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Subchronic toxicity and hepatocyte apoptosis of dietary olaquindox in common carp (*Cyprinus carpio*)

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ABSTRACT

Olaquindox as one of the effective antimicrobial agents and growth-promoting feed additives, had been widely used in animal and fish production. However, few studies have been done to unveil its possible toxic effect and tissue injury on aquatic animal. In this study, the toxic effect and underlying mechanisms of olaquindox toxicity were investigated in common carp when feed with different doses of olaquindox for 90 days. The morbidity and mortality, pathological changes, hematology parameters, residue concentration in the tissues of common carp were assessed, hepatocyte apoptosis was detected through ultrastructural observation and flow cytometry methods. The results showed that the morbidity and mortality increased with the increasing dosages of dietary olaquindox, subchronic exposure to olaquindox caused remarkably pathological changes, including congestion and bleeding, intramuscular edema, vacuolar degeneration, degeneration and deformation in renal tubules architecture, respiratory epithelium fusion and intestinal epithelial microvilli disintegration. Besides, dietary olaquindox led to significant changes in blood biochemical parameters including red blood cell, hemoglobin, alanine aminotransferase and aspartate aminotransferase, an elevated residue concentration of olaquindox was detected in liver and kidney after exposure, hepatocyte apoptosis and necrosis were observed. Moreover, insulin-like growth factor I (IGF-I) mRNA level in liver was higher than normal level with the dose below 25 mg/kg olaquindox and was lower than normal level with the dose above 50 mg/kg. Our results demonstrated that dietary olaquindox may pose subchronic toxicity and residue in fish organs and provided scientific data for the safe application of olaquindox in fish.

1. Introduction

Quinoxalines, part of benzene and pyrazine heterocyclic compounds, having kinds of biological activities including antibacterial, anti-candida, insect-resistant and antineoplastic (Carta et al., 2005). Olaquindox as one of quinoxaline derivatives, has been widely used as antimicrobial growth feed additive around the world since 1970s owing to the beneficial effects in animal husbandry (Wu et al., 2007). However, with the increasingly use of olaquindox, more and more studies about its adverse effects has been reported. Olaquindox was reported to be phototoxic in animals, cause photosensitization in pig breeders (Schauder et al., 1996) and show signs of mutagenicity in mice (Wang et al., 2010). Related studies also have suggested that olaquindox has genotoxic effects in vivo and vitro (Awais et al., 2013; Chen et al., 2009), it can cause denaturation and necrosis in majority tissues and organs based on the long-term toxicity studies of olaquindox in rats (De et al., 1990), chickens (Liu et al., 2011), pigs (Tao et al., 2009) and beagle dogs (Wang et al., 2015), and induce apoptosis in human HepG2 cells (Zou et al., 2011).

Due to the side effects and its possible dangers as well as the widely concerns over food safety, some European areas had banned or limited the usage of olaquindox (Wilkin, 1999), but its illegal use in fish or livestock has never vanished from sight worldwide (Corcoran et al., 2010). Incorrect or excessive use of olaquindox in fish have caused lots of adverse effects on the environment (Rabølle and Spliid, 2000), which even results in health risks to human consumers through the food chain and olaquindox contaminated water sources (Abdelkhalek, 2015). However, systemic study of the tissues injury caused by olaquindox in

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fish was few. With exposure to contaminations, the host animals are capable of decreasing toxic effects of chemicals by regulating their internal biochemical responses. Thus, early biochemical reaction of fish is also fairly important information for the assessment of olaquindox potential adverse effects.

On the other hand, olaquindox as low doses antibiotics has been used as growth promoters in livestock, and dietary supplementation with antibiotics has been shown to improve growth and feed efficiency in fish (Li et al., 2014). However, the dosage of olaquindox used as dietary supplementation in fish is still unclear. Insulin-like growth factor-I (IGF-I) is a very potent mitogenic growth factor that has been shown to affect proliferation and differentiation of a wide variety of cell types (D Ercole et al., 1996). Liver expression of IGF-I mRNA has been found closely related with the growth rate in several fish species. Thus, the IGF-I gene may be a possible indicator of growth rate in fishes (Yarmohammadi et al., 2013).

In this study, to investigate the effects of olaquindox on tissues injury and the underlying mechanism of olaquindox toxicity in common carp (*Cyprinus carpio*) when feed with different doses of olaquindox for 90 days, the subchronic toxicity of olaquindox was assessed from different aspects including morbidity and mortality, pathological changes, hematology parameters, residue concentration in common carp. Hepatocyte apoptosis was also detected through ultrastructural observation and flow cytometry methods to understand olaquindox toxicity mechanisms. Besides, the safe dosage of dietary olaquindox was evaluated through the detection of IGF-I mRNA level in liver. Our results not only demonstrates that dietary olaquindox may pose subchronic toxicity and residue in fish organs, which leads to health risks to human consumers through the food chain, but also provides scientific data for the safe application of olaquindox in fish, even in animals.

2. Material and methods

2.1. Fish, diet and olaquindox

Healthy common carp (average weight 60 ± 5 g) were purchased from a fish farm in Chengdu, Sichuan Provence, China. Fish were feed in aquarium tanks (1 m × 1.5 m × 2 m), 24 h continuous aeration under the circumstance of pH 6.8–7.2, temperature 25–28 °C and dissolved oxygen of 8–12 mg/L. Other indicators meet the fishery water quality standard (GB11607-89). The basal diet for common carp were produced according to “The nutritional needs of NRC fish” (Table 1). The olaquindox was analytical grade (purity 99.7%) and provided by Chinese Veterinary Supervision Department (Batch NO.: 9590).

2.2. Subchronic toxicity experiment

Common carp (n = 280) were randomly divided into seven groups. Each group of fish were administered orally with basal diet mixed with 10, 25, 50, 100, 150, 200 mg/kg (group I-VI) and 0 mg/kg (control group) of olaquindox respectively. The test lasted for 90 days. Fish were anaesthetized with MS222 (Sigma-Aldrich, USA) prior to experiments involving serum collection and sacrifice. All the animal experiments complied with ethical standards as determined by the ethical committee of Sichuan Agriculture University (No. XF201418).

Table 1
The composition and nutrient levels of basal diet of common carp.

Components	Content (%)	Nutrient level	Content (%)
Peruvian fishmeal	25	Crude protein	33.4
soybean meal	25	Crude fat	6
wheat middling	10	Crude fibre	5.9
wheat bran	15	Calcium	1.24
rapeseed cake	10	Phosphorus	1.42
fine rice bran	13	Lysine	1.74
vitamin[a] premix	1	Methionine	0.95
Mineral[b] premix	1	Arginine	1.31

[a] Mineral element addition level (mg/kg): Fe 150, Zn 50.00, Mn 20.00, Cu 3.00, I 0.50.

[b] Vitamin addition level (mg/kg): V_A 4000 IU, V_D 1000 IU, V_K 3.00, V_C 100.00, V_{B1} 5.00, V_{B2} 7.00, V_{B6} 10, VB_{12} 12, V_{B1} 5 V_{B2} 7, Inositol 440, Nicotinic acid 28, Pantothenic acid 30, Choline 1500.

2.3. Clinical symptoms and pathological changes

Fish in each group were observed for daily clinical signs during the experimental period. All the organs/tissues carefully examined and gross lesions were recorded, the liver, kidney, muscle, brain, intestine and gill were collected from four freshly dead or moribund fish per group at 15th, 30th, 45th, 60th, 75th and 90th days, respectively, then fixed with Bouin's and subsequently dehydrated using a series of graded alcohols and embedded with Paraffin. Ultimately, 5 μm of sections were stained with haematoxylin and eosin (H.E) for histopathological examination. Six histopathological parameters were observed to detect the tissues injury degree from normal to severe and determined by the same pathologists. The total score was calculated by dividing the number of fish.

2.4. Hematology and clinical chemistry

Blood samples were collected from fish caudal vein (n = 5) for hematology and serum biochemistry at 30th, 60th, 90th day post-poisoning, respectively. According to the physiology experimental course (Heng, 2004), red blood cell count (RBC) and hemoglobin (Hb) counts were performed by direct microscopecount and spectrophotometry, respectively. Serum chemistry parameters including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed according to the manufacturer's directions (Huang et al., 2006).

2.5. Olaquindox extraction and quantification

Olaquindox residue in muscles, livers and kidneys were measured by high-performance liquid chromatography (HPLC) at the end of subchronic toxicity test (at 90th day). To establish a standard curve, tissues (liver, kidney and muscle) of the control group were added into olaquindox standard solution to obtain the different olaquindox concentrations at 0.5, 1, 2, 4, 6, 8 and 16 μg/g respectively. The chromatographic peak area and olaquindox concentration were used to make a linear regression and standard curve (R = 0.984–0.986). The extraction of olaquindox in fish was done as following: four fish were randomly chosen to extract the olaquindox in liver, kidney and muscle. Tissue (1 g) was homogenated with 1 ml saturated ammonium sulfate, followed by centrifuging for 15 min (4000*g*), the supernatant was then collected, 1 ml 0.1 M KH_2PO_4 and 5 ml extracting solution which contained acetonitrile and ethyl acetate (3:2) were added, after 20 min of vibrating, the organic phase was collected by centrifuging for 10 min (4000*g*), and 1 ml 0.2 M NaOH was added. After vibrating for 10 min and centrifuging for 10 min (4000*g*), the supernatant was used to analysis the olaquindox concentration by HPLC (LC-6A, Shimadzu, Japan).

The treated sample was initially dissolved with 100 μl mobile phase, then vortex for 2 min, 15 μl of the mixture was injected into HPLC for analysis. Finally, the olaquindox concentration was calculated basis upon the standard curve. Specimens were conducted on an analytical reversed-phase Shperisorb-C18 column (4 mm × 200 mm, 5 μm) at a mobile phase with the flow rate of 1.0 ml/min. The column temperature was maintained at 25 °C. The mobile phase in the preparative clean-up step was methanol-water (15/85, v/v). The speed of record paper was 2 mm/min and the UV-detector operated at a 266 nm.