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Serum immune responses in common carp (*Cyprinus carpio* L.) to paraquat exposure: The traditional parameters and circulating microRNAs

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ABSTRACT

Paraquat (PQ) is a herbicide used worldwide, and it was shown to be a high-risk compound to aquatic organisms. This study was conducted to investigate the effects of PQ on traditional serum parameters and circulating microRNAs (miRNAs) in common carp to further elucidate the mechanism of PQ toxicity in fish. In the current study, a subacute toxicity test of common carp exposed to PQ at 1.596 and 3.192 mg/L for 7 d was conducted under laboratory conditions. The results showed that PQ exposure generally reduced the levels of T-AOC, SOD, CAT, and GST, but significantly increased MDA levels in the serum, indicating that PQ exposure induces oxidative stress and lipid peroxidation in the fish. The results of biochemical assays showed that PQ exposure not only significantly altered the activities of LDH, AST, ALT, ACP, AKP, and lysozyme and the contents of IgM and complement 3 but also promoted the expression of pro-inflammatory cytokines, including IFN-γ, IL-1β, IL-6, IL-8, and TNF-α. Additionally, PQ inhibited the levels of the anti-inflammatory cytokines IL-10 and TGF-β, suggesting that PQ exposure may cause fish tissue injury and promote immune inflammatory responses. Furthermore, we found that serum circulating miRNAs, such as ccr-mir-122, ccr-mir-125b, ccr-mir-146a, and ccr-mir-155, were generally promoted in fish following PQ exposure. Based on our results and reports on miRNA-based diagnosis of tissue damage and inflammatory responses in mammals, we suggest that serum ccr-mir-122, ccr-mir-125b, ccr-mir-146a, and ccr-mir-155 could be new biomarkers of PQ toxicity in fish.

1. Introduction

Paraquat (PQ, 1- 1′dimethyl-4-4′- bipyridinium dichloride) is a fast-acting and non-selective quaternary nitrogen herbicide that is used worldwide to manage the growth of weeds by interfering with their intracellular electron transport systems, thereby inhibiting reduction of NADP to NADPH during photosynthesis [1]. However, PQ is a highly toxic herbicide for animals and humans [2] as it undergoes a redox cycling reaction that directly or indirectly triggering reactive oxygen species (ROS) and nitrogen species (RNS) production and oxidative stress, leading to organ injury [3]. Epidemiological studies identified PQ exposure as a potential risk factor for the onset of Alzheimer disease, Parkinson's disease, and spinocerebellar atrophy [4,5]. Therefore, PQ is a banned substance in the European Union, and the PQ aqueous solution has also been banned in China since 2016s. Additionally, the US Environmental Protection Agency (EPA) has included PQ in a priority list of hazardous chemicals [6].

PQ is polar and has high water solubility and low volatility; thus, PQ may enter surface water via spray drift or run off from agricultural farmlands with extensive use of this chemical [7]. Meanwhile, PQ has also has been used as an aquatic herbicide with direct application in static or slow-moving waters [8]. Currently, increasing research has confirmed the presence of PQ in the aquatic ecosystems, with a concentration range of 0.01–3.95 μg/L in some bodies of water in the Valencian community (Spain) [9]; concentrations of 1.5–18.9 μg/L and 9.3–87.0 μg/L have been found in the ground and surface water, respectively, in the Thailand [10], which are higher than the drinking water limits recommended by the World Health Organization (WHO) (10 μg/L) and the EPA (3 μg/L) [6]. Thus, this chemical is a threat to the aquatic environment and aquatic organisms.

Blood characteristics of fish are important indicators of their physiological processes and reflect the relationship between aquatic ecosystem characteristics and their health status [11–13]. Fish serum parameters could be important bio-indicators for determination of the toxic effects in target organs to evaluate the physiological status of fish exposed to pollutants [14]. For example, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are considered important indicators of liver damage [15]. In recent years, there have been many reports about the adverse effect of PQ on fish [16–18], but information about the effect of PQ on blood biochemical and

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immunological parameters is still scarce.

MicroRNAs (miRNAs) are a class of endogenous, evolutionarily conserved, small single-stranded noncoding RNAs that play crucial roles in the regulation of gene expression to control a wide range of biological processes [19]. Interestingly, many microRNAs have been found in the blood of various organisms, including humans, mammals, and fish [20–22]. These findings triggered interest in their use as potential diagnostic indicators. Accumulating research indicates that miRNAs play crucial roles in responses to environmental chemicals, causing various toxicant-associated conditions and diseases [23,24], and the serum or plasma miRNAs could be used as precocious, sensitive biomarkers for organic damage caused by various xenobiotics or toxicants [23,25]. However, there is still a lack of research on the role of circulating miRNAs in toxicant-induced toxicity of non-mammalian species, such as fish.

In the present study, common carp was adopted to determine the changes in serum biochemical and immunological parameters and the circulating miRNAs in the fish following 7 days of PQ exposure. The results of this study will help further elucidate the PQ toxicity on fish and these parameters, especially the circulating miRNAs in fish serum, which can be helpful indicators for monitoring contaminants in aquatic organisms.

2. Materials and methods

2.1. Paraquat

Paraquat (PQ) as a commercial formulation (200 g/L, w/v) was obtained from Anhui Fengle Agrochemical Co. Ltd. China. It was first dissolved in double distilled water for stock solution and then diluted with dechlorinated tap water to obtain the experimental concentrations.

2.2. Experimental fish

Common carp (23.26 ± 2.53 g) were originally purchased from a local fish farm (Feilong aquarium fishery, Xinxiang, China). The fish's domesticated conditions were described previously [16], and the present study was handled under the strict control of the China Law for Animal Health Protection and Instructions for Granting Permits for Animal Experimentation for Scientific Purposes (Ethics approval No. SCXK (YU) 2005-0001).

2.3. PQ exposure and sampling

Based on the LC_{50} value obtained from an acute toxicity test described previously [16], PQ concentrations of 0, 1.596 (1/10 of the 72 h LC_{50}), and 3.192 mg/L (1/5 of the 72 h LC_{50}) were used for the subacute exposure on common carp, with 18 fish in each of the above treatments. The experimental conditions were described previously by Ma et al. [16]. All experiments were performed in triplicate, and no fish death was observed during the period of the test.

Sampling was performed on 1, 3, and 7 d of PQ treatment, and blood samples were withdrawn from the caudal vein of 6 fish selected at random from every group at each time. No anaesthetic was used to avoid any possible effect of the anaesthesia agent on serum parameters. The blood samples were incubated in the laboratory for 1–2 h and stored overnight at 4 °C, and then, they were centrifuged at 4000 g for 15 min. The supernatant obtained was stored at –20 °C for serum biochemical and immunological parameter assays and RNA isolation.

2.4. Lactate dehydrogenase (LDH) assay

LDH activity in the fish serum was detected by the LDH assay kit (Nanjing Jiancheng Bioengineering Co. Ltd.) following the manufacturer's instruction. The LDH level was calculated by measuring the absorbance at 450 nm using an ELISA microplate reader.

2.5. Determination of aminotransferase activities

The AST and ALT activities in serum were determined by NADH consumption and its conversion to NAD^+ at 340 nm using commercially available standard kits (Nanjing Jiancheng Bioengineering Co. Ltd.) according to the manufacturer's instructions.

2.6. Phosphatase activity

The acid phosphatase (ACP) and alkaline phosphatase (AKP) activities were determined by a colorimetric method using diagnostic reagent kits purchased from Nanjing Jiancheng Bioengineering Co. Ltd. China. The King unit of ACP or AKP activity (1 King unit = 7.14U/L) was defined as the ability to produce 1 mg phenol at 37 °C and 30 min or 15 min for 100 mL serum, respectively.

2.7. Antioxidant capacity

The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione *S*-transferases (GST) and total antioxidant capacity (T-AOC) in fish serum were determined using commercially available standard kits purchased from the Nanjing Jiancheng Bioengineering Co. Ltd. China, according to the manufacturer's instructions following the description by Ma et al. [26]. Lipid peroxidation was measured by the thiobarbituric acid (TBA) method [27]. The assay was monitored for the appearance of the conjugated complex of TBA and malondialdehyde (MDA) at 532 nm.

2.8. Lysozyme (LYZ) activity assay

LYZ activity in the carp serum was determined using *Micrococcus lysodeikticus* following the description by Ma et al. [16]. One activity unit of LYZ (U) was defined as the amount of enzyme that caused a decreased of 0.001 in OD 520 per min.

2.9. IgM and complement 3 (C3) assays

The IgM and C3 content in fish serum were determined using kits from the Wuhan ColorfilGene Biological Technology Co. Ltd. China according to the kit's instructions.

2.10. Cytokine content assay

The contents of IL-1β, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and TGF-β in fish serum were evaluated using the kits from Wuhan ColorfilGene Biological Technology Co. Ltd. China according to the manufacturer's instructions.

2.11. Determination of miRNA expression by qPCR

To normalize sample-to-sample variation in the RNA isolation procedure, an exogenous spike-in control of 3 μL of synthetic 5 nM *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p) was added into each serum sample. miRNA isolation from fish serum, synthesis of first-strand cDNA, and qPCR were performed with a miRNA Purification Kit (CoWin Biosciences, Beijing, China), a miRNA cDNA Synthesis Kit (CoWin Biosciences, Beijing, China), and a miRNA qPCR Assay Kit (CoWin Biosciences, Beijing, China), respectively, as described previously [17]. The upstream primers, miRNA target specific primers, were designed based on sequences from miRbase: ccr-mir-122 (MI0023311), ccr-mir-125b (MI0023312), ccr-mir-146a (MI0023331), ccr-mir-155 (MI0023336), and cel-miR-39-3p (MIMAT0000010) (Table 1). The downstream primers were obtained from the miRNA qPCR Assay Kit (CoWin Biosciences, Beijing, China). For serum