Full length article

Atrazine exposure triggers common carp neutrophil apoptosis via the CYP450s/ROS pathway

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ABSTRACT

Due to the excessive pursuit of crop yields and the abuse of herbicides, water pollution caused by atrazine (ATR) has become one of the most severe environmental issues threatening the health of fish and aquatic animals. However, no detailed report has been conducted on the mechanisms of ATR immunotoxicity in fish neutrophils. To investigate these mechanism, we exposed peripheral blood neutrophils to 25 μg/ml atrazine for 1, 2, and 3 h. The results showed that ATR induced the mRNA expression of CYPs enzymes (CYP1A1, CYP1B1, CYP1C and CYP3A138), which increased the ROS levels, and inhibited the SOD and CAT activities, GSH content and spurred the accumulation of MDA. Additionally, a significant decline in the OXPHOS, Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities of mitochondria was observed after ATR exposure. Concurrently, ATR activated Caspase3 and induced apoptosis by changing the expression of mitochondrial pathway factors (Bcl-2, BAX, Caspase9) and death receptor pathway major genes (TNF-α, TNFR, Fas, FasL, and Caspase8). The results reported here indicate that the oxidative stress and mitochondrial damage caused by ATR metabolism may play a crucial role in the apoptosis of carp neutrophils, and enrich the immunotoxicological mechanisms of ATR observed in fish.

1. Introduction

Atrazine (ATR), a selective herbicide, is widely used around the world to limit the growth of weeds and to ensure crop yields [1,2]. However, due to its excessive use and irregular handling, ATR has been distributed into aquatic ecosystems by percolating through groundwater, threatening the health of aquatic animals [3]. It has been reported that ATR levels reached 31–41 ng/L in extractives drawn from Istanbul and Dardanelles [4]. Moreover, concentrations of ATR in rainwater and surface water in Iowa have been measured at 154 μg/L and 300 μg/L, respectively, exceeding limits outlined by the EU (0.1 μg/L) [5,6]. In recent years, negative effects of ATR on fish and amphibians have received extensive attention. ATR can spur diverse forms of toxicity in aquatic animals, such as sexual alterations by ATR for larval *Xenopus laevis* and reproductive dysfunction for adult zebrafish [7,8]; endocrine toxicity for anuran larvae [9], biochemical toxicity for *Prochilodus lineatus* [10], and histological changes in pacu fish (*Piaractus mesopotamicus*) [11]. In addition, the most recent report has shown that ATR induces apoptosis in spleen cells of Nile tilapia (*Oreochromis*

niloticus), disrupting the normal immune response [12].

Cytochrome P450s (CYPs) plays a crucial role in the metabolisms of many detrimental substances including drugs, xenobiotics and herbicides [13,14]. It has been reported that levels of CYP450 and enzyme activities observed in common carp gills are up-regulated after ATR exposure [15]. In addition, the activation of CYPs induces the generation of excessive reactive oxygen species (ROS), spurring the emergence of oxidative stress [16]. For example, endosulfan (EDS) spurred oxidative stress in liver tissues by activating CYP1A [17]. A hallmark of ATR toxic injury is the generation of oxidative stress in organic cells [18]. In many cases, when cells undergo irreversible and severe oxidative stress, cells undergo apoptosis [19]. Generally speaking, apoptosis can be induced by certain toxic chemicals through the participation of distinct intracellular signaling pathway molecules and transmembrane receptors which ultimately activate executioner caspase3 [20,21]. The destruction of mitochondrial structures and functions may activate apoptosis via the mitochondrial pathway, which involves the changes of related gene expression (Bcl-2, BAX, and Caspase9) [22,23]. Meanwhile, disorders of mitochondrial energy

metabolism are closely related to apoptosis. The disturbance of oxidative phosphorylation (OXPHOS) in mitochondria induces the apoptosis of human embryonic kidney cells [24]. Mitochondrial injury induced by rotenone inhibits ATPase activity and promotes apoptosis in Jurkat cells [25]. In addition, cells can initiate apoptosis by activating death receptor pathways, such as the TNF-α/TNFR1 pathway, which is targeted by mycobacterium avium in macrophages, and the Fas/FasL pathway, which is induced by LPS in the lung of mice [26,27].

Neutrophils are key members of innate immunity that are capable of killing invading germs through migration and phagocytosis [28]. However, the impacts and toxicological mechanisms of ATR on carp neutrophils remain unclear. In the present study we examined neutrophils of carp peripheral blood to observe changes in CYP450s, ROS production, anti-oxidation enzymes, mitochondrial morphologies and functions, and apoptosis related indicators after ATR exposure and, to identify the potential mechanisms of cytotoxicity in fish neutrophils resulting from ATR exposure. The results of this study enrich our understanding of who the immune systems of ATR in common carp are damaged by ATR.

2. Materials and methods

2.1. Ethics statement and experimental animals

All procedures applied in this study were authorized by the Institutional Animal Care and Use Committee of the Northeast Agricultural University. Thirty common carp (*Cyprinus carpio* L., mean body weight of 115 ± 10 g; mean body length of 15.5 ± 1.49 cm) were purchased from local fish farm that specializes in freshwater fish species. To avoid unnecessary interferences, the species were individually acclimated for a week in a 200-L stock tank at 21 °C programmed with a 12 h light/dark cycle prior to the experiments. Circulating tap water was taken from a local water supply company, aeration was uninterrupted, and food was provided twice a day. All procedures were conducted according to the European Communities Council Directive (86/609/EEC) and were approved by a local ethics committee.

2.2. Cell isolation and ATR exposure

All thirty fish were experimented on and each test was conducted on five samples of neutrophils pooled from 5 carp (n = 5). The fish were anesthetized with 0.2 g/L of tricaine methane sulphonate buffered with 0.4 g/L of NaHCO₃. We punctured the caudal vein to collect peripheral heparinized blood (twice/fish) and 10 ml of blood was obtained from each fish. The neutrophils were obtained from a fish peripheral blood neutrophil separation kit (BeiJing Solarbio Biological Manufacture CO., China). Cells were resuspended in RPMI⁺ (RPMI 1640 supplemented with 10% Fetal bovine serum) and diluted to a density of 1×10^6 cells/ml. The neutrophils were then incubated with 25 μg/ml ATR (98% purity, Superlco, Bellefonte, PA, USA) for 1, 2, and 3 h at 25 °C in a humidified atmosphere with 5% CO₂, and neutrophils not treated with ATR were used as a control group.

2.3. Morphological observation

After 3 h of ATR treatment, neutrophils of the control group and ATR-treated groups were obtained by centrifugation at $400 \times g$ for 8 min and were rinsed with PBS three times. The cells were then fixed with 2.5% glutaraldehyde for 2 h, rinsed twice for 15 min in 0.2 M of phosphate buffer (pH 7.2) and treated with 1% osmium tetroxide for 1 h. Ultrathin sections stained with uranyl acetate were observed through a transmission electron microscope.

2.4. Real-time measurement of OXPHOS activity

We used a CLARIOstar bioenergy metabolism analyzer (BMG LABTECH, Co, Germany) to monitor real-time cellular OXPHOS activity, according to the manufacturer's instructions. Control and ATR-treated neutrophilic granulocytes were cultured on a 96 well assay culture plate (1×10^5 cells/well) for 3 h. Meanwhile, cells were treated the complex I/III inhibitors rotenone (1 μM) and antimycin A (1 μM) to assess the lowest oxygen consumption rate (OCR), facilitating our observations of changes in the oxidative phosphorylation of ATR-treated cells.

2.5. Apoptosis assay

We employed acridine orange/ethidium bromide (AO/EB) dual staining to analyze cell apoptosis processes. Neutrophils (1×10^6 cells/ml) were grown in 6-well plates and were incubated with ATR (25 μg/ml) for 3 h. Cells cultured with pure RPMI + medium were used as controls. We removed the medium and rinsed the cells with PBS. The cells were stained with 10 μl AO/EB (1 mg/ml) for 3 min and we employed fluorescence microscopy (Thermo Fisher Scientific, USA) to visualize the apoptosis fluorescence images.

2.6. ROS detection

To observe and analyze levels of oxidative stress of the neutrophils, fluorescent staining imaging and fluorescent intensity quantification were used to measure levels of ROS production occurring in different cell groups using commercial assay kits (E004 Nanjing Jiancheng Biological Manufacture CO., China). Neutrophils (1×10^6 cells/ml) attached to Poly-L-Lysine-coated μ-slides were incubated with ATR for 1, 2, and 3 h and then we removed the culture media and the cells were cultured with RPMI⁺ containing ROS probe 2,7-dichlorofuorescin diacetate (DCFH-DA) for 30 min. We then rinsed the neutrophils with PBS and observed ROS production under fluorescent microscopy. ROS quantification procedures applied were more concise. After ATR treatment, neutrophils of the different treatment groups were obtained by centrifugation. The cells were then incubated with DCFH-DA for 30 min and washed with PBS twice. Neutrophils resuspended in PBS were distributed across 96 well opaque plates and fluorescence intensity levels were determined using a fluorescent microplate reader (Bio-TEK, USA).

2.7. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from neutrophils in the control group and in groups treated with ATR for 1, 2, and 3 h using the TRIzol reagent. The reverse transcription of mRNA was performed via First-Strand cDNA Synthesis Kit (Tiangen Biotech Co. Ltd., Beijing) and qRT-PCR was carried out using the LightCycler® 480 II Detection System (Roche, Switzerland). Primers of gene qRT-PCR for detoxification and stress (CYP1A1, CYP1B1, CYP1C and CYP3A138), mitochondrial apoptosis pathway signaling molecules (BAX, Bcl-2, Caspase9, and Caspase3) and five death receptor pathway major factors (TNF-α, TNFR, Fas, FasL, and Caspase8), along with housekeeping gene β-actin are shown in Table 1.

2.8. Biochemical assays

After 1, 2, and 3 h of ATR treatment, respectively, the activities of catalase (CAT) and superoxide dismutase (SOD), the contents of glutathione (GSH) and malonic dialdehyde (MDA) and ATPase activity observed in neutrophils were analyzed using detection kits (A007, A006, A001, A003 and A069, Nanjing Jiancheng Bioengineering Institute, P.R. China) according to the manufacturer's instructions.

Table 1
Gene-special primers used in the real-time quantitative reverse transcription PCR.

Gene	Accession number	Primer (5′→ 3′)
BAX	XM_019078270.1	TCCACTCTTCAACCAACTC
		GCCAATAGTCTGCCATGT
Bcl-2	XM_019067767.1	ATGCGTGAATAAGGAGATGA
		AGACCGAAGACCGTTACT
Caspase9	XM_019066459	AGGCAACTGGTGACAGACTTAGAGA
		GACAGAAGGAGGCAGACGAACAC
Caspase3	XM_019110173.1	GCTGTGCTTCGTTAGTGT
		GAACCAAGAACCGCTCAT
Caspase8	XM_019098019.1	GCTGGTTACAGATGGTGCTGGTC
		GTCCTGGCCTTGTTCACTTCCTTC
TNF-α	AJ311800	GCTGTCTGCTTCACGCTCAA
		CTTGGAAGTGACATTTGCTTTT
TNFR1	CAFS_CommonC_G_033453	TGTGCTGGACGAGGTGCCTATC
		TCCACTGCCGCTGTCACTCC
Fas	XM_019079188.1	ACATCCACAATCCACAAGCACAGG
		AACACCACTGCACCAACACCAG
FasL	XM_019081039.1	ACAGCCACAACCGGACAATTATGG
		AGCCAATCGGATTCCTTGAGATGC
CYP1A1	XM_019122549.1	ATGAGCAGAAATCGAGGGCTCTC
		ACAGCCCTCAGAGGAGCCCTT
CYP1B1	XM_019075104.1	TCTTCACATTCTTCCGAGTT
		CCGAGGTAGTCCAGTTCT
CYP1C	AY437776.1	AGCAGCGGGTGGAGGATGTA
		CCTCAGAAATGGCGGTGGAC
CYP3A138	KF790756	GACCTTCGCCCTCCACAG
		CCTCATCCCGATGCATGTTC
β-actin	M24113.1	GAGGTGGAGGCTAACAAC
		GATGTGAGGAGAGGATTCG

2.9. Western blot analysis

To understand the change in protein levels observed in neutrophils after 3 h of ATR exposure relative to the control group, the western blot method was applied as described previously [29]. In brief, 5×10^6 neutrophils were spun at $400 \times g$ for 8 min, and pellets were resuspended and lysed in 500 μl of ice-cold buffer (20 mM of Tris-HCl, pH 7.4, 2 mM of EDTA, 2 mM of EGTA, 1 mM of PMSF, 30 mM of NaF, 30 mM of sodium pyrophosphate, 0.1% SDS, 1% Triton X-100, and protease inhibitor cocktail). Supernatants were harvested by centrifugation at 4 °C, $10,000 \times g$. The concentration of protein obtained was determined with a Bicinchoninic acid assay and protein lysates (30 μg) were subjected to SDS–polyacrylamide gel electrophoresis. The antibodies used in this study include Caspase3 (1:500, produced by our lab), Caspase9 (1:1000, Santa Cruz, CA), Caspase8 (1:1000, Santa Cruz, CA), TNFR1 (1:1200, Santa Cruz, CA), Fas (1:800, Santa Cruz, CA), FasL (1:800, Santa Cruz, CA), GADPH antibody (1:1000, Santa Cruz, CA), HRP-conjugated secondary antibodies against rabbit IgG (1:5000, Santa Cruz, USA) and HRP-conjugated goat anti-mouse IgG (1:3000, Santa Cruz, CA).

2.10. Statistical analysis

Data were collected from at least three independent experiments and all results are expressed as the means ± standard deviations. A statistical analysis was performed with a paired *t*-test followed by least significant difference determination using GraphPad Prism software 5. Values of P < 0.05 were considered to denote a significant differences.

3. Result

3.1. ATR induced apoptosis and mitochondrial injury of neutrophils

We first used a transmission electron microscope (TEM) and AO/EB staining to observe effects of ATR on the apoptosis of neutrophils in common carp. As is shown in Fig. 1A, without ATR treatment, the nuclei of control cells presented a normal architecture. After 3 h of 25 μg/ml ATR exposure, neutrophils exhibited typical features of apoptosis, such as chromatin condensation (electron-dense nucleus), nuclear fragmentation, intact cell membranes, and disorganized cytoplasmic organelles. In addition, AO/EB staining results showed that parts of cells treated with ATR appeared bright orange, indicating the emergence of apoptosis. No morphological changes were observed in the control cells, which appeared green. We then detected the changes in mitochondrial structures of neutrophils in common carp by TEM. In the control group, the edges of mitochondrial membranes remained intact, the distribution of cristaes was uniform and soigne, showing that the mitochondrial morphologies of the control group did not vary (Fig. 1B). ATR exposure resulted in the destruction of mitochondrial integrity, as shown by the tumidness of cristae spacing and by blurred mitochondrial membranes. Meanwhile, we examined OCR and ATPase activities to evaluate changes in mitochondrial energy metabolism levels of the cells after ATR exposure (Fig. 1C and D). The results show that ATR significantly decreased OCR levels relative to the control group, damaged OXPHOS activity and disrupted Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities (P < 0.05).

3.2. ATR activated mitochondrial apoptotic and death receptor pathways

We next determined the type of apoptosis caused by 3 h of ATR exposure. Fig. 2A illustrates our quantitative analysis of mRNA expression in major molecules (BAX, Bcl-2, Caspase9) that mediated apoptosis through the mitochondrial pathway, death receptor pathway related factors (TNF-α, TNFR, Fas, FasL, and Caspase8) and markers of apoptosis (Caspase3). We found that ATR generally enhanced BAX Caspase 9 and Caspase3 expression, but limited the mRNA expression of Bcl-2 (P < 0.05). Meanwhile, ATR significantly increased the transcriptional expression of TNF-α, TNFR1, Fas, FasL, and Caspase8 relative to the control group. As shown in Fig. 2B, western blot results show protein expressions of TNFR1, Fas, FasL, Caspase8, Caspase9, and Caspase3 consistent with the results of RT-PCR (P < 0.05).

3.3. Transcriptional alterations of CYPs in neutrophils after ATR exposure

As cytochrome P450s can participate in the metabolism of various compounds including drugs and poisons, we determined whether these detoxification genes play a rule in carp neutrophils after ATR exposure. As is shown in Fig. 3, CYP1A1, CYP1B1, CYP1C and CYP3A138 mRNA levels were markedly promoted by ATR relative to those of the control cells (P < 0.05). We also found that the transcriptional elevation of above-mentioned genes caused by ATR reached the highest levels after 2 h.

3.4. Effects of ATR on ROS production

ATR increased the production of reactive oxygen species above control levels (Fig. 4). We used DCFH-DA staining to observe changes in ROS production after ATR exposure for 1, 2, and 3 h. Fluorescence images show that ROS production in fish neutrophils was promoted by ATR in a time-dependent manner (Fig. 4A). In addition, quantitative analysis results of ROS fluorescence intensity levels are consistent with our FM observations (Fig. 4B).

3.5. ATR affected the antioxidant capacities of neutrophils

We next measured effects of ATR on antioxidant capacities of carp neutrophils. As is shown in Fig. 5, we found significant changes in the antioxidant systems of neutrophils due to ATR. For example, ATR markedly inhibited the activities of CAT, content of GSH and promoted the MDA levels in neutrophils, compared with control group (Fig. 5A, B, D). The results also show that SOD activity levels were significantly higher (P < 0.05) in neutrophils exposed to ATR than that in control
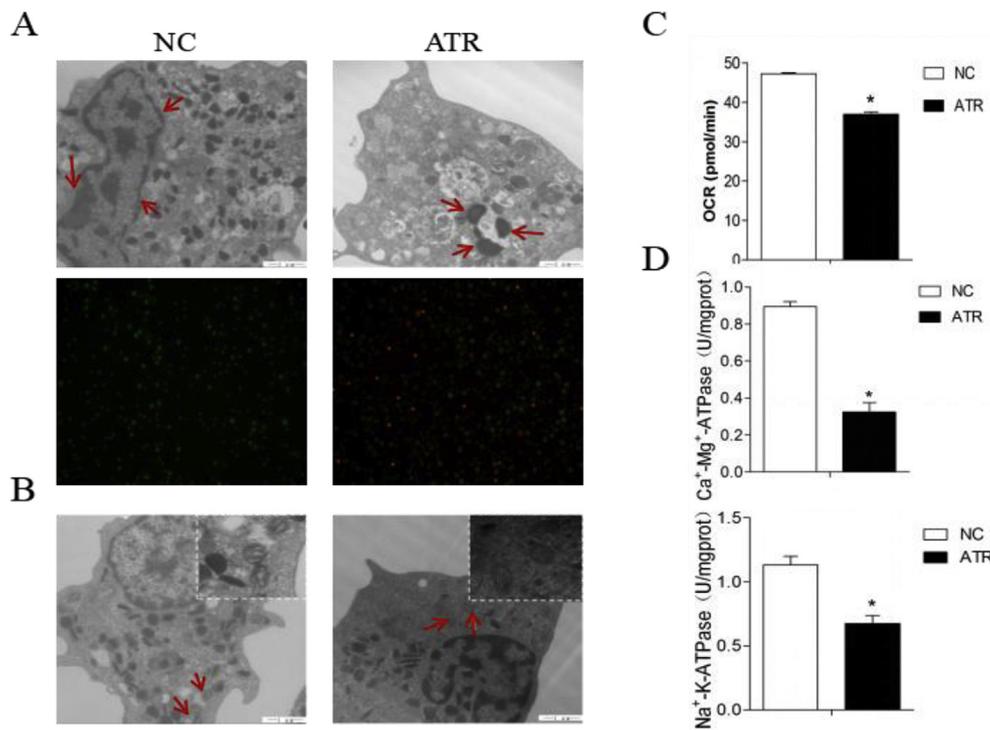
Fig. 1. Effects of ATR on the apoptosis and mitochondria of neutrophils. Neutrophils were treated by ATR for 3 h. A: Morphological changes in neutrophils observed via scanning electron microscopy and AO/EB staining, changes observed in nuclei (red arrow), normal cells (green), and apoptosis cells (bright orange). B: Effects of ATR on mitochondrial structures of neutrophils. C: Oxygen consumption rates (OCRs) observed in common carp neutrophils from an extracellular flux analyzer (normalized to cell number). D: Na$^+$-K$^+$-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities. All experiments were performed at least three times, and data are shown as the mean ± SD values. *P < 0.05, comparing with the corresponding control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells after 1 h but were lower after 3 h (Fig. 5C).

4. Discussion

As major environmental pollutants, potential threats of atrazine pesticides to aquatic life have long been underestimated [30]. Although has been regarded as short-lived phagocytic cells, neutrophils play a crucial role in immune modulation, tissue damage and host protection, and are very sensitive to water pollution in fish [31]. Most effects of ATR exposure on the morphological and functional organization of animals involve the participation of neutrophils, such as the ATR-induced inflammation of the liver and head kidneys and lowered lysozyme and bactericidal activity levels in the blood of common carp [32,33]. However, relatively little is known of the impacts of ATR exposure on neutrophils. In this study we observed the apoptosis of carp neutrophils after ATR exposure. In addition, our results show that ATR exposure activated four major CYPs enzymes; induced ROS accumulation by disrupting SOD, GSH and CAT functions; undermined mitochondrial energy metabolism and altered the related factor expression of mitochondrial apoptotic and death receptor pathways in
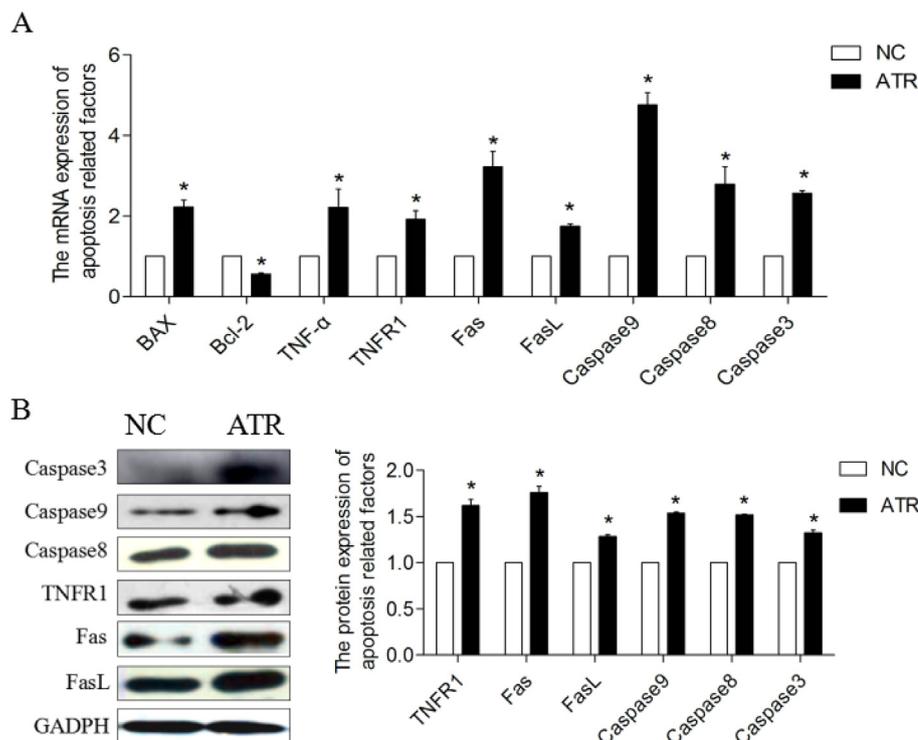

Fig. 2. Effects of ATR on the mitochondrial apoptosis pathway and death receptor pathway of neutrophils. Neutrophils were treated by ATR for 3 h. A: The mRNA expressions of mitochondrial pathway and death receptor pathway related genes. B: The protein expressions of mitochondrial pathway and death receptor pathway related genes. All experiments were performed at least three times, and the data are shown as the mean ± SD values. *P < 0.05, comparing with the corresponding control group.
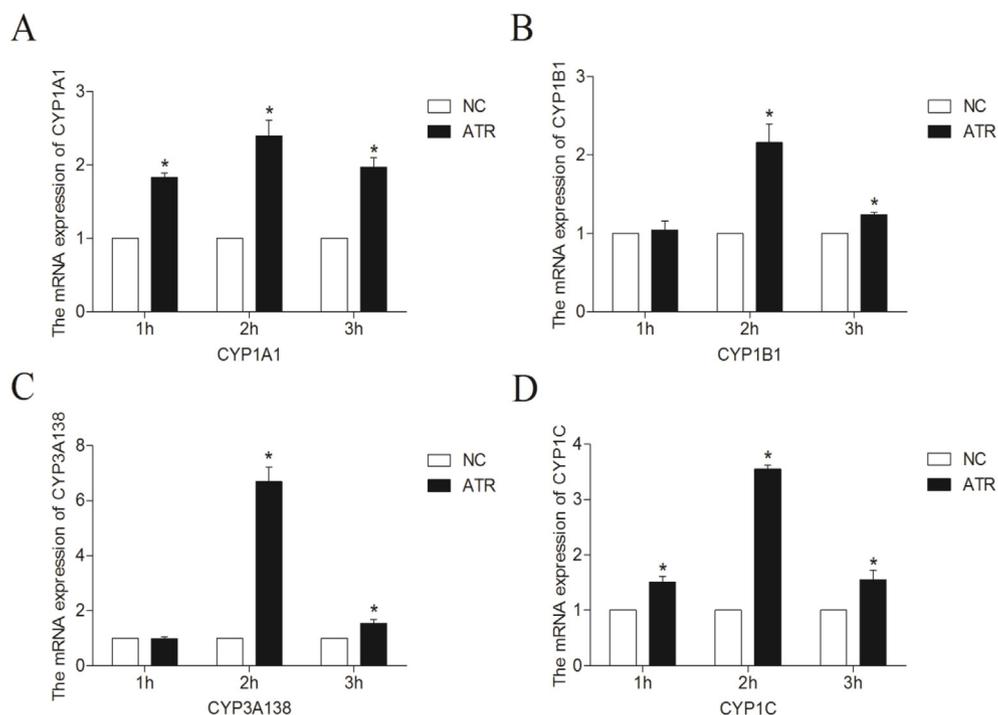
Fig. 3. Effects of ATR on CYP450s expression in neutrophils of common carp. Cells were treated with 25 µg/ml for 1, 2, and 3 h, respectively. A: CYP1A1 mRNA level. B: CYP1B1 mRNA level. C: CYP3A138 mRNA level. D: CYP1C mRNA level. All experiments were performed at least three times, and the data are shown as the mean ± SD values. *$P < 0.05$, comparing with the corresponding control group.
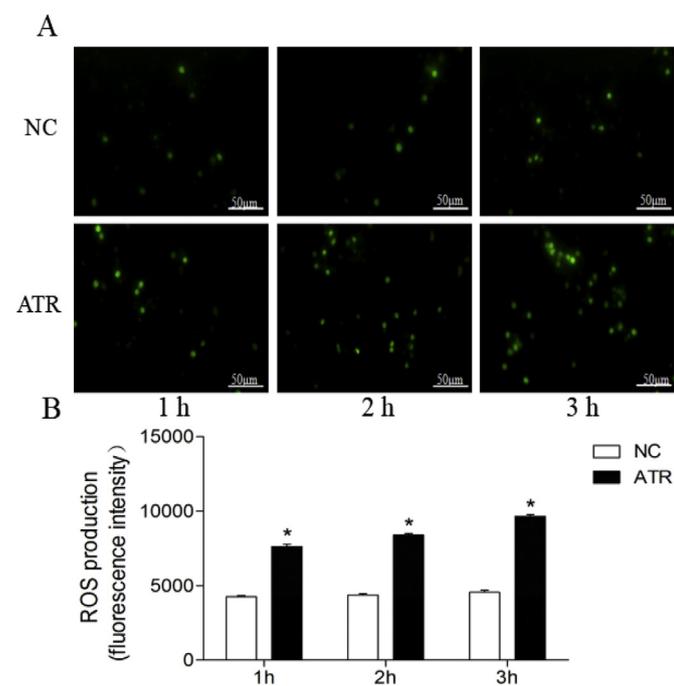

Fig. 4. Effects of ATR on ROS production. A: Cells were treated by 25 µg/ml ATR for 1, 2, and 3 h, and the ROS production was detected using a fluorescent microplate reader after stained with DCFH-DA. B: Cell groups were treated with ATR for 1, 2, and 3 h, and ROS production was analyzed through fluorescent microplate reader after stained with DCFH-DA. *$P < 0.05$, comparing with the corresponding control group.

neutrophils of common carp.

The CYP1 subfamily is the most widely studied CYP form in fish and has been considered the most sensitive RNA biomarker for evaluating environmental pollution [34]. Previous studies show that the combined exposure of ATR and chlorpyrifos significantly increases mRNA levels of CYP1 (CYP1A, 1B and 1C) in common carp gills [35]. Similarly, in this study we found that CYP1A, 1B and 1C expressions are up-

regulated in carp neutrophils after ATR treatment. CYP3A is involved in drugs metabolism and its cloned gene in common carp is identified as CYP3A138 [36]. It has been reported that paraquat exposure spurs the transcription of CYP3A138 in the livers of common carp [14]. Through qPCR detection we found that CYP3A138 mRNA expression to be enhanced in the ATR-treated group, indicating that CYP3A may take part in the metabolism of ATR in neutrophils of common carp. Additionally, the biotransformation of toxicants by the CYP family inevitably leads to a rapid increase in intracellular ROS, destroying the anti-oxidation functions of cells and thus creating oxidative stress. In antioxidant enzyme systems, SOD is regarded as the first line of defense against oxidative stress by converting superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) [37]. CAT and GSH are responsible for clearing and blinding H_2O_2 by redox reactions and MDA content serves as a chemical marker of oxidative damage [38,39]. It has been reported that nitenpyram inhibits the activities of SOD and CAT, thus causing oxidative stress and DNA damage in zebrafish livers [40]. Narra, M. R. proved that chlorpyrifos disrupts the antioxidant defense systems of metabolically active tissues of freshwater crab, Barytelphusa guerini [41]. Our analysis of antioxidant functions of neutrophils shows that SOD activity levels significantly increase in early stages of ATR exposure (1 h) and that CAT activity and GSH content levels did not decrease during the same period of ATR treatment, which may be why MDA content levels did not change compared with the control group. In addition, with the extension of ATR exposure time (2–3 h), SOD, CAT activity and GSH content levels decreased and failed to take responsibility for removing excess superoxide anion, resulting in excess ROS accumulation [42]. Our results echo the conclusion that carbamate pesticide Carbaryl restricts antioxidant defense capability and boosted risks of oxidative stress experienced in terrestrial snail Cantareus apertus [43].

From morphological observations of cells after exposure to ATR, we identified typical characteristics of mitochondrial damage and apoptosis that may contribute to pro-oxidative stress effects of ATR [44]. Mitochondrial ultrastructural changes play an important role apoptosis. The main function of mitochondria is to provide energy to cells through the coupling of OXPHOS and ATPase and to prevent apoptosis by up-regulating intracellular ATP levels [45,46]. It has been reported that insecticide Fipronil decreases aerobic respiration rates, mediates the mitochondrial apoptosis pathway, activates Caspase3 and induces the
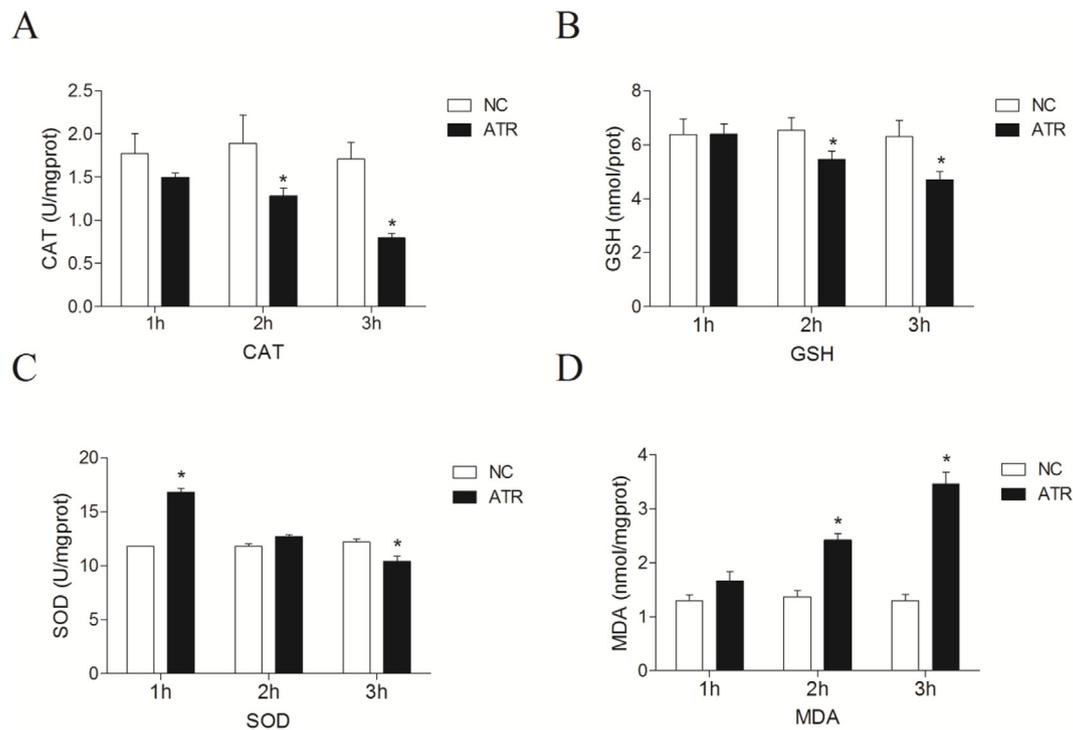
Fig. 5. Effects of Atrazine on antioxidant functions of carp neutrophils. Cells were treated with ATR for 1, 2, and 3 h and then the antioxidant capacities were measured. A: CAT activity. B: GSH content. C: SOD activity. D: MDA content. The data are shown as the mean ± SD values. *$P < 0.05$, comparing with the corresponding control group.

apoptosis of human neuronal cells [47]. Endosulfan induces the apoptosis of spermatogenic cells in testicles by inhibiting the activities of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase [48]. Similarly, ATR exposure significantly restrains OXPHOS, Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activates in carp neutrophils in agreement with findings showing that aflatoxin B_1 induce apoptosis by undermining mitochondrial respiration in broiler cardiomyocytes [49]. The process of mitochondrial apoptotic pathway activation mainly involves the interaction and activation of several key factors, such as the inhibition of Bcl-2, the transference of BAX from cell fluid to mitochondria, the release of Cyt-C and the activation of Caspase9 [50,51]. Jin Y et al. showed that Cis-bifenthrin (cis-BF) exposure induced oxidative stress promotes apoptosis occurrence via the Bcl-2/BAX pathway in zebrafish during the embryonic stage [52]. In addition, several reports show that environmental pollutants of different sources can induce cell apoptosis by binding to death receptors on cell surfaces and by activating Caspase8. It has been reported that the Fas/FasL pathway plays a critical role in the apoptosis of testicular cells extracted from 18-to 22-day-old rats exposed to Bisphenol-A [53]. Meanwhile, multiple herbicides have been found to induce apoptosis through the TNF/TNFR1 signal transduction pathway, such as acetochlor and 2,4-dichlorophenoxyacetic acid [54,55]. We found a similar result that ATR treatment markedly vary the expression of the above factors in cells. These results suggests that mitochondrial and death receptor pathways may serve as the main internal mechanisms of apoptosis in peripheral blood neutrophils of common carp induced by ATR.

In brief, these results reveal that ATR exposure can induce carp neutrophilic granulocyte apoptosis by disturbing xenobiotic metabolism, restraining fish antioxidant enzymes activities, causing oxidative stress and promoting mitochondrial damage. We thus show that the mitochondrial apoptotic pathway and death receptor pathway are the direct causes of immune cell apoptosis induced by ATR. The present study refines the mechanistic theory of ATR immunotoxity in fish and complements risk assessments of aquatic animal health.

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