Study on Detoxification of Biochemical Fulvic Acid on *Quasipaa boulengeri* Tadpoles under Copper Stress

Zhang Xiaqin¹, Qin Xianfeng¹, Zhang Qinya¹, Zhang Hangyue¹, Yang Yiman¹, Yang Kuo²*

¹College of Resources and Environment, ABa Teachers College, Wenchuan, Sichuan Province 623002, China
²Institute of Applied Physics, ABa Teachers College, Wenchuan, Sichuan Province 623002, China

Abstract: To understand the detoxification effect of biochemical fulvic acid (BFA) on *Quasipaa boulengeri* (*Q. boulengeri*) tadpoles under copper stress, effects of different concentrations of BFA on growth and physiological indexes of tadpoles. The results showed that the survival rate of tadpoles increased with the increase of BFA concentration. With the increase of BFA concentration, the growth rate of body weight and body length reached the peak at 45 mg/L; The superoxide dismutase (SOD) showed a downward trend in the control group (CK) and an upward trend in each treatment group, with significant difference between the treatment groups (P < 0.05); The content of the malondialdehyde (MDA) and the treatment time and concentration of BFA showed an upward trend in CK group. With the increase of BFA concentration and the content of the MDA decreased, there was significant difference between the treatment groups (P < 0.05). In conclusion, BFA can be used as a detoxification agent under copper stress, and the suitable concentration of BFA is 45 mg/L. This study provides a theoretical basis for the artificial breeding and scientific protection of *Q. boulengeri* tadpoles.

1. Introduction

Heavy metal pollution is serious due to the leakage of chemical materials and the destruction of environmental protection facilities caused by industrial and agricultural production and geological disasters. The appearance and internal organs of tadpoles is greatly harmed. When the heavy metals in soil water exceeded the tolerance of aquatic animals, especially the physiology and behavior of tadpoles had significant negative impact. Compared the tolerance of fish and tadpoles to heavy metals in aquatic animals, and found that heavy metal poisoning was more harmful to tadpoles, indicating that heavy metal pollution was more urgent to protect tadpoles. At present, most scholars focus on the toxicological study of heavy metals on tadpoles to provide scientific basis for water environment detection and tadpole protection[2]. From these studies, it was found that the heavy metal poisoning of tadpoles was manifested in the changes of initial state behavior, body color and enzyme activity until death. Some scholars in China and abroad have also found that aquatic animals themselves have detoxification ability through heavy metal stress[3-4]. However, there are relatively few studies on the substances that have detoxification effect on heavy metal poisoning of aquatic animals. Consulting extensive literature[5-6]. The substances that have detoxification effect on heavy metal poisoning of aquatic animals include BFA, vitamins, antibiotics, probiotics, fungal melanin electrospun membrane, activated carbon, etc. BFA is a kind of green bioactive substance with non-toxic side effects. The fermentation of special microorganisms forms main BFA and a variety of polycondensations of bioactive substances by people. Wang[7] points out that BFA has significant advantages compared with antibiotic additives. It not only has strong chelating ability to heavy metal elements, but also has the advantages of long-term use without drug resistance, rich resources and low price[8]. In summary, *Q. boulengeri* tadpoles were used as experimental subjects, the effects of BFA on the growth of tadpoles under heavy metal copper stress and the changes of superoxide dismutase (SOD) and malondialdehyde (MDA) contents in the tadpoles were simulated in the wild environment. The effects of BFA on the growth performance and physiological and biochemical indexes of *Q. boulengeri* tadpoles, the aim is to evaluate whether BFA can be used as a detoxification agent for heavy metal copper ions, and provide scientific basis for the scientific breeding and protection of tadpoles.

2. Materials

2.1 Animal and experimental materials

*Q. boulengeri* tadpoles were purchased from Fuchao Stone Frog Farm in Shifang City, Sichuan Province. The spawning time is June 2020, and tadpoles of about 3 months old are selected as the experimental objects in this laboratory. After entering the laboratory, the tadpoles were temporarily reared in a transparent box.
with the size of 80 cm × 50 cm × 42 cm. The water used in the experiment was tap water after 2 days of aeration. The water was changed every 24 hours, and the tadpoles were temporarily reared for 2 days. During the temporary rearing period, fasting was forbidden, and the dead individuals were recovered in time. Until the mortality rate of tadpoles reared in the laboratory was less than 5%.

2.2 BFA and basic feed

BFA provided by Inner Mongolia Yongye Nongfeng Biotechnology Co., Ltd. component content: BFA ≥ 95%, pH 5-6, water insoluble ≈ 1%. The test basic feed was artificially prepared according to the tadpole feeding standard. The composition and nutritional level of basic feed are shown in Table 1.

<table>
<thead>
<tr>
<th>Basal diet</th>
<th>Content(%)</th>
<th>Nutritional level</th>
<th>Content(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fish meal</td>
<td>35</td>
<td>Crude protein</td>
<td>32.15</td>
</tr>
<tr>
<td>high-gluten flour</td>
<td>25</td>
<td>Crude fat</td>
<td>6.23</td>
</tr>
<tr>
<td>bean meal</td>
<td>10</td>
<td>Crude fiber</td>
<td>6.96</td>
</tr>
<tr>
<td>starch</td>
<td>20</td>
<td>Crude ash</td>
<td>11.22</td>
</tr>
<tr>
<td>Others (fish oil, vitamins, minerals, etc.)</td>
<td>10</td>
<td>Moisture</td>
<td>9.28</td>
</tr>
</tbody>
</table>

2.3 Test drugs and reagents

CuSO4·5H2O, glacial acetic acid (analytically pure, acetic acid concentration ≥ 99.5%), CH3CH2OH, NaCl, rapid detection kit for heavy metal copper (Luheng Biotech), SOD test kit, and MDA test kit (Nanjing Jianscheng Biotechnology Institute).

2.4 Main test instruments

TGL-20BR high-speed freeze centrifuge (Shanghai Anting Science Instrument Factory). UV-1800PC UV-visible spectrophotometer (Shanghai Jinghua Technology Instrument Co., Ltd.). DW-HL218 Ultra Low Temperature Refrigerator (CMB), etc.

3. Test design

A total of 210 Q.boulengeri tadpoles with similar quality, sensitive activity and good health were randomly selected as experimental subjects. The average body weight was 0.86 ± 0.04 g and the average body length was 45.59 ± 0.53 mm. The tadpoles were randomly cultured in a transparent box of 28 cm × 19.5 cm × 16.5 cm (the volume of reagent water was 1 L). This experiment was divided into seven groups. According to the results of the acute study of heavy metal copper on the Q.boulengeri tadpoles, 2 mg/L heavy metal copper ions were set in each group. The CK group was not added with BFA, and the other groups were added with BFA of 15, 30, 45, 60, 75 and 90 mg/L, respectively. Three replicate groups were set up, and 10 tadpoles were placed in each square box. Other breeding conditions such as temperature, operation, and water change were the same. In this experiment, after 48 hours of heavy metal copper ion stress, BFA with different mass concentrations was added as an antidote, and 24, 48, 72 hours of detoxification were used as measurement points. At each measurement time point, 3 test tadpoles were randomly selected from each experimental gradient. The SOD activity and MDA content in the tadpoles of each group were detected.

3.1 Determination indexes and methods

3.1.1 Determination of growth performance index

Stop feeding one day before the beginning and end of the experiment, let the tadpoles starve for one day, weigh the tadpoles with an electronic balance, accurate to 0.01 g. The formula is as follows:

\[ WGR(\%) = \frac{(M_2 - M_1)}{M_1} \times 100 \]  

\[ LGR(\%) = \frac{(L_2 - L_1)}{L_1} \times 100 \]  

\[ SR(\%) = \frac{(N_2 - N_1)}{N_1} \times 100 \]

WGR is body weight growth rate, \(M_1\) is the initial average mass (g), \(M_2\) is the terminal average mass (g), LGR is body length growth rate, \(L_1\) is the initial average body length (mm), \(L_2\) is the terminal average body length (mm), SR is survival rate, \(N_1\) is initial number of tadpoles, \(N_2\) is number of surviving tadpoles.

3.1.2 Determination of antioxidant indexes in tissues

\[ SOD(U/mg prot) = \frac{[I + (1 - I) \times V_1] \times (V_2 \times Cpr) \times F}{I} \]  

\[ MDA(nmol/mg prot) = \frac{[\Delta A_3 \times V_1 + (\varepsilon \times d) \times 109] \times (V_2 \times Cpr) \times F}{\Delta A_2} \]

(Note: according to the instruction of SOD activity detection kit)

\(I\) is inhibition percentage, \(V_1\) is the total volume of the reaction, \(V_2\) is the volume of the sample added to the reaction system, \(F\) is the dilution ratio of the sample, \(Cpr\) is the protein concentration of the sample (mg/ml) \(\Delta A_1\) is the difference between the absorbance of blank group 1 and blank group 2 during the reaction time, \(\Delta A_2\) is the difference between the absorbance of the measurement group and the control group during the reaction time, \(\Delta A_3\) is the difference in absorbance between the measured group and the blank group during the reaction time, \(\varepsilon\) is the molar absorption coefficient of MDA, \(d\) is the diameter of cuvette light.

3.1.3 Activity determination

3.1.3.1 Sample preparation

Three-tailed tadpoles were randomly selected from each parallel unit of each experimental group. After numbering respectively, the weight of the tissue to be
tested was accurately weighed by an electronic balance, and 9 times the volume of normal saline (0.65%) was added according to the ratio of weight (g): volume (ml) = 1:9. Under the condition of ice water bath, mechanical homogenate was prepared into 10% homogenate, the supernatant was stored in a Ultra-low temperature refrigerator at -80 °C and a high-speed freezing centrifuge with 2500 r/min with centrifugal 10 min, which was used to determine the activity of antioxidant enzymes.

3.1.3.2 Definition and determination of activity

Definition of SOD activity: when the SOD inhibition rate reaches 50% in 1 ml reaction solution, the corresponding amount of SOD per mg of tissue protein is one SOD activity unit (U). The content of MDA can reflect the degree of lipid peroxidation in tadpoles and indirectly reflect the degree of cell injury. The determination method of enzyme activity determined the activities of various antioxidant enzymes according to the requirements of the corresponding enzyme kit of Nanjing Jiancheng Bioengineering Research Institute, and calculated the value of enzyme activity.

3.2 Statistical analysis of data

After the preliminary collation of the experimental data, SPSS25.0 software was used to detect the normality and variance homogeneity of the data. When analyzing the growth and physiological indexes of tadpoles with different concentrations of BFA, the data were analyzed by one-way analysis of variance and two-way analysis of variance, and multiple comparisons were made by the method of minimum significant difference. The data were expressed as "mean ± standard deviation ", and the difference was significant.

4. Results and analysis

4.1 Effects of BFA on the growth and development of tadpoles

See Table 2, single factor analysis results of weight and length growth rate of tadpoles treated with different concentrations of BFA under acute copper stress: there are significant differences in the body weight growth rate of tadpoles at 30, 45 mg/L and other experimental groups. There were significant differences between CK, 15 and 60, 75, 90 mg/L, 60 and 75, 90 mg/L, but there was no significant difference between CK and 15 mg/L, 75 and 90 mg/L. The growth rate of body length of *Q. boulengeri* tadpoles was significantly different from that of other experimental groups at 45 and 60 mg/L. There were significant differences between CK, 30, 75 and 15, 60, 90 mg/L groups. There were no significant difference among CK, 30, 75 mg/L groups, 15 and 90 mg/L groups. After *Q. boulengeri* tadpoles were treated with different concentrations of BFA at the same toxicological concentration, the growth rate of body weight and body length increased at first and then decreased with the increase of the concentration of BFA. When the concentration of BFA was 45 mg/L, the growth rate of body weight and body length reached the peak. In conclusion, BFA as a heavy metal poisoning and detoxification of tadpoles have a greater impact on their weight and body length growth rate.

Table 2. The growth rate of body weight and length of *Q. boulengeri* tadpoles under different concentration of BFA treatments

<table>
<thead>
<tr>
<th>BFA concentration/ (mg/L)</th>
<th>Weight gain/%</th>
<th>Growth rate of body length/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>7.27±0.03c</td>
<td>5.90±0.10b</td>
</tr>
<tr>
<td>15</td>
<td>11.00±0.20c</td>
<td>4.83±0.15d</td>
</tr>
<tr>
<td>30</td>
<td>11.66±0.30b</td>
<td>5.90±0.10b</td>
</tr>
<tr>
<td>45</td>
<td>15.60±0.17a</td>
<td>8.33±0.25a</td>
</tr>
<tr>
<td>60</td>
<td>6.26±0.64d</td>
<td>5.63±0.20c</td>
</tr>
<tr>
<td>75</td>
<td>3.23±0.15c</td>
<td>5.96±0.47b</td>
</tr>
<tr>
<td>90</td>
<td>3.50±0.12c</td>
<td>5.10±0.36d</td>
</tr>
</tbody>
</table>

Note: CK represents the blank control group, with the same column of data lowercase English letters are different expressed significant difference (P < 0.05) (Tukey’s test, α = 0.05, a > b > c > d > e).

4.2 Effect of BFA treatment on survival rate of tadpoles

See Table 3, *Q. boulengeri* tadpoles poisoned by copper ions treated with different concentrations of BFA. With the increase of the concentration of BFA, the survival rate of tadpoles increased, and the survival rate reached the peak when the concentration of BFA was 90 mg/L. There were significant differences among groups 75, 90 and 15, 30, 45, 60 mg/L, 45 and 15, 30 mg/L, but there was no significant difference between 75, 90 and CK, 15, 30 and 45, 60, 75, 90 mg/L groups, indicating that higher concentration of BFA had a great effect on the survival rate of tadpoles.

Table 3. Significant difference in survival rate of tadpoles under the treatment of BFA concentration in each group

<table>
<thead>
<tr>
<th>BFA concentration/ (mg/L)</th>
<th>Survival rate/%</th>
<th>Significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>0.30±0.10</td>
<td>b</td>
</tr>
<tr>
<td>15</td>
<td>0.33±0.05</td>
<td>b</td>
</tr>
<tr>
<td>30</td>
<td>0.23±0.05</td>
<td>b</td>
</tr>
<tr>
<td>45</td>
<td>0.60±0.20</td>
<td>a</td>
</tr>
<tr>
<td>60</td>
<td>0.66±0.15</td>
<td>a</td>
</tr>
<tr>
<td>75</td>
<td>0.76±0.15</td>
<td>a</td>
</tr>
<tr>
<td>90</td>
<td>0.96±0.05</td>
<td>a</td>
</tr>
</tbody>
</table>

4.3 Effects of BFA on SOD and MDA in *Q. boulengeri* tadpoles under acute copper stress

The results of two-factor analysis showed that MDA content in tadpoles after BFA treatment was related to BFA concentration and detoxification time under acute copper stress. With the increase of detoxification time and BFA concentration, the activity of SOD increased significantly, and the content of MDA decreased. From
Fig 1, it can be seen that with the increase of BFA concentration, the enzyme activity of each treatment group increased significantly, while the SOD activity of CK group decreased significantly, and there were significant differences between each treatment group (P < 0.05). Within 24, 48 and 72 h after detoxification with BFA, the activity of SOD in each treatment group was significantly higher than that in CK group, and the activity of SOD in CK group decreased with the passage of time, which may be inhibited in the process of no BFA treatment.

It can be seen from Fig 2 that the MDA content in BFA treatment group was significantly lower than that in CK group, and there was significant difference in MDA content among different treatment groups. With the increase of BFA concentration, the content of MDA in treatment group decreased gradually, and with the passage of detoxification time, the content of MDA in CK group gradually increased. The results showed that the damage of lipid peroxidation of tadpoles poisoned by acute copper stress could be reduced or protected from lipid peroxidation, and the degree of injury decreased with the increase of detoxification concentration and detoxification time of BFA under the influence of BFA treatment. This shows that the different concentrations of BFA can increase the activity of SOD and decrease the content of MDA in tadpoles, protecting the biofilm structure, thus promoting the growth of tadpoles and reducing the survival number of tadpoles due to heavy metal poisoning under acute copper stress.

![Fig. 1. The effect of BFA treatment on SOD activity in Q. boulengeri tadpoles under heavy metal stress](image1)

![Fig. 2. The effect of BFA treatment on MDA content in Q. boulengeri tadpoles under heavy metal stress](image2)

5. Discussion

Zhang et al.[9] showed that in the toxicity test of copper ions on *Q. boulengeri* tadpoles, the mortality rate of the tadpoles gradually increased with the time under the same mass concentration. In the same time, the higher the mass concentration of heavy metal copper ions, the greater the mortality of tadpoles. The acute toxicity test of heavy metals has a great impact on tadpoles' behavioral morphology, growth and development, enzyme activity, tissues and organs, heredity, etc., which causes tadpoles to be poisoned. The effects on tadpoles' behavioral morphology are the most obvious, mainly manifested in: slow swimming ability and uncertain swimming direction; Skin ulceration, test solution cloudy. Amphibians have amphibious specificity in life cycle, there is skin respiration in physiological characteristics, studies have shown that tadpoles rely on skin to absorb about 60% of oxygen. The *Q. boulengeri* skin is the main place for gas exchange with the outside world. It can promote animal growth, enhance animal immune function, prevent diseases and so on. The addition of a certain amount of BFA to the feed of aquatic animals can accelerate their growth rate and increase the activities of intestinal digestive enzymes and immune enzymes. Under the conditions of this experiment, BFA with different mass concentrations was added, and the growth rate of body weight and body length of the tadpoles increased first and then decreased when the *Q. boulengeri* tadpoles were poisoned by copper. The growth rate of body weight and body length of *Q. boulengeri* tadpoles both reached their peaks when the concentration of BFA was 45 mg/L, indicating that 45 mg/L of BFA was more suitable for the growth of tadpoles. In this experiment, the survival rate of each group was significantly decreased due to copper stress. The survival rate increased with the increase of BFA concentration when treated with different concentrations of BFA. The survival rates of tadpoles in 45 and 90 mg/L groups were significantly different (P < 0.01). This is at odds with the findings of Mei[10], which change trends of body weight growth and survival rate of *Oryzias latipes* exposed to pickle and PCB-153 with different doses of BFA for two weeks were different. It may be because the fish and tadpoles belong to different species, or the time of BFA detoxification is not long enough, there is no obvious difference.

*Q. boulengeri* tadpoles will rapidly respond to stress under the stress of copper. The SOD in the body will be the first line of defense for antioxidant defense system, and its activity will be correspondingly improved to eliminate cell damage and promote phagocytosis, and catalyze the transformation of superoxide anion free radicals in the body into peroxides to eliminate free radicals in the body. MDA is one of the products of lipid oxidation in animal body, which can react with free amino groups and cross-link protein molecules, resulting in cell damage, indirectly reflecting the degree of lipid peroxidation in vivo. The determination of SOD activity often cooperates with the determination of MDA. The level of SOD activity indirectly reflects the ability of the body to scavenge oxygen free radicals, while the level of MDA indirectly reflects the severity of the body cells attacked by free radicals. The analysis of the results of SOD and MDA is helpful to judge the detoxification of heavy metal stress.
tadpoles. The *Q. boulengeri* tadpoles exposed to heavy metal copper, CK with the extension of exposure time under the experimental conditions. The SOD activity of each treatment group was significantly increased by adding the same concentration gradient of BFA, and the SOD activity of CK control group was significantly decreased. The ability of self-repair was enhanced and the activity of SOD was increased which compared with CK. Under the stress of toxic substances, SOD activity decreased again with the prolongation of exposure time, and the decrease of SOD activity was caused by the serious damage to the immune function of the body. This is consistent with the findings of Zhao et al. That BFA can improve the activity of SOD in shrimps, and improve the immune performance and survival rate of shrimps. In the CK, under copper stress and prolonged exposure time, the content of malondialdehyde in *Q. boulengeri* tadpoles increased significantly, indicating that the lipid peroxidation in the tadpoles was aggravated. This was consistent with the results of Hong et al. when they studied the acute copper stress on *Fejervarya cancrivora* tadpoles. The results showed that the ion exchange, adsorption, complexation and chelation could be effectively carried out by adding BFA when the tadpoles were exposed to heavy metals, so as to eliminate heavy metals and achieve detoxification effect.

6. Conclusions

The skin and liver of *Q. boulengeri* tadpoles are the main target organs for the induction and accumulation of external heavy metal stress. BFA can effectively improve the survival rate of tadpoles saving cost for intensive artificial breeding of *Q. boulengeri* tadpoles and improving economic benefit of farmers. Under the test conditions, under the same mass concentration of heavy metal copper ions exposure, Growth performance and physiological and biochemical indexes of *Q. boulengeri* tadpoles comprehensive analysis, BFA can be used as detoxification agent of heavy metal copper ions, the suitable concentration of BFA was 45 mg/L, which provided scientific basis for scientific breeding and protection of *Q. boulengeri* tadpoles.

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