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Atrazine induces necroptosis by miR-181–5p targeting inflammation and glycometabolism in carp lymphocytes

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ABSTRACT

Atrazine (ATR) causes environmental problems and damages the health of fish and aquatic animals. MicroRNAs (miRNAs) play important roles in immune regulation. However, the immunotoxicity mechanism of ATR in fish lymphocytes and the role of miRNA in this process remain unclear. To further study these mechanisms, spleen lymphocytes were exposed to 20, 40 and 60 μg/ml ATR for 18 h. Fluorescence staining and flow cytometry showed that the number of necrotic lymphocytes increased after ATR exposure. Compared with the control group, the mRNA expression of miR-181–5p was inhibited and the mRNA levels of TNF-α and HK2 were increased after ATR exposure. Additionally, the NF-κB inflammatory pathway and the levels of glycometabolism-related genes were upregulated. These results suggest that ATR induces inflammation and elevates glycometabolism in lymphocytes. We further found that the mRNA levels of receptor-interacting serine-threonine kinase 1 (RIP1), receptor-interacting serine-threonine kinase 3 (RIP3), mixed lineage kinase domain-like pseudokinase (MLKL), cylindromatosis (CYLD) and Fas-Associated protein with Death Domain (FADD) and the protein levels of RIP3 and MLKL in the treatment groups were significantly increased compared to those in control group, suggesting that ATR causes lymphocyte necroptosis. We conclude that miR-181–5p plays a key role in necroptosis in carp lymphocytes exposed to ATR by downregulating the expression of HK and TNF-α, which increases the level of glycometabolism and induces the inflammatory response, respectively.

1. Introduction

ATR is a common pesticide pollutant in groundwater, surface water and precipitation [1]. In eroded soil, the content of ATR was as high as 49000 μg/kg [2]. In addition, large quantities of ATR will also enter surface runoff and groundwater through farmland, resulting in irreversible water pollution. It was reported that the content of ATR in water was up to 1000 μg/L [3–5]. Studies have shown that ATR is an environmental stimulant that affects the central nervous system, endocrine system and immune system [6–8]. One report has shown that the resistance of goldfish to pathogen invasion was reduced after mixed herbicides (including ATR) exposure [9]. ATR affected the immune system of young mice by reducing the number of immune cells and changing the distribution of lymphocytes [10]. Another study has shown that ATR has immunotoxicity in carp [11]. Long-term exposure of carp to ATR could lead to an imbalance of pro-inflammatory and anti-inflammatory factors [12] and disrupting biochemical indexes in immune organs and tissue damage by increasing the synthesis of IL-1β and IL-1R1 [13,14]. ATR also induced oxidative stress in the immune organs of carp, which promoted autophagy [15]. Recent reports have shown that ATR induced apoptosis of neutrophils in carp peripheral blood through oxidative stress and mitochondrial damage [16] and inhibited the formation of neutrophil extracellular traps [17].

MiRNAs comprise a large family of noncoding single-stranded RNA molecules whose major function is to negatively regulate gene expression at the posttranscriptional level. MiRNAs are important participants in biogenesis and cellular function [18]. MiR-181 is associated with inflammation and metabolism. It has been reported that microglia activation and inflammation-induced neurotoxicity can be effectively regulated by miR-181 in persistent neuroinflammation [19]. In addition, miR-181 also plays an important role in the regulation of vasculitis and airway inflammation [20,21]. In a study of Senegalese sole development, researchers found that the expression of miR-181 was associated with muscle formation [22]. It is noteworthy that TNF-α has

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been shown to be a key target of miR-181. In dendritic cells, the study confirmed that miR-181a-5p targeted the 3′UTR region of TNF-α [23]. The potential targeting relationship between TNF-α and miR-181–5p was also observed in human smooth muscle cells [24]. In a study of sepsis-induced immunoparalysis, it was also found that the binding site of miR-181 was frequently distributed in the 3′UTR of many inflammatory cytokines, such as TNF-α [25]. In particular, miR-181 is also a target miRNA of HK2 in rat mesenchymal stem cells [26].

Necroptosis is a new model of cell death [27] called receptor-interacting protein kinase (RIP) - mediated cell necrosis, caspase-independent, which is different from the traditional mode of apoptosis and necrosis. There is a close relationship between miRNAs and necroptosis. Studies have shown that miR-200a-5p and miR-500a-3p regulate necroptosis by targeting ring finger protein 11 (RNF11) and MLKL, respectively [28,29]. Necroptosis involves in various stages of many inflammatory diseases. There was almost no tissue damage caused by inflammation with acute pancreatitis in RIP3 knockout mice, thereby, the development of pancreatitis was inhibited [30]. The high expression of RIP3 in mouse intestinal inflammatory diseases indicated that these conditions were associated with RIP3-dependent necroptosis [31]. It has been reported that activation of the NF-κB signaling pathway is associated with the inflammatory response. Thymic inflammation injury induced by ammonia exposure was associated with increased levels of downstream genes of the NF-κB signaling pathway (IL-1β, IL-6, IL-8, and iNOS) in the chicken thymus [32]. Studies have shown that ammonia harmed the immune response in chicken respiratory inflammation, which was manifested by irregular rupture and vacuolation of the tracheal mucosa, and led to tracheal inflammation injury and activation of the NF-κB pathway [33]. There are connections between necroptosis and the NF-κB signaling pathway. In cisplatin-treated mice, the signal transduction of NF-κB was inhibited; inflammatory cytokines and oxidative stress were reduced with Nec-1 (a necrosis inhibitor) pretreatment, which alleviated the nephrotoxicity [34]. Other studies have shown that necroptosis signal transduction induced by TNF-α mediated by RIP1 and RIP3 activation promoted the expression of cytokines, which involved the NF-κB signaling pathway [35]. There are also connections between necroptosis and intracellular energy metabolism. In human colorectal cancer, cell proliferation mediated by RIP1 through the mitochondrial Ca^{2+} monotransporter provided a key therapeutic target for the treatment of colorectal cancer [36]. In a model of rats with cerebral ischemia-reperfusion injury, the levels of RIP1 and RIP3 protein were increased, accompanied by an increase in activity of glutamate dehydrogenase (GLUD1), which is the downstream gene of RIP3 [37].

The development of fish lymphocytes and the study of immunobiology have great significance in comparative immunology, which can help us to better understand the immunobiology of mammalian innate lymphoid cells and the relationship with human diseases [38]. Previous studies of the toxicity of ATR have been carried out, but there are still gaps in our understanding of the mechanism of necroptosis in carp lymphocytes induced by ATR exposure. In this study, necroptosis, inflammation, and glycometabolism were examined in carp spleen lymphocytes after ATR exposure, and connected TNF-α and hexokinase 2 (HK2) with miR-181–5p. This experiment will further enrich our understanding of the mechanisms of the immune system damage in carp exposed to ATR.

2. Materials and methods

2.1. Ethical statement and laboratory animals

Twenty carps (average body weight 1150 ± 100 g, average body length 37 ± 2.45 cm) were purchased from a local freshwater fishery. To avoid unnecessary interference, the carps were adaptively fed for 7 days in a 200-L stock tank at 21 °C under a 12 h light/dark cycle before the experiment. Circulating water was continuously ventilated and food was provided twice a day [16]. All procedures were carried out following the directive of the Council of the European Communities (86/609/EEC) and approved by the Committee on Animal Management and Use of Northeast Agricultural University (SRM-11).

2.2. Cell isolation and ATR exposure

All twenty fish were experimented on and each test was conducted on five samples of lymphocytes pooled from 5 carp. The fish were anesthetized with 0.2 g/L of tricaine methane sulphonate buffered with 0.4 g/L of $NaHCO_3$. We dissected the abdominal cavity of the fish and separated the spleen. The lymphocytes were separated using a fish spleen lymphocyte separation kit (Beijing Solarbio Biological Manufacture CO., China). Cells were resuspended in $RPMI^+$ medium (RPMI 1640 supplemented with 10% fetal bovine serum) and diluted to a density of 1×10^6 cells/ml. According to previous studies [16,17,39] and pre-experimental results, we decided to incubate lymphocytes with 20, 40, and 60 μg/ml ATR (purity 98%, Superlco, Bellefonte, PA, USA) for 18 h at 25 °C in a humidified atmosphere with 5% CO_2, lymphocytes without ATR treatment were used as the control group.

2.3. AO/EB staining

Cell suspensions treated with ATR at different concentrations were collected and the sediment was collected after centrifugation for 5 min at 1500 rpm. 1×10^6 cells/ml were collected and washed twice with PBS (1500 rpm centrifugation for 5 min each time) and resuspended in PBS. Acridine orange (AO) penetrate the whole cell membrane, inserts into nuclear DNA and emits bright green fluorescence. Ethyl bromide (EB) only penetrates damaged cell membranes, inserts into nuclear DNA and emits orange fluorescence. The cells were stained with AO/EB working fluid (20 μL/ml) for 3 min, removed by centrifugation, and washed twice with PBS (1500 rpm centrifugation for 5 min each time) to remove the residual dyes. The cells were resuspended in fresh PBS and fluorescence microscopy (Thermo Fisher Scientific, USA) was used to visualize the necrotic fluorescence images [28].

2.4. Flow cytometry

Cells in each group were collected and centrifuged for 5 min at 1500 rpm. Cells were washed twice with PBS (centrifugation for 5 min at 1500 rpm each time). $1–10 \times 10^5$ cells/ml were collected and suspended in PBS. The assay was carried out following the instructions of the Apoptotic Necrosis Kit (Jiangsu Kaiji Biotechnology Co., Ltd.). Flow cytometry was used for detection within 1 h after the addition of dye solution [28].

2.5. RNA isolation and real-time quantitative PCR analysis

Total RNA was separated from lymphocytes in the control group and ATR treatment groups by Trizol reagent at 18 h. The reverse transcription of the cDNA was based on the manufacturer's instructions (Roche, Shanghai, China). Reverse transcription of miRNAs was carried out with the miRcute microRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech Co. Ltd., Beijing, China), and cDNA was synthesized according to the instructions of the Trans' Kit. The expression level of related genes was detected by qRT-PCR using the LightCycler®480 II detection system (Roche, Basel, Switzerland) [40]. The primers for detection are shown in Table 1.

2.6. Protein extraction and western blot analysis

To further understand the changes in protein expression after ATR exposure for 18 h in lymphocytes, Western blotting was used to detect the protein changes. An average of 10^6 cells/ml was treated with 100 μl cell lysate and 1 μl PMSF, shaken for 30 min on ice and then centrifuged

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

Gene	Forward 5′to'3	Reverse 5′to'3
miR-181–5p	GCGAACATTCAACGCTGTCGGTGAGT	
U6	CTCGCTTCGGCAGCACA	
TNF-α	AGGTGATGGTGTCGAGGAGGAAG	AGACTTGTTGAGCGTGAAGCAGAC
NF-κB	AGGAGCAGTGTAGTGGCCGTAC	CTCTGGATCTCGCTGTTGTGTCTG
TAK1	GGCGTGACTGGCTTCCTGTTG	ACGATCTCCTGGTTGACCTGCTC
IL-6	CCGCATGGACTCGCAAGACG	CCTCCTGCTCATGTAGTTGATGGC
IL-8	CTGGCTGTAGATCCACGCTGTC	CCAGTTGTCATCAAGGTGGCAATG
IKKβ	CAGATCCAGTCTTACAGCAGCA	TCGACACTGTTTGATGGCGA
iNOS	TCCAGTGACACTCGTGTTCG	TGGGTTGTCCAGTCTGCCTA
RIP1	TACGAGAGCCACACGGTCACC	TTACGAGCCACTTGCTTCCAGTTG
RIP3	CACTACTGCCAACTCCACCATCAG	GATTCGTCATCGTCCTCACTGCTC
MLKL	GGAACCGCTGTCACATGACTGTC	GTCCACCAACACTCCAGCAGAAG
FADD	CGGCATACAGGAGAAGCACAGC	TGAGATCCTCCACTCTGGCGTTC
CYLDa	AACAGCCTCGGACGCACAATC	TCATCCACGCTCACCACTACATTG
HK2	GCTGCTCATCTCTGTGCTGCTTC	AGACTGATCCATCCACGCCGATAG
PDHX	CCAAGACCACCATACCTCATGCG	CCGGACCACATCACATTCACCTC
SDHB	ATGGTTCTGGACGCACTCATCAAG	TTCATAGCACACGAGCCACAGATG
PK	AGGATCTACATCGACGACGGACTC	CTGCTGCTCCACACCGAACTG
β-actin	GGCTCTCTTCCAGCCTTCCT	AGCACGGTGTTGGCATACAG

Table 2
antibody dilution multiples.

Antibody	Dilution multiple
β-actin	1:1000
RIP3	1:500
MLKL	1:500

at 4 °C (12000 rpm centrifugation for 25 min). Next we absorbed supernatant, added 1/4 amount of SDS, boiled for 10 min, and preserved the samples at - 20 °C for Western blot detection. The total protein was separated by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was carried out by blocking the membranes in 5% non-fat milk for 4 h and incubated with diluted primary antibody overnight. The dilution multiple of the primary antibody is shown in Table 2. Then, the membranes were washed in PBST three times, incubated with secondary antibody, and washed again in PBST three times. The protein bands were examined using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China) and X-ray films (TransGen Biotech Co., Beijing, China). The relative abundance of the proteins was normalized to β-actin [41].

2.7. Data analysis

Statistical analysis of all data was carried out with one-way analysis of variance (ANOVA) using GraphPad Prism Version 5.0 software, and Tukey's Multiple Comparison Test was used to analyze the differences. All the experiments were performed at least three times. The results were expressed as the means ± standard deviations. There were significant differences between two or more groups with different letters, $P < 0.05$; the same letter indicated that there was no significant difference between different groups, $P > 0.05$.

3. Results

3.1. AO/EB staining

AO/EB staining was used to observe the effect of ATR on lymphocyte necrosis in carp. As shown in Fig. 1, AO/EB staining showed that necrotic cells appeared red, apoptotic cells appeared bright orange, and normal cells appeared green after ATR treatment. The results of each group were from five randomly selected visual fields for counting, and the number of necrotic cells was determined. The results showed that with the increase in ATR concentration, the proportion of red cells increased gradually and there were significant differences ($p < 0.05$).

3.2. Flow cytometry analysis

The lymphocytes are suspended cells and the results of fluorescence staining are not enough to accurately describe cell death. To further establish the proportion of necrotic cells induced by ATR, flow cytometry was used to detect the degree of lymphocyte necrosis after ATR treatment, as shown in Fig. 2 (Q1: Necrotic cells, Q2: Late apoptotic cells, Q3: Early apoptotic cells, Q4: normal cells). The results showed that the percentage of lymphocyte necrosis increased significantly after ATR treatment compared with the control group ($p < 0.05$). There was no significant difference in the percentage of necrotic cells between the 20 and 40 μg/L ATR groups.

3.3. The expression of mir-181–5p and its target genes

According to the predictions of the website (http://www.targetscan.org/fish_62/) , we selected the common miRNA of two target genes, which was miR-181–5p. As shown in Fig. 3A, qRT-PCR was used to evaluate the expression levels of miR-181–5p, HK2, and TNF-α in splenic lymphocytes. The results showed that ATR significantly inhibited the expression of miR-181–5p ($p < 0.05$). On the contrary, ATR exposure significantly increased the expression of TNF-α and HK2 ($p < 0.05$). We searched the target gene of different species on NCBI (https://www.ncbi.nlm.nih.gov/pubmed/), and compared these 3′UTR sequences of the target gene with the seed region of miR-181–5p in order to find the binding sites, as shown in Fig. 3B. These findings suggest that ATR exposure significantly alters the expression patterns of miR-181–5p, TNF-α, and HK2 in splenic lymphocytes.

3.4. The mRNA levels of the inflammation-related genes

We further examined the expression of NF-κB signaling pathway-related genes (TAK1, NF-κB, IKKβ) and downstream related genes (iNOS, IL-6, IL-8) in lymphocytes after ATR exposure for 18 h, as shown in Fig. 4. We found that the mRNA levels of NF-κB, TAK1, IKKβ, iNOS, IL-6, and IL-8 were significantly increased after ATR exposure ($p < 0.05$). In general, the expression level of related genes was positively correlated with the concentration of ATR. In particular, at 60 μg/L ATR concentration, the enhancement was more significant than that in the control group ($p < 0.01$).

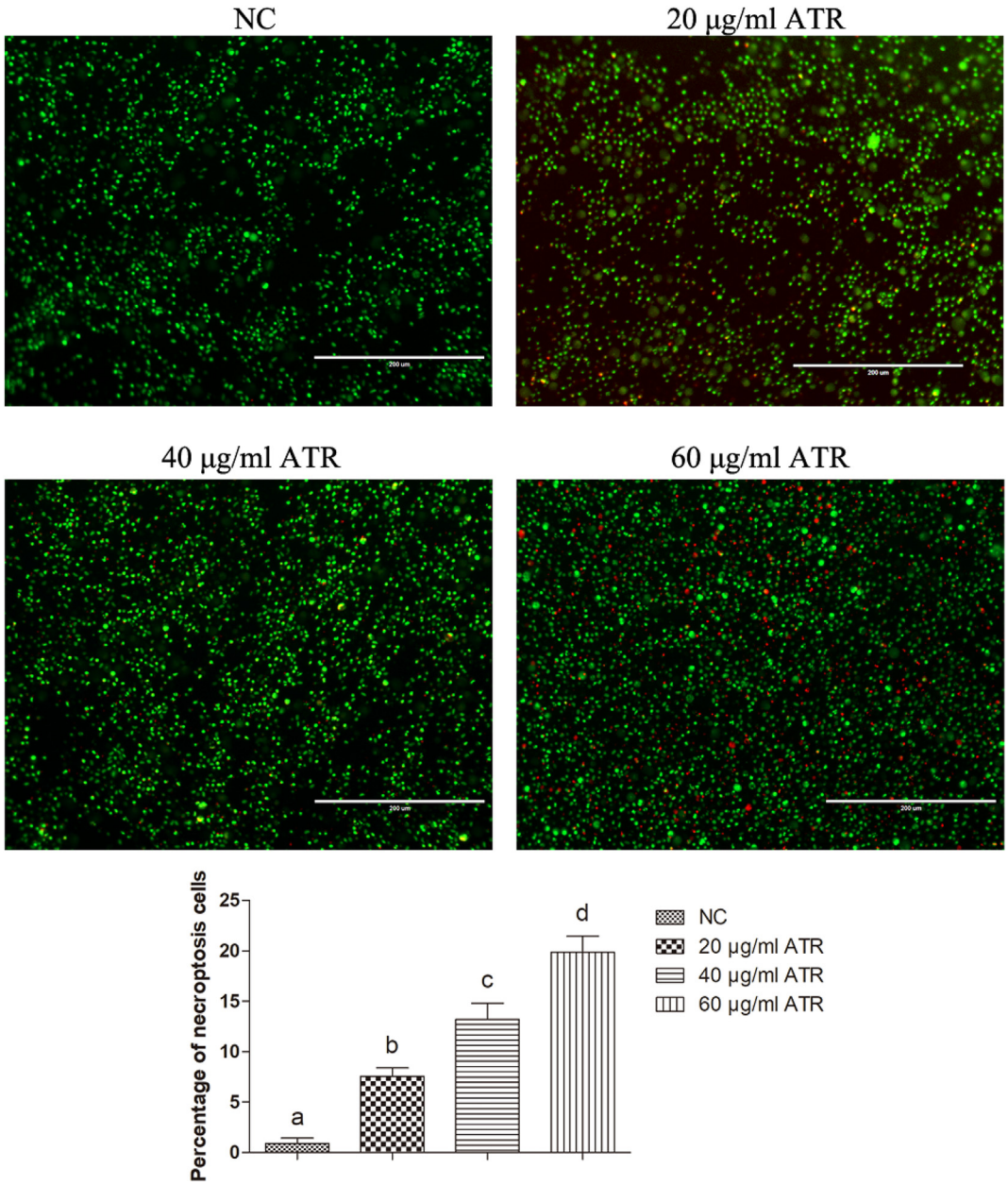

Fig. 1. Effect of ATR on lymphocyte necrosis by AO/EB staining. Lymphocyte were treated with different concentrations (20, 40, and 60 μg/ml) of ATR for 18 h. AO/EB staining showed that necrotic cells appeared red, apoptotic cells appeared bright orange, and normal cells appeared green. The results of each group were from five randomly selected visual fields for counting, and the number of necrotic cells was determined. The results were expressed as the means ± standard deviations. There were significant differences between two or more groups with different letters, $P < 0.05$; the same letter indicated that there was no significant difference between different groups, $P > 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. The mRNA levels of glycometabolism-related genes

We further examined the mRNA expression of glycometabolism-related genes, including pyruvate dehydrogenase complex X (PDHX), succinate dehydrogenase B (SDHB), and pyruvate kinase (PK), as shown in Fig. 5. The results showed that the mRNA levels of PDHX, PK, and SDHB in lymphocytes were significantly increased after ATR exposure ($p < 0.05$). The levels of related genes were positively correlated with the concentration of ATR. In particular, at 60 μg/L ATR concentration, the enhancement was more significant than that in the control group ($p < 0.01$).

3.6. The mRNA and protein levels of necroptosis markers

To confirm that the necroptosis signaling pathway was activated after ATR exposure for 18 h, we analyzed the mRNA expression of the major molecules FADD, MLKL, RIP1, RIP3, and CYLD in the necroptosis signaling pathway, as shown in Fig. 6A. We found that the mRNA levels of necroptosis-related genes were significantly increased after ATR exposure. The expression level of related genes was positively correlated with the concentration of ATR. In particular, at 60 μg/L ATR concentration, the enhancement was more significant than that in the control group ($p < 0.01$). Then, the key proteins in the necroptosis pathway were analyzed by Western blotting, as shown in Fig. 6B. The Western blot results showed that the protein levels of RIP3 and MLKL were consistent with that of qRT-PCR. The protein levels of RIP3 and

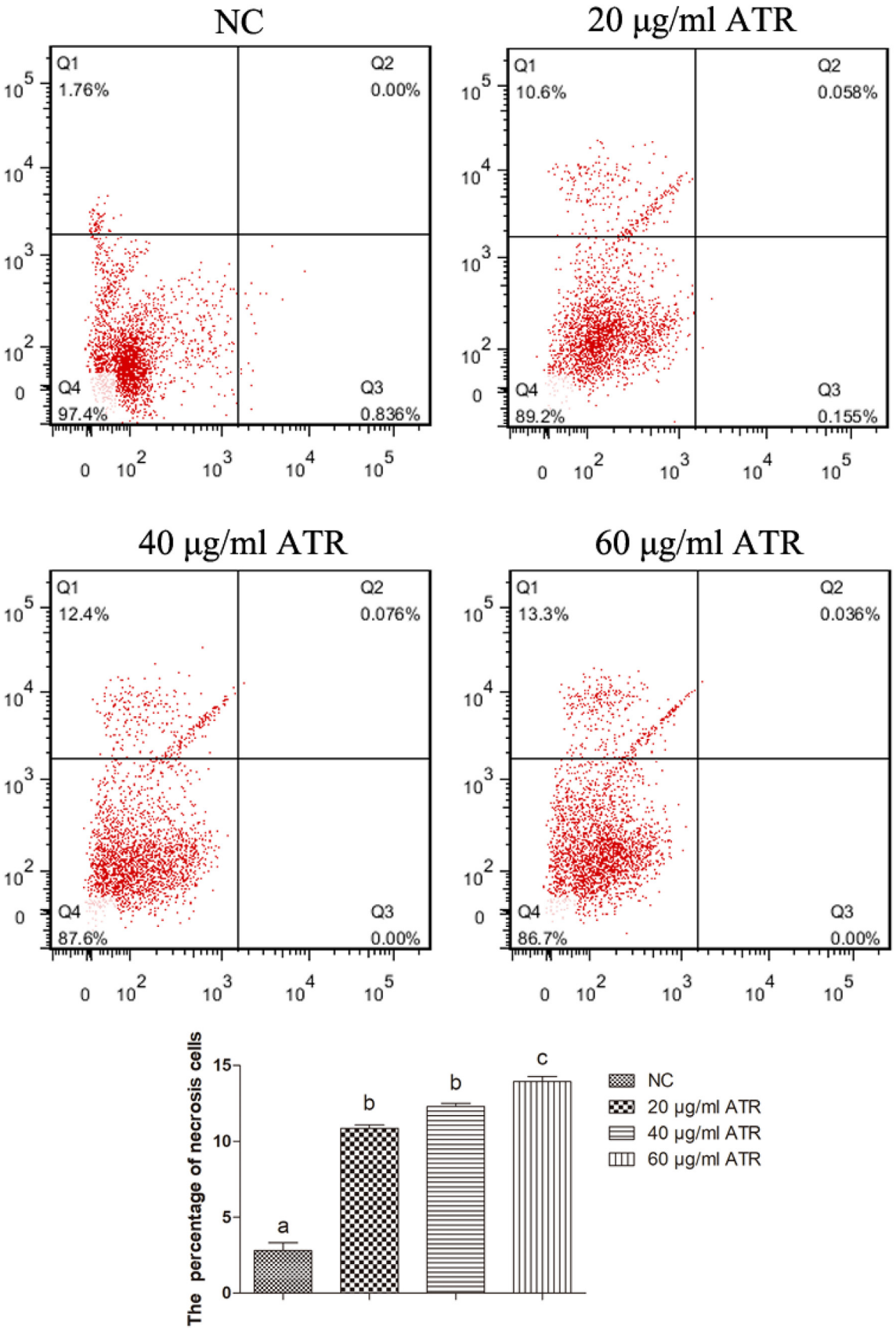

Fig. 2. Effect of ATR on lymphocyte necrosis by flow cytometry analysis. Lymphocyte were treated with different concentrations (20, 40, and 60 μg/ml) of ATR for 18 h. Flow cytometry was used to detect the degree of lymphocyte necrosis after ATR treatment(Q1: necrotic cells, Q2: late apoptotic cells, Q3: early apoptotic cells, Q4: normal cells). The results were expressed as the means ± standard deviations. There were significant differences between two or more groups with different letters, $P < 0.05$; the same letter indicated that there was no significant difference between different groups, $P > 0.05$.

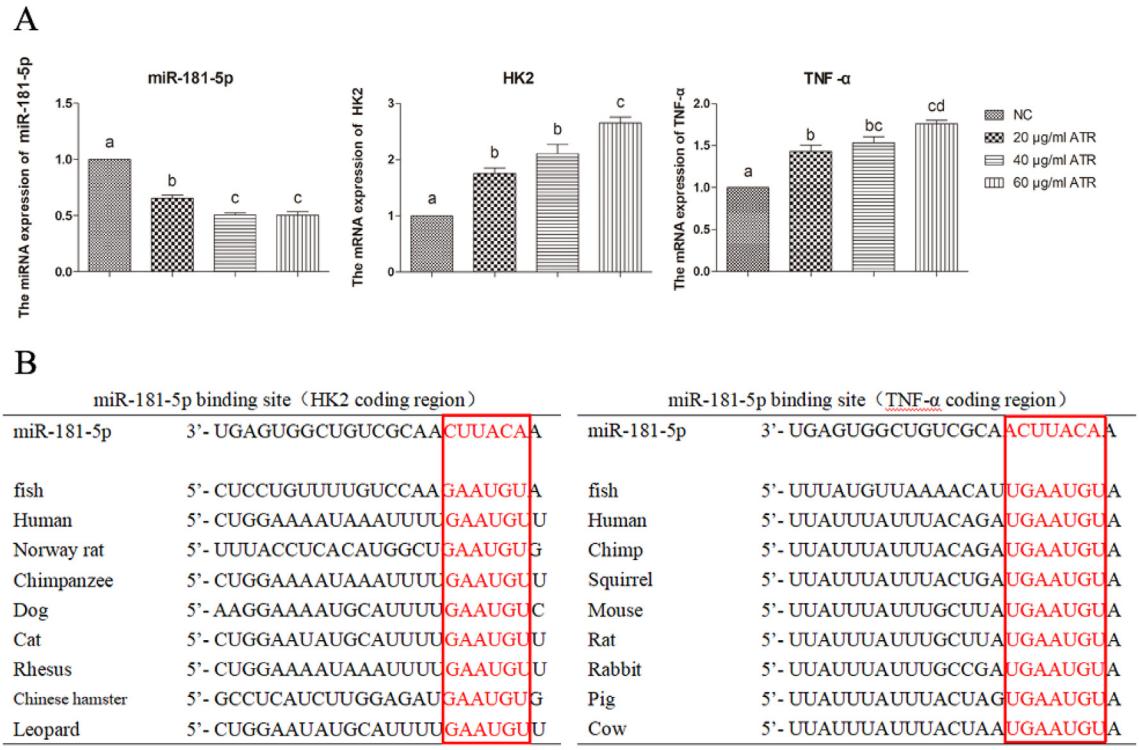

Fig. 3. A: Expression levels of miR-181–5p, TNF-α and HK2 in lymphocytes exposed to ATR. Lymphocyte were treated with different concentrations (20, 40, and 60 μg/ml) of ATR for 18 h. B: The conserved sequence in the 3′UTR of TNF-α and HK2 is predicted to be the target of miR-181–5p.

MLKL were significantly increased after ATR exposure. In particular, at 60 μg/L ATR concentration, the enhancement was more significant than that in the control group ($p < 0.01$).

4. Discussion

Necroptosis, as a newly defined type of cell death, has far-reaching significance in modern toxicological research. It is reported that triptolide caused necroptosis by upregulating the expression of MLKL and RIP1, thereby exerting its toxicity to the liver and inducing abnormal liver function and cell death in mice [42]. Chlorpyrifos damaged the immune function of carp [43], causing necroptosis in peripheral blood neutrophils of carp by inducing high levels of CYLD, RIP3, RIP1, and MLKL [44]. In our experiment, the levels of CYLD, RIP3, RIP1, FADD, and MLKL were increased in splenic lymphocytes after ATR exposure, flow cytometry and fluorescence staining, which also indicated that ATR exposure leads to an increase in the number of necrotic cells. Our results are consistent with previous studies suggesting that ATR causes

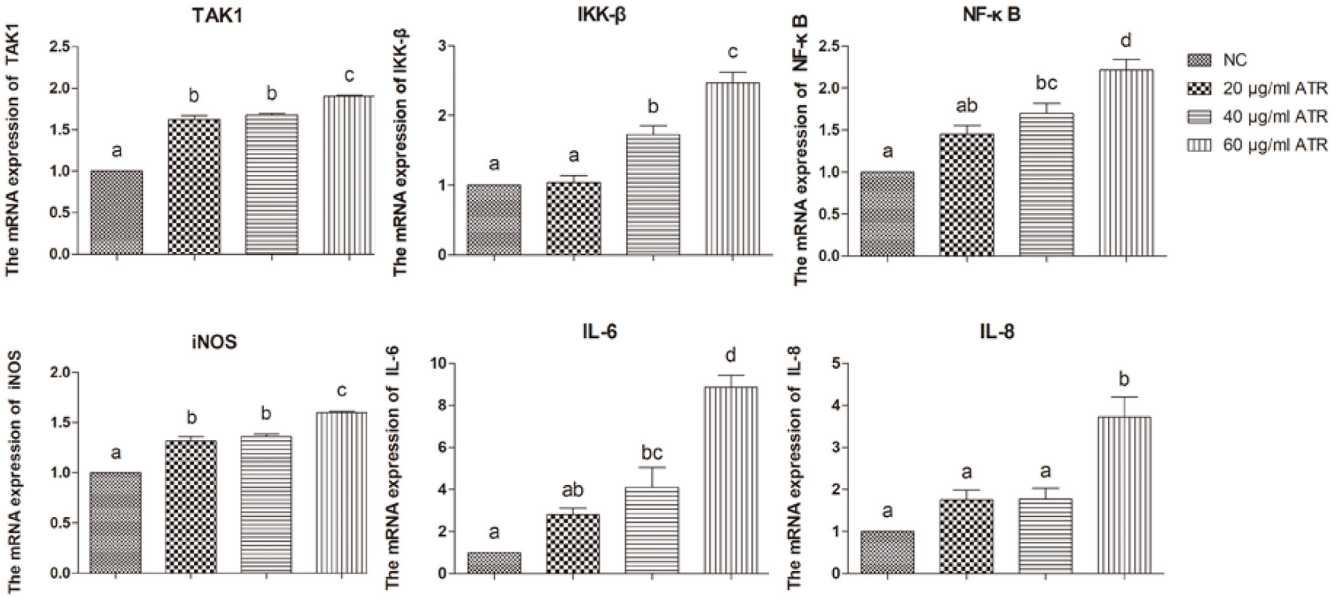

Fig. 4. mRNA levels of the NF-κB pathway after ATR exposure. Lymphocyte were treated with different concentrations (20, 40, and 60 μg/ml) of ATR for 18 h. The results were expressed as the means ± standard deviations. There were significant differences between two or more groups with different letters, $P < 0.05$; the same letter indicated that there was no significant difference between different groups, $P > 0.05$.

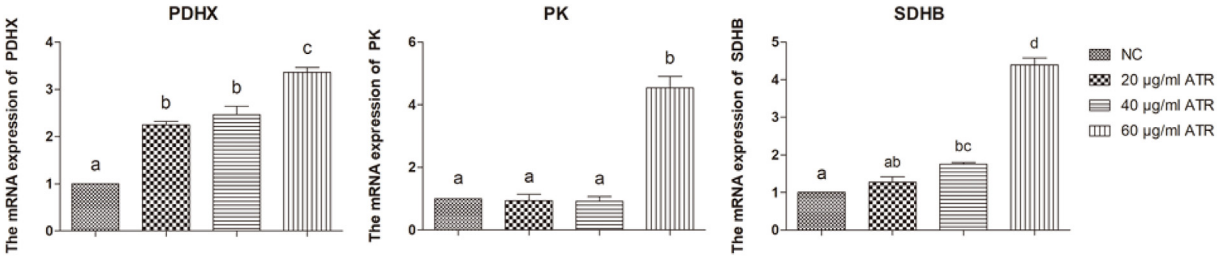

Fig. 5. mRNA levels of some glycometabolism-related genes after ATR exposure. Lymphocyte were treated with different concentrations (20, 40, and 60 μg/ml) of ATR for 18 h. The results were expressed as the means ± standard deviations. There were significant differences between two or more groups with different letters, $P < 0.05$; the same letter indicated that there was no significant difference between different groups, $P > 0.05$.

necroptosis in carp lymphocytes, damages the immune system of carp and then exerts its immunotoxicity.

The study has shown that the expression of miRNAs related to angiogenesis, cancer, and neurodevelopment was affected by ATR in humans and zebrafish [45]. Seahyoung Lee et al. found that miR-181 targeted 3′UTR of the HK2 gene and then regulated HK2 in rats [26]. In this experiment, we observed a significant decrease in the expression of miR-181–5p in lymphocytes exposed to ATR, which was accompanied by an increase in the expression of HK2, consistent with previous studies. In addition, as shown in Fig. 3B, by comparing and analyzing the 3′UTR gene sequences of different species of HK2, it was found that the 3′UTR of HK2 in fish shared a common binding site of miR-181–5p with a variety of organisms, which proved that miR-181–5p is targeted to regulate HK2 from another point of view. Energy metabolic dysfunction is closely related to immune function. The mechanism of metabolism in the immune cell would provide effective treatment opportunities for changes in immune response, targeted infection, vaccine response, cancer monitoring, autoimmune and inflammatory diseases [46]. Glycometabolism is an important part of energy metabolism, and HK2 is a key enzyme in glycometabolism [47]. The results showed that the downregulation of energy metabolism-related genes in chicken peripheral blood lymphocytes was induced by increasing reactive oxygen species after H_2S exposure [48]. Cadmium caused disordered expression of energy metabolism-related genes in spleen [49–51]. Many studies have shown that necroptosis is associated with energy metabolism in cells. It has been reported that the glycolysis pathway was related to the anti-necrosis mechanism. Glycolysis metabolism eliminated mitochondrial free radicals partly by pyruvic acid and prevented the

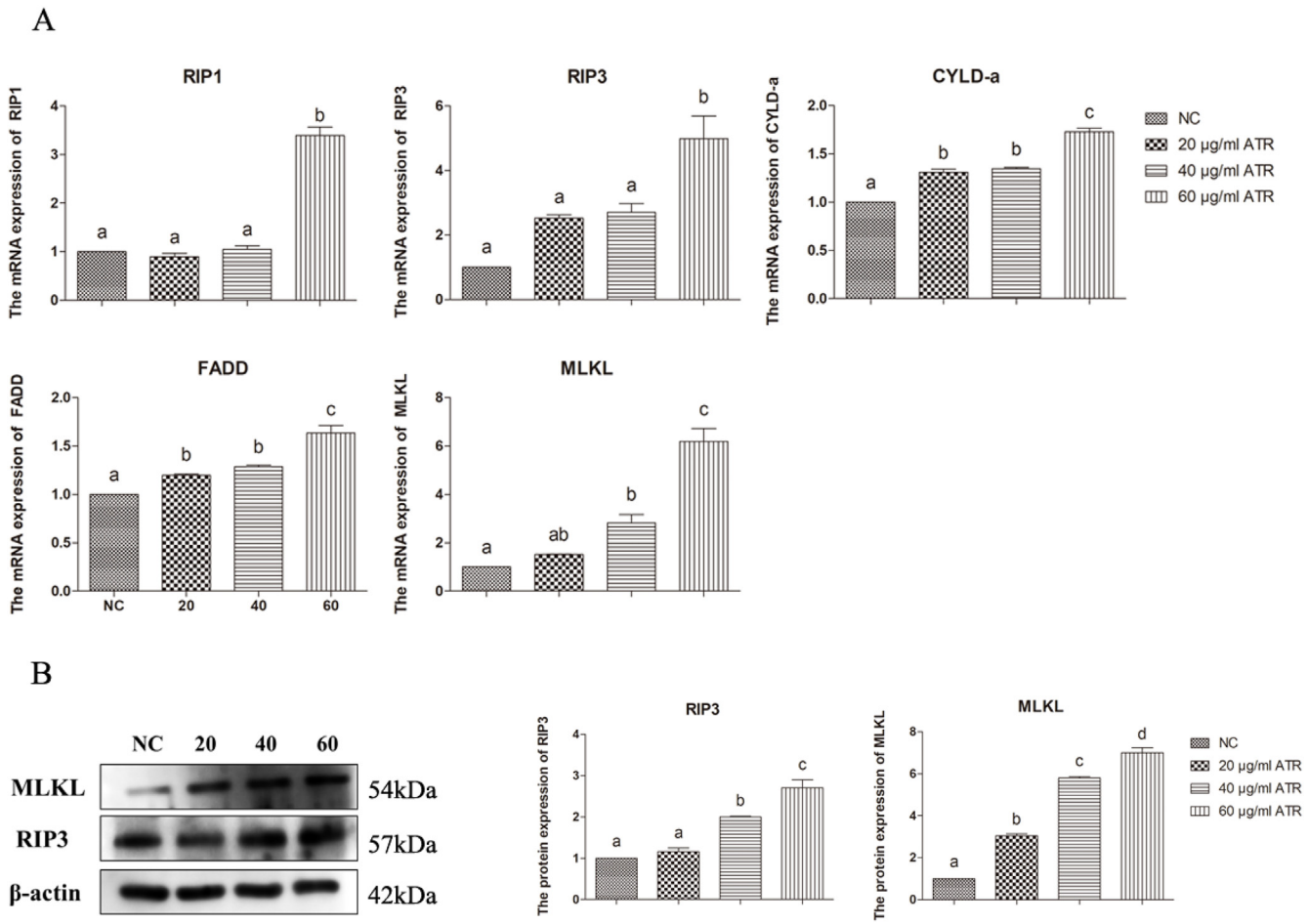

Fig. 6. A: mRNA levels of necroptosis markers after ATR exposure. B: Protein levels of necroptosis markers after ATR exposure. Lymphocyte were treated with different concentrations (20, 40, and 60 μg/ml) of ATR for 18 h. The results were expressed as the means ± standard deviations. There were significant differences between two or more groups with different letters, $P < 0.05$; the same letter indicated that there was no significant difference between different groups, $P > 0.05$.

necrosis in hypoxic cancer cells [52]. Hiroshi Kanda et al. found that reduced expression of energy production-related genes did not affect cell death induced by the apoptotic genes. Energy production-related genes played a special role in non-apoptotic cell death, which indicates that the downregulation of energy production-related genes inhibited the pattern of cell death induced by this characteristic; i.e., necroptosis [53]. In our experiment, the mRNA levels of PK, PDHX, and SDHB in carp lymphocytes were increased after ATR exposure. Therefore, we could infer that ATR further induced necroptosis in carp lymphocytes by increasing the level of energy metabolism. Our results are consistent with previous studies. Yang et al. have shown that RIP3 targeted the pyruvate dehydrogenase complex (PDH) to increase aerobic respiration in TNF-induced necroptosis [54]. Liu and Rizza et al. have shown that necroptosis was accompanied by increased expression of succinate dehydrogenase (SDH) [55,56]. PK, HK, PDHX, and SDHB are key genes in cell energy metabolism, and their expression levels reflect the energy metabolism level of cells [48]. We can further confirm that ATR can increase the expression of HK2 by inhibiting the expression of miR-181–5p, thereby enhancing the level of glycometabolism, which is one of the mechanisms of necroptosis in lymphocytes.

MiRNAs have multiple targeting functions, which simultaneously regulate multiple target genes and play corresponding biological functions. Studies have shown that miR-155 had 147 target genes involved in the regulation of breast cancer, including apoptosis, differentiation, angiogenesis, proliferation and epithelial-mesenchymal transformation [57]. MiR-223 regulated the pathophysiological state of liver diseases such as the inflammatory response of the liver by targeting a variety of target genes, including the NF-κB pathway and the nucleotide-binding oligomerization domain-like receptor protein 3 inflammasome [58]. In this experiment, the results also confirm that miR-181–5p targeted TNF-α while regulating HK2. That is to say, the expression of miR-181–5p in lymphocytes decreases significantly after ATR exposure, accompanied by an increase in the expression level TNF-α. The previous study has shown that miR-181c inhibited IL-6-mediated beta cell apoptosis by targeting TNF-α [59]. Reduction in TNF-α was observed in human major trauma models with a significant increase in miR-181 [60]. In the lipopolysaccharide-induced astrocyte inflammation model, knockdown of miR-181 caused increased expression of TNF-α [61]. Our results are consistent with previous studies suggesting that ATR exposure increases the expression of TNF-α by targeting miR-181–5p. In addition, as shown in Fig. 3B, by comparing and analyzing the 3′UTR gene sequences of different species of TNF-α, it was found that the 3′UTR of TNF-α in fish shared a common binding site for miR-181–5p with a variety of organisms, which proved that miR-181–5p is targeted to regulate TNF-α from another point of view. TNF-α is an important upstream gene of the NF-κB signaling pathway [62]. The classical NF-κB signaling pathway plays an important role in the immune response and inflammatory injury. Inhalation of hydrogen sulfide induced bursal inflammation and aggravated pneumonia in broiler chickens by regulating downstream genes of the NF-κB signaling pathway, resulting in an imbalance of Th1/Th2, thereby causing immune damage [63,64]. Selenium deficiency would aggravate inflammatory injury in the liver by increasing the expression of downstream genes of NF-κB [65]. Inflammation is also closely related to necroptosis. It is reported that TNF-α, as a downstream target gene of the classical NF-κB signal pathway, induced necroptosis in bronchial epithelial cells [66]. Yang et al. showed that inflammation caused necroptosis in chicken cardiomyocytes [28]. In our experiments, ATR exposure led to elevated expression levels of the related genes TAK1, IKKβ, NF-κB, iNOS, IL-6 and IL-8 in the inflammation. Previous studies showed that TNF binds to its receptor (TNFR1) to form a complex that could lead to necroptosis [67]. TAK1 regulated oxidative stress and activity of RIP1 kinase through the NF-κB signal pathway, inducing cell necroptosis [68]. H_2S induced jejunal inflammation in broiler chickens by activating the NF-κB pathway, increasing the levels of NF-κB, TNF-α and other genes [69]. Our results are consistent with these reports that ATR increased the expression of TNF-α by inhibiting the expression of miR-181–5p, and then activated the inflammatory pathway of NF-κB to induce lymphocyte necroptosis.

In conclusion, our results suggest that ATR exposure upregulates the expression of TNF-α and HK2 by downregulating the expression of miR-181–5p, which further induces inflammation and increases glycometabolism, leading to necroptosis in carp lymphocytes and destroying the immune function in carp. Our experiments linked miR-181–5p, inflammation, glycometabolism, and necroptosis. We may indirectly change the level of lymphocyte necroptosis by regulating the expression of miR-181–5p. The present study refines the mechanistic theory of ATR immunotoxicity in fish and complements risk assessments of aquatic animal health.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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