Acute and subacute toxicities effect of oxytetracycline pharmaceutical wastewater on Zebrafish

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Abstract. Oxytetracycline wastewater is a major category of pharmaceutical wastewater, and its toxic effects on aquatic organisms have aroused people's attention. In this study, Zebrafish were separately exposed to four Oxytetracycline wastewater treatments (20%, 40%, 60%, 80%) and a control group were sampled on days 3, 6, 9, 12, and 15. Superoxide dismutase (SOD) activities showed significant inhibition, but the highest SOD activity was found in 20% and 40% the treatment groups (195.12U/mgprot, 187.43U/mgprot, respectively) on the 12th day. MDA contents increased significantly compared with control group. MDA contents showed that the higher the volume concentration, the higher the contents of MDA with the increase of exposure time. The highest MDA content shown in 60% exposure group (5.49nmol/mgprot) on the 12th day. And SOD activities and MDA contents showed a trend of "Λ" type. In conclusion, Oxytetracycline wastewater induced oxidative stress and toxicity in Zebrafish muscle tissue.

1. Introduction

According to Wastewater characteristics and sources, wastewater is divided into many types, such as chemical wastewater, hospital wastewater, pharmaceutical wastewater etc. Pharmaceutical wastewater is considered as a kind of wastewater with a greater risk, especially antibiotic wastewater. Antibiotics have been used in large quantities for several decades, and scholars have a growing concern about potential effects of antibiotics wastewater. Approximately 100-200 thousands tons of antibiotics are used globally by one year, and 20% of the world's consumption used in China, so a large amount of antibiotic pharmaceutical wastewater will be generated[1, 2]. In addition, most of wastewater treatment plants (WWTPs) have no process for antibiotics wastewater and do not remove antibiotics wastewater effectively[3]. Therefore, Chinese antibiotics wastewater is higher concentrations than other countries[4]. And antibiotics wastewater will be a big risk for aquatic ecosystem in China.

Oxytetracycline (OTC) is one of the broad-spectrum antibiotics, and belongs to a member of the tetracycline family[5]. OTC is often used as a human and animal medicine in developing countries, because it has antimicrobial activity and low cost[6]. Nowadays, OTC is one of the main fish drugs to protect bacterial disease. Most of OTC used for fish through the feed, but organism has very little absorption of OTC. And 70-80% of OTC are excreted into the water[7]. However, OTC pharmaceutical

wastewater is also an important pathway to discharge into surroundings. It is reported that OTC pharmaceutical industry wastewater discharge up to 50mg/L[6]. Nowadays, Many scholars began to study the ecotoxicity of oxytetracycline by simulating the biological effects of oxytetracycline in wastewater environment in the laboratory. But nobody uses real pharmaceutical wastewater to study.

It is well-known that Shijiazhuang is a medicine city in Hebei Province, So it is necessary to study on the impact of pharmaceutical wastewater to aquatic organisms. Zebrafish are commonly used to determine ecotoxicity because of body length and environmental sensitivity. And Zebrafish genes have a high degree with people(up to 86%). So our study use Zebrafish as research object. OTC wastewater of this experiment comes from local pharmaceutical company. In this study, Zebrafish were used to examine the acute toxicity of OTC wastewater, and determine SOD activities and MDA contents by Zebrafish muscle tissue. The aims to indicate the ecological risks of OTC wastewater in the aquatic ecosystem.

2. Materials and Methods

2.1 Experimental Materials

2.1.1 Experimental Equipments and Instruments. UV-visible light spectrophotometer (UV-2550) were purchased from SHIMADZU（Japan）. Microplate reader (SpectraMax190) were purchased from Molecular Devices (Silicon Valley, USA). Acidity meter (FE20); Pipette (Pipet-Lite; TopPette Pipettor); Low temperature refrigerator (BBC-226STV); Glass homogenizer; High-purity water distiller (SYZ-A); analytical balance (EL204); water bath (DK-S26);High speed centrifuge (TG16-WS); filter; aeration pump; bucket; dissolved oxygen analyzer (JPBJ-608); vortex mixer (XW-80A); water hardness meter (YD300). Other are laboratory basic equipment.
The kit of coomassie brilliant blue (CBB), SOD and MDA were purchased from a biological engineering research institute.

2.1.2 Test Organisms. Zebrafish is a small fish that is very environmentally sensitive and very easy to raise. Zebrafish were purchased from aquafarm in Shijiazhuang. In the study, body length and weight of Zebrafish are strictly controlled through the selection before the experiment. The body length of the fish is controlled at 25±3mm. The weight of the fish is controlled at 0.20±0.05g. After purchase, Zebrafish are disinfected with 5% saline, and then fish are domesticated for a week in aerated water. In addition, experimental water accord with “Fishery water quality standards" (GB11607-1989), and the Zebrafish mortality rate should be less than 5% within the week.

2.1.3 Experimental wastewater. Oxytetracycline wastewater comes from the secondary sedimentation tank effluent of local pharmaceutical company. The wastewater sample is light yellow with a small amount of gray floccule and stored in a freezer at 0-4℃. Before the experiment, regular projects of wastewater were determined, and the data is shown on table 1.

Table 1. Determination of Oxytetracycline wastewater quality

Item	Data	Water quality standards (Y/N)	Methods
COD	50 mg/L	Y	Microwave sealed digestion COD rapid detector
NH_3-N	4.54 mg/L	Y	Nessler reagent - spectrophotometry
TOC	77.21 mg/L	N	Shimadzu TOC-Vcpn analyzer
TN	42.36 mg/L	Y	Shimadzu TOC-Vcpn analyzer
pH	7.36	Y	Laboratory pH meter

Y show reaching the standard, N show surpassing standard.

2.2 Experimental Methods

2.2.1 Acute toxicity test method. Pre-experimental use Baptist bath toxin method. The experiment set five different volume concentration group (0, 25%, 50%, 75% and 100%, respectively). Experiment use 5L glass aquarium, and every glass aquarium raise 6 Zebrafish with 4L configured test solution. The experimental solution was adjusted to pH 7.5 with 5% HCl, and the experiment time is 96h.Then, the deaths and abnormalities of Zebrafish were observed at 0h, 24h, 48h, 72h and 96h, respectively. The median lethal concentration of OTC wastewater is calculated at different times(24h LC_{50}, 48h LC_{50}, 72h LC_{50}, 96h LC_{50}, respectively).

2.2.2 Subacute toxicity test methods. The subacute toxicity experiment also set five different concentration group (0, 20%, 40%, 60% and 80%, respectively) by geometric sequence, and every concentration set three parallel experiment. The experimental period is 15d. Before Zebrafish added to each concentration group, they must be cultured more than 7 days in a laboratory. Every group added 25 fish. SOD activities and MDA contents in muscle tissue were respectively determined at 3d, 6d, 9d, 12d and 15d after exposure.

2.3 Assay Methods

The experimentally exposed fish were dissected to obtain muscle tissue, then the muscle tissue is homogenized in a homogenizer until sufficient grind. After homogenization, it is poured into a centrifuge tube and centrifuged. And the experiment use supernatant liquid.

2.4 Statistical Analysis

All data were checked using One-way Anova and Significant difference analysis uses the method of least significant difference (LSD). P value of <0.05 was considered statistically different, and $p<0.01$ statistically very significantly different. All statistical analyses were performed using SPSS software (IBM SPSS Statistics 22.0 software; IBM, Chicago, IL, USA). All figures are drawn using software Origin 8.5.

3 Results and Discussions

3.1 Acute toxicity test results

The experimental results are shown in Table 2. All concentrations of Zebrafish did not show any signs of death or abnormalities. According to the United States wastewater toxicity standards[8], the study got 96h LC_{50} for this OTC wastewater on Zebrafish. 96h $LC_{50}>1.0$TU (TU is the original wastewater concentration). It shows that OTC wastewater is a low toxic material to Zebrafish. So OTC wastewater can be directly determined by subacute toxicity test.

Table 2. Acute toxicity pre-experimental of OTC wastewater.

Concentration (volume percentage)%	Temperature℃	Fish amount	Mortality rate%
0	22	6	0
25	22	6	0
50	22	6	0
75	22	6	0
100	22	6	0

3.2 Effect of OTC Wastewater on the SOD Activities in Zebrafish Muscle Tissue

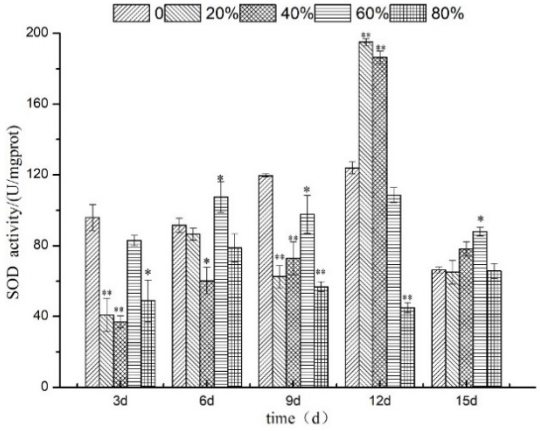

Figure 1. SOD activities in Zebrafish muscle tissue after 15d exposure to OTC pharmaceutical wastewater. Values are presented as means ± SD. *p < 0.05 and **p < 0.01.

As shown in Fig 1, in the pre-exposure period (3~9d), the exposed group had a relatively stable SOD activities in Zebrafish muscle tissue by 6 days (0.01＜P＜0.05). On the 12th day, SOD activities of the exposure group reached its maximum (195.12U/mgprot). On the 15th day, the SOD activities of the exposed group recovered normal. Liu et al. exposed the earthworms using Metalaxyl-M showed similar changes in SOD activities, and concluded that there was a dynamic equilibrium between ROS production and elimination[9]. Compared with the control group, the SOD activities produced some fluctuations. This may result in changing in the degree of oxidative stress by the foreign substances just entering the fish body. The Zebrafish was adapting to OTC wastewater environment. Furthermore, zhang et al. also showed ROS levels were dose-dependent through a similar experiment[10]. Overall, SOD activities in different OTC wastewater exposure groups showed a "Λ" type change trend compared with control group, and finally there was no significant difference compared with control group. This phenomenon can be considered as a self-defense reaction of the body against foreign substances.

3.3 Effect of OTC Wastewater on the MDA contents in Zebrafish muscle tissue

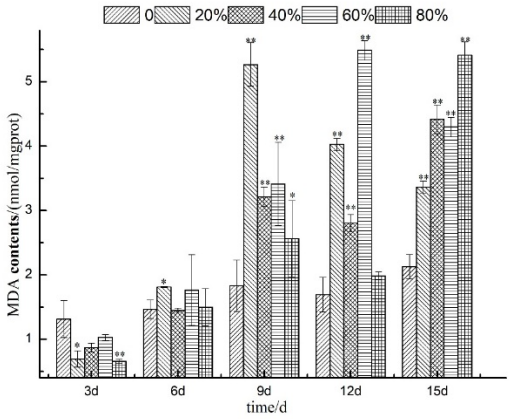

Figure 2. MDA contents in Zebrafish muscle tissue after 15d exposure to OTC pharmaceutical wastewater. Values are presented as means ± SD. *p < 0.05 and **p < 0.01.

MDA is an indicator of lipid peroxidation[11]. The results presented in Fig 2 demonstrate the effects of OTC wastewater exposure on the MDA contents in the Zebrafish muscle tissue. On the third day, compared with the control group, MDA contents in the experimental group had an inhibitory effect, and MDA contents is the minimum during the experiment (0.66 nmol/mgprot). On the 6th day, there was no significant difference in the contents of MDA between the exposed group and the control group ($P > 0.05$). On the 9th day, the 20% exposed group suddenly reached the maximum during the experiment (5.27 nmol/mgprot). On the 15th day, all the exposed groups compared with the control group showed extremely significant induction ($P<0.01$). Zhang et al used 1-butyl-3-methylimidazolium chloride to perform subacute toxicological exposure tests on Zebrafish and found that there was the same induction trend in the Zebrafish liver[12]. Besides, Han et al. tested Zebrafish with Azoxystrobin, showing a "Λ" pattern of change in the exposed group, similar to the MDA profile obtained in this study[13].

4. Conclusion

In the pre-experiment, all fish in the 25-100% OTC exposure group showed no abnormalities and death, and 96h LC_{50}>1.0TU. So OTC pharmaceutical wastewater was a low toxicity substance to Zebrafish. In this study, OTC wastewater was used for subacute toxicity test by semi-static laboratory exposure method, and experiments determined the Zebrafish muscle tissue SOD, MDA. Under the different concentrations of OTC wastewater, the activities of SOD and the contents of MDA in the exposed groups showed a "Λ" type. The SOD activities was not severely damaged during the whole exposure period. MDA contents（9~15d）were highly significantly induced ($P<0.01$), which eventually led to the membrane lipid peroxidation system severely damaged. Therefore, OTC pharmaceutical wastewater has some toxicity to aquatic organisms, and should arouse people's attention.

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