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RESEARCH PAPER

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Antioxidative and Inflammatory Responses in Spleen and Head Kidney of Yellow Catfish (*Pelteobagrus fulvidraco*) Induced by Waterborne Cadmium Exposure

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Abstract

Cadmium (Cd) is an environmental contaminant that presents significant threat to aquatic organisms. This study was conducted to examine histological structure as well as antioxidative and inflammatory responses in spleen and head kidney of yellow catfish after 8 weeks of exposure to 50 and 200 μg/L Cd^{2+}. It showed that Cd^{2+} exposure induced Cd accumulation and impaired histological structure in the spleen and head kidney. For antioxidative response: in the spleen, *sod1* expression was significantly up-regulated by 50 and 200 μg/L Cd^{2+}, while *cat*, *gpx1*, *gstω1* and *nrf2* expressions were up-regulated by 50 μg/L Cd^{2+} but not by 200 μg/L Cd^{2+}; in the head kidney, these genes were down-regulated by Cd^{2+} exposure. For inflammatory response: in the spleen, the expression of *il-8*, *tnf-α* and *il-10* were up-regulated by 50 μg/L Cd^{2+} but not by 200 μg/L Cd^{2+}, while the expression of *tor* and *tgf-β* were significantly up-regulated only by 200 μg/L Cd^{2+}; in the head kidney, Cd^{2+} exposure down-regulated the expression of *il-1β*, *il-8*, *tor*, *tnf-α*, *il-10* and *tgf-β*. Therefore, this study suggested that Cd^{2+} exposure could cause Cd accumulation in the spleen and head kidney, and induced histological lesions, antioxidative and inflammatory responses in both tissues.

Introduction

Cadmium (Cd) is one of the most hazardous environmental pollutants, having no known beneficial biological function (Swarup *et al.*, 2007). Duo to widely used in industry such as smelting, mining and electroplating, Cd has been emitted to the aquatic environment in large quantities, resulting in several waters in different areas have been polluted by Cd (Coles, Farley, & Pipe, 1995; Guo, Zheng, Yuan, Zhu, & Wu, 2017). For example, the highest Cd concentration detected was 70 μg/L in water of Kali River, India (Fatima *et al.*, 2015), 194.5 μg/L in the surface water of Linglong Gold Mining area, China (Liang, Yang, Dai, & Pang, 2011) and even 513 μg/L in unfiltered water of Tarapaya River, South America (Smolders, Lock, Van der Velde, Hoyos, & Roelofs, 2003), which were much higher than those in normal freshwaters (< 500 ng/L) (Jones, Kille, & Sweeney, 2001). In Cd-contaminated areas, fish are continually exposed to ambient Cd through both water and food, which can adversely affect the growth of fish and endanger the health of human beings via food chain.

In fish, both the spleen and head kidney are major immune organs that can trap and clear foreign particulate material and maintain stable internal environment (Rønneseth, Wergeland, & Pettersen, 2007; Wang *et al.*, 2013). Meanwhile, the spleen and head kidney play a vital role in haematopoiesis (Rønneseth *et al.*, 2007). Since the majority of metals (including Cd) absorbed by fish are transported within the body by blood (Bonda, Włostowski, & Krasowska, 2007; Kondera, Ługowska, & Sarnowski, 2014), metals accumulation in these hematopoietic organs can produce toxic action on many important physiological processes (Kondera *et al.*, 2014), which subsequently

disturb homeostatic mechanisms, such as the antioxidant system and the immune system of fish (Coles *et al.*, 1995; Liu *et al.*, 2011).

The antioxidant defense system, containing antioxidant enzymes and low-molecular-weight antioxidants, can interact with various kinds of reactive oxygen species (ROS) directly and maintain cellular homeostasis to protect biological targets (Kohen & Nyska, 2002; Zhang, Wang, Guo, Wu, & Xue, 2004). It is well documented that Cd exposure could induce abnormal high amounts of ROS formation (Bertin & Averbeck, 2006). The ROS can damage important molecules such as proteins, lipid and DNA, and alter biochemical compounds and corrode cell membranes, accordingly exhibiting a severe threat to health status of fish (Wiseman & Halliwell, 1996; Jo, Choi, & Choi, 2008). When increase of ROS production exceeds natural antioxidant system ability to scavenge these reactive species, oxidative stress occurs, thereby damaging the constituents of a living body (Lee *et al.*, 2004). Besides the antioxidant defense system, the immune system of fish can also be injured by high levels of ROS (Bartosz, 2009), and has been used as an indicator to reflect the degree of pollutant stress and evaluate effects of contaminants (Coles *et al.*, 1995). Inflammation is an immune response to injury and is regulated by many cytokines, including pro- and anti-inflammatory cytokines (Danesh *et al.*, 2008; Xu, Yang, Qiu, Pan, & Wu, 2013). To date, although extensive studies have been carried out to investigate the toxic effects and mechanisms of Cd, little is known about antioxidative and inflammatory responses to waterborne Cd exposure in immune organs of fish at the molecular levels.

Yellow catfish (*Pelteobagrus fulvidraco*) is an omnivorous freshwater fish, being popular with consumers in China because of the excellent meat quality and high nutritional values (Chen *et al.*, 2013). It has also been employed to monitor environmental pollution due to its advantages such as wide distribution and easy cultivation (Chen *et al.*, 2012). In this study, yellow catfish was used as a model to evaluate the effects of Cd exposure on immune organs in fish. Cd accumulation and histology were examined in both the spleen and head kidney after 8 weeks of exposure to 50 and 200 μg/L Cd^{2+}. Moreover, the expression of antioxidant- and inflammation-related genes in these two tissues was further analyzed to elucidate the potential mechanisms of Cd-induced toxicity.

Materials and Methods

Chemicals

Cadmium chloride ($CdCl_2$, purity≥99%) was purchased from Shanghai Sinopharm Group Corporation (Shanghai, China). RNAiso Plus, PrimeScript® RT reagent Kit with gDNA Eraser and 2 × SYBR Premix Ex Taq™ were procured from TaKaRa (Dalian, China). All other chemicals and solvents were also obtained from Shanghai Sinopharm Group Corporation.

Fish Maintenance, Exposure and Sampling

Juvenile yellow catfish (about 3 months old) were obtained from a commercial farm (Beibei, Chongqing, China). Prior to the experiments, fish were acclimatized for two weeks in indoor 250-L polyethylene tanks. During the acclimatization period, fish were fed twice a day with commercial pellets, and food residues and metabolic wastes were removed daily. After acclimation, a total of 180 fish with the similar size (6.26±0.22g) were randomly assigned into three gradient Cd^{2+} concentration groups (0, 50, and 200 μg/L, respectively) for 8 weeks, three tanks per level. Both 50 and 200 μg/L were environmentally realistic Cd concentrations, which were comparable to those reported in water of Kali River, India (Fatima *et al.*, 2015) and in the surface water of Linglong Gold Mining area, China (Liang *et al.*, 2011), respectively. The experiment was conducted at natural photoperiod (approximately 14 h light/10 h darkness). Fish were fed at 4% of their body weight in two divided doses daily. During the experiment, half of the water in aquariums was changed and fresh water with corresponding Cd concentration was added every 24 h. The measured Cd^{2+} concentrations for the control, 50, and 200 μg/L groups were 0.23±0.09, 55.22±3.10, and 184.58±7.66 μg/L, respectively. Water quality parameters were as follows: temperature, 25.1±0.8°C; pH, 7.6±0.1; dissolved oxygen, 6.4±0.2 mg/L; hardness, 131.8 ± 6.8 mg/L as $CaCO_3$. The experimental design and procedure were approved by the Committee of Laboratory Animal Experimentation at Chongqing Normal University.

After 8 weeks of exposure, fish were anesthetized by submersion in 100 mg/L MS-222. Then, fish were dissected on ice to obtain spleen and head kidney samples. The obtained samples were divided into two parts: one was fixed in Bouin's solution for histological examination, and the other immediately frozen in liquid nitrogen and stored at -80 °C for later determination of Cd accumulation and gene expression.

Samples Analysis

Cd Accumulation Measurement

The samples were weighed (approximate 0.3 g wet weight) separately and put in digestion pot, and then digested in High-throughput microwave digestion instrument (Master 40, Shanghai, China) with 10 mL HNO_3. Digestive power is 1000 watts (digestion procedure: 150 °C, 10 min, 1000 W; 180 °C, 10 min, 1000 W; 130 °C, 10 min, 1000 W). The digested samples were diluted to appropriate concentrations, and measured Cd content by graphite furnace atomic absorption spectrometry (Shimadzu, AA-6880, Kyoto, Japan).

Histological Examination

Obtained spleen and head kidney from the control and treated groups (6 specimens per group) were fixed for 24 h in Bouin's solution. After dehydration in graded ethanol concentrations, the tissue specimens were embedded in paraffin and cut into sections of 4–5 µm thickness. The sections were stained using routine hematoxylin-eosin (H&E) and then scanned with Pannoramic MIDI (3D Histech, Hungary) for examination.

Gene Expression Analysis

Gene expression levels were measured by quantitative real-time PCR (qPCR) method described in Sun, Li, Rao, Liu, and Chen (2018). Total RNA was isolated from tissues (three fish per tank, three tanks for each concentration) following the protocol provided by RNAiso Plus (TaKaRa, Dalian, China), size-separated on 1% agarose gel to assess quality, and concentration determined spectrophotometrically. To synthesize a single-stranded cDNA, 1 µg of total RNA along with PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) were used. qPCR was performed in triplicate using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Amplifications were performed in the presence of 2 × SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) using 1 µL diluted cDNA template (10-fold) and 0.4 µL gene-specific primers (10 mM) with the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30s. Each sample had three technical repeats. The melting curve analyses were performed to confirm the precision and accuracy of the specific amplification. Each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis and have no significant differences in amplification efficiencies. The quantification cycle between samples was normalized by 18s ribosomal RNA (*18s rRNA*) gene as an endogenous control which was stable under the experimental conditions. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The primers for qPCR are presented in Table 1, which were referred to our recent study (Sun *et al.*, 2018) and were designed based on the transcriptome sequence (SRX1838469).

Statistical Analysis

All data were analyzed statistically by one-way ANOVA with Tukey's multiple comparison tests using SPSS 22.0 software package. Data are expressed as the mean value with standard deviation (mean±SD). Differences were considered statistically significant at $P<0.05$.

Results

Cd Accumulation

As shown in Figure 1, Cd accumulation in spleen and head kidney enhanced with increasing exposure concentration. Significant increases were observed in 200 µg/L Cd^{2+} group of spleen and in 50 and 200 µg/L Cd^{2+} groups of head kidney. In spleen, Cd concentrations were 0.23±0.02 and 0.32±0.03 mg/kg wet mass in 50 and 200 µg/L Cd^{2+} groups, respectively, approximately 1.49- and 2.03-fold of that in the control (0.16±0.06 mg/kg). In head kidney, Cd concentrations were 0.32 ± 0.01 and 0.38±0.03 mg/kg wet mass in 50 and 200 µg/L Cd^{2+} treatments, respectively, approximately 1.98- and 2.41-fold of that in the control (0.16±0.02 mg/kg).

Histological Structure

After 8 weeks of exposure, obvious histological alterations in both tissues were observed (Figure 2). In spleen, it appeared red cell loss in the splenic sinus and increasing melano-macrophage centers (MMCs) in Cd^{2+}-exposed fish as compared to the control fish (Figure 2 A-C). In head kidney, Cd^{2+} exposure elicited a reduction in lymphocyte numbers, and an increase in MMCs as well as multinucleate giant cells (Figure 2 D-F).

Table 1. Primers used for qPCR analysis

Gene	Forward primers (5'-3')	Reverse primers (5'-3')	Size (bp)
sod1	TTGGAGACAATACAAATGGGTG	CATCGGAATCGGCAGTCA	129
cat	CTGCATCAGGTGTCGTTCCT	GCAGTAGACAGGGTTGCCTT	120
gpx1	ACAACCCTAAGGCTTTGATGAC	TGGTCTGGACGCTCTTGCT	144
gstω1	AAGCTGCTCCTTATCCACATTC	TGACCACAGGGTGTCTCCAAT	123
nrf2	TCTCGCCCAGTTACAGCTTG	GTTCCGTGAACGCCACATTC	128
il-1β	ATTACTCTGAAAGGTGGAAATGAAG	TTTGGTGGGTTGTAGGCTGA	68
il-8	ACTGACTGCGATGCTTTGTG	AGGAGCCACTTGGAGGGAATA	131
tnf-α	ATCAGGTGAACGCTGATGCT	GTGTTGAGGGAAGGGGTCTG	98
tor	CGGTCCGATGAAGAAGTTGC	ATGTAGGTCTGGGCGAGGG	177
il-10	CTCCTCCCCCTGAGGATTCA	CGGATCACGGCGTATGAAGA	227
tgf-β	ATCTTCTCGCTGTCCACTTGC	CGGTCTCGGTGTTGTCCTG	235
18s rRNA	CGGTGGTCTTCTTCCACTCTG	TCAGCGGGTCGTCTCGTC	179

Expressions of Genes Involved In Antioxidant Response

In spleen (Figure 3), Cd^{2+} exposure up-regulated the mRNA levels of *cat*, *gpx1*, *gstω1* and *nrf2* at 50 µg/L, while had no significant effect at 200 µg/L. Besides, *sod1* expression was significantly up-regulated at both Cd^{2+} doses. In head kidney, the transcriptional levels of *sod1*, *cat*, *gpx1*, *gstω1* and *nrf2* in Cd^{2+}-exposed fish were obvious lower than those in the controls.

Expressions of Genes Involved In Inflammatory Response

In spleen (Figure 4), Cd^{2+} exposure produced a significant increase in *il-8*, *tnf-α* and *il-10* expression at 50 µg/L, while had no significant effect at 200 µg/L. Besides, obvious elevation in *tor* and *tgf-β* mRNA levels was observed in 200 µg/L group. However, Cd^{2+} exposure did not significantly impact the expression of *il-1β* at any concentration. In head kidney, Cd^{2+} exposure significant down-regulated the expression levels of *il-1β*, *tnf-α*, *tor*, *il-10* and *tgf-β* at 200 µg/L as well as *il-8* at both Cd^{2+} doses.

Discussion

Heavy metal accumulation in organs of fish depends on the ratio of uptake and depuration, and high level of Cd concentration may be accumulated when the uptake rate exceeds the degradation rate (Suresh, Sivaramakrishna, & Radhakrishnaiah, 1993). As demonstrated in this study, Cd accumulation in both spleen and head kidney increased with increasing waterborne Cd concentrations. Similarly, Karaytug, Erdem, and Cicik (2007) and Salazar-Lugo, Vargas, Rojas, and Lemus (2013) also found obvious accumulation in spleen and head kidney after Cd exposure, respectively.

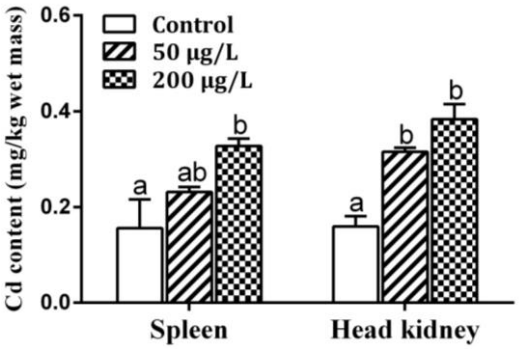

Figure 1. Cd accumulation in spleen and head kidney of yellow catfish after 8 week exposure to Cd^{2+}. Values are mean±SD (n = 3). Statistically significant differences among groups are denoted by different letters (P<0.05).

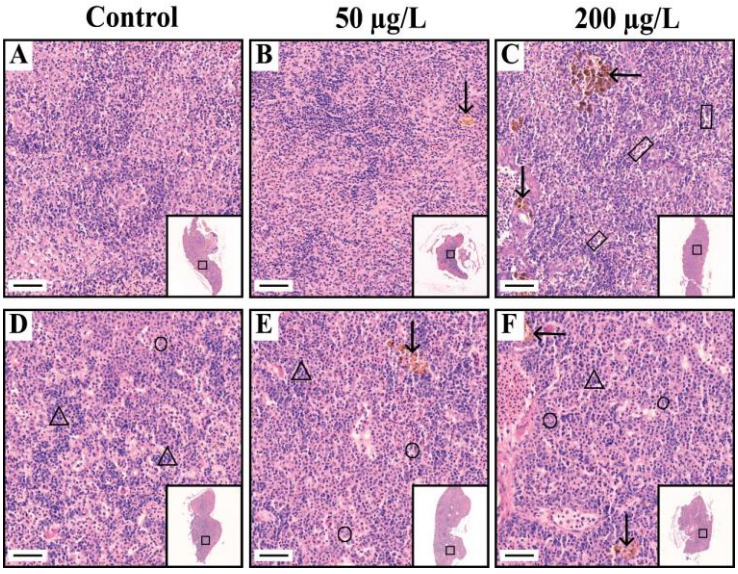

Figure 2. Histology (stained with H&E) of spleen (the top row, A–C) and head kidney (the bottom row, D–F) of yellow catfish after 8 week exposure to Cd^{2+}. (A, D) Control; (B, E) 50 µg/L Cd^{2+}; (C, F) 200 µg/L Cd^{2+}. Triangle: lymphocyte; rectangle: splenic sinus; arrow: melano-macrophage center; roundness: multinucleate giant cell. Bar = 400 µm.

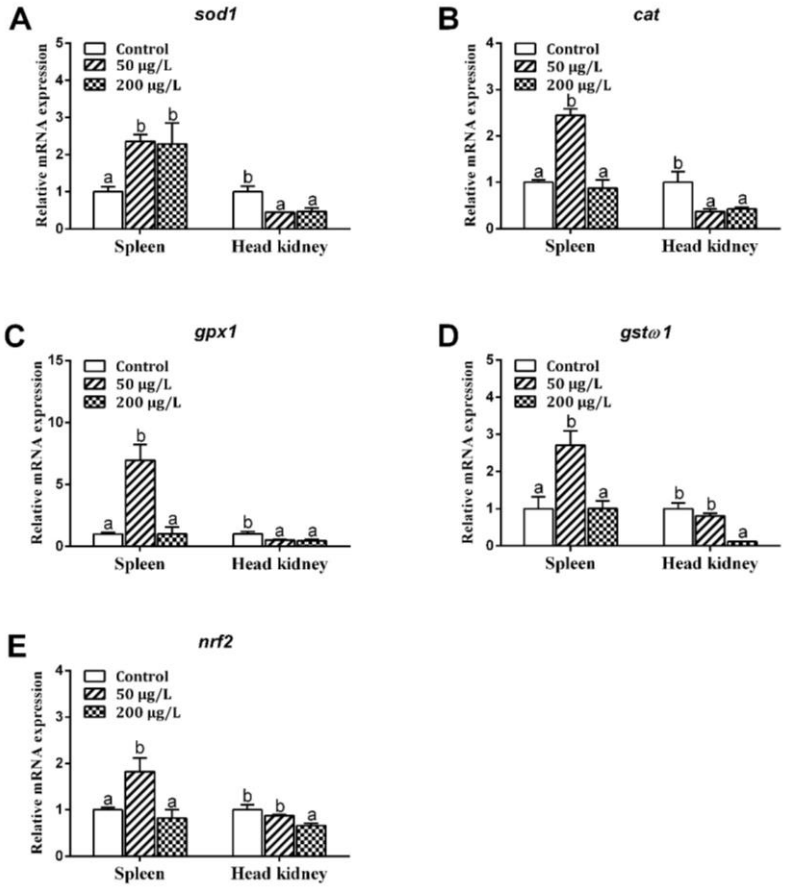

Figure 3. Expression of antioxidant-related genes in spleen and head kidney of yellow catfish after 8 week exposure to Cd^{2+}. Values are mean±SD (n = 3). Statistically significant differences among groups are denoted by different letters (P<0.05).

When heavy metals accumulate in organs and/or tissues excessively, dysfunction of physiological metabolism and homeostasis can be induced, thereby exerting an influence on growth and development of fish (Gupta & Srivastava, 2006; Vinodhini & Narayanan, 2008; Liu *et al.*, 2011). Due to different magnitude of absorption or adsorption, heavy metals accumulation and distribution in fish organs and/or tissues occur in a tissue-specific manner under the same exposure condition (Suresh *et al.*, 1993; Li, Yan, & Xie, 2018). In this study, head kidney accumulated higher amount of Cd than spleen at the same exposure dose, similar to our recent observation in yellow catfish subjected to mercury exposure (Sun *et al.*, 2018), indicating that head kidney might play a more active role in Cd handling.

In the present study, Cd^{2+} exposure altered the histological structure of immune organs, characterized by red cell loss in the splenic sinus of spleen, increased multinucleate giant cells and decreased lymphocyte numbers in head kidney, and increased MMCs in both tissues, which probably produced negative influence on normal immune function of fish. In general, increase of multinucleate giant cells might be an inflammatory response to Cd exposure (Sado & Matushima, 2008). Since lymphocyte is an important component in immune system, the decrease in lymphocyte counts might lead to downregulation of the immune response against xenobiotic. Melano-macrophages, repository for toxic contaminants and cellular degradation products, mainly involve in antigen processing and presentation to resident lymphocytes, and sequestration of potential toxic tissue materials (Agius & Roberts, 2003). It has been reported that MMCs would increase in the number and size during environmental contamination, detoxification processes and immune responses (reviewed by Ali *et al.*, 2014). Thus, increased MMCs suggested that phagocytosis and ability of elimination enhanced to resist damage caused by Cd. The function and structure of fish immune organs are related to disease resistance which shows a positive correlation with fish growth (Ni *et al.*, 2016). Therefore, histological injury in spleen and head kidney might cause growth retardation induced by Cd exposure.

Oxidative stress has been documented as a mechanism of toxicity in fish exposed to heavy metals (Jin *et al.*, 2015), which was related to abroad variety of diseases such as mutagenesis, cancer and chronic inflammatory diseases (Martínez-Álvarez, Morales, & Sanz, 2005). In the antioxidant system, antioxidant enzymes such as SOD, CAT, GPx and GST play a key role in maintaining cellular homeostasis and counteracting the toxicity of ROS under unfavorable environments (Jo

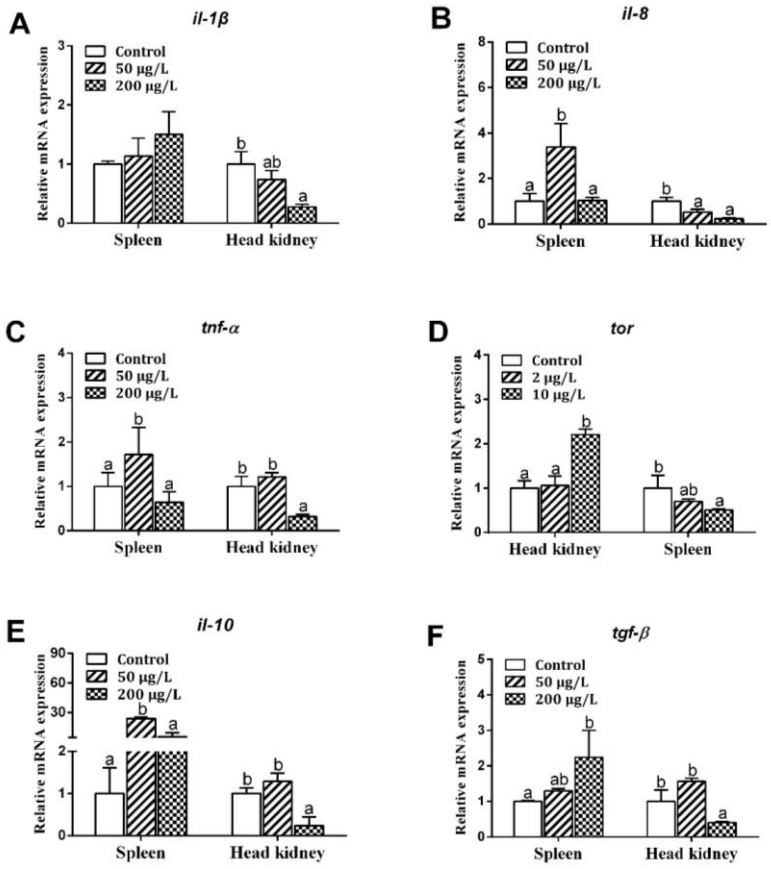

Figure 4. Expression of inflammation-related genes in spleen and head kidney of yellow catfish after 8 week exposure to Cd^{2+}. Values are mean±SD (n = 3). Statistically significant differences among groups are denoted by different letters (P<0.05).

et al., 2008; Tabrez & Ahmad, 2009). Analysis of the expression levels of the genes encoding these antioxidant enzymes is an important marker for evaluating antioxidant responses and an indication of environmental pollution or the degree of stress in fish tissues (Olsvik *et al.*, 2005; El-Sayed, El-Gazzar, El-Nahas, & Ashry, 2016). As presented in our study, all tested antioxidant enzyme genes in spleen were up-regulated in 50 μg/L Cd^{2+} group while returned back to the control level at 200 μg/L Cd^{2+}, except for *sod1* that was up-regulated at both Cd^{2+} doses. Interestingly, in head kidney, both Cd^{2+} doses decreased *sod1*, *cat* and *gpx1* transcriptional levels. In general, under relatively low oxidation stress, the antioxidant system would be activated to scavenge ROS, and thus the up-regulation of antioxidant enzyme gene expression was observed. However, excess accumulation of Cd in a living body could induce strong oxidative stress that dramatically weakened the metabolic capacity, leading to decreased mRNA levels of genes encoding antioxidant enzymes (Jo *et al.*, 2008). It is reported that elevation of antioxidant gene transcriptional levels may be attributed to induce of antioxidant-related signaling molecules, such as Nrf2 (Wang *et al.*, 2015). In the presence of ROS, Nrf2 can translocate into the nucleus where it accumulates and thereafter activates an array of genes expression to regulate antioxidant response through binding antioxidant responsive element (ARE) sequence in the promoter region of antioxidant enzyme genes (Kobayashi & Yamamoto, 2006; Wang *et al.*, 2015). In this study, expression changes of *cat*, *gstω1* and *gpx1* in spleen as well as *sod1*, *cat*, *gstω1* and *gpx1* in head kidney were in parallel with alteration in *nrf2* expression, suggesting that Cd^{2+} affected antioxidant status of immune organs via Nrf2 signaling. Similarly, a positive correlation between *nrf2* expression and the mRNA levels of antioxidant enzyme genes was also reported by other studies in fish after Cd exposure (Wang & Gallagher, 2013; Zheng, Yuan, Wu, & Li, 2016).

Cytokines play a prominent role in maintaining normal immune status of fish, including pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α, and anti-inflammatory cytokines such as IL-10 and TGF-β (Zhao *et al.*, 2014; Li *et al.*, 2015). Pro-inflammatory cytokines are able to initiate inflammatory processes and improve additional inflammatory processes by inducing other inflammatory molecules, while anti-inflammatory cytokines can inhibit production of pro-inflammatory cytokines and initiate processes of tissue recovery (Liu *et al.*, 2009; Verburg-Van Kemenade, Stolte, Metz, & Chadzinska, 2009). Changes in expression levels of genes encoding cytokines after treatment with chemicals have been considered as an inflammatory response (Park & Park, 2009; Liu *et al.*,

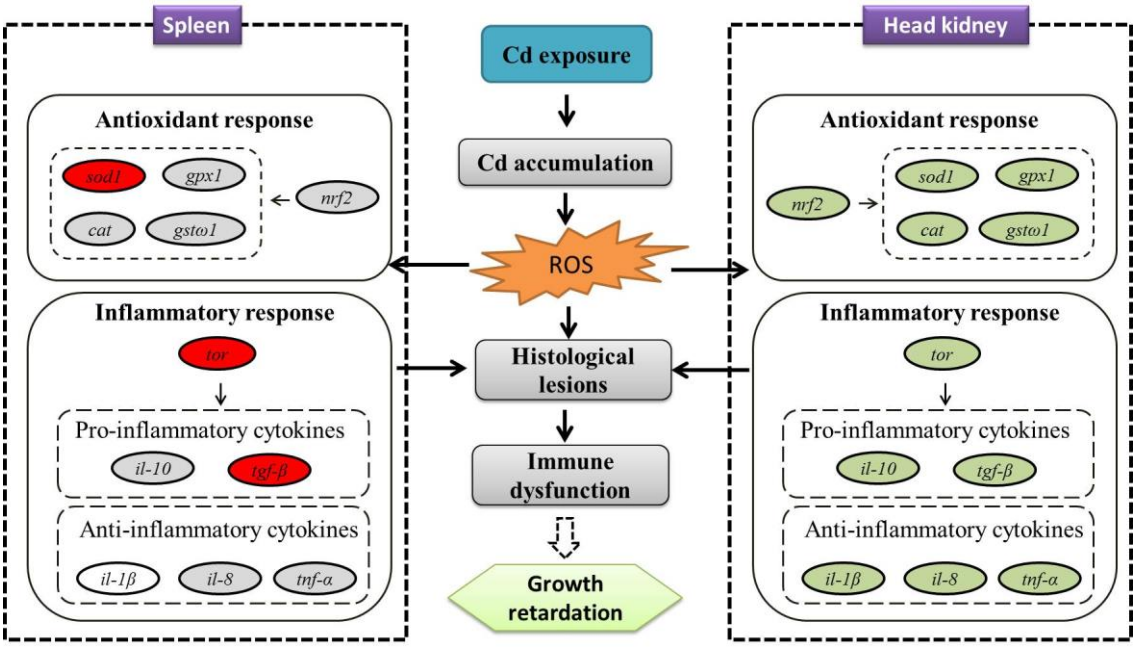

Figure 5. Schematic diagram of waterborne Cd^{2+} exposure on antioxidative and inflammatory responses in spleen and head kidney of yellow catfish. Genes with different background color mean different regulation induced by Cd^{2+} exposure: green (down-regulation); red (up-regulation); gray (up-regulation in 50 μg/L Cd^{2+} but no significant changes in 200 μg/L Cd^{2+}); no color (no significant changes).

2015). In the current study, *il-8* and *tnf-α* transcription levels had significantly higher expression at 50 μg/L Cd^{2+} in spleen; in head kidney, however, *il-1β*, *il-8* and *tnf-α* expressions were down-regulated by Cd^{2+} exposure, reflecting a tissue-specific inflammatory response. The up-regulation of pro-inflammatory cytokine expression might result in inflammation, while the down-regulation of pro- and/or anti-inflammatory cytokine expression could be the immunosuppressed effects induced by Cd (Zhang *et al.*, 2017). Due to decrease of *il-1β* and *tnf-α* expression in immune organs might cause lower ROS generation (Wu *et al.*, 2014), the down-regulation of these two genes expression observed in this study could be a defense mechanism to ROS overproduction induced by Cd exposure. TOR, a highly conserved serine/threonine protein kinase, controls transcription and translation in response to environmental stress (Arsham, Howell, & Simon, 2003; Corradetti & Guan, 2006). Previous studies have shown that TOR can regulate the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Li *et al.*, 2016; Feng *et al.*, 2017; Pan *et al.*, 2017). In this study, Cd-induced alterations of *tgf-β* expression in spleen as well as *il-10* and *tgf-β* expression in head kidney were parallel with *tor* mRNA levels, probably implying that Cd altered the expression of anti-inflammatory cytokines by affecting the transcription of *tor*. Nevertheless, alteration of *il-10* transcription level did not present similar tendency with *tor* in spleen, which could be related to other transcription factor such as *NFκB* that could also regulate anti-inflammatory expression (Li *et al.*, 2016). Overall, both pro-inflammatory and anti-inflammatory cytokine genes in head kidney were down-regulated by Cd (mainly in the high dose group), reflecting the immunosuppressed effects caused by Cd. On the contrary, in spleen, these genes were up-regulated at low or high Cd dose, indicating that inflammation was induced to protect itself from immunotoxicity. Besides, inappropriate inflammatory response could also result in histological lesions in immune organs (Bridle, Morrison, & Nowak, 2006), and thus histological impairment observed in spleen and head kidney might also attributed to Cd-induced changes in expression levels of genes involved in inflammatory response.

In summary, Cd^{2+} exposure caused Cd accumulation and impaired histological structures in the spleen and head kidney. Moreover, Cd^{2+} exposure could induce antioxidative and inflammatory responses, affecting expression of genes involved in antioxidant defense system and immune system in both tissues (Figure 5).

Acknowledgments

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