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Immunological and histopathological responses of the kidney of common carp (*Cyprinus carpio* L.) sublethally exposed to glyphosate

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ABSTRACT

Glyphosate is a broad-spectrum herbicide frequently used world widely in agricultural and non-agricultural areas to control unwanted plants. Health risk of chronic and subchronic exposure of glyphosate on animals and humans has received increasing attention in recent years. The aim of this study was to evaluate the effects of glyphosate on the immunoglobulin M (IgM), complement C3 (C3), and lysozyme (LYZ) in the kidney of common carp exposed to 52.08 or 104.15 mgL⁻¹ of glyphosate for 168 h. The results showed that the transcriptions of IgM, C3, or LYZ were altered due to glyphosate-exposure, for example, IgM and C3 initially increased at 24 h later it decreased (except for a increase of C3 in higher dose group at 24 h) while the expression of G-type LYZ were not affected at 24 h, then increased at 72 h, but decreased at the end of test, however C-type LYZ expression was initially up-regulated (24–72 h) but down-regulated at the end of exposure (168 h). However, glyphosate-exposure generally decreased the contents of IgM and C3 or inhibited LYZ activity in the kidney of common carp. In addition, glyphosate-exposure also caused remarkable histopathological damage, mainly including vacuolization of the renal parenchyma and intumescence of the renal tubule in fish kidney. The results of this study indicate that glyphosate causes immunotoxicity on common carp via suppressing the expressions of IgM, C3, and LYZ and also via damaging the fish kidney.

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1. Introduction

Glyphosate is a broad-spectrum herbicide that is frequently used in agricultural and non-agricultural areas to control unwanted plants, including terrestrial plants and hydrophyte, in the world (Nedelkoska and Low, 2004). It is considered that the possible biochemical mechanism of glyphosate is the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an important enzyme of shikimate pathway,

which exists in plants or some microorganisms but not in animals (Steinrucken and Amrhein, 1980; Tomlin, 2006; Mottier et al., 2013; Sandrini et al., 2013). Therefore, glyphosate is thought to be non-toxic or relatively low toxic to humans and animals (Giesy et al., 2000; Williams et al., 2000). Nevertheless, there have been a number of reports concerning glyphosate occurrence in contaminated soil and water, bioaccumulation in animals, intoxication or health risk of humans by conducting wild detections or controlled laboratory studies (Bolognesi et al., 2009; Kwiatkowska et al., 2014), for

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example, the death of umbilical, embryonic, and placental cells of humans caused by glyphosate-exposure (Benachour and Seralini, 2009), genotoxic damage (Bolognesi et al., 2009) and cutaneous toxicity of glyphosate (Penagos et al., 2004, Nagami et al., 2005), and Parkinsonism linked to glyphosate-exposure (Gui et al., 2012). Furthermore, Peruzzo et al. (2008) reported that glyphosate concentrations in surface water were between 0.1 and 0.7 mgL⁻¹ within the nucleus area of soybean sowing in Argentina. Glyphosate has also been detected in the blood of people who were not directly exposed to glyphosate at mean concentrations of 73.6 ± 28.2 µgL⁻¹ (Aris and Leblanc, 2011), and in the case of glyphosate poisoning, blood content was in the range from 0.6 to 150 mgL⁻¹ while it was from 690 to 7480 mgL⁻¹ in moderate intoxication (Zouaoui et al., 2013). US EPA has recommended the highest level of glyphosate of 0.7 mgL⁻¹ allowed in water for human consumption (US EPA, 2011) and the drinking water standard for glyphosate has been set at 0.1 µgL⁻¹ in Europe (Yue et al., 2008).

Particularly, concerns about glyphosate-toxicity on aquatic organisms and safety to the aquatic environment have been accumulating increased in the recent time due to its high water solubility and extensive use in agriculture (Baer, 2005; Battaglin et al., 2005; Peruzzo et al., 2008; Bolognesi et al., 2009; Kwiatkowska et al., 2014). Glyphosate toxicity on various kinds of fish have been considerably evaluated for the past few years, for example, acute toxicity and biochemical lesions in *Cyprinus carpio* (Cattaneo et al., 2011; Gholami-Seyedkolaei et al., 2013), genotoxicity in *Anguilla Anguilla* (Guilherme et al., 2012), biochemical toxicity on *Danio rerio* and *Jenynsia multidentata* (Sandrini et al., 2013; Armiliato et al., 2014), and hepatotoxicity in *Piaractus mesopotamicus* (Shiogiria et al., 2012). However, relatively little is known about its effect on the immune system of fish to the best of our knowledge (Kreutz et al., 2010, 2011).

Common carp (*C. carpio* L.) is distributed in the freshwater all over the world and it is also an important aquatic food product consumed by people in China. Furthermore, they are frequently adopted as bio-indicators of environmental pollution and served as the model animal for toxicological tests to determine the toxicity of chemicals in aquatic environment (Lakra and Nagpure, 2009; Wang et al., 2011). The aim of this study was to determine the acute toxicity of glyphosate and to evaluate the immunological and histopathological effects of glyphosate on common carp exposed to sublethal concentrations of glyphosate (52.08 or 104.15 mgL⁻¹) for 168 h.

2. Materials and methods

2.1. Experimental fish, chemicals, and assay kits

Common carp (8.14 ± 1.37 g) were originally obtained from a local fish farm (Feilong aquarium fishery, Xinxiang, China). The fish were subjected to a prophylactic treatment by bathing twice in 0.05% potassium permanganate for 2 min before raised in a 200-L tank under laboratory conditions for at least two weeks before the test. The water quality characteristics were detected according to the Standards for Drinking Water Quality, China (GB5479–2006) and the determined parameters were as follow: total hardness of water 340 mgL⁻¹, pH 7.6, turbidity 1.5 nephelometric turbidity units, and total dissolved

solid content 660 mgL⁻¹. Fish were exposed to a 16 h light/8 h dark photoperiod and the tank water was partially changed every day with aerated tap water. During the acclimatization, the fish were fed on a mixture of commercial food (Wannong Fishery Company, China) at a day-rate of 1–1.5% of fish body weight. The fish were handled according to the guidelines in 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).

Glyphosate was obtained from Anyang Anlin Agrochemical Co., Ltd., China as a commercial formulation (50% soluble powder). It was first dissolved in distilled water for stock solutions and then diluted to obtain the various concentrations for the following toxicity experiments.

The assay kits for the content determinations of immunoglobulin M (IgM) and complement C3 (C3) were obtained from the Shanghai BOYAO Biotechnology, China. Other reagents used in this study were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade.

2.2. Determination of glyphosate LC₅₀ and subacute glyphosate exposure and sampling

The acute toxicity test of glyphosate on common carp and the design of exposure concentrations of glyphosate were conducted according to the Spearman–Kärber method (Kärber, 1931) with modification (Zhang and Liu, 1997). Briefly, a total of 160 healthy fish were used in acute toxicity tests to determine the median lethal concentration (LC₅₀) of glyphosate for common carp. Experiments were performed in 30 L glass jars containing 10 fish each and the test groups consisted of 7 groups of fish exposed to seven concentrations of glyphosate (771.70, 714.52, 661.58, 612.55, 567.22, 525.18, and 486.26 mgL⁻¹) and a control group exposed to aerated tap water without the herbicide. During the period of test, no food was provided, but saturated oxygen was maintained in the solution for every group, and the water and glyphosate solution were completely changed daily. Each test was conducted in duplicate. Fish behavior was observed and dead fish were counted and removed from the glass jar. The LC₅₀ value was calculated by using the Spearman–Kärber method (Kärber, 1931).

For the subacute exposure of glyphosate, 54 fish with the same body weight as above were randomly divided into three groups, in which two groups were as the glyphosate-treatment groups and one as the control with aerated tap water, and 18 fish were included in each group. The fish of the treatment-groups were exposed to glyphosate solution at the concentrations of 1/10 or 1/5 of 96 h LC₅₀ under semi-static condition for 168 h (7 d). During the period of glyphosate-exposure, no food was provided to avoid interference or adverse effect on the following biochemical assay owing to the difference in ingestion and digestion between the fish from treatment group and control group. Saturated oxygen was maintained for fish and the water of the three groups was completely changed daily. No fish death occurred during the period of test. Each test was conducted in duplicate.

After 24, 72, or 168 h of glyphosate-exposure, 6 fish from every group was taken each time. The fish were anaesthetized

Table 1 – The primers used for qPCR.

Primers	Sequences
IgM F	5'-TCTTGTGTTTAGCCAGCAG-3'
IgM R	5'-CGGTTACTGAGCCTTTCTTCTC-3'
C3 F	5'-CCCTGGACAGCATTATCACTC-3'
C3 R	5'-GATGGTCGCCTGTGTGGT-3'
G-type LYZ F	5'-AGCGGTCTGAAGAATGGAT-3'
G-type LYZ R	5'-GCATTGCTCTTGTGTCCACTT-3'
C-type LYZ F	5'-AAGACCCACAGAGTGGCTTC-3'
C-type LYZ R	5'-GAACGCACTCTGTGGGTCTT-3'
β -actin F	5'-GCTATGTGGCTCTTACTTCG-3'
β -actin R	5'-CCGTCAGGCAGCTGATAGCT-3'

F, forward; R, reverse.

with 100 mg L⁻¹ MS-222 (Tricaine), dissected, and then the kidney were washed with cold physiological saline solution (PBS). One part of the tissue was stored at -80 °C for biochemical assay and the rest was placed in the solution of 10% neutral-buffered formalin for histopathological examination.

2.3. Transcription analysis of IgM, C3, and lysozyme in the kidney of fish after subacute exposure of glyphosate

Total RNA was isolated from fish kidney by using a TRIzol Reagent Kit (Cwbiotech, Beijing, China) according to manufacturer's instructions. Total RNA concentration and purity were determined spectrophotometrically according to the method of Sambrook and Russel (2001). A total of 2 μ g of RNA was used as a template for the first-strand cDNA synthesis by using the HiFi-MMLV cDNA Kit (Cwbiotech, Beijing, China) with oligo (dT) as the primer.

The expressions of IgM, C3, and lysozyme (LYZ) at mRNA level in fish kidney were detected by quantitative real-time PCR (qPCR) using UltraSYBR Mixture (With ROX) (Cwbiotech, Beijing, China) according to manufacturer's instructions. The primers were designed based on the sequences retrievable from GenBank: IgM (AB004105), C3 (AB016211), G-type LYZ (AB084624), C-type LYZ (AB027305.1), and β -actin (M24113) (endogenous reference gene) (Table 1). The cycling reaction conditions of qPCR were as following: 1 cycle at 94 °C for 30 s; 40 cycles at 94 °C for 5 s, 60 °C for 34 s; and at dissociation stage 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s. The reaction was performed in duplicate. The amount of target mRNA, normalized to β -actin mRNA, was given by the formula. $2^{-\Delta\Delta Ct}$. (Livak and Schmittgen, 2001); where ΔCt value was determined by subtracting the average β -actin Ct value from the average target gene Ct value. $\Delta\Delta Ct = (Ct(\text{target}) - Ct(\beta\text{-actin}))_{\text{target}} - (Ct(\text{target}) - Ct(\beta\text{-actin}))_{\text{standard}}$.

2.4. Content assays of IgM and C3 and activity determination of LYZ in the kidney of fish after subacute exposure of glyphosate

The fish kidney (0.1 g) were homogenized to a 1/10 (w/v) ratio in cold PBS (pH 7.2), then each homogenate was centrifuged at 3000 \times g for 10 min at 4 °C and the supernatants obtained were stored at -20 °C for the following biochemical assays.

Contents of IgM and complement C3 in fish kidney were determined by using the kits from the Shanghai

BOYAO Biotechnology, China, according to the manufacturer's instructions.

LYZ activities in the kidney of carp were determined by the method of Binuramesh and Michael (2011). Bacterial suspension of *Micrococcus lysodeikticus* was obtained by dissolving cold dried bacterial powder with 0.04 M PBS (pH 6.2 and OD 520 nm = 0.3–0.5). A 25 μ L of sample or standard were added to the wells of a flat-bottomed 96-well microplate, and then 175 μ L of the bacterial suspension was added to each well and quickly mixed by vortex, and absorbance was monitored at 520 nm with a microplate reader (MSS multiskan spectrum, SkanIt software 2.2). The reaction was carried out at 25 °C and OD 520 was measured after 0.5 and 4.5 min of reaction, respectively. Each reaction was conducted in triplicate. One activity unit of LYZ (U) was defined as the amount of enzyme that caused a decreased 0.001 of OD 520 per min.

2.5. Renal histopathological examination

Sections of kidney were fixed in 10% neutral-buffered formalin for 24 h and then dehydrated and embedded in paraffin. These sections were cut at 5–6 μ m thickness and stained with hematoxylin and eosin (H & E) before examination under a light microscope.

2.6. Statistical analyses

Data were analyzed using a one-way analysis of variance (Tukey) followed by a least significant difference determination using SPSS 13.0 for Windows. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Glyphosate LC₅₀

The 24, 48, 72, and 96 h-LC₅₀ values of glyphosate for common carp were calculated to be 683.47, 602.61, 557.64, and 520.77 mg L⁻¹, respectively, according to the result of acute toxicity test. Additionally, we also observed that fish behavior in the treatment group was different from that of control, for example, the fish in the treatment groups became agitated and swam quickly or jumped at the early periods of exposure while gradually became dull and weak and swam feebly and slowly and vomited a white floc at the later time of test, showing the obvious symptom of toxication.

3.2. IgM expression at mRNA level and IgM content in the kidney of common carp

The mRNA levels of IgM and its contents in the kidney of carp after 52.08 or 104.15 mg L⁻¹ of glyphosate-exposure are demonstrated in Fig. 1. IgM expression in the treatment group was first up-regulated (24 h), but then down-regulated (72–168 h) (Fig. 1A). However, IgM contents of the treated fish were all lower than that of control group except for an increase in 104.15 mg L⁻¹ group at 72 h (Fig. 1B).

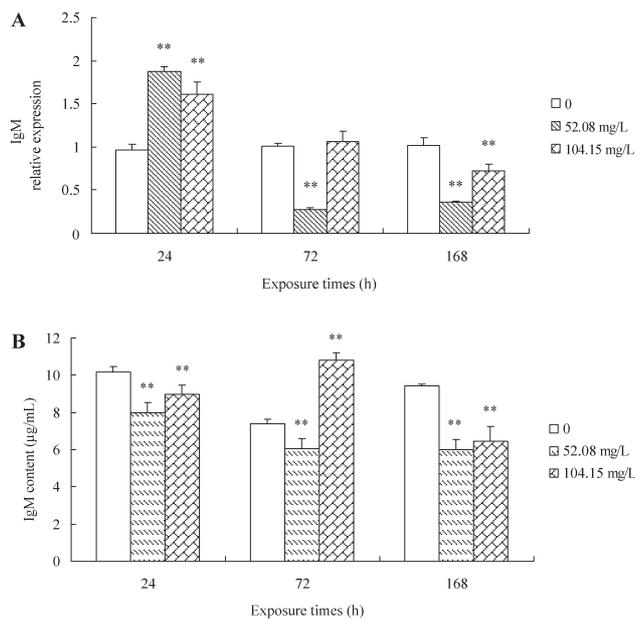


Fig. 1 – IgM mRNA levels and contents in the kidney of common carp exposed to 52.08 or 104.15 mg L⁻¹ of glyphosate for 168 h. Glyphosate-exposure and the determinations of IgM mRNA level and content were as described in Sections 2.3 and 2.4, respectively. All of the experiments were performed in duplicate and data were shown as the means ± SD. Asterisks denote a response that is significantly different from the control ($p < 0.05$, ** $p < 0.01$). (A) IgM mRNA level. (B) IgM content.

3.3. Complement C3

Transcription of complement C3 was totally down-regulated in the kidney of the treatment groups when compared to the control group, with only one exception of 52.08 mg L⁻¹ group at 24 h (Fig. 2A). Regarding C3 content, no change was found after 24 h of exposure between the treatment and control groups, but it significantly decreased at 72 and 168 h of testing time intervals, except for an increase in 104.15 mg L⁻¹ at 72 h (Fig. 2B).

3.4. LYZ

The expression of G-type LYZ in the treated group was up-regulated in lower concentration group at 72 h, but repressed in the treatment groups at 168 h compared to the control (Fig. 3A). However, C-type LYZ expression was initially up-regulated (24–72 h) but down-regulated at the end of exposure (Fig. 3B). In addition, LYZ activities in treatment groups basically decreased in comparison with that of control group except for an increase in 52.08 mg L⁻¹ group at 24 h.

3.5. Histological observation

The result of histopathological examination revealed that remarkable damage such as vacuolization of the renal parenchyma and intumescence of the renal tubule in the

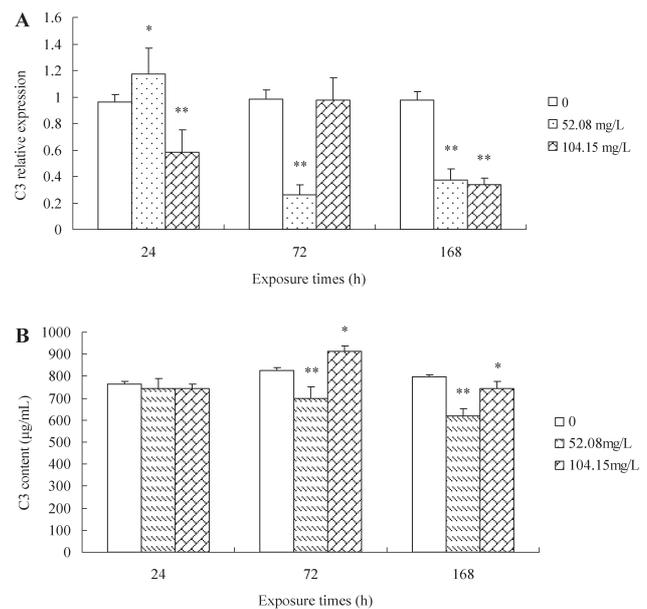


Fig. 2 – C3 mRNA levels and contents in the kidney of common carp exposed to 52.08 or 104.15 mg L⁻¹ of glyphosate for 168 h. Glyphosate-exposure and the determinations of C3 mRNA level and content were described in Sections 2.3 and 2.4, respectively. The experiment was performed in duplicate and data were shown as the means ± SD. Asterisks denote a response that is significantly different from the control ($p < 0.05$, ** $p < 0.01$). (A) C3 mRNA level. (B) C3 content.

kidney from glyphosate-exposed fish were observed as shown in Fig. 4.

4. Discussion

LC₅₀ is the most important index determined in an acute toxicity test because it can generally represent the degree of toxicity of toxicants (Eaton and Klaassen, 2001). In the present study, the 24–96 h LC₅₀ values of glyphosate in common carp were determined to be from 683.47 to 520.77 mg L⁻¹, indicating that glyphosate has only relatively low toxicity on common carp according to the toxicity degree classification of toxicants (Eaton and Klaassen, 2001). Moreover, the 96 h LC₅₀ value in the our study was much higher than those obtained in neotropical fish (3.74 mg L⁻¹) (Shiogiria et al., 2012), *Prochilodus lineatus* (13.7 mg L⁻¹) (Langiano and Martinez, 2008), and *Oreochromis niloticus* (16.8 mg L⁻¹) (Jiraungkoorskul et al., 2002) while a little lower than that of common carp (620 mg L⁻¹) (Neskovic et al., 1996), suggesting that common carp may be not sensitive to glyphosate toxicity (Vera-Candioti et al., 2013).

Fish immunologic system mainly includes indicators of innate immunity such as LYZ and complements and acquired immunity such as immunoglobulin, that not only play a key role in preventing fish from pathogen infection, but also in defending fish against xenobiotics from the aquatic environment (Magnadóttir, 2006). These immunologic substances usually make an early and rapid response to the stress of environmental toxicant by representing alterations in their activity

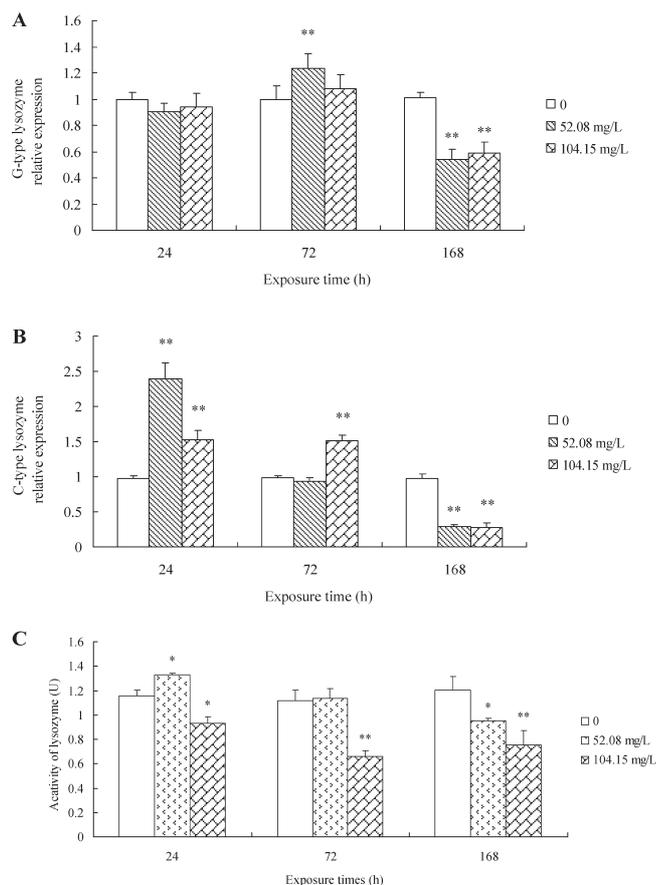


Fig. 3 – LYZ mRNA levels and activities in the kidney of common carp exposed to 52.08 or 104.15 mg L⁻¹ of glyphosate for 168 h. Glyphosate-exposure and the determinations of LYZ mRNA level and activity were described in Sections 2.3 and 2.4, respectively. The experiment was performed in duplicate, and data were shown as the means \pm SD. Asterisks denote a response that is significantly different from the control ($p < 0.05$, ** $p < 0.01$). (A) G-type LYZ mRNA level. (B) C-type LYZ mRNA level. (C) LYZ activity.

or contents. IgM is one of the most important antibodies against pathogens and it is also the primary immunoglobulin mediating humoral adaptive immunity in fish (Wilson and Warr, 1992; Bag et al., 2009). IgM can respond sensitively to xenobiotics and hence it can be used as a valuable biomarker for humoral immune response to aquatic pollution (Chen et al., 2013). Bag et al. (2009) reported that herbicide exposure up-regulated the gene expression of IgM in European flounder (*Platichthys flesus*). In Nile tilapia, Girón-Pérez et al. (2007) found that plasma IgM level in diazinon-treated group (1.96 mg L⁻¹) was significantly higher than that of control. In our study, the expression or content of IgM were found to be generally repressed or decreased in kidney from the glyphosate-treated common carp, suggesting that subacute glyphosate-exposure may disturb the humoral immune response of common carp. This result is in agreement with a study in common carp exposed to chlorpyrifos (Li et al., 2013).

Fish complement C3 is a key component in the cascade activation of complement system and plays a major role in linking innate and adaptive immunity to defend pathogenic infection and xenobiotic stress (Nakao et al., 2000; Magnadóttir, 2006; Dunkelberger and Song, 2009; Prado-Alvarez et al., 2009). Several evidences indicate that toxin-treatment affects C3 content and disturbs the innate immune system of fish. Jin et al. (2011) showed that the mRNA level of C3 was altered in newly hatched zebrafish when exposed to 3 or 10 μ g L⁻¹ cypermethrin. Furthermore, Li et al. (2012) observed the decreased C3 content in ionic liquid treated brocaded carp. In the present study, glyphosate as a whole either repressed C3 transcription or decreased C3 content in the kidney from the glyphosate-exposed common carp, as shown in Fig. 2. This result suggests that glyphosate may disturb the innate immune system of common carp via suppressing complement C3.

LYZ is another important component of innate immunity in fish, which is produced by several types of leukocytes, such as neutrophils and macrophages (Magnadóttir, 2006). It is involved in a broad range of defense mechanisms, such as antimicrobial, antiviral and opsonization, etc. (Chipman and Sharon, 1969; Losso et al., 2000). LYZ activity is usually modulated to improve the immune defense when fish encounter the increasing pathogens and other various stress factors (Zhao et al., 2010). In this study, both G-type and C-type LYZ

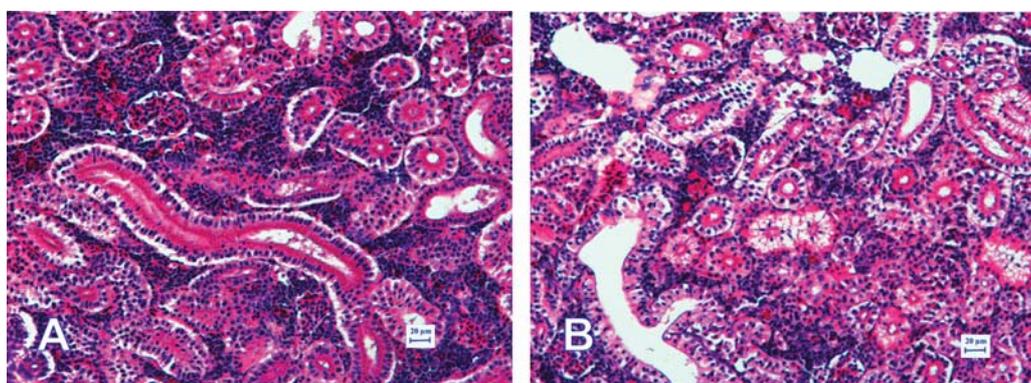


Fig. 4 – Kidney histopathology from the glyphosate-treated common carp and control fish. The treatment fish were exposed to 104.15 mg L⁻¹ of glyphosate for 168 h and histopathological examination was described in Section 2.5. (A) Kidney from control fish. (B) Kidney from the glyphosate-treated fish.

exhibited a similar tendency of transcriptional change in the kidney from the glyphosate-treated fish, that is, up-regulation at the early period of treatment but down-regulation at the end of exposure, as shown in Fig. 3. However, its activity in the treatment groups was totally lower than that of control group except for an increase in 52.08 mgL⁻¹ group at 24 h, which is similar to a report in common carp exposed to chlorpyrifos (Li et al., 2013). This result indicates that LYZ is involved in glyphosate toxicity on common carp.

Head kidney is the most important immune organ of teleost fish and the histopathological alterations in kidney usually imply moderate to severe and irreparable damage and result in dysfunction of immune capacity (Shiogiria et al., 2012). In this study, we also observed that glyphosate-exposure caused obvious histopathological injury in fish kidney, for example, vacuolization of the renal parenchyma and intumescence of the renal tubule, which was in accordance with the results obtained in lindane treated fishes by Ortiz et al. (2003), in common carp exposed to atrazine and chlorpyrifos (Xing et al., 2012), and in paraquat-treated common carp (Ma et al., 2014). The histopathological damage may affect the normal physiological functions of the immune organs and lead to immune dysfunction in common carp. Integrating the above results, we think that glyphosate indeed has immunotoxicity on common carp although it has only weaker acute toxicity to the fish.

There have been increasing evidences indicate that glyphosate can exert a cytotoxic effect on human cell lines at very low sub-agricultural dilutions (Gasnier et al., 2009). Moreover, cases of cutaneous toxicity of glyphosate from chemical burns to allergic contact dermatitis were also reported according to epidemiological studies on glyphosate (Penagos et al., 2004, Nagami et al., 2005). Nevertheless, little is known about the immunotoxicity of glyphosate in humans to the best of our knowledge. Our results reveal that glyphosate-exposure alters the transcription or content of IgM, C3, and LYZ in the kidney of common carp and causes remarkable histopathological damage to the kidney of common carp, suggesting that intensive using of glyphosate in agriculture may be a potential threat to human health due to its existence or bioaccumulation in the food chain (US EPA, 1993; Larsen et al., 2012). Therefore further work is greatly necessary to evaluate the harmful effect of glyphosate on susceptible population, to conduct risk assessment, and establish the safety standard of glyphosate for humans.

In conclusion, our results reveal that glyphosate-exposure alters the transcription or content of IgM, C3, and LYZ and causes remarkable histopathological damage to the kidney of common carp, which may disturb the function of the fish immune system.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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