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MicroRNA-155 and microRNA-181a, via HO-1, participate in regulating the immunotoxicity of cadmium in the kidneys of exposed *Cyprinus carpio*

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ABSTRACT

Cadmium (Cd) is a nonessential metal that is a contaminant in aquatic ecosystems. Cd can accumulate in aquatic animals, leading to detrimental effects in tissues, and Cd exposure can induce immunotoxicity in fish. MicroRNAs (miRNAs) play critical roles in immune responses, yet the participation of miRNAs in Cd-induced immunotoxicity remains poorly understood. The present study evaluated the effects of Cd exposure on the immune responses and the mRNAs and miRNAs expressions of immune-related genes in *Cyprinus carpio* (*C. carpio*). Then, microRNA-155 (miR-155) was overexpressed and microRNA-181a (miR-181a) was knocked down to determine which miRNA plays a key role in the immune response to Cd. The results showed that 0.5 mg/L Cd^{2+} significantly decreased the activity of alkaline phosphatase (AKP) and acid phosphatase (ACP) in the kidneys of *C. carpio*. Cd exposure upregulated the mRNA expressions of interleukin (IL)-1β, IL-8, nuclear factor-kappa B (NF-κB), tumour necrosis factor-α (TNF-α), and Toll-like receptor 4(TLR-4) and downregulated those of IL-10 and heme oxygenase-1 (HO-1) in *C. carpio* kidneys. Cd exposure also led to upregulation of miR-155 and miR-181a expressions. Furthermore, AKP and ACP activity in the kidneys was markedly changed after intraperitoneal injection of *C. carpio* with miR-155 agomir and miR-181a antagomir. All detected mRNA expressions were significantly decreased after injection of miR-155 agomir, and IL-10, NF-κB, TNF-α, and HO-1 mRNA expressions were markedly increased after injection of miR-181a antagomir. The results of this study demonstrate that Cd exposure can immunocompromise *C. carpio* by targeting HO-1 through miR-155 and miR-181a. This is the first study to reveal that Cd exposure induces immunotoxicity through miR-155 and miR-181a in the kidneys of *C. carpio*.

1. Introduction

Cadmium (Cd) is a known noxious heavy metal that is highly persistent in the aquatic environment and even in drinking water. Since Cd has a biological half-life of 15–20 years in organisms, it can cross the blood-brain barrier and placenta and is then systemically distributed to all bodily tissues; however, the kidneys are target organs particularly affected by Cd toxicity [1]. Experimental and epidemiological studies have shown that Cd can induce carcinogenesis [2], teratogenesis [3], reproductive toxicity [4], genotoxicity [5], nerve toxicity [6] and immunotoxicity [7].

The immunotoxicity of Cd has been demonstrated in diverse aquatic animals. Several studies have reported that Cd exposure can induce both non-specific and specific immunity in fish [8–11]. For example, the immune responses induced by Cd include changes in immune enzyme activities, serum immunoglobulin M (IgM) levels [10,12], immune gene expressions [13], and pro-inflammatory and anti-inflammatory cytokine levels [9,14].

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate target gene expression by binding to the 3′ untranslated regions (3-'UTRs) of target mRNAs. MiRNAs are involved in many biological processes. Recent studies have indicated that miRNAs serve as important regulators of immune responses [15]. MiR-146, miR-155, miR-21, miR-210, miR-181a, miR-223, miR-9, miR-132 and other miRNAs play key roles in regulating the inflammatory process [15–17]. In addition, miR-146, miR-155, miR-21, miR-10, miR-36, miR-184 and miR-184b have been found to play critical roles in animal responses to Cd stress [7,18,19].

In the network of miRNAs and target genes involved in Cd stress, miRNA-146a expression is regulated by the transcription factor nuclear

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factor-kappa B (NF-κB), which is considered an important regulatory factor of the transcription of inflammatory genes [20–22]. Cd can activate the transforming growth factor-β (TGF-β) pathway targeting miR-155 in human placental trophoblast cells [19]. Inflammatory cytokines include both pro-inflammatory cytokines (e.g., interleukin [IL]-1β, IL-8, tumour necrosis factor [TNF]-α) and anti-inflammatory cytokines (e.g., IL-10, TGF-β) [14]. In addition, miR-210, miR-181, miR-223, and miR-21 have been reported to regulate Toll-like receptor (TLR) expression [15]. The immunotoxicity of Cd has been studied extensively, but the regulatory network of fish immune responses has been less thoroughly explored.

The present study aimed to explore which miRNA plays a key role in the immunotoxicity of Cd to *Cyprinus carpio* (*C. carpio*). Cd can bioaccumulate in the fish body, and fish tissues can accumulate high concentrations of Cd. Cd^{2+} levels and alkaline phosphatase (AKP) and acid phosphatase (ACP) activity were measured in the kidneys, livers, and spleens of *C. carpio* exposed to Cd, and the tissues exhibiting the most severe immune function injuries were selected for further study. The expressions of select immune-related genes (e.g., IL-1β, IL-8, IL-10, NF-κB, TNF-α, TLR-4, heme oxygenase-1[HO-1]) and miRNAs (e.g., miR-146a, miR-155, miR-181a, miR-210, miR-182) were measured in the kidneys of *C. carpio*. Furthermore, *C. carpio* were intraperitoneally injected with miR-155 agomir and miR-181a antagomir, and the activity of AKP and ACP and the mRNA expressions of immune-related genes were detected.

2. Materials and methods

2.1. Chemicals

$CdCl_2$ (99% purity) was obtained from Aladdin Industrial Corporation (Shanghai, China). All other chemicals used in the present study were of analytical grade and were obtained from local companies.

2.2. Cd exposure and sample collection

Common carp (with an average weight of approximately 80 g) were purchased from a fish farm in Zhengzhou and maintained in tanks (200 L) for 2 weeks prior to the experiment. After acclimation, forty healthy fish were randomly divided into four groups. Water quality standard for fisheries of China (GB11607-89) stipulated Cd concentrations not exceeding 0.005 mg/L. This study chose 0.005 mg/L Cd^{2+} as low concentration, 0.05 mg/L Cd^{2+} as middle concentration, 0.5 mg/L Cd^{2+} as high concentration. The fish in each of three Cd exposure groups were incubated in water containing 0.005 mg/L Cd^{2+}, 0.05 mg/L Cd^{2+}, or 0.5 mg/L Cd^{2+}, and the fish in the control group were incubated in clean water. The experiments were carried out in triplicate. Half of the water was exchanged every 3 days and Cd^{2+} was added to the water every time. The fish were fed 1.5% body weight twice a day with the commercial pellet, and after 15 min the remaining food was removed. The water temperature was maintained at 18 ± 2 °C, and the pH was controlled at 7.0 ± 0.1. After 30 days, the fish were anesthetized using MS-222 (40 mg/L), then were placed on ice and carefully dissected to isolate tissues, the tissues were excised immediately and then stored at −80 °C pending analysis.

The animal experiments were carried out in accordance with the protocols of the 'Guidelines for Experimental Animals' of the Ministry of Science and Technology (Beijing, China) and was approved by Animal Care and Use Ethics Committee of the Henan Normal University.

2.3. Cd accumulation in tissues

Head kidney, liver, kidney and spleen (0.5 g) tissues were mixed in Teflon crucibles with 10 mL of HNO_3 and 4 mL of $HClO_4$. After overnight digestion, they were hated at 200 °C for 1 h or longer until a transparent solution was obtained. After dilution, the concentrations of cadmium were analysed by flame atom absorption spectrophotometry (FAAS, Analytik Jena, ZEEnit 700P) under the following conditions: type of flame air-acetylene; wavelength 228.8 nm; slit width 1.2 nm; gas flow 1.0 L/min; lamp current 2.0 mA.

2.4. Measurement of enzyme activity

The tissues were rinsed with phosphate-buffered saline (PBS) and then homogenized in 10% (w/v) PBS in an ice bath. The crude homogenates were centrifuged at 2,500 rpm for 10 min at 4 °C, and the supernatants were stored on ice for enzyme activity assays. AKP and ACP activity was measured using a standard assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The AKP activity was determined through a spectrophotometric method. Briefly, 5 μL supernatants were incubated in 96-well plates with 50 μL 0.5% disodium phenyl phosphate and 50 μL 0.05 mol/L borax sodium hydroxide buffer (pH 10.0), then incubated for 15 min at 37 °C. Next, 150 μL of 2.5% potassium ferricyanide containing0.5% 4-Aminoantipyrine were added. The absorbance was measured at 520 nm wavelength by using a micro plate spectrophotometer (Thermo Fisher, USA). The ACP activity was determined through a spectrophotometric method. Briefly, 5 μL supernatants were incubated in 96-well plates with 50 μL 0.5% disodium phenyl phosphate and 50 μL 0.05 mol/L sodium acetate buffer (pH 5.0), then incubated for 30 min at 37 °C. Next, 100 μL 0.05 mol/L borax sodium buffer (pH 9.0) and 150 μL of 2.5% potassium ferricyanide containing0.5% 4-Aminoantipyrine were added. After incubated at room temperature for 10 min, the absorbance was measured at 520 nm wavelength by using a micro plate spectrophotometer (Thermo Fisher, USA). The supernatants were tested for protein content using a BCA Protein Assay Kit (Beyotime Biotechnology, China) for calculation of AKP and ACP activity. One unit of AKP activity was defined as the amount required to liberate 1 mg of phenol per gram tissue protein in 15 min at 37 °C. One unit of ACP activity was defined as the amount required to liberate 1 mg of phenol per gram tissue protein in 30 min at 37 °C.

2.5. Intraperitoneal administration of miR-155 agomir and miR-181a antagomir

Common carp (25–28 g) were purchased from a fish farm in Zhengzhou and maintained in tanks (100 L) for 2 weeks prior to the experiment. After acclimation, twenty-four healthy fish were randomly divided into four groups (the miR-155 agomir group, the miR-155 agomir negative control group, the miR-181a antagomir group, and the miR-181a antagomir negative control group). MiRNA agomir, antagomir and negative controls were synthesized by RIBOBIO Company (Guangzhou, China). MiRNA agomirs are chemically synthesized dsRNA oligonucleotides used to mimic miRNAs. MiRNA antagomirs are single-stranded oligonucleotides used to silence endogenous miRNAs. The universal negative controls for both the agomir and antagomir were based on the sequence of cel-miR-239b-5p in *Caenorhabditis elegans*, which has been confirmed to have minimal sequence identity with miRNAs in *C. carpio*.

MiR-155 agomir (5 nmol), miR-155 agomir negative control (5 nmol), miR-181a antagomir (12.5 nmol), and miR-181a antagomir negative control (12.5 nmol) were diluted in 100 μL of physiological saline and intraperitoneally administered to fish for 3 days in succession. After 3 days, all fish were dissected; the tissues were excised immediately and then stored at −80 °C pending analysis.

2.6. mRNA expression

Total RNA was isolated and reverse-transcribed into cDNA using an RNA LA PCR Kit (TaKaRa, Japan). Quantitative PCR (qPCR) was performed with a qPCR System instrument (Bio-Rad, USA) using a SYBR PrimeScript™ Kit (TaKaRa) according to the manufacturer's

Table 1
Primers for mRNAs.

Names	Primers	Efficiency
IL-1β	Forward: 5′-CCAATTTACAATAAGACCAGC-3′ Reverse: 5′-CTTTGTATTGCATTGCTCCA-3′	95.4%
IL-8	Forward: 5′-ATGAGAATATCATTGCCACA-3′ Reverse: 5′-CCTCAGAAATGGCGGTGGAC-3′	93.2%
IL-10	Forward: 5′-CCAGCATAAAGAACTCGTA-3′ Reverse: 5′-TTGATGCCAAATACTGCTC-3′	98.1%
NF-κB	Forward: 5′-TATGAAGCAGACCTACAG-3′ Reverse: 5′-TCTTGGCATCAGGAATA-3′	99%
TNF-α	Forward: 5′-ATCATCATTCCTACTGACGG-3′ Reverse: 5′-TCATATGCACAAGATCGTGGT-3′	91.9%
TLR-4	Forward: 5′-TTTACTTGATCTACATGCCAA-3′ Reverse: 5′-AAATGTCTTTGAAAGCTCC-3′	94.7.%
HO-1	Forward: 5′- GCTCATGCATATACGCGCTA Reverse: 5′- CAGAATTCCCTTGTTGCCACT	92.4%
β-actin	Forward: 5′-GATGGACTCTGGTGATGGTGTGAC-3′ Reverse: 5′-TTTCTCTTTCGGCTGTGGTGGTG-3′	97.9%

instructions. The mRNA primer sequences used for qPCR are shown in Table 1. The qPCR procedure was carried out with denaturation at 95°Cfor 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The expression levels of β-actin were used as endogenous controls within each sample, and the relative gene expression levels were calculated. Each sample was run in three parallel reactions.

2.7. MiRNA expression

Small RNA was isolated using RNAiso for Small RNA (TaKaRa, Japan) and reverse-transcribed using a Mir-X miRNA First-Strand Synthesis Kit (TaKaRa, Japan) according to the manufacturer's protocol. Subsequently, miRNA qPCR was performed on a LightCycler 96 Real-time PCR system with a Mir-X miRNA qPCR SYBR Kit (TaKaRa, Japan). The forward primer sequences of the tested miRNAs are shown in Table 2. The expression levels of U6 were used as endogenous controls within each sample, and the relative gene expression levels were calculated. Each sample was run in three parallel reactions.

2.8. Statistical analysis

The data are expressed as the mean ± standard deviation (SD) of the mean. The data were analysed by one-way analysis of variance to determine the significance of differences between the control and Cd^{2+}-treated groups. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using SPSS 18.

Table 2
Primers of miRNAs.

miRNAs	Primers
ccr-miR-146	Forward: 5′-CTGAGAACTGAATTCCATAGATGG-3′ Reverse: Takara universal primer
ccr-miR-155	Forward: 5′-TTAATGCTAATCGTGATAGGGG-3′ Reverse: Takara universal primer
ccr-miR-181a	Forward: 5′-AACATTCAACGCTGTCGGTGA-3′ Reverse: Takara universal primer
ccr-miR-210	Forward: 5′-CTGTGCGTGTGACAGCGGCT-3′ Reverse: Takara universal primer
ccr-miR-182	Forward: 5′-TTTGGCAATGGTAGAACTCACAC-3′ Reverse: Takara universal primer'
U6	Forward: 5′-TGGAACGCTTCACGAATTTGCG-3′ Reverse: 5′-GGAACGATACAGAGAAGATTAGC-3′

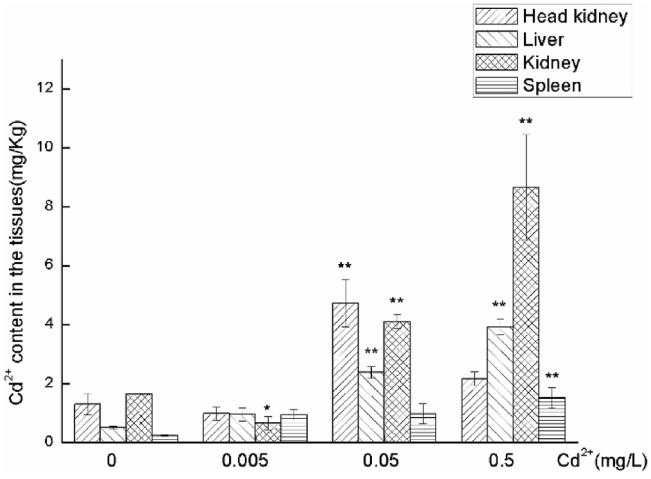

Fig. 1. Cd^{2+} accumulation in the head kidneys, livers, kidneys and spleens of *C. carpio* exposed to Cd. The results are expressed as the mean ± SD. *P < 0.05 compared with the control group, **P < 0.01 compared with the control group.

3. Results

3.1. Cd^{2+} accumulation in the tissues

To explore Cd^{2+} accumulation in the tissues, we detected Cd^{2+} levels in the head kidneys, livers, kidneys and spleens of *C. carpio* exposed to 0.005 mg/L, 0.05 mg/L, and 0.5 mg/L Cd^{2+} for 30 days. In tissues of fish from the control group, the levels of Cd^{2+} ranged from 0.23 to 1.65 mg/kg, and the highest concentrations were detected in the kidneys. After 30 days of exposure, the concentrations of Cd^{2+} in the head kidneys, livers and spleens were not significantly different between the 0.005 mg/L Cd^{2+} group and the control group ($P > 0.05$, Fig. 1). However, the levels of Cd^{2+} in the head kidneys, liver and kidneys were significantly higher ($P < 0.05$, Fig. 1) in the 0.05 mg/L Cd^{2+} group than in the control group. Furthermore, the Cd^{2+} concentrations in the liver, kidneys and spleen were significantly higher in the 0.5 mg/L Cd^{2+} group than in the control group ($P < 0.05$, Fig. 1). The maximum cadmium level in fish tissues was observed in the kidneys (8.67 mg/kg) of fish exposed to 0.5 mg/L Cd^{2+}.

3.2. Cd^{2+} induced changes in AKP and ACP activity in tissues

To investigate the immune response induced by Cd, we detected AKP and ACP activity in the head kidneys, livers, kidneys and spleens. As shown in Fig. 2, AKP activity in the kidneys was significantly lower in *C. carpio* exposed to 0.005 mg/L Cd^{2+} than in control *C. carpio* ($P < 0.01$), and AKP activity in the livers and kidneys of *C. carpio* was significantly lower in the 0.05 mg/L Cd^{2+} group than in the control group ($P < 0.05$). In addition, AKP activity in the kidneys of *C. carpio* was significantly decreased in the 0.5 mg/L Cd^{2+} group compared with that in the control group ($P < 0.01$), while AKP activity in the livers was much higher in the 0.5 mg/L Cd^{2+} group than in the control group ($P < 0.01$). Regarding AKP activity in the head kidneys and spleens of *C. carpio*, no significant differences between the control and Cd^{2+} treatment groups were detected ($P > 005$). AKP activity in the livers of *C. carpio* was significantly lower in the 0.05 mg/L Cd^{2+} group than in the control group ($P < 0.05$); however, livers AKP activity was significantly higher in the 0.5 mg/L Cd^{2+} group than in the control group ($P < 0.01$). AKP activity in *C. carpio* kidneys was significantly decreased ($P < 0.01$) in the Cd^{2+} treatment groups compared with that in the control kidneys (see Fig. 2).

The results showed that ACP activity in the head kidneys of *C. carpio* exposed to 0.005 mg/L Cd^{2+} was significantly higher than that in the

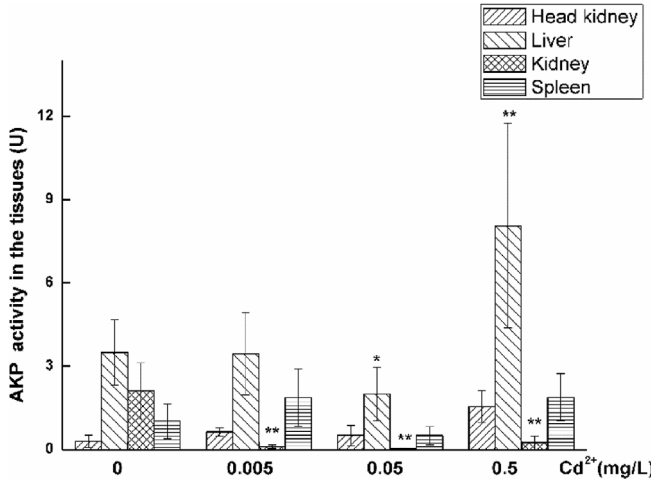

Fig. 2. AKP activity in the head kidneys, livers, kidneys and spleens of *C. carpio* exposed to Cd. The results are expressed as the mean ± SD. *P < 0.05 compared with the control group, **P < 0.01 compared with the control group.

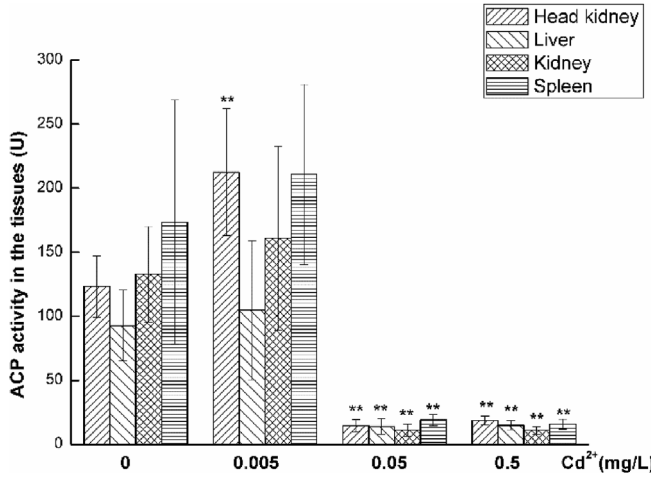

Fig. 3. ACP activity in the head kidneys, livers, kidneys and spleens of *C. carpio* exposed to Cd. The results are expressed as the mean ± SD. **P < 0.01 compared with the control group.

head kidneys of *C. carpio* in the control group (P < 0.01, Fig. 3). ACP activity in the livers, kidneys and spleens of *C. carpio* exposed to 0.005 mg/L Cd^{2+} was not significantly different from that in the livers, kidneys, and spleens of *C. carpio* in the control group (P > 0.05, Fig. 3). However, ACP activity in the head kidneys, livers, kidneys and spleens was significantly lower in *C. carpio* treated with 0.05 mg/L Cd^{2+} and 0.5 mg/L Cd^{2+} for 30 days than in control *C. carpio* (P < 0.01, Fig. 3). The kidneys were thus considered target organs associated with the immune response given the findings regarding the activity of AKP and ACP.

3.3. Cd^{2+} changed the mRNA expressions of immune genes in the kidney

To uncover the molecular mechanism of the immune response induced by Cd^{2+}, we measured the mRNA expressions of IL-1β, IL-8, IL-10, NF-κB, TNF-α, TLR-4, and HO-1 in the kidneys of *C. carpio*. Here, 0.5 mg/L Cd^{2+} significantly elevated the mRNA expressions of IL-1β, IL-8, NF-κB, TNF-α, and TLR-4 (Fig. 4, P < 0.01) in the kidneys of *C. carpio*. Kidney IL-10 and HO-1 mRNA expressions in *C. carpio* exposed to 0.5 mg/L Cd^{2+} were decreased to 15.1% and 51% of control levels (Fig. 4, P < 0.01).

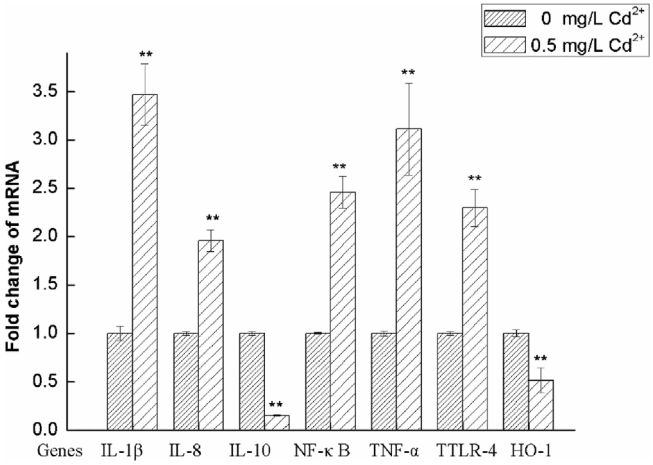

Fig. 4. mRNA expressions of IL-1β, IL-8, IL-10, NF-κB, TNF-α, TLR-4, and HO-1 in the kidneys of *C. carpio* exposed to 0.5 mg/L Cd^{2+}. The results are expressed as the mean ± SD. **P < 0.01 compared with the control group.

3.4. Cd^{2+} changed miRNA expression in the kidney

To explore which miRNAs participate in the immune response in the kidneys of *C. carpio* exposed to Cd^{2+}, the kidney miRNA expressions of miR-146a, miR-155, miR-181a, miR-210, and miR-182 were measured. The results showed that 0.5 mg/L Cd^{2+} significantly elevated miR-155 and miR-181a expressions (Fig. 5, P < 0.01). MiR-146a, miR-210 and miR-182 expressions were not significantly different in the 0.5 mg/L Cd^{2+} group compared with that in the control group.

3.5. MiRNA expression and AKP and ACP activity in the kidneys after intraperitoneal administration of agomir and antagomir

As shown in Fig. 6A, kidney miR-155 expression was significantly higher in the group receiving intraperitoneal administration of miR-155 agomir than in the corresponding negative control group (P < 0.01), and kidney miR-181a expression in the group receiving intraperitoneal administration of miR-181a antagomir was 2% of that in the miR-181a antagomir negative control group (P < 0.01). Moreover, AKP and ACP activity in the kidneys was affected by intraperitoneal administration of agomir and antagomir. AKP activity was significantly elevated in the miR-155 agomir group (P < 0.01) and markedly decreased in the miR-181a antagomir group (P < 0.01) compared to that in the

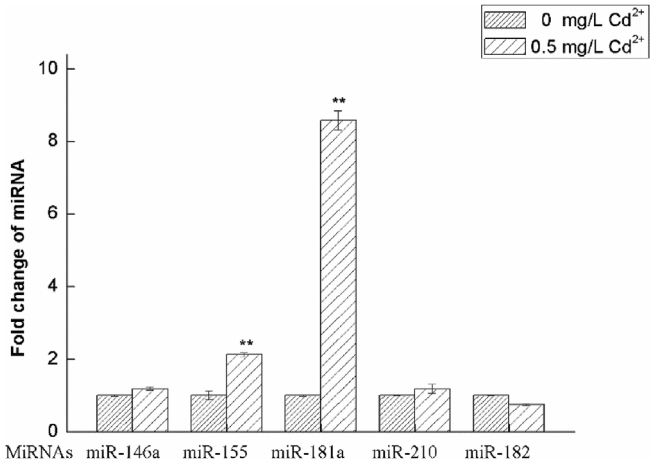

Fig. 5. MiRNA expressions of miR-146a, miR-155, miR-181a, miR-210, and miR-182 in the kidneys of *C. carpio* exposed to 0.05 mg/L Cd^{2+}. The results are expressed as the mean ± SD. **P < 0.01 compared with the control group.

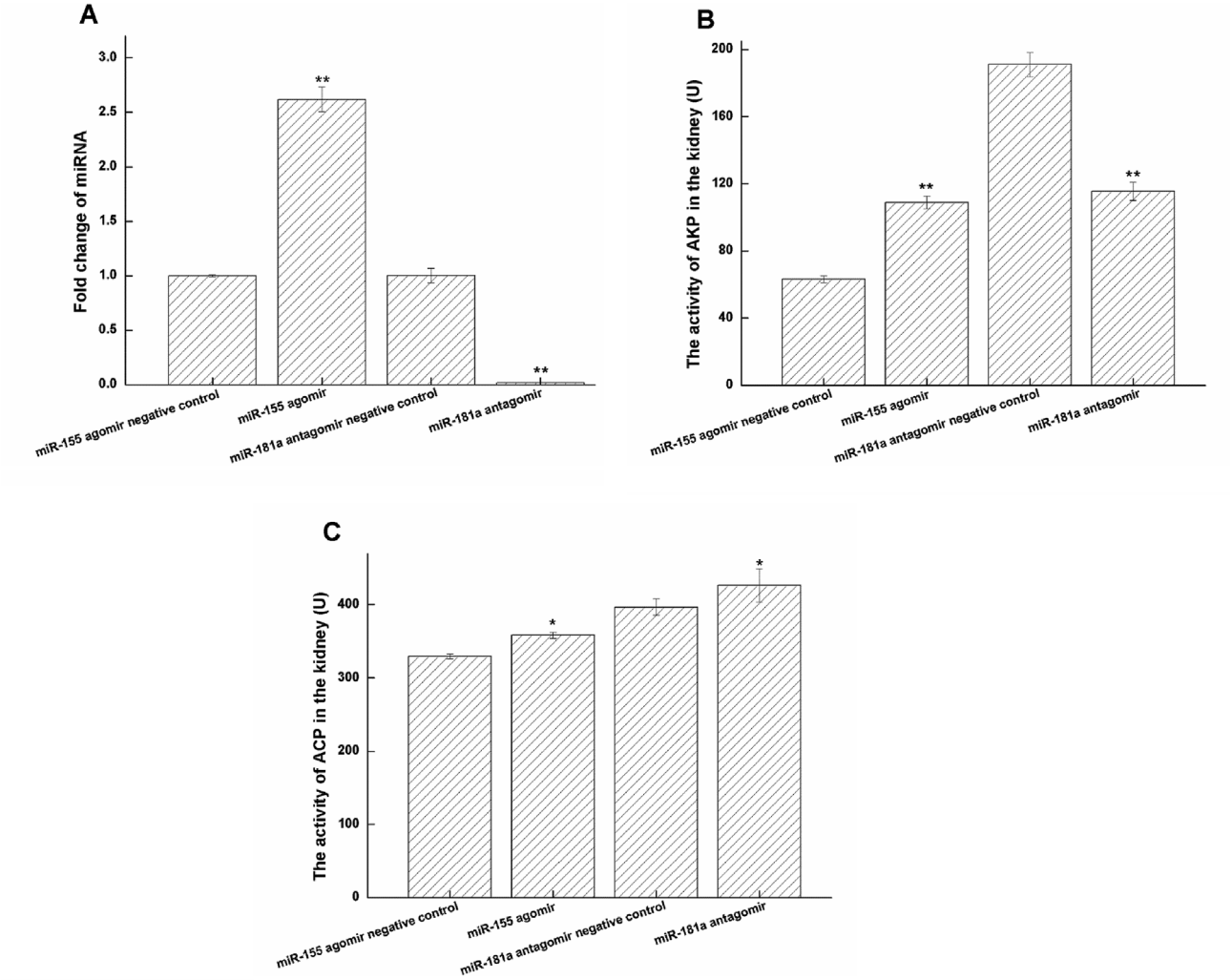

Fig. 6. Kidney miRNA expression and AKP and ACP activity after intraperitoneal administration of agomir and antagomir. A, MiR-155 expression in the miR-155 agomir and miR-155 agomir negative control groups and miR-181a expression in the miR-181a antagomir and miR-181a antagomir negative control groups. B, Kidney AKP activity after intraperitoneal administration of agomir and antagomir. C, Kidney ACP activity after intraperitoneal administration of agomir and antagomir. The results are expressed as the mean ± SD. *P < 0.05 compared with the control group, **P < 0.01 compared with the control group.

corresponding negative control groups (Fig. 6B). ACP activity was also significantly increased in the miR-155 agomir group (P < 0.05) and miR-181a antagomir group (P < 0.05) compared to that in the corresponding negative control groups (Fig. 6C).

3.6. Kidney immune gene expression after intraperitoneal administration of agomir and antagomir

The expressions of select immune genes were investigated to identify target genes of miRNAs. The mRNA expressions of IL-1β, IL-8, IL-10, NF-κB, TNF-α, TLR-4, and HO-1 were dramatically decreased in the miR-155 agomir group compared with that in the miR-155 agomir negative control group (P < 0.01) (Fig. 7A). In addition, IL-10, NF-κB, TNF-α, and HO-1 mRNA expressions were markedly higher in the miR-181a antagomir group than in the miR-181a antagomir negative control group (P < 0.01) (Fig. 7B). The results showed that miR-155 and miR-181a target multiple mRNAs to exert their effects.

4. Discussion

Several studies have shown that an immune response is induced by Cd in rohu (*Labeo rohita*) [12], zebrafish (*Danio rerio*) [11], European sea bass (*Dicentrarchus labrax*) [10], rainbow trout (*Oncorhynchus mykiss*) [13], and common carp (*C. carpio*) [23]. Cd induces immunotoxicological effects by affecting immunoenzyme activity, antibacterial activity, IgM levels and immune gene expression [12]. However, the detailed mechanisms of Cd-induced immunotoxicological effects are still poorly understood. In this study, we found that accumulation of Cd in the tissues of *C. carpio* induced immunotoxicological effects. Cd changed AKP and ACP activity, immune-related gene expression, and immune-related miRNA expression in the kidneys of *C. carpio*. After intraperitoneal injection of *C. carpio* with miR-155 agomir and miR-181a antagomir, AKP activity, ACP activity and immune-related gene expression were significantly changed. The results demonstrated that miR-155 and miR-181a target HO-1 to regulate the immune response to Cd.

Increasing accumulation of heavy metals, particularly Cd, in seafood has become a food safety and public health concern [24]. An estimated 25,000 tons of Cd per year are dumped into the environment, approximately half of which is released into rivers [23]. In aquatic ecosystems, Cd can accumulate in the gills, kidneys, livers, and spleens of fish. However, Cd is not uniformly distributed in the tissues, and the kidneys are prime target organs for Cd [25]. Indeed, in this study, irrespective of exposure concentration, the maximum cadmium levels in fish tissues were observed in the kidneys.

Exposure to Cd increases stress sensitivity in fish, which may

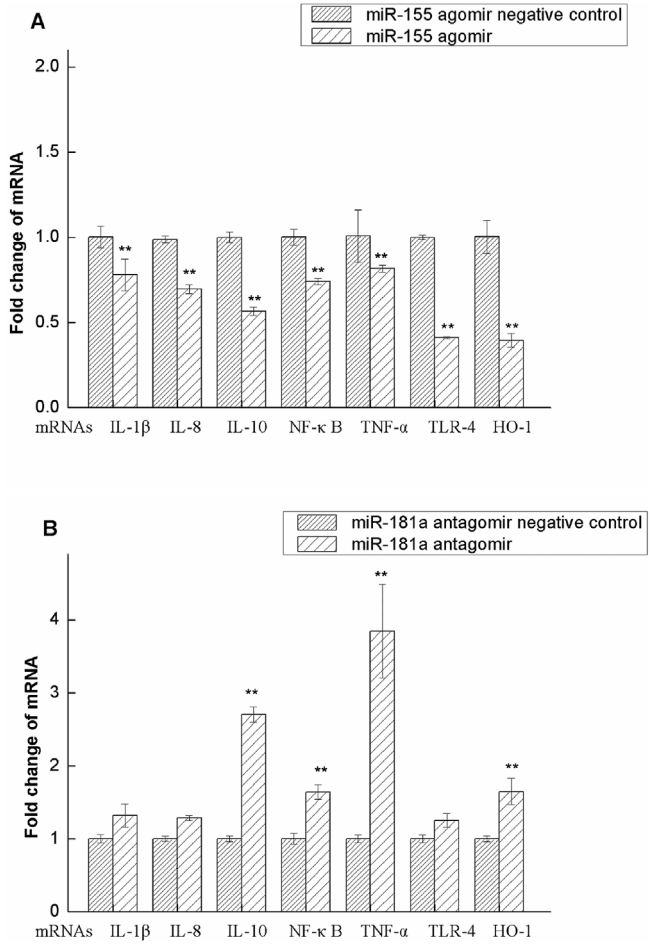

Fig. 7. Kidney immune gene expression after intraperitoneal administration of miR-155 agomir (A) and miR-181a antagomir (B).

thereby stimulate anti-inflammatory and immune responses [26]. AKP and ACP are vital lysosomal enzymes that play roles in non-specific immune defence against ambient stressors [27]. AKP is an important phosphomonoesterase that mitigates energy requirements during stress conditions to detoxify contaminants [42]. In the present study, AKP activity in the head kidneys and spleens of *C. carpio* was not significantly changed following Cd exposure; however, in the kidneys, AKP activity was significantly decreased. ACP activity can be used as a reliable tool for the assessment of environmental pollution [28,29]. For example, in hybrid tilapia (*Oreochromis niloticus* × *O. aureus*), Cd exposure greatly reduces ACP activity [33]. Likewise, in this study, the ACP activity in tested tissues was significantly decreased after 0.05 mg/L and 0.5 mg/L Cd exposure. Taken together, the decreases in AKP and ACP activity in the Cd-exposed fish indicated immune function impairment in the kidneys.

Cytokines, including both pro-inflammatory cytokines (e.g., IL-1β, IL-8, TNF-α) and anti-inflammatory cytokines (e.g., IL-10) [14], also play important roles in the immune functions of fish. Upregulation of pro-inflammatory cytokine expression and downregulation of anti-inflammatory cytokine expression can initiate and accelerate additional inflammatory processes [30]. Cd is known to induce upregulation of some mediators and markers of inflammation and to possess pro-inflammatory properties [31]. Sub-chronic exposure to Cd (75 μg/L $CdCl_2$) triggers inflammation in rare minnow (*Gobiocypris rarus*) gills, specifically inducing increases in the pro-inflammatory cytokines TNF-α and IL-1β [32]. When an organism suffers injury, IL-1β stimulates immune responses by activating lymphocytes and inducing the release of other cytokines, which in turn activate immune cells. Then, the chemokine IL-8 induces leucocyte activation, thereby mitigating tissue injury. Finally, IL-10 mediates anti-inflammatory functions via induction of IL-10-inducible genes, including HO-1 and suppressor of cytokine signalling 3 [33]. HO-1 plays important anti-inflammatory roles in a number of chronic inflammatory diseases and is critical for the suppression of *Tlr4*-stimulated TNF-α expression [34,35]. As key components of the innate immune system, TLRs mediate innate immune responses for host defence. TLRs are expressed on various immune cells, and the expression of TLRs is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses [36]. TLRs induce the activation of NF-κB, which subsequently mediates the inducible expression of a variety of genes involved in immune and inflammatory responses, including TNF α, IL-1 and IL-6 [37]. The present study showed that Cd exposure increased the mRNA expression of pro-inflammatory cytokines, including IL-1β, TNFα, and IL-8, and decreased the mRNA expression of the anti-inflammatory cytokines IL-10 and HO-1, suggesting that Cd induce an inflammatory response in the kidneys of *C. carpio*.

MiRNAs are components of innate immune responses that can regulate inflammatory signalling [38–40]. MiR-155 plays an important role in innate immune responses, through its ability to directly and indirectly to increase the rate of translation of TNF-α transcripts and thus regulate the response to endotoxin shock [41]. Zheng et al. demonstrated that miRNA-155 plays a critical role in the regulation of IL-10 production by B cells and impairs B cell function in patients with Crohn's disease [42]. Elevated miR-155 expression has also been found to be associated with high levels of inflammatory cytokines such as IL-1β, IL-6, and IL-8 [43]. MiR-181a has an important function in regulating inflammatory responses. This miRNA regulates inflammatory responses by directly targeting the 3′-UTR of IL-1a and downregulating IL-1a levels in Raw264.7 cells [44]. In lipopolysaccharide (LPS)-stimulated BV2 cells, the expression levels of TLR-4, p63, and miR-181a are upregulated [45]. Song et al. discovered that miR-181a expression increases significantly in cells after TLR-4 activation and then determined that TLR-4 upregulates miR-181a expression in microglia [46]. Our results also showed that miR-155 and miR-181a expression was significantly increased after Cd exposure, indicating that miR-155 and miR-181a are involved in the immune response to Cd in the kidneys of *C. carpio*. MiRNAs function to repress the expression of target mRNAs by binding to the targets. MiR-155 and miR-181a expressions were significantly increased after Cd exposure; however, in the same group, the expressions of IL-10 and HO-1 were obviously reduced, suggesting that miR-155 and miR-181a may target IL-10 and HO-1 to control the immune response to Cd.

Furthermore, miR-155 agomir and miR-181a antagomir were administered to *C. carpio* by intraperitoneal injection. AKP and ACP activity was significantly changed in the miR-155 agomir group and the miR-181a antagomir group compared to that in the corresponding negative control groups, indicating that miR-155 and miR-181a are key regulators of the immune response in the kidneys of *C. carpio*. It is known that miRNAs can bind many different mRNAs, and miR-155 regulates immunity by targeting TNF-α [41], IL-10 [42,47], NF-κB [48], and TLR-4 [49], among other mRNAs. MiR-155 and miR-181a often play immunoregulatory effects together [17,50], and miR-181a, by targeting IL-1a [44], TLR-4 [51], and other molecules, regulates inflammatory responses. The mRNA expressions of IL-1β, IL-8, IL-10, NF-κB, TNF-α, TLR-4, and HO-1 were dramatically decreased in the miR-155 agomir group compared to that in the corresponding negative control group. After *C. carpio* were exposed to Cd, miR-155 expression was increased; IL-1β, IL-8, NF-κB, TNF-α, and TLR-4 mRNA expressions were elevated; and IL-10 and HO-1 mRNA expressions were decreased. In addition, the mRNA expressions of IL-10, NF-κB, TNF-α, and HO-1 were markedly increased in the miR-181a antagomir group compared to that in the corresponding control group. In *C. carpio* exposed to Cd, miR-181a expression was increased; IL-1β, IL-8, NF-κB, TNF-α, and TLR-4 mRNA expressions were elevated; and IL-10 and HO-1 mRNA

expressions were decreased. MiRNAs can inhibit the expressions of target mRNAs. Therefore, these findings imply that miR-155 and miR-181a target IL-10 and HO-1 to regulate the immune response to Cd in the kidneys of *C. carpio*. However, other miRNAs and mRNAs also regulate the immune response to Cd. IL-10 is an immunomodulatory cytokine with potent anti-inflammatory properties that decreases the production of pro-inflammatory cytokines, including TNF-α and IL-1β [33]. Drechsler et al. found that treatment with IL-10 reduces the acute effects of ethanol in monocytes through an HO-1-dependent mechanism [52]. HO-1 plays key roles in defence mechanisms against inflammation and is considered to be a protective factor in various inflammatory conditions [53]. Macrophages from HO-1 ± mice show decreased expression of the anti-inflammatory cytokine IL-10 [54]; moreover, overexpression of HO-1 in macrophages induces high levels of endogenous IL-10 production by macrophages [55]. The results of this study suggest that miR-155 and miR-181a, via HO-1, participate in the immune response to Cd in the kidneys of *C. carpio*.

Taken together, these results suggest that 0.5 mg/L Cd^{2+} exposure can induce immunotoxicological effects in *C. carpio*. The immune responses in the kidneys of *C. carpio* exposed to Cd are regulated by miR-155 and miR-181a via HO-1.

5. Conclusion

In conclusion, the present study demonstrated that Cd induced immunotoxicological effects in *C. carpio* kidneys. This process was accompanied by immune function impairment, upregulation of IL-1β, IL-8, TNF-α, TLR-4, and NF-κB expression, and downregulation of IL-10 and HO-1 expression. Additionally, the expression of immune-related miRNAs (miR-155, miR-181a) was increased upon Cd exposure. The results obtained upon overexpression of miR-155 and knockdown of miR-181a suggested that miR-155 and miR-181a play key roles in the immune response to Cd. Furthermore, miR-155 and miR-181a target HO-1 to participate in the immunotoxicological effects in *C. carpio* kidneys caused by exposure to Cd.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.11.010.

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