Bioaccumulation and Toxicity Effects of Flubendiamide in Zebrafish *(Danio Rerio)*

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Abstract

As a diamide insecticide, flubendiamide is widely used and has many adverse effects on environmental organisms. In this study, bioaccumulation and toxicity effects of flubendiamide in zebrafish (*Danio rerio*) were studied. Specifically, the results showed that the concentrations of flubendiamide increased in the early stage and achieved steady stages at 14 days and the bioconcentration factors (BCFs) of flubendiamide in zebrafish were 1.125 - 2.011. Furthermore, flubendiamide had no significant effects on the growth phenotypes of zebrafish. However, zebrafish hepatic somatic index (HSI) of zebrafish had changed significantly with exposure. Histopathological analysis showed that exposure to flubendiamide could cause structural damage to liver tissue of zebrafish. Further physiological and biochemical analysis showed that flubendiamide could significantly change the activity of CAT and the contents of MDA and GSH in liver of zebrafish. In particular, exposure to flubendiamide could also cause significant changes in the mRNA expression levels of cell apoptosis-related genes involving *p53*, *puma*, *caspase-3*, *caspase-9*, *apaf-1* and *bax* in liver of zebrafish. In general, these results indicated that exposure to flubendiamide could induce liver damage by inducing oxidative stress and apoptosis in liver of zebrafish. The results of this study will help to further comprehensively evaluate the safety of flubendiamide to aquatic organisms.

1. Introduction

As a new type of broad-spectrum and high-efficiency pesticides, diamide insecticides are widely used in agricultural production (Teixeira &Andaloro 2013), making them ubiquitous in the natural environment (Caboni et al. 2008, Sharma et al. 2014, Song et al. 2019), thereby bringing potential toxicity risks to environmental organisms. Among them, the toxicity risks of diamide insecticides in aquatic organisms has received more and more attention (Barbee et al. 2010), but the current research is very limited. As one of the earliest applied diamide insecticides, the impacts of flubendiamide on environmental organisms has attracted more and more attention (Sarkar et al. 2014). It has been confirmed that chronic sub-lethal flubendiamide exposure could induce cell apoptosis in in larval imaginal discs of *Drosophila melanogaster* (Sarkar et al. 2017). In addition, chronic flubendiamide exposure also could induce oxidative stress in water buffalo (*Bubalus bubalis*) calves (Ranjan et al. 2018). Consistently, exposure to flubendiamide could also cause oxidative stress and DNA damage in earthworms (*Eisenia fetida*) (Liu et al. 2017). In particular, previous studies had shown that flubendiamide was high toxicity to *Daphnia magna* during acute and chronic exposure. In addition, the μg/L level of flubendiamide could seriously affect the survival, reproduction and growth of *Daphnia magna* (Cui et al. 2017). The above study suggests that flubendiamide may have potential adverse effects on aquatic organisms. Therefore, it is necessary to study the toxicological effects of flubendiamide on aquatic organisms.

As a representative aquatic organism, zebrafish (*Danio rerio*) has the characteristics of easy availability, low price, short life cycle, and sensitivity to chemicals. In addition, zebrafish has a high degree of conservation in the structure and function of genes and proteins, and is widely used in the toxicity assessment of environmental pollutants (Jia et al. 2020, 2021, Liu et al. 2021, Tian et al. 2021).

Importantly, the toxicological effects of flubendiamide on zebrafish has not been reported. In this study, we aimed to study the bioaccumulation behaviors of flubendiamide in zebrafish by using LC-MS/MS analysis and aimed to explore the toxic effects of flubendiamide on zebrafish via physiological and biochemical analysis, histopathological analysis and gene expression analysis. These results of our study will help to further understand the potential risks of flubendiamide potential risks to aquatic organisms and provide a theoretical basis for comprehensive assessment the environmental health risks of flubendiamide.

2. Materials And Methods

2.1 Reagents

Flubendiamide (analytical standards, purity > 99.0%) were purchased from Sigma-Aldrich (Sigma, USA). All other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) at analytical grade levels.

2.2. Zebrafish maintenance

Adult male zebrafish of four month old (AB wild-type strain) were obtained from Yangzhou aquarium supermarket (Yangzhou, China) and maintained in a regular light cycle of 14/10 h at 26 ± 2℃ with a commercial diet once a day. And every 15 zebrafish were randomly distributed in a 10 L glass tank containing 5 L oxygen-enriched water. All zebrafish acclimated for 2 weeks prior to the start of experiments.

2.3 Zebrafish exposure and sample collection

The zebrafish were exposed to flubendiamide at doses of 0.1, 0.5, and 1.0 mg/L based on the 96 h LC_{50} value (> 30 mg/L) of our previous study. The control group was exposed to 0.1% DMSO. For bioaccumulation of flubendiamide in zebrafish, each concentrations exposure contained 60 zebrafish, respectively. And each concentrations exposure were repeated 3 times. The exposure media was changed every 48 h and the experimental conditions were consistent with the adaptation period. In each treatment group, 5 zebrafish and 5 mL water samples were collected as one sample on 1, 3, 5, 7, 10, 14, 17, and 21 days after the start of the bioaccumulation experiment and stored at -20℃. In addition, zebrafish used for toxicological effect analysis were sampled on the 7th and 14th days, respectively. For biochemical analysis, 2 zebrafish per sample were collected and stored at -20℃. For histopathological analysis, 3 zebrafish from each treatment groups were anesthetized on ice and dissected. And the liver tissue of zebrafish were collected and stored in tissue fixative. For the qPCR analysis, at least six fish of each treatment groups were anesthetized on ice and dissected and the liver sample were pooled as one sample and stored at -80℃.

2.4 Extraction and determination of flubendiamide

Sample extraction and purification

The zebrafish (0.5 g per samples) samples were weighed into 10 mL centrifuge tubes and freeze-dried using a vacuum freeze-dryer. Then, 5 mL of acetonitrile were added and ground for 5 min with a tissue grinder. And 0.2 g of sodium chloride and 1 g of anhydrous magnesium sulfate were added in the samples. The mixtures were agitated for 5 min in a rotary shaker at 5000 rpm. The 2 mL of supernatant were collected into a new glass tube containing 50 of mg C18, and agitated for 5 min in a rotary shaker at 5000 rpm. The 1mL of supernatant was collected and concentrated to dryness. Finally, the extracts were re-dissolved into 1 mL of acetonitrile was filtered through a 0.22 mm membrane and subjected to LC-MS/MS chromatographic analysis. Moreover, the water samples (5mL per samples) were collected into 50 mL centrifuge tubes. Subsequently, 5mL of n-hexane was used for shaking extraction, which was repeated 3 times. The organic phases were combined and collected in new centrifuge tube and concentrated with nitrogen to dry. Finally, the extracts were re-dissolved into 2 mL of acetonitrile was filtered through a 0.22 mm membrane and subjected to LC-MS/MS chromatographic analysis

Determination of flubendiamide by LC-MS/MS

The concentrations of PEN in zebrafish and water were analyzed by liquid chromatography-mass spectrometry (LC-MS/MS) according to our previous research (Chen et al. 2012). The detailed analysis method of flubendiamide was listed in the supporting information S1.

2.5 Biochemical indicators and histopathology analysis

The contents of MDA and GSH and the activities of SOD, CAT and GPx in liver samples of zebrafish were detected using the corresponding kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. The liver tissues of each treatment group were randomly selected for histopathological analysis. The liver tissues were fixed in 4% formaldehyde solution. Subsequently, paraffin embedding, tissue sectioning and Hematoxylin-eosin (H&E) staining were performed.

2.6 RNA extraction and qPCR analysis

TRIzol reagent was used to extract total RNA from the 20 mg of liver samples of zebrafish. Then, the 1.5 μg RNA sample was reverse transcribed into cDNA using the Fast Quant RT kit. After two-fold dilution with dd-H2O, the SuperReal PreMix Plus (SYBR Green) kit was used to perform real-time fluorescent quantitative PCR analysis by the Bio-Rad CFX 96 PCR system (Bio-Rad, USA). Each samples were subjected to 3 technical replicates. The above analysis methods were carried out in accordance with the manufacturer's instructions. *β-actin* was used as an internal reference gene, and the cycle threshold (*Ct*) method was used for homogenization. The PCR primers were obtained from Sangon Biotech (Shanghai, China.) and the sequences of primers were listed in Table S1.

2.7 Statistical analysis

All data were expressed as mean ± standard deviation (SD). In order to compare the statistical differences between different treatment groups, one-way ANOVA was performed using SPSS 19.0 (IBM, USA).

Graphical illustrations were performed with GraphPad Prism version 6.0 (Graph Pad). Asterisk (*) indicated statistically significant difference between the control (CK) and treatment group ($p < 0.05$).

3. Results

3.1 Optimal conditions for the Detection of flubendiamide using LC- MS/MS

The results showed that based on optimal MS parameters, the highest sensitivity could be achieved for flubendiamide using methanol and 10 mmol/L ammonium acetate aqueous solution containing 0.1% acetic acid as mobile phases and using the MRM negative mode. And m/z 214 was selected as the qualitative ion of flubendiamide, and m/z 254 was selected as the quantitative ion of flubendiamide. The collision energies of ion pairs 681/214 and 681/254 were − 45 eV and − 20 eV, respectively. The selected ionization chromatography of flubendiamide were shown in Fig. 1. Furthermore, the fortified recoveries of flubendiamide from the zebrafish and water samples were listed in Table 1. The fortified recoveries of flubendiamide in water samples were 82.58%-97.36% and the relative standard deviation were 2.95%-3.29%. And the fortified recoveries of flubendiamide in zebrafish samples were 80.16%-87.95% and the relative standard deviation were1.55%-3.24%. The results indicate that the method could be adopted to study the bioaccumulation of flubendiamide in zebrafish.

Table 1
Recoveries of flubendiamide in zebrafish and water samples

Samples	Fortification levels (mg/kg or mg/L)	Recoveries (%)			Average recoveries (%)	Standard deviation (%)
Zebrafish	0.1	82.65	81.05	80.16	81.29	1.55
	1.0	87.55	82.62	83.04	84.40	3.24
	10	83.85	87.95	84.58	85.46	2.56
Water	0.1	97.36	91.34	93.15	93.95	3.29
	1.0	93.52	92.46	88.16	91.38	3.11
	10	86.15	87.45	82.58	85.39	2.95

3.2 Bioaccumulation of flubendiamide in zebrafish

In the 0.1, 0.5, and 1.0 mg/L flubendiamide treatment groups, the bioaccumulation behaviors of flubendiamide in zebrafish showed a similar trend. The concentrations of flubendiamide in zebrafish rose first from 1 to 5 days and then decreased from 5 to 7 days. Subsequently, the concentrations of flubendiamide in zebrafish continued to increase after 7 days and reached a steady stage at 14 days of

exposure (Fig. 2A). The changes in the concentration of flubendiamide in zebrafish may be attributed to the balance between bioaccumulation and metabolism. In addition, the concentrations of flubendiamide in water was particularly stable. The actual concentrations of flubendiamide in the 0.1, 0.5, and 1.0 mg/L treatment groups were 0.0912 ~ 0.1033, 0.4825 ~ 0.5083 and 0.9124 ~ 1.1367 mg/L, respectively (Fig. 2B). In particular, the bioconcentration factors (BCFs) of flubendiamide in zebrafish in the0.1, 0.5, and 1.0 mg/L treatment groups were 2.011, 1.125 and 1.444, respectively (Table 2).

Table 2
Bioconcentration factors(BCFs)of flubendiamide in zebrafish

Treatment	C_{fish}(mg/kg)	C_{water}(mg/L)	BCFs
0.1 mg/L	0.194	0.097	2.011
0.5 mg/L	0.564	0.501	1.125
1.0 mg/L	1.472	1.019	1.444

3.3 Effects of flubendiamide on the growth phenotypes of zebrafish

Subsequently, the effects of flubendiamide on the growth phenotypes of zebrafish were analyzed. The results showed that body weight and body length of zebrafish did not change significantly after 7 days and 14 days of 0.1, 0.5, and 1.0 mg/L flubendiamide exposure compared to control group (Fig. 3A and Fig. 3B). In addition, the effect of flubendiamide on the growth phenotype of zebrafish was further evaluated through growth factors (K-factors). Consistently, there was no adverse effects on K-factors of zebrafish after exposure to 0.1, 0.5 and 1.0 mg/L flubendiamide (Fig. 3C). In particular, hepatic somatic index (HSI) of zebrafish increased significantly in 1.0 mg/L flubendiamide treatment group after 14 days compared to control group (Fig. 3D). And the hepatic somatic indexes of zebrafish did not change significantly in other treatment groups. These results indicated that flubendiamide exposure may adversely affect liver tissue of zebrafish. Therefore, histopathological analysis of liver samples in zebrafish with flubendiamide exposure after 14 days was performed (Fig. 4). In the control group, the cytoplasm of the liver in zebrafish was uniform, with regular round nucleus located in the center of the liver cells. However, the liver tissues of zebrafish showed obvious damage after flubendiamide exposure. A large number of massive micro or macro vesicular intracellular lipid droplets were apparent in the flubendiamide treatment groups. Furthermore, the degrees of liver cell pathology became more and more obvious with the increase of the concentration of flubendiamide. These results further proved that flubendiamide exposure could cause liver function in zebrafish.

3.4 Effects of flubendiamide on liver oxidative stress of zebrafish

Then, we evaluated the effect of exposure to flubendiamide on liver oxidative stress of zebrafish by measuring the enzymatic activities of SOD, CAT and GPx and the contents of MDA and GSH. After 7 and

14 days of flubendiamide exposure, there were no significantly changes in the activities of SOD and GPx (Fig. 5). However, the activity of CAT was significantly increased and the contents of GSH was significantly decreased after 14 days of 1.0 mg/L flubendiamide exposure compared to control group (Fig. 5B and Fig. 5E). After 7 days of flubendiamide exposure, the content of MDA was significantly increased in the 1.0 mg/L flubendiamide treatment group. Moreover, the contents of MDA were significantly increased after 14 days of 0.1, 0.5 and 1.0 mg/L flubendiamide exposure compared to control group (Fig. 5E). These results implied that flubendiamide could cause oxidative stress in liver tissue of zebrafish.

3.5 Effects of flubendiamide on liver cell apoptosis of zebrafish

Next, the effects of flubendiamide on cell apoptosis in liver of zebrafish were explored by detecting the mRNA expression levels of apoptosis related genes involving in a*paf-1*, *p53*, *puma*, *caspase-3*, *caspase-9*, *bcl-2* and *bax* (Fig. 6). After 7 days of flubendiamide exposure, the mRNA expression levels of a*paf-1* and *bax* were significantly increased in 1.0 mg/L flubendiamide treatment group (Fig. 6A and Fig. 6G). And the mRNA expression levels of *caspase-9* were significantly increased with 0.5 and 1.0 mg/L flubendiamide exposure (Fig. 6E). In addition, the mRNA expression levels of *puma* were significantly increased in the 0.1, 0.5 and 1.0 mg/L flubendiamide treatment groups compared to control group (Fig. 6C). After 14 days of flubendiamide exposure, the mRNA expression levels of a*paf-1* and *caspase-9* were significantly increased with 0.5 and 1.0 mg/L flubendiamide exposure (Fig. 6A and E). Particularly, the mRNA expression levels of *p53*, *puma*, *caspase-3* and *bax* were significantly increased in the 0.1, 0.5 and 1.0 mg/L flubendiamide treatment groups compared to control group (Fig. 6B, C, D and G). Moreover, flubendiamide exposure had no significant effects on the mRNA expression levels of *bcl-2* after 7 and 14 days.

4. Discussion

As an efficient broad-spectrum insecticide, flubendiamide is widely used in agricultural production. Recently, the toxicological effects of flubendiamide on non-target organisms have received more and more attention (Nareshkumar et al. 2017, Ranjan et al. 2018). Some studies have reported the adverse effects of flubendiamide on non-target organisms such as earthworms (*Eisenia fetida*), *Drosophila melanogaster* and *Daphnia magna* (Cui et al. 2017, Liu et al. 2017, Sarkar et al. 2017). Unfortunately, the bioaccumulation and toxic effects of flubendiamide on zebrafish have not been reported. Therefore, the bioaccumulation behavior of flubendiamide on zebrafish was analyzed by using LC-MS/MS. In addition, the toxic effects involving growth phenotypes, oxidative stress and cell apoptosis of flubendiamide on zebrafish were explored in this study.

First of all, the results of the bioaccumulation experiment showed that the concentrations of flubendiamide could reach bioaccumulation equilibriums in zebrafish after 14 days of flubendiamide exposure. In addition, the BCFs of flubendiamide in zebrafish were 1.125–2.011. These results indicated

that zebrafish had a low bioaccumulation capacity for flubendiamide according to the classification standards of bioaccumulation capacities (Jia et al., 2017). Meanwhile, there were no significantly change in body weight, body length and K-factors with flubendiamide exposure. However, the HIS was increased significantly after 14 days of 1.0 mg/L flubendiamide exposure. This implied that flubendiamide exposure may affect liver functions of zebrafish and short-term exposure to flubendiamide could not adversely affect the growth of zebrafish. The liver tissue is the largest gland in vertebrates, and it plays an important role in the metabolism of carbohydrates, fats, proteins, vitamins and hormones (Qiu et al., 2019). Furthermore, the effects of flubendiamide on liver tissue of zebrafish was studied by histopathological analysis. After 14 days of 0.1, 0.5 and 1 mg/L flubendiamide exposure, a series of changes occurred in liver tissue structure of zebrafish including lymphocytic infiltration, hepatocellular vacuolization and cell necrosis. These results further confirmed that flubendiamide can cause liver damage in zebrafish.

A large number of studies have shown that the damage of liver tissue is often accompanied by the occurrence of oxidative stress (Meng et al. 2021, Meng et al. 2019, Wang et al. 2015). Next, we analyzed the impacts of flubendiamide on the activities of SOD, CAT and GPx and the contents of MDA and GSH in liver of zebrafish. The results showed that activity of CAT in liver of zebrafish had changed significantly after flubendiamide exposure. As an important antioxidant enzyme, CAT can efficiently catalyze H_2O_2 into water and oxygen, which has the function of scavenging free radicals and protecting cells from damage in organisms (Zhang et al. 2018).This implied that flubendiamide could destroy the antioxidant enzyme system of zebrafish. In addition, exposure to flubendiamide caused a significant decrease in the content of GSH in the liver of zebrafish. GSH is an important regulatory metabolite in cells. The decrease of content of GSH is a potential early activation signal of apoptosis, and the subsequent generation of oxygen free radicals could promote cell apoptosis (Meng et al. 2019). Importantly, as a product of lipid peroxidation, the content of MDA is usually used to reflect the degree of oxidative damage (Gupta et al. 2009).In our study, the content of MDA in liver of zebrafish had increased significantly with flubendiamide exposure. It is indicated that the oxidative stress in liver of zebrafish induced by flubendiamide. In addition, the increase in the content of MDA may severely damage cell membranes (Teng et al. 2019). Therefore, we observed structural damage to the liver tissue of zebrafish in the flubendiamide treatment groups.

The appearance of oxidative stress and lipid peroxidation may lead to apoptosis has been confirmed (Teng et al. 2019). It was necessary to further explore the effects of flubendiamide on the cell apoptosis in liver tissue of zebrafish. Therefore, the mRNA expression of a series of cell apoptosis related-genes were determined by qPCR analysis. In a series of genes that promote apoptosis, the increased mRNA expression of *p53* and *puma* can induce apoptosis (Wang et al. 2004, Yu &Zhang 2008). In addition, *caspase-3* and *caspase-9* are the key executive genes in the process of apoptosis (Soengas et al. 1999). However, *apaf-1* can activate the mRNA expression of *caspase-3* and *caspase-9* (Zimmermann et al. 2001). In particular, the mRNA expression levels of *p53*, *puma*, *caspase-3*, *caspase-9* and a*paf-1* in liver of zebrafish were significantly increased after flubendiamide exposure. Moreover, as the target gene of *p53*, *bax* can promote the release of cytochrome C from mitochondria. In addition, *bcl-2* plays an important

role in inhibiting cell apoptosis. The ratio of expression levels of *bcl-2/bax* expression has a decisive influence on the release process of cytochrome C in mitochondria (Zimmermann et al. 2001). Therefore, the decrease of the ratio of expression levels of *bcl-2/bax* can be used as a sign of the occurrence of apoptosis process (Whiteman et al. 2007). Consistently, the ratios of expression levels of *bcl-2/bax* in liver of zebrafish were significantly decreased in the flubendiamide treatment groups. This resulted suggested that exposure to flubendiamide could induce apoptosis in liver of zebrafish. In conclusion, our results from this study showed that flubendiamide may cause liver damage of zebrafish by inducing oxidative stress and cell apoptosis.

4. Conclusion

In conclusion, the results of our study indicated that flubendiamide had a low bioaccumulation ability in zebrafish. In addition, the activity of CAT and the levels of GSH and MDA in liver of zebrafish changed significantly after flubendiamide exposure. In addition, the mRNA expression of apoptosis-related genes (*p53*, *puma*, *caspase-3*, *caspase-9*, *apaf-1* and *bax*) had also changed significantly in liver of zebrafish after flubendiamide exposure. Based on these results, the study provided better understandings of the toxic effects of flubendiamide on zebrafish involving oxidative stress and apoptosis. Therefore, this study provided data support for better evaluation of the biological effects of flubendiamide on aquatic vertebrates.

Declarations

Ethics approval

All animal experiments were in accordance with the current Chinese legislation and were approved by the independent Animal Ethical Committee at Yangzhou University.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing Interest

The authors declare that they have no competing interests.

Authors' contributions

Zhiyuan Meng: Conceptualization, Methodology, Writing-review &editing. Zhichao Wang: Methodology, Validation, Formal analysis. Xiaojun Chen: Writing- review & editing, Supervision, Project administration. Yueyi Song: Methodology, Data curation, Writing-original draft. Miaomiao Teng: Methodology, Data curation. Tianle Fan: Methodology, Data curation. YangZheng: Resources, Writing-review &editing. Jiajia Cui: Validation, Supervision. Wangjing Xu: Methodology, Data curation.

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Figures

Fig.1

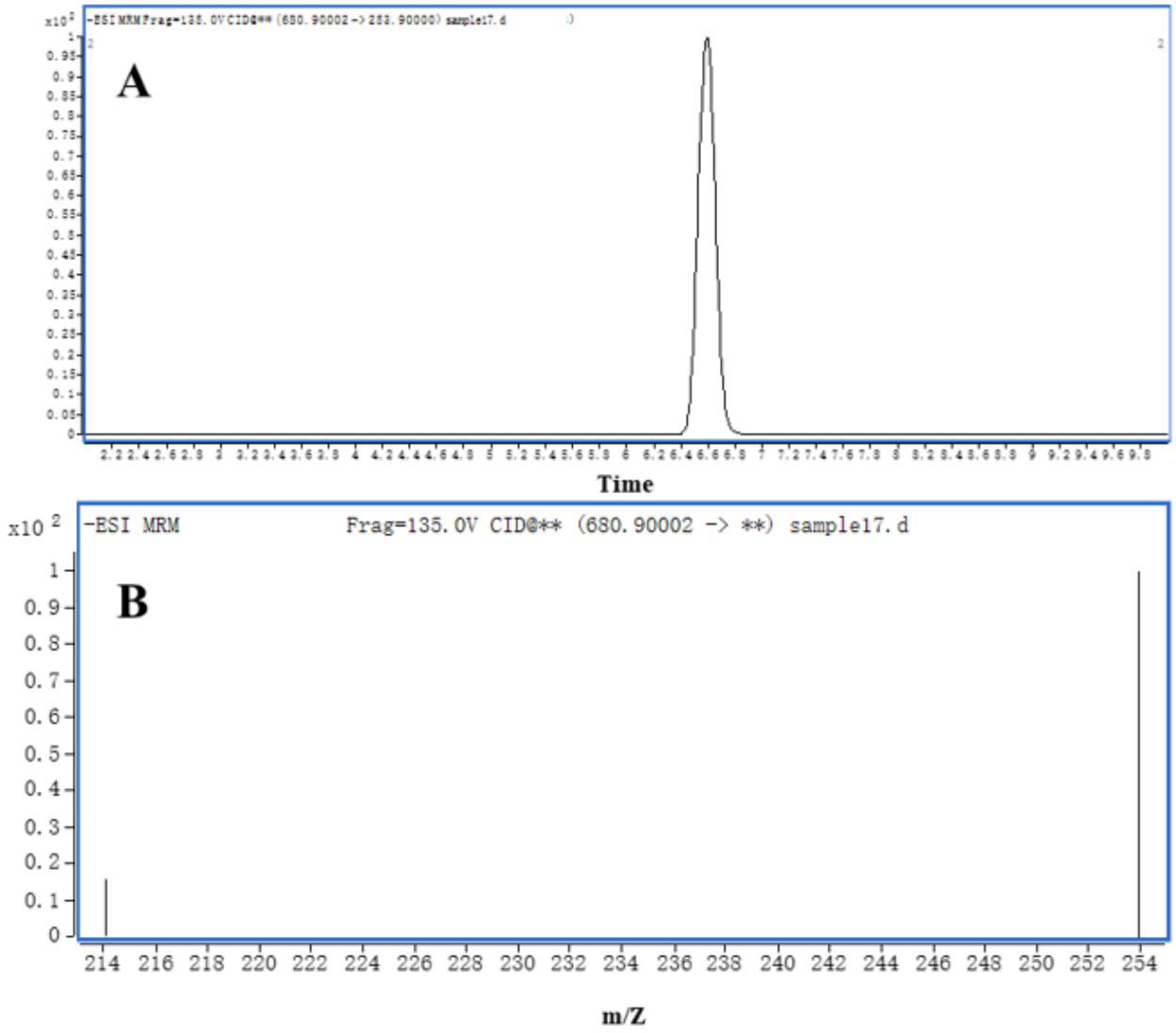

Figure 1

(A) Selected ionization chromatography of flubendiamide standard by MRM mode (m/z 214) and (B) MS-MS spectrum of flubendiamide standard by MRM mode.

Fig.2

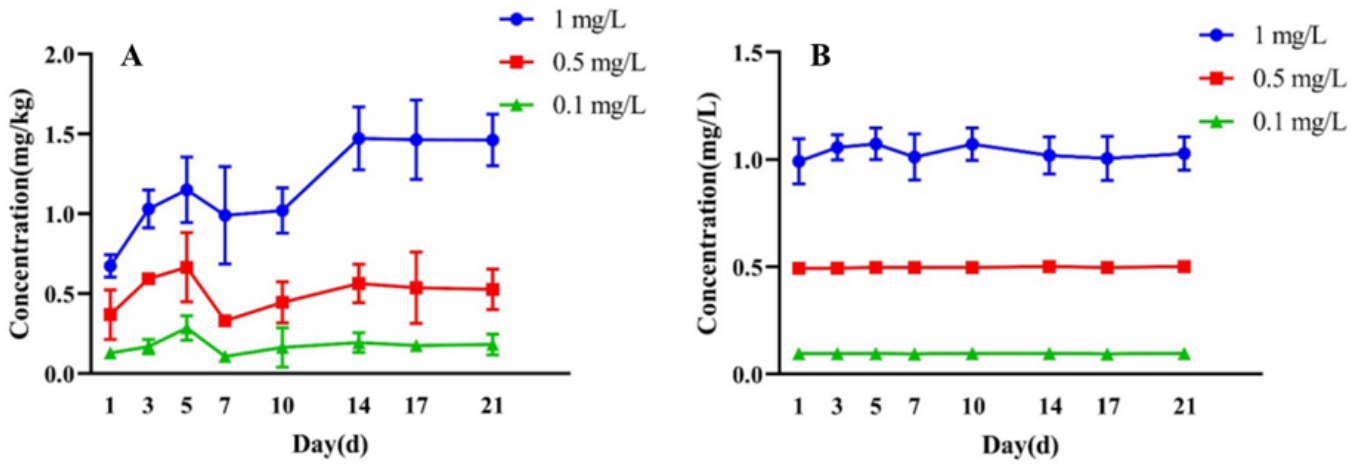

Figure 2

The concentration of flubendiamide in zebrafish (A) and water (B) samples. Data were expressed as mean ± SD.

Fig.3

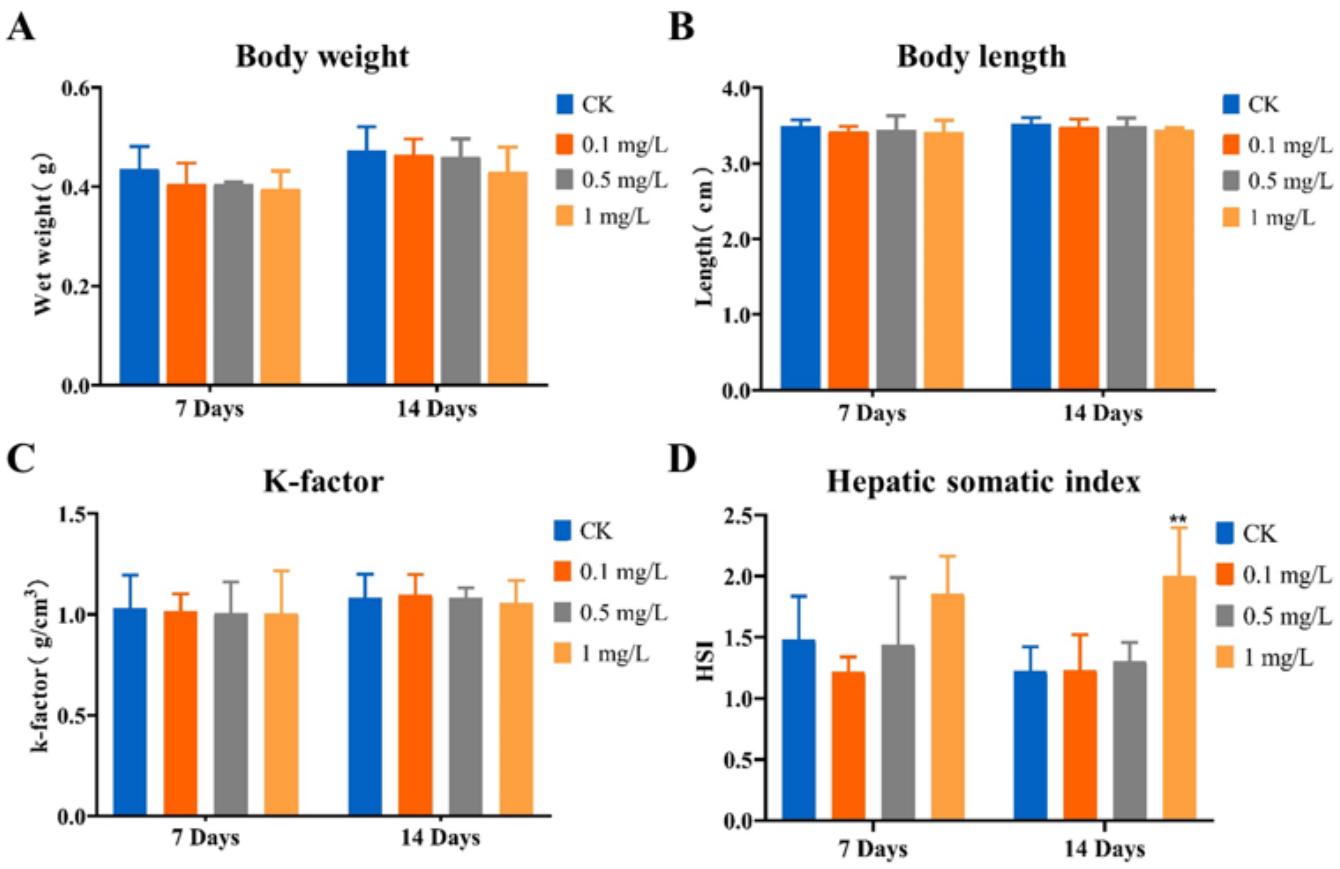

Figure 3

Effects of flubendiamide exposure on growth phenotypes of zebrafish. (A) Body weight, (B) Body length, (C) K-factors and (D) Hepatic somatic index (HSI). Data were expressed as mean ± SD. *p < 0.05 compared with the control group.

Fig.4

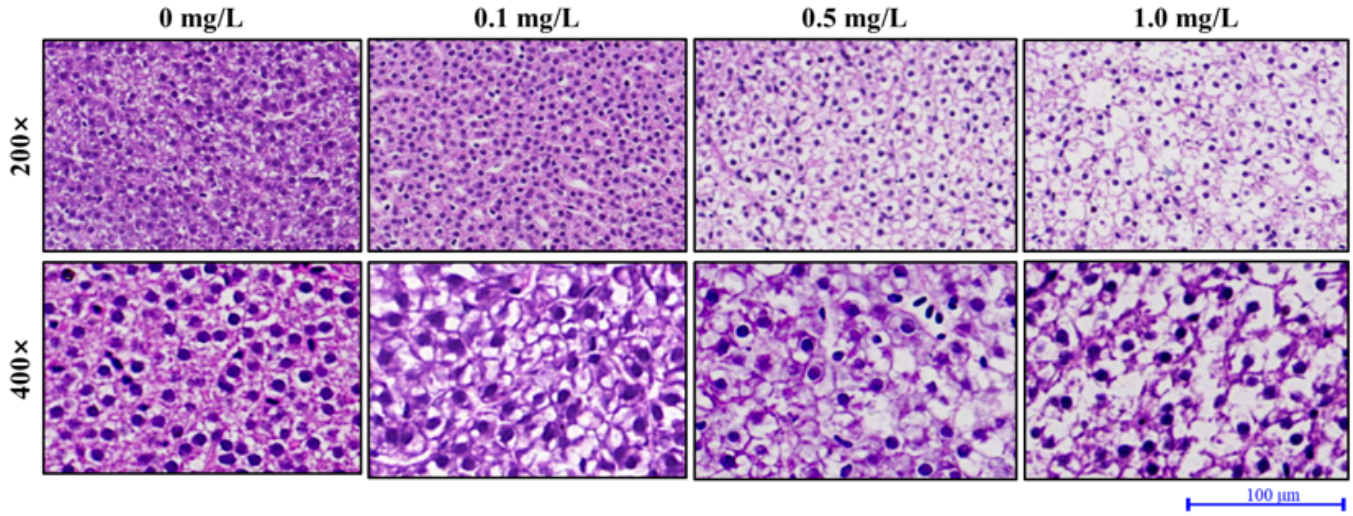

Figure 4

Histological changes in livers from zebrafish exposure to flubendiamide.

Fig.5

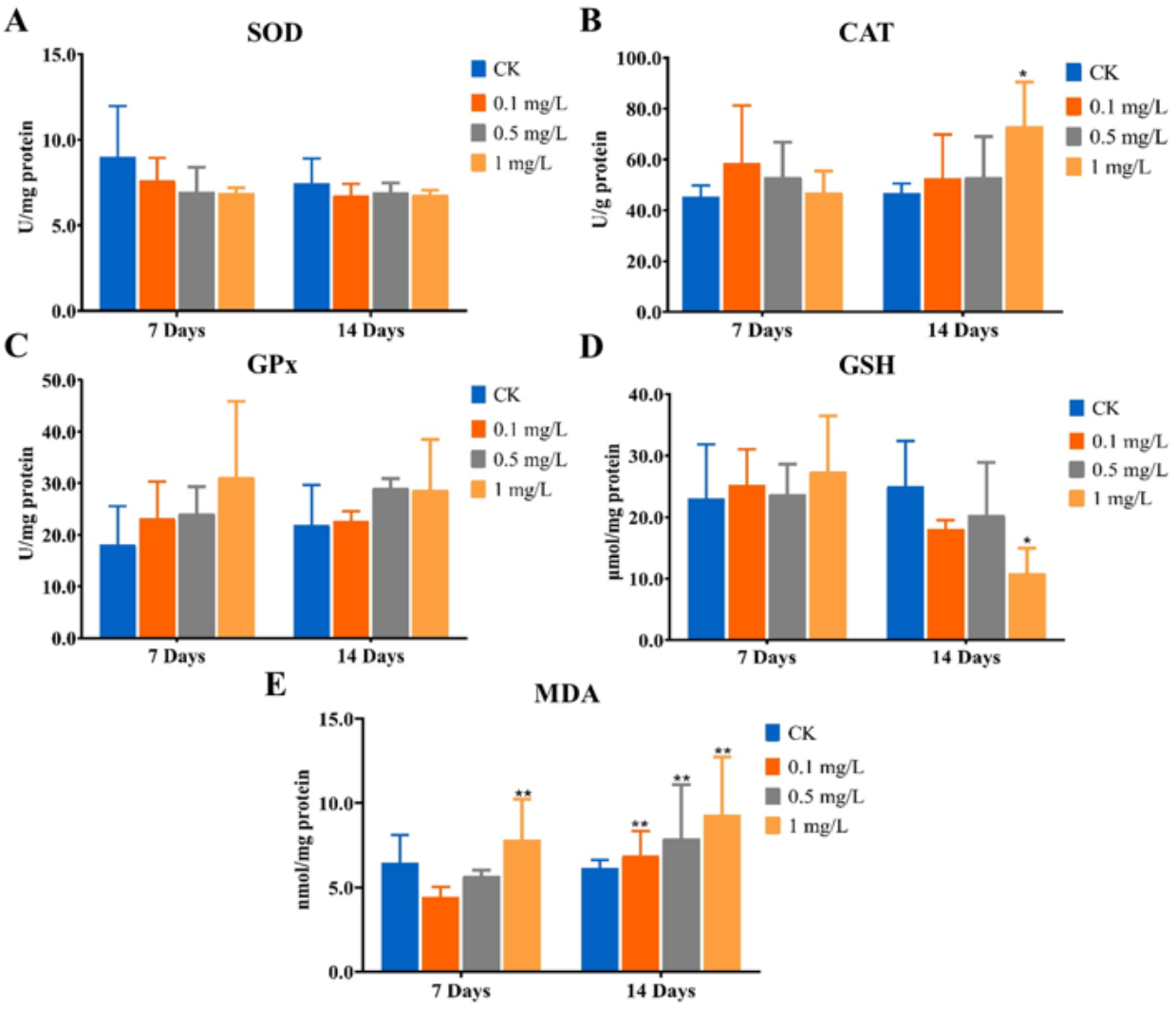

Figure 5

Effects of flubendiamide exposure on oxidative stress in liver of zebrafish. (A, B and C) the activities of SOD, CAT and GPx, (D and E) the contents of GSH and MDA. Data were expressed as mean ± SD. *p < 0.05 compared with the control group.

Fig.6

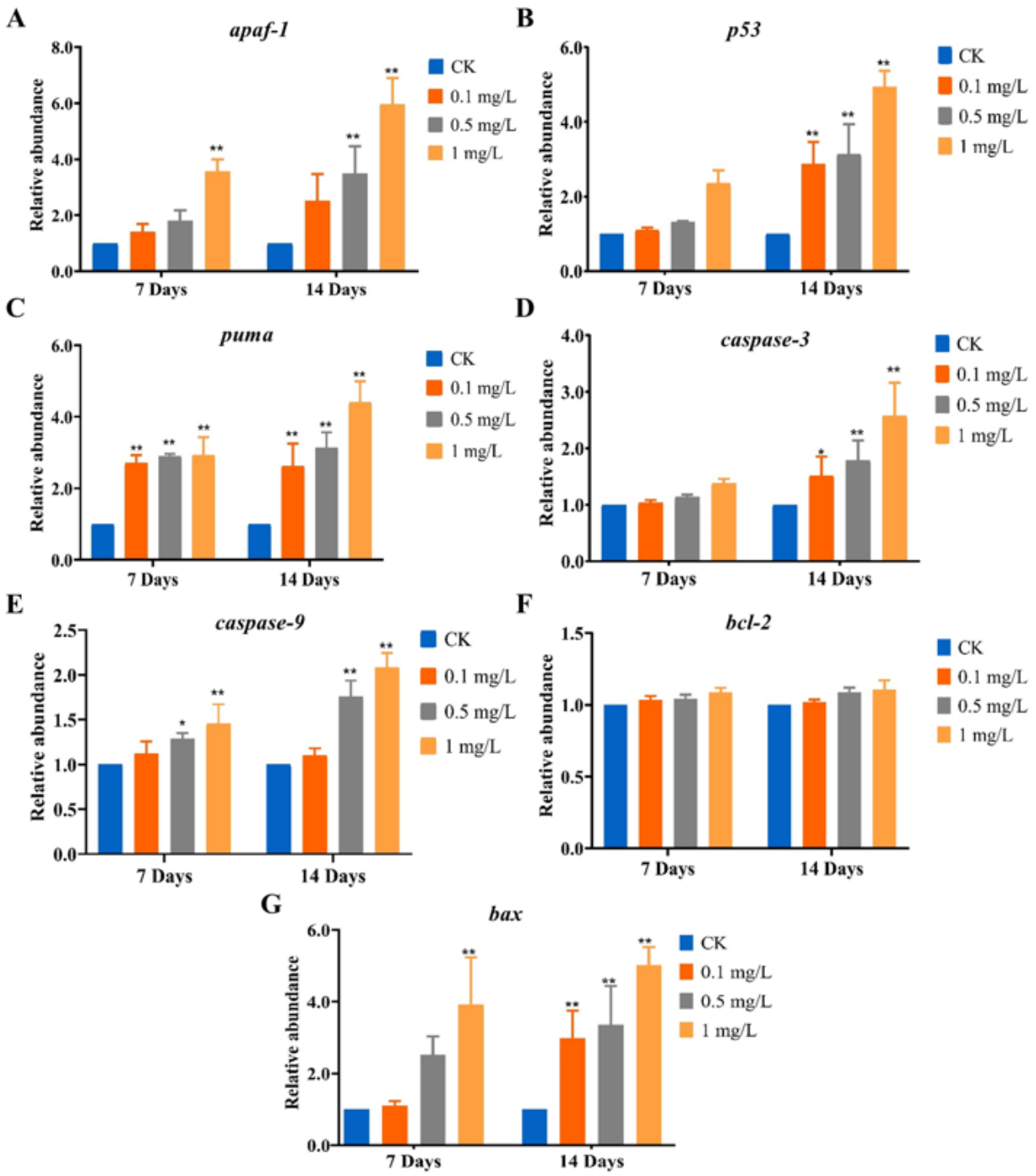

Figure 6

Effects of flubendiamide exposure on the mRNA expressions of apoptosis related genus in liver of zebrafish. (A) apaf-1, (B) p35, (C) puma, (D) caspase-3, (E) caspase-9, (F) bcl-2 and (G) bax. Data were expressed as mean ± SD. *p < 0.05 compared with the control group.

Supplementary Files

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- 20210830Supportinginformation.docx