies have reported that several NSAIDs influence thyroid homeostasis in humans (Bishnoi et al., 1994), and recent *in silico* research indicated that NSAIDs have the potential to bind to thyroid receptors (Zloh et

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al., 2016). Aquatic vertebrates have enzymes and receptors of the thyroid system that are very similar to those of humans (Evans, 1993; Huggett et al., 2003). For example, thyroid homeostasis in vertebrate species is controlled primarily by the hypothalamic-pituitary-thyroid (HPT) axis, which regulates the synthesis, secretion, transport and metabolism of thyroid hormones (THs) (Cheng et al., 2017; Jia et al., 2016; Yu et al., 2011). Taking into consideration the relatively high conservation of the thyroid axis between humans and fish (Segner, 2009), it is reasonable to hypothesize that NSAIDs may induce similar thyroid disruption in fish by influencing the HPT axis.

Naproxen (NPX) is one of the most frequently detected NSAIDs in the aquatic environment. The residue concentration of NPX in a Canadian sewage treatment plant (STP effluents) was found to be 7.6 μg/L (Brun et al., 2006), with a maximum level of 33.9 μg/L (Metcalfe et al., 2003). Similar levels of residue were also found in Sweden (Bendz et al., 2005). NPX has been detected in surface water at concentrations of up to 32 μg/L in Pakistan (Kwak et al., 2018) and 4.5 μg/L in China (Zhao et al., 2010). In addition, high bioconcentrations of NPX have been detected in wild fish *via* steady accumulation from the environment, resulting in an increased chronic risk (Brozinski et al., 2011 and 2013; Brown et al., 2007). Previous studies have shown that NPX can negatively affect the behavior, development and expression of antioxidant genes of fish (Sehonova et al., 2017; Neal and Moore, 2017; Stancová et al., 2015) and modulate sex hormone production in H295R cells (Kwak et al., 2018). However, to date the available chronic ecotoxicity data on NPX are not sufficient (Straub and Stewart, 2007) and the long-term effects of NPX at environmentally relevant concentrations are still unclear, especially its endocrine effects on nontarget organisms.

Based on the results of previous studies, we hypothesized that long-term exposure to environmental concentrations of NPX would result in bioconcentration and cause thyroid disruption in fish. Therefore, in this study, we used zebrafish larvae as the model organism for a 60-day exposure experiment. The objectives of this study were to 1) investigate the bioconcentration of NPX and 2) elucidate the thyroid-disrupting effect and associated mechanisms in zebrafish. Particularly, enzyme activity and gene expression involved in the metabolism of NPX were studied, and thyroid hormone concentrations and transcription levels of genes along the HPT axis were analyzed. The findings of this study will complement the current understanding of the risks of NPX and emphasize the importance of considering thyroid disruption following long-term exposure.

2. Materials and methods

2.1. Chemicals and reagents

Naproxen (purity >98%), beta-naphthoflavone (*β*NF), ellipticine, 7-ethoxyresorufin, dicumarol, resorufin and 3-amino-9-ethylcarbazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of NPX (1000 mg/L) was prepared in methanol and stored under dark conditions at 4 °C. Fluorinated ethylene propylene (FEP) centrifuge tubes were purchased from Thermo Fisher Scientific (Rochester, NY, USA), and Florisil SPE cartridges were obtained from Anpel Scientific Instrument Co., Ltd. (Shanghai, China). All other reagents utilized in this study were of HPLC grade and purchased from Tedia Company Inc. (Fairfield, OH, USA).

2.2. Zebrafish maintenance, embryo collection and larvae husbandry

Zebrafish maintenance and embryo collection were carried out according to the protocol described in our previous study (Xu et al., 2018). Briefly, adult zebrafish were maintained at 27 ± 1 °C under a 14/10 h light/dark photoperiod in a flow-through system and fed *Artemia nauplii* twice daily. Adult male and female zebrafish (the male/ female ratio was 2/1) were separated by nylon nets in spawning boxes overnight. Spawning was induced in the morning when the light was turned on. The collected embryos were washed with 10% Hank's solution and examined under an inverted dissecting microscope (Leica Micosystems, Wetzlar, Germany). Fertilized embryos (embryos with normal development to the blastula stage) were allowed to hatch and were fed laboratory-grown paramecia and rotifers for 14 days post-hatching (dph), after which they were fed *A. nauplii*. Healthy larvae with stable survivorship at 21 dph were used for the subsequent NPX exposure experiments, because the larval survivorship prior to 20 dph was not stable, even without any treatment, which may mask the possible effects of pollutant exposure (Chou et al., 2010).

2.3. Experimental protocol

The test concentrations of NPX in this study were 0.1, 1, 10 and 100 μg/L. NPX in methanol stock solution was dissolved in test water (the same charcoal-filtered water used for zebrafish maintenance) to give a final methanol concentration of 0.01%. Solvent controls were set with the equivalent methanol concentration in water. Each experimental group consisted of 40 larvae zebrafish (21 dph) exposed in 50 L of water and were replicated in three aquariums (n = 3). The exposure solutions were renewed completely every 24 h. During the exposure, the fish were fed *A. nauplii* twice daily. The exposure was stopped on the 60th day (before sexual maturation) (Maack and Segner, 2003). At the end of the exposure, the survival rates were recorded, and the body weight (mg) and length (mm) of each fish were determined to calculate the condition factor indices ($K = [\text{weight} \times 100]/\text{length}^3$) (Jones et al., 1999).

2.4. Determination of NPX concentration in water and fish

The concentrations of NPX in the exposure solutions were measured at the beginning of the exposure (T_0), after 12 h of exposure (T_{12}) and after 24 h of exposure (T_{24}), before water renewal. A water sample (5 mL for each group, except for the 0.1 μg/L group, where the volume was 20 mL) was acidified (pH < 2.0) with 1 mol/L of HCl, and the internal standard ibuprofen-d3 was added at 10% of the NPX nominal concentration. The pretreated sample was extracted by mechanical shaking for 1 min with 1:1 (v/v) *n*-hexane/ethyl acetate (1 mL each time). The extraction was repeated twice. The total organic phase was evaporated under a stream of nitrogen gas, and the residue was subjected to a derivatization procedure, as described in a previous study (Shin and Oh, 2012).

To determine the concentration of NPX in the fish, six fish of each replicate were homogenized and extracted using the method described above, and the crude extract was dissolved in 0.5 mL of *n*-hexane. The solution was extracted twice with acetonitrile (0.5 mL each time) to remove the lipids, it was then evaporated to dryness and reconstituted in 1 mL of methylene chloride/ acetone/ methanol (7.5:1.5:1). The dissolved extract was cleaned by passing it through a Florisil SPE column, which was conditioned with 5 mL of methylene chloride and 5 mL of methylene chloride/acetone/ methanol (7.5:1.5:1). The analyte was then eluted with 1.5 mL of methanol, evaporated to dryness, and reconstituted in 0.1 mL of *n*-hexane: ethyl acetate (1:1) for derivatization. After the derivatization, the solution was extracted twice with 1 mL of *n*-hexane/ethyl acetate (1,1). The

organic layer was evaporated to dryness and dissolved in 1 mL of *n*-hexane for analysis (Fig. S1).

The concentration of NPX was quantified using a gas chromatography mass spectrometry (GC/MS) system, according to the method described by Shin and Oh (2012). The precision, recovery and detection limit of the analytical techniques were optimized by spiking the water and fish samples (3 replicates each) with NPX. The recovery rates of NPX were 95 ± 3.7% and 86 ± 5.2% in the water and fish samples, respectively. The limit of quantification (LOQ) and the limit of detection (LOD) were defined as the concentrations of NPX having a signal-to-noise (S/N) value of 10 and 3, respectively on GC/MS. The LOQ and the LOD values of NPX in zebrafish in the present study were 12.5 ng/m and 3.4 ng/mL, respectively.

The bioconcentration of NPX was evaluated using the bioconcentration factor (BCF), which is expressed as $BCF = C_{fs}/C_{ws}$, where C_{fs} and C_{ws} are the concentrations of NPX in the fish and water samples, respectively.

2.5. Thyroid hormone assay

The thyroid hormone assay was carried out according to the method described in our previous study (Xu et al., 2019b). Briefly, after the 60-day exposure to NPX, the juvenile fish were anesthetized in 0.03% MS-222 (3-Aminobenzoic acid ethyl ester methane sulfonate) before tail cutting. Immediately, their blood was collected with glass capillaries saturated in phosphate buffered saline (PBS) buffer containing heparin (1000 unites/mL). The blood samples from sixteen fish from each group were pooled to form one replicate (approximately 40 μL). The sample was then centrifuged at 3000 rpm for 20 min, and the plasma was collected in an Eppendorf tube (EP) and stored at −80 °C until analysis. The total T4 and T3 levels were measured using enzyme-linked immunosorbent assay (ELISA) test kits (Wuhan EIAab Science Co. Ltd. Wuhan, China) following the manufacturer's instructions.

2.6. Cytochrome P450 monooxygenase activity assay

The P450 monooxygenase activity was measured in terms of the enzyme 7-ethoxyresorufin-O-deethylase (EROD) activity in the zebrafish gills (Jönsson et al., 2009; Xu et al., 2019a). Briefly, two gill arches were placed in duplicate wells of a 12-well tissue culture plate containing an HC buffer. The HC buffer was then replaced with 0.5 mL of a reaction buffer containing 7-ethoxyresorufin (1 μM), dicumarol (10 μM), and 0.2% DMSO (volume/volume, v/v) in an HC buffer. The gill preparations were preincubated under continuous shaking at 20 °C, and after 10 min the buffer was replaced with 0.7 ml of fresh reaction buffer. After 10 min of incubation, 0.2 ml of the buffer in each well was sampled and transferred to a 96-well plate. The fluorescence was determined at the wavelengths of 544 nm (ex) and 590 nm (em) in an FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

2.7. Quantitative real-time PCR

Five zebrafish from each treatment were over-anesthetized with MS222. Zebrafish were kept on ice during the entire procedure. Their brains and livers were rapidly removed and rinsed with ice-cold 0.68% physiological saline solution, and then homogenized using TRIzol reagent (TaKaRa, Japan). Total RNA quantification and verification were carried out as previously reported (Xu et al., 2019b). Reverse-transcription reactions to synthesize cDNA were performed according to the manufacturer's protocols. The quantitation of target genes (Table S1), using the SYBR Green PCR kit, was performed on the Mastercycler ep realplex (Eppendorf, Hamburg, Germany). The RT-RCP conditions were as follows: initial denaturation at 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. With the most stable expression, rpl8 was selected as the reference gene to normalize the expression of the target genes. The mRNA levels were normalized to the corresponding rpl8 value and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For each exposure concentration, samples from three biological replicates were measured. Five technical replicates of each measurement were obtained.

2.8. Protein extraction and Western blot analysis

Protein extraction and concentration determination were performed with a BCA protein assay kit (CWBio, Beijing, China), according to the manufacturer's instructions. Briefly, zebrafish livers (20 juvenile fish, n = 3) from each treatment were homogenized in 0.5 mL of a lysis buffer containing proteinase inhibitors (1%, v/v), phosphatase inhibitors (0.1%, v/v) and phenylmethanesulfonyl fluoride (PMSF) (1%, v/v). The samples were then centrifuged at 1000*g* for 10 min at 4 °C. The supernatants were collected and stored at −80 °C before Western blot analysis. Protein concentrations were measured using the Bradford method (Kruger, 2009), and Western blot analysis was performed according to the technique described in previous studies (Tu et al. 2016a and 2016b). Briefly, protein fragments were separated by gel electrophoresis, followed by transfer to a polyvinylidene difluoride membrane. The membrane was probed with primary antibodies against TTR (1500; Abcam, Cambridge, UK) and stained with horseradish peroxidase (HRP)-conjugated secondary antibodies. It has been verified that the sheep TTR antibody is reactive and suitable for zebrafish studies (Zhu et al., 2014). The protein expression levels were quantified by densitometry of the chemiluminescence signal, and the results were normalized to GADPH expression using Quantity One v4.6 software (Bio-Rad, Hercules, CA, USA).

2.9. Data analysis

The Kolmogorov-Smirnov test was used to examine the normality of the data, and the homogeneity of the variances was analyzed by Levene's test. The differences between the control and exposure groups were evaluated by one-way analysis of variance (ANOVA) and followed by LSD tests using SPSS 12.0 software (SPSS Inc., USA). A p value <0.05 was considered statistically significant. All data were expressed as the mean ± standard deviation (SD). The histogram was plotted using Origin 8.5 software (OriginLab Corporation, Northampton, MA, US).

3. Results and discussion

3.1. NPX concentration in exposure solution

The mean measured concentrations of NPX were between 95.3 and 107.1% of the nominal concentrations within 24 h (Table S2), indicating that the NPX concentrations in the exposure solutions were stable during the entire experiment, due to the regular renewal of the exposure solutions.

3.2. Growth and survival of zebrafish

The survival and growth parameters of the zebrafish after the 60-day exposure are shown in Table 1. Compared to the control group, the survival rate, body length and weight were not significantly different in the 0.1 and 1 μg/L groups. However, the survival rates were reduced by 5% and 7.5% in the 10 and 100 μg/L groups, respectively. Similar reductions were also found in length and weight of the zebrafish in the NPX exposure groups. The length was significantly reduced from 18.3 mm to 17.1 mm and from 18.3 mm to 16.2 mm in the 10 and 100 μg/L groups, respectively, while the weight of the zebrafish was significantly reduced from 88.9 mg to 76.4 mg and from 88.9 mg to 63.9 mg in the 10 and 100 μg/L groups, respectively.

Previous studies have suggested that NSAIDs have a developmental toxicity to fish in their early life stages (Sehonova et al., 2017; Stancova et al., 2014). Subchronic exposure to an NPX and tramadol hydrochloride mixture may influence the hatching, developmental rate, morphology and histopathology of common carp embryo-larvae (Sehonova et al., 2017). Although a 35-day exposure to a mixture of ibuprofen, diclofenac, and carbamazepine showed no toxic impairment of *Tinca tinca* at environmentally relevant concentrations (0.02–0.2 μg/L), a significant increase in mortality and malformations and a reduction in weight and length were found at a concentration of 60 μg/L (Stancova et al., 2014). This developmental toxicity might be induced by oxidative stress that suggested by Stancová et al., who found a significant effect of NPX on the gene expression of antioxidant enzymes in zebrafish (Stancová et al., 2015). In addition, another NSAID drug ibuprofen was also found to have the ability to modulate the hormone production and related gene transcription of the hypothalamic-pituitary-gonad (HPG) axis in zebrafish and thus could cause adverse effects on the reproduction and development of offspring (Ji et al., 2013). Similarly, NPX showed a significant influence on the survival and growth of zebrafish at an even lower concentration (10 μg/L) after a 60-day exposure in this study. These results indicate that chronic exposure could significantly increase the developmental toxicity risk of NPX in aquatic organisms.

3.3. Bioconcentration of NPX in zebrafish

The bioconcentrations and BCF values of NPX in the zebrafish after the 60-day exposure are shown in Fig. 1 and Table S3. No NPX was detected in the control group. The bioconcentrations ranged from 252.65 to 400.67 ng/g in the 0.1 to 10 μg/L groups (Fig. 1). In the 100 μg/L group, the bioconcentration significantly increased to 2052.69 ng/g. The BCF values deceased with the increasing exposure concentrations, with the highest BCF value of 1684 in the lowest concentration exposure group (0.1 μg/L) and the lowest value of 20.6 in the highest concentration exposure group (100 μg/L) (Fig. 1 and Table S3).

Bioconcentration of NSAIDs in aquatic organisms has been found in previous studies (Brown et al., 2007; Fick et al., 2010; Bhandari and Venables, 2011; Cuklev et al., 2012; Molina-Fernandez et al., 2017). With short-term exposure, NSAIDs have little potential for bioconcentration in fish (Bhandari and Venables, 2011; Nallani et al., 2011). For example, after a 48-h laboratory exposure to 5–100 μg/L ibuprofen, the highest BCF value in the bluntnose minnow (*Pimephales notatus*) was only 1.3 (Bhandari and Venables, 2011). Similar BCFs (0.08–1.4) were also found in the fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*) after exposure to 250 μg/L ibuprofen for 28 days, followed by a 14-d depuration phase (Nallani et al., 2011). However, high plasma BCFs of NSAIDs (*e.g.*, ibuprofen, diclofenac and NPX) were reported in rainbow trout from sewage effluents in Sweden (Fick et al., 2010). In addition, the concentrations of diclofenac, NPX, and ibuprofen in the bile of the fish were approximately 1000 times higher than those found in the lake water that received the treated municipal wastewater (Brozinski et al., 2013). Similar to the results of these field investigations, significant bioconcentrations and high BCF values of NPX in zebrafish were found in our study, which might be partly attributable to the longer exposure time. In addition, the decreasing BCF values of NPX with increasing exposure concentrations are also consistent with previous studies (Fick et al., 2010; Cuklev et al., 2012). These results indicate that long-term exposure to NSAIDs could pose high bioconcentration risks in aquatic organisms, even at low environmental concentrations.

3.4. CYP450-related gene expression and enzyme activity

Cytochrome P450 enzymes have been reported as the primary contributor that responsible for the metabolism of pharmaceuticals in fish (Burkina et al., 2015; Ribalta and Sole, 2014). Therefore, we hypothesized that the bioconcentration of NPX in zebrafish might be related to the suppressed detoxification enzymes involved in the metabolism. In this study, gene expression of CYP1A and CYP3A and enzyme activity of ethoxyresorufin-O-deethylase (EROD) were utilized to study the NPX metabolization in zebrafish. As shown in Fig. 2A and B, after a 60-day exposure, the expressions of CYP1A and CYP3A were significantly decreased in all of the NPX exposure groups. For example, the expression of CYP1A decreased by 0.64- and 0.46-fold in the 0.1 and 100 μg/L groups, respectively, compared to the control. Similarly, the expression of CYP3A decreased by 0.72- and 0.35-fold in the 0.1 and 100 μg/L groups, respectively, compared to the control. The EROD activities in the fish gills were suppressed in all of the NPX exposure groups (Fig. 2C). An 0.83- and a 0.59-fold decrease of EROD was observed in the 0.1 μg/L and 100 μg/L groups, respectively.

CYP2C9 and CYP1A2, which belong to CYP450 family, together accounted for the majority of NPX metabolism in human liver (Miners et al., 1996). Aquatic toxicity studies also indicated that the activity of CYP450 played a key role in the formation of NSAID metabolites in fish (Gomez et al., 2011; Thibaut and Porte, 2008; Ribalta and Sole, 2014). However, there may be different kinds of CYP enzymes involved in the metabolism of NPX in fish. For exam-

Table 1
Mean growth, survival, and condition indices (±SD) of zebrafish after a 60-day exposure to NPX (n = 3).

Concentration (μg/L)	Survival (%)	Length (mm)	Weight (mg)	Condition factor (K)
Control	85.0	18.3 ± 0.9	88.9 ± 19.3	1.44 ± 0.08
0.1	85.0	18.0 ± 1.0	86.5 ± 12.1	1.43 ± 0.10
1	82.5	17.5 ± 0.8	82.2 ± 22.4	1.51 ± 0.08
10	80.0*	17.1 ± 0.7*	76.4 ± 18.6*	1.52 ± 0.12
100	77.5*	16.2 ± 0.8*	63.9 ± 15.4*	1.50 ± 0.11

Means with superscript asterisk (*) designations within columns are significantly different ($p < 0.05$).

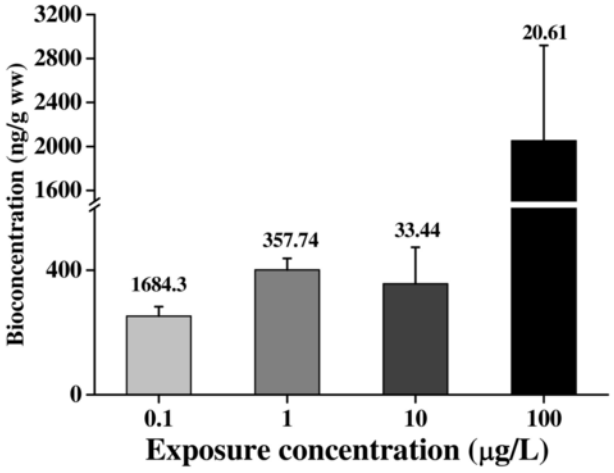

Fig. 1. Bioconcentrations in zebrafish after exposure to naproxen for 60 days. The results are the mean ± SD of three replicate samples (six fish of each replicate). No NPX was detected in the control group. BCF values are present at the top of columns.

ple, the metabolic pathway of NSAID ibuprofen in rainbow trout (*Oncorhynchus mykiss*) was found to be through CYP1A isozymes (Gomez et al., 2011; Thibaut and Porte, 2008). In addition, the exposure to NSAID diclofenac could inhibit the EROD activity and the CYP3A-mediated reaction in *Trachyrincus scabrous*, *Mora moro* and *Amphilophus rostratus* (Gomez et al., 2011; Ribalta and Sole, 2014). Similarly, in this study, we found that the CYP1A and CYP3A transcriptional levels and EROD activity were inhibited after long-term exposure to NPX. Although metabolites of NPX metabolites, which were found in rainbow trout in previous studies (Brozinski et al., 2011; Gomez et al., 2011), were not measured in this study, the significant enrichment of NPX in zebrafish is consistent with the suppression of detoxification enzymes involved in its metabolism. These results might, to some extent, explain the significant bioconcentration of NPX in zebrafish, even at environmentally relevant concentrations.

3.5. Content of THs in zebrafish

The changes in T3 and T4 levels caused by the 60-day NPX exposure in zebrafish are shown in Fig. 3. A concentration-dependent decrease in T4 level was observed. The T4 level was significantly decreased from 72.3 ng/g in the control group to 54.4 ng/g in the 100 μg/L group (Fig. 3). Likewise, a significant decrease in T3 level was also observed in the 10 and 100 μg/L exposure groups compared to the controls. The T3 level was decreased from 7.0 in the control group to 5.5 and 5.1 ng/g in the 10 and 100 μg/L exposure groups, respectively.

THs control diverse cellular processes, such as cellular differentiation and metabolism, in all vertebrates throughout their life. They are particularly important to fish in the early life stages, because proper TH signaling controls normal growth, development, differentiation and metabolism (Power et al., 2001). Many previous studies have found that the influence on TH levels in the early life stages induced by toxicants can lead to significant developmental toxicity in zebrafish (Cheng et al., 2017; Tu et al., 2016a; Xu et al., 2019b). Although the literature on thyroid disruption by pharmaceuticals in aquatic organisms is limited, there are recent reports indicating that some NSAIDs may have thyroid-disrupting effects in fish (Saravanan et al., 2014; Zloh et al., 2016). The T4 and T3 levels decreased in *Cirrhinus mrigala* after exposure to two NSAIDs, clofibric acid and diclofenac, for 35 days; however, diclofenac showed no influence on the T4 level after a short-term exposure (96 h) (Saravanan et al., 2014). The reduction of TH levels in zebrafish found in our study indicates that the side effect of NPX found in clinical studies may be a valuable reference in fish and that NPX may have a similar influence

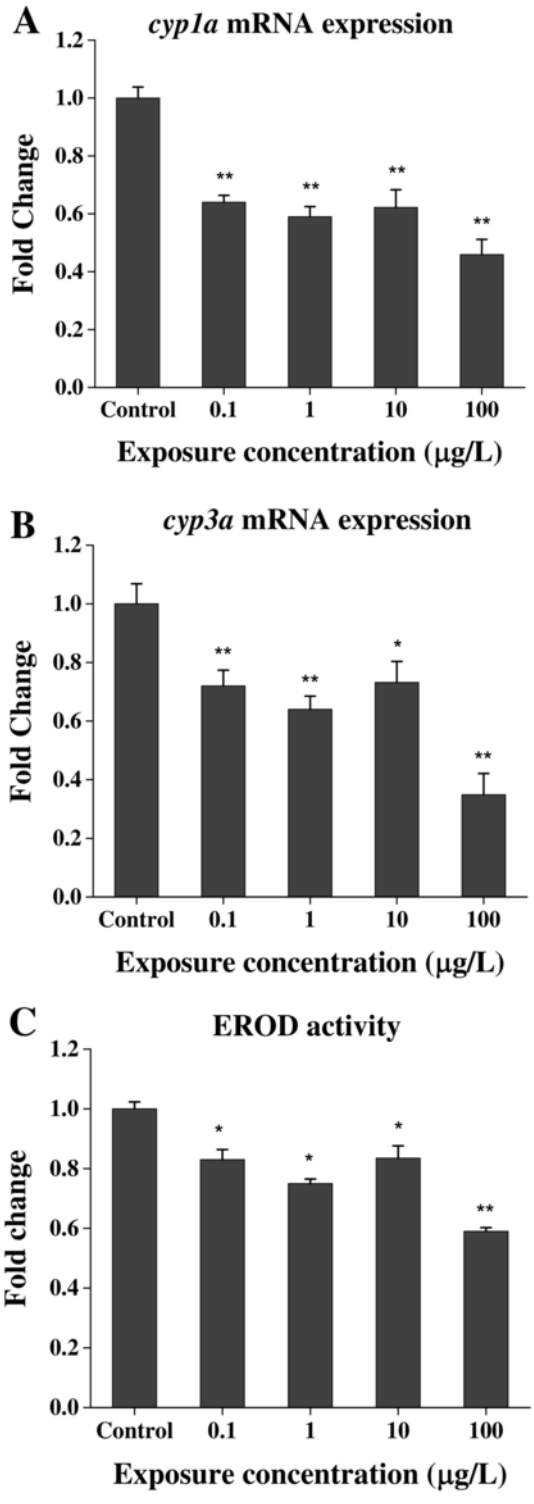

Fig. 2. Relative expression of *cyp1a* (A) and *cyp3a* (B) in zebrafish (five zebrafish of each replicate) and relative EROD activity in the gills of zebrafish (C) after exposure to naproxen for 60 days. The results are the mean ± SD of three replicate samples. * indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the control.

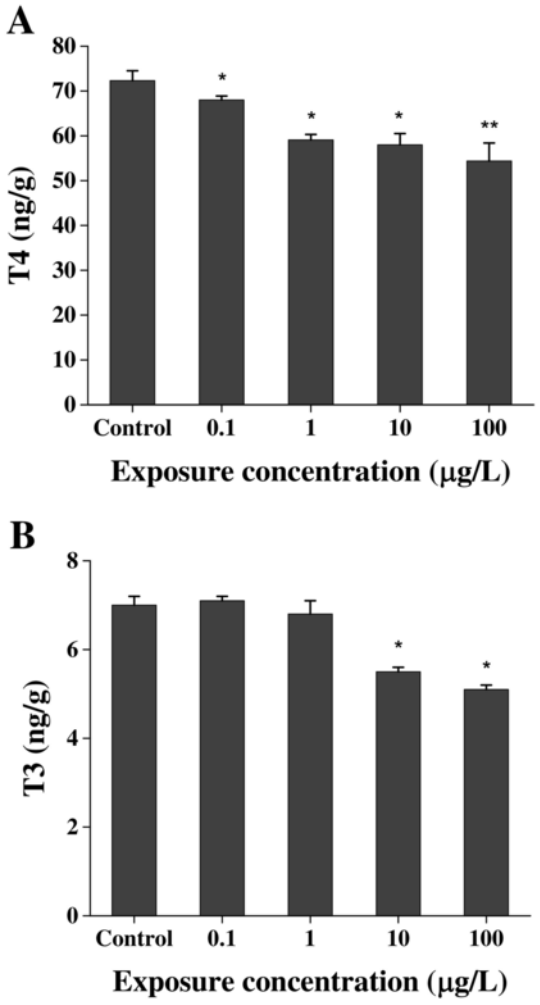

Fig. 3. Levels of T4 (A) and T3 (B) in zebrafish (sixteen fish of each replicate) after exposure to naproxen for 60 days. Values are expressed as the mean ± SD of three replicate samples. * indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the control.

on the thyroid systems of other vertebrates. The results are consistent with the thyroid-disrupting effects caused by other NSAIDs after long-term exposure (Saravanan et al., 2014; Zloh et al., 2016). Concerning the growth inhibition found in this study, thyroid disruption caused by long-term exposure to NPX may play an important role in the developmental toxicity of zebrafish.

3.6. Gene transcription profile along the HPT axis

Thyroid homeostasis results from a multiloop system controlled by the HPT axis. The influence on TH levels in zebrafish caused by NPX exposure could thus be associated with the change in gene transcription along the HPT axis. As shown in Fig. 4, the mRNA expression of key genes associated with the HPT axis was assessed. The expression of most genes was downregulated after exposure to NPX for 60 days compared to control levels. For instance, the transcriptional levels of *nkx 2.1* were significantly downregulated compared to controls at NPX concentrations higher than 0.1 μg/L, while the transcriptional levels of *dio2*, *nis*, *pax8*, *tg*, *tpo*, *trβ*, *ttr and ugt1ab* genes decreased significantly by 0.36-, 0.53-, 0.63-, 0.53-, 0.32-, 0.60-, 0.20- and 0.37-fold, respectively, even in the 0.1 μg/L exposure group. However, a 1.78- and 1.52-fold upregulation of *tshβ* compared to controls was observed in the 1 and 10 μg/L groups, respectively.

Thyroid stimulating hormone (TSH) stimulates the thyroid gland to produce T4 (Cheng et al., 2017; Jia et al., 2016; Yu et al., 2010). The transcription of *tshβ* genes can also be modulated by alterations in TH levels through negative feedback mechanisms. Previous studies of decabromodiphenyl ether (BDE-209) and pyrethroid insecticide exposure in zebrafish showed a decrease in TH level and an increase in *tshβ* gene expression (Chen et al., 2012; Tu et al., 2016a). Increased TSH level and decreased TH concentration were also observed in fish exposed to another NSAID, clofibric acid (Saravanan, 2014). In this study, NPX exposure led to a remarkable upregulation of *tshβ* genes at concentrations of 1 and 10 μg/L compared to controls. Therefore, the upregulation of *tshβ* genes observed in zebrafish after exposure to NPX might also be attributable to the decrease in T4 concentration, which may act as a negative feedback.

Thyroid hormone receptors (TRs) are members of a large nuclear receptor superfamily that acts as ligand modulated transcription factors.TH performs its biological activity by binding to TRs. Previous studies have suggested that abnormal mRNA levels of TRs contribute to thyroid dysfunction in zebrafish larvae (Chen et al., 2012; Yu et al., 2011), which results in the disruption of TH's ability to bind and

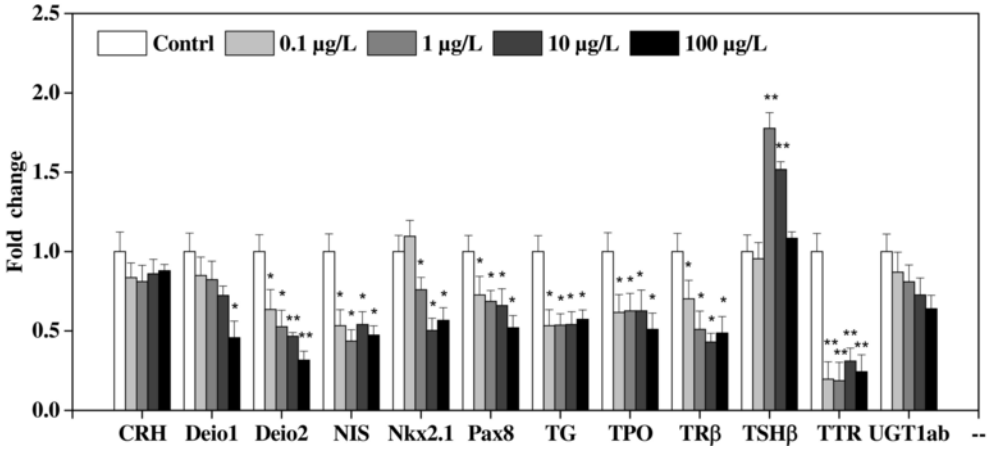

Fig. 4. Relative expression of HPT-related genes in zebrafish (five zebrafish of each replicate) after exposure to naproxen for 60 days. The results are the mean ± SD of three replicate samples. * indicates $p < 0.05$ and ** indicates $p < 0.01$ relative to the control.

activate the proper cascade of responses (Cheng et al., 2017). *In silico* and *in vitro* studies have confirmed that the NSAIDs celecoxib and diclofenac, but not NPX, can bind to TR*β* and exhibit off-target TR*β* antagonist behavior (Zloh et al., 2016). The difference in the interaction of TR*β* with NPX from that with celecoxib and diclofenac might be caused by their different chemical structures. Celecoxib and diclofenac have similarities in shape and differ in comparison to the small and flat NPX (Zloh et al., 2016). However, the results of the present study show that, compared to controls, there is a downregulation of the transcript levels of *trβ* in zebrafish after exposure to NPX for 60 days. This finding is consistent with a recent study showing that exposure to benzophenones leads to significant decreases in T3 and T4, accompanied by a decline in *trβ* expression in GH3 and FRTL-5 cells, which suggests a T3 activity of benzophenones (Lee et al., 2018). Therefore, we deduced that the significantly decreased transcription levels of *trβ* and the high bioconcentration of NPX in the zebrafish may also result from possible T3-like activities.

Diodinases are important regulators of circulating TH levels in vertebrates. In fish, T3 levels in the target organs are primarily controlled by the activities of the two Diodinases, DIO1 and DIO2, which convert T4 into biologically active T3. Compared to DIO1, DIO2 plays a major role in plasma TH homeostasis by exclusively catalyzing outer-ring Diodination and producing active T3 (Yu et al., 2010). In fish, Diodinase activity is sensitive to environmental chemicals, and its transcriptional level is regarded as a sensitive biomarker of thyroid disruption (Picard-Aitken et al., 2007). In this study, the observed significant downregulation of *dio1* and *dio2* is indicative of a state of hypothyroidism. One possible explanation for this finding is the failure of autoregulation of thyroid hormone levels and the strong stimulating effect of NPX on the HPT axis.

Transcription factors, such as *nkx2.1* and *pax8*, play essential roles in thyroid development in fish, which might explain the transcriptional downregulation of *nkx2.1* observed in the zebrafish in our exposure experiments. Additionally, *nkx2.1* and *pax8* could also regulate the expression of *nis* and *tg*, which are involved in TH synthesis (Chen et al., 2012). In this study, the suppressed expression of *nis* and *tg* was accompanied by the decreased transcription of *nkx2.1* and *pax8*, suggesting the decreased T4 levels might be related to a disruption of thyroid development. Meanwhile, the downregulation of the enzyme thyroperoxidase (TPO), which is responsible for the synthesis of thyroid hormones (Baumann et al., 2016), was also consistent with the observed decreased TH levels.

3.7. TTR protein expression

TTR protein levels were further assessed *via* Western blotting (Fig. 6). Upon exposure to 0.1, 1, 10 and 100 μg/L of NPX, the TTR protein was significantly downregulated by 0.82-, 0.8-, 0.72- and 0.56-fold, respectively, compared to controls. As an important transport protein of THs, TTR binds and transports THs to various target tissues and plays a crucial role in teleost fish and amphibians (Morgado et al., 2007). TTR is suggested to be one of the molecular targets of thyroid endocrine disruptors; chemicals interfering with the TH binding ability to TTR may directly affect the concentration of free THs and the clearance rate in fish (Tu et al., 2016a; Zhu et al., 2014). Administration of T3 and T4 can significantly increase the plasma levels of TTR in sea bream (Morgado et al., 2007), and downregulation of the *ttr* gene is regularly coincident with the reduction of THs, which indicates that TTR activity may be directly or indirectly mediated by THs (Kim et al., 2015, Yu et al., 2013). The observed decrease in *ttr* mRNA (Fig. 5) and protein expression in this study might suggest a mechanism for the reduction of TH levels and validate TTR as a potential target for NPX-mediated disruption of thyroid function in zebrafish.

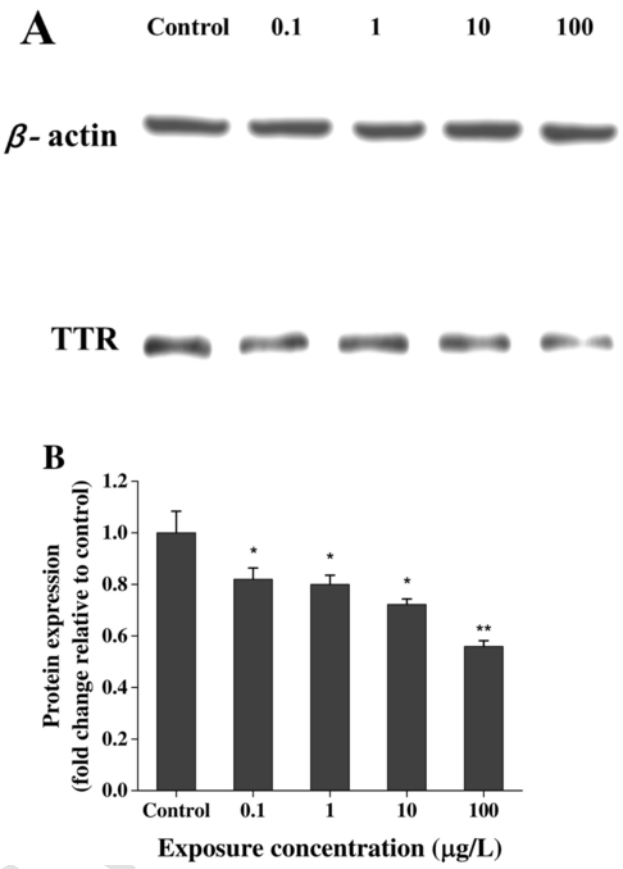

Fig. 5. Protein abundance of transthyretin (TTR) in zebrafish after exposure to naproxen for 60 days. A representative Western blot of TTR is shown (A), with the relative quantification of TTR protein expression (B). * indicates $p<0.05$ and ** indicates $p<0.01$ relative to the control.

4. Conclusions

While acute toxicity of NPX to aquatic organisms has been reported in previous studies, its chronic effects have been recognized more recently. The results from this study clearly show that long-term exposure to NPX can cause bioconcentration, growth inhibition and significant thyroid disruption in zebrafish. The end points assessed in zebrafish exposed to NPX were summarized in a mechanistic scheme (Fig. 6) based on the adverse outcome pathway (AOP), in order to link the effects on TH function with effects on growth at organism-level that can affect the population (Ankley et al., 2010; Moreira et al., 2018). Suppression of the metabolizing enzyme activity might be the major reason for the bioconcentration, while the disturbance of gene transcription along the HPT axis and the significant reduction in TTR played important roles in decreasing the TH levels. These responses are consistent with the effects observed on growth. Since the concentrations used in this study are environmentally relevant, these findings highlight the importance of evaluating the thyroid disruption in aquatic organisms of various kinds by nonhormone pharmaceuticals found in the environment. However, it should be noted that the biotransformations of NPX were not considered. Future research is needed to identify the metabolites and their contribution to the thyroid-disrupting effect. Given the worldwide increasing consumption

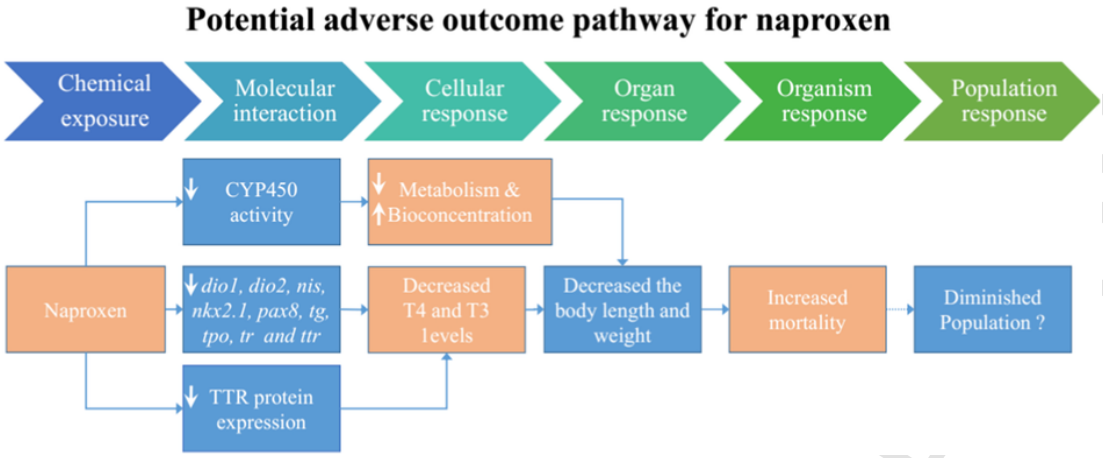

Fig. 6. Potential adverse outcome pathway for zebrafish exposed to naproxen.

and emission of NSAIDs, the ecological risks of their long-term exposure in aquatic organisms warrants further investigation.

Uncited references

Barcelo and Petrovic, 2007
Segner, 2009

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.04.323.

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