ANOVA of Cerium Chloride on Morphology and Metamorphosis Development in Tadpoles of *C. melanogaster*

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**ABSTRACT**

In this experiment, the effects of 0.004 mg·L\(^{-1}\), 0.02 mg·L\(^{-1}\), 0.1 mg·L\(^{-1}\), and 0.5 mg·L\(^{-1}\) cerium chloride solutions on the growth and metamorphosis development of the tadpoles of the black spotted frog were investigated. The experimental results showed that cerium chloride solution at 0.5 mg·L\(^{-1}\) significantly reduced the survival rate of tadpoles, which was 6.67% at 168h (7d) in the 0.5 mg·L\(^{-1}\) treatment compared with 93.3% in the blank control (lake water). The growth condition (body weight) at 168h (7d) (0.56 g) was different from that of the blank control (lake water) tadpoles. The growth (mass) of tadpoles at 0.5 mg·L\(^{-1}\) slowed down significantly in early development compared to the blank control (lake water) tadpoles (0.67g ± 0.05g); at the same time, we noticed that the tadpoles at 0.5 mg·L\(^{-1}\) concentration were in a dormant state of growth, and the mass was not significantly different compared to the beginning of the experiment. The metamorphosis time of tadpoles in the high concentration of cerium chloride salt solution (31 ± 5 days) was greater than that of the blank control tadpoles (26 ± 3 days). Tadpoles at concentrations of 0.02 mg·L\(^{-1}\), 0.1 mg·L\(^{-1}\), and 0.5 mg·L\(^{-1}\) were significantly heavier than the blank control tadpoles at metamorphosis. The results of this experiment indicated that moderate concentration levels of cerium chloride (0.004 mg·L\(^{-1}\), 0.02 mg·L\(^{-1}\)) could significantly accelerate the growth and promote the metamorphosis development of the tadpoles of *C. melanogaster*.

**Keywords:** ANOVA, cerium chloride, *C. melanogaster*, metamorphosis development, tadpoles.

I. INTRODUCTION

In the world, more than 90% of the rare earth resources are in China. Rare earth resources are used in a wide range of applications, from electronic products, chemical products, and household appliances, to energy applications. Rare earths are known as "growth regulators" in agriculture and fisheries and "vitamins" in the industry because of their unique physical and chemical properties. Still, as the application of rare earths in agriculture and industry is expanding, rare earths are also widely entering the ecological environment and entering the body through the food chain and other channels. Cerium, one of the lanthanide metals, is the most abundant rare earth element in the earth's crust. Cerium is an iron-gray, dactile metal (Atul & William, 2018). Cerium metal is very reactive and is a strong oxidizer, and is stable when associated with oxygen ligands (Gentili & Candito, 2020). Cerium exists in the trivalent state (Ce\(^{3+}\), cerous) and the tetravalent state (Ce\(^{4+}\), ceric) when present in compounds. Cerium is also currently used extensively in the manufacture of cerium-iron alloy lighters (Cui et al., 2019). Cerium chloride is an inorganic substance with the chemical formula CeCl\(_3\), which often exists in the form of a heptahydrate compound, showing white powder-like crystals at room temperature, and is irritating. The substance is harmful to the environment and has an accumulation effect in groundwater. It is used as an additive in steam lamp yarn covers, as a catalyst in petrochemicals, and also in pharmaceuticals, which are closely related to life.

The property that the juvenile stages of plants and animals are more sensitive to salt than adults has been previously demonstrated. For example, two fundamental processes, seed germination (Nadjafi, 2010) and seedling development (Ellen & Lucie, 2020), are adversely affected at low salt concentrations compared to mature plants. The reason why tadpoles are more susceptible to salt stress than frogs is that they are confined to aquatic environments and do not have the ability to change habitats. For example, in an intermittent wetland in southeastern Australia, salt concentrations increased nearly fivefold as water levels dropped dramatically with the massive evaporation of summer water (Webster & Jacqui, 2015). In addition, almost immediately after egg release and fertilization, the membranes surrounding the eggs of anurans absorb water and swell, thereby increasing the chances of salt absorption around them (Ringler et al., 2015). Tadpoles are susceptible to surrounding salts because their outer skin is highly permeable (Benjamin, 2010). Although the kidneys and regulatory mechanisms are fully developed in adult frogs, in juveniles, the kidneys...
develop gradually. They do not reach full development until later juvenile stages, increasing the likelihood of kidney failure in juveniles at high salt concentrations compared to older juveniles or adults (Haywood & Lorren, 2001).

II. TEST MATERIAL AND INSTRUMENT

A. Instrument

MA200 type electronic balance: Shanghai Second Instrument Factory

Microscope: Chongqing Zhongxian Photoelectric Instrument Co.

Double-barrel body microscope: state-owned Jinan Bayi Optical Instrument Factory

B. Main Reagent

Cerium chloride heptahydrate: State Pharmaceutical Group Chemical Reagent Co., Ltd (SCRC)

Lake water: taken from Hongde Lake, Weifang University

C. Preparation of Different Concentrations of Cerium Chloride Solutions

The concentration gradient of cerium chloride for the test was 0.004, 0.02, 0.1, 0.5 mg·L⁻¹ from the pre-test results.

Because it is heptahydrate cerium chloride, it needs to consider the mass fraction of bound water, so the cerium chloride is 0.004, 0.02, 0.1, 0.5mg·L⁻¹ converted to heptahydrate cerium chloride as multiplied by (372.58/246.6) to get 0.006, 0.03, 0.15, 0.75 mg.

Weigh 0.75 mg of cerium heptahydrate into a beaker, add the lake water to 1000 ml and prepare 0.5 mg·L⁻¹ solution of cerium chloride. Similarly, prepare 0.1 mg·L⁻¹ and 0.02 mg·L⁻¹ of cerium chloride solution.

D. Test Animal

The tadpoles (C. melanogaster) were purchased from Pengcheng Agricultural Frog Breeding Base. The tadpoles were staged according to Gosner's staging criteria (Lihong et al., 2016), and the tadpoles at stage G26 were selected as the test material to ensure uniform growth and developmental status (Jie et al., 2017). During the test period, the tadpoles were fed with green vegetable juice and egg yolk at 48-hour intervals, the water was changed, and the excreta was cleaned for later tests.

III. TEST METHOD

A. Experimental Design

There is no physiological toxicokinetic model for cerium chloride or other cerium compounds, so GB/T 31270 was used as the basis to design the test.

The cerium chloride salt solution was prepared with heptahydrate cerium chloride from State Pharmaceutical Group Chemical Reagent Co., Ltd (SCRC), which complies with the Q/CYDZ2614-2005 standard. The cerium chloride salt treatment consisted of four concentrations (0.004, 0.02, 0.1 and 0.5 g·L⁻¹) of cerium chloride solution and a set of freshwater controls (lake water). The choice of salt concentrations was based on pre-tests: the pre-tests were used to determine the approximate range of concentrations of cerium chloride for the formal animal toxicity tests and to get an idea of the reasonableness of the concentrations of cerium chloride solutions required for the tests, as well as changes in other physicochemical indicators such as acidity and alkalinity of the solutions during the tests. The pre-test was designed by combining the toxicity of congeners and referring to the literature to estimate the approximate toxicity of cerium chloride (Ling Lu & Fu Song, 2002).

The pre-test was performed by selecting 0.004, 0.02, 0.1, 0.5, 2.5 mg·L⁻¹ and five concentration gradients with five tadpoles in each concentration group in hydrostatic mode, without parallel groups. The test lasted from 24 to 96h. The number of dead tadpoles in each container was recorded at least twice a day, and the dead tadpoles were removed promptly. The 100% mortality concentration at 24h (24h LC₉₀) and no mortality concentration at 96h (96h LC₀) were obtained.

Five groups of different concentration gradients of cerium chloride solutions were designed for testing in the formal experiment, including the lowest concentration that caused all tadpoles to die or nearly all die at 24h (24h LC₉₀) and the highest concentration that caused all tadpoles to survive or nearly all survive at 96h (96h LC₀).

After the pre-test, it was observed that all tadpoles in the solution of cerium chloride salt at a concentration of 2.5 mg·L⁻¹ died because the concentration of rare earth element salt was too high, so this concentration was less effective for the study of growth and metamorphosis development and development of black-spotted frog tadpoles, so it was discarded and the remaining four groups of concentrations were chosen for the formal test.

Lake water was used because sodium chloride alone does not provide the necessary ions for normal osmoregulation and is toxic to amphibians (Marika et al., 2020). The tadpoles were randomly divided into five bottles containing the salt solution described above. Each bottle contained five tadpoles, with three parallel trials for each treatment.

B. Measurement of Test Results

Effect of cerium chloride concentration on tadpole body weight: Recorded three times per week and then again at the time of final metamorphosis (defined as completion of tail uptake). Tadpoles were gently removed from the water with a crib sheet, dried on filter paper by plucking the tadpoles with a glass minute needle, then weighed (±0.0001 g) using an electronic balance, and readings were taken and recorded when the readings were stable (Bin et al., 2016).

Effect of cerium chloride concentration on the morphology of developmental periods: Tadpoles were placed in Petri dishes and plucked with a glass splitting needle under binocular dissecting microscope, and the developmental periods of tadpoles were identified and recorded according to Zhao Ermi's method for identification of developmental stages of tadpoles combined with the characteristics of tadpoles.

The effect of cerium chloride concentration on body length: The tadpoles with the identified developmental stages were placed on filter paper, and their bodies were stretched into a straight line with a glass splitting needle and measured and recorded with a straightedge of 0.5 mm index (Guoqiang & Lizhi, 2021).
C. Processing of Test Results

Effect of cerium chloride on tadpole survival: The survival rate of tadpoles in all treatment groups for the first 72h was 73.33% (n = 75). In the 0.5 mg L⁻¹ treatment, tadpole survival was significantly lower than in the other groups (P<0.001), with more than 50% of tadpoles in the 0.5 mg L⁻¹ treatment dying in the first three days, compared to 93.33% in the control group and more than 80% in all other treatment groups. A total of 9 mortalities occurred in the following 48h (1 in the control group and 8 in the 0.5 mg L⁻¹ concentration treatment group).

| TABLE I: RESULTS OF THE CHI-SQUARE TEST FOR MORTALITY AT 72H |
|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | Actual value    | Actual value    | Total            | Theoretical      | Theoretical      |
|                  | positive inverse| negative inverse|                  | positive inverse | negative inverse |
| Control group    | 15              | 14              | 29              | 8.88            | 5.62            |
| Experimental group | 15            | 5              | 20              | 6.13            | 3.88            |
| Total            | 30              | 19              | 98              |                  |                  |
| P-value          | <0.001          |                |                  |                  |                  |

| TABLE II: RECORDED RESULTS OF TADPOLE MORTALITY |
|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | 24h              | 48h              | 72h              | 96h              | 120h             | 144h             | 168h             |
| Blank            | 0.59            | 0.63            | 0.67            | 0.70            | 0.76            | 0.76            | 0.79            |
| Control group    | 0.58            | 0.62            | 0.67            | 0.69            | 0.73            | 0.77            | 0.80            |
| 0.004 mg L⁻¹     | 0.59            | 0.61            | 0.65            | 0.67            | 0.69            | 0.72            | 0.75            |
| 0.02 mg L⁻¹      | 0.56            | 0.58            | 0.59            | 0.60            | 0.61            | 0.62            | 0.63            |
| 0.1 mg L⁻¹       | 0.57            | 0.56            | 0.56            | 0.57            | 0.57            | 0.57            | 0.56            |
| 0.5 mg L⁻¹       | 0.56            | 0.58            | 0.60            | 0.61            | 0.62            | 0.63            | 0.63            |
| 1 mg L⁻¹         | 0.57            | 0.58            | 0.59            | 0.60            | 0.61            | 0.62            | 0.63            |
| 5 mg L⁻¹         | 0.57            | 0.58            | 0.59            | 0.60            | 0.61            | 0.62            | 0.63            |

| TABLE III: RESULTS OF ONE-WAY ANALYSIS OF VARIANCE (ANOVA) TEST FOR MORTALITY IN TADPOLES |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Source of difference | SS   | df | MS      | F    | P-Value | F crit |
| Intergroup        | 0.0979 | 4  | 0.0244  | 8.0679 | 0.0001 | 2.6896 |
| Within the group  | 0.0910 | 30 | 0.0030  |       |        |        |
| Total             | 0.1889 | 34 |        |       |        |        |

Cerium chloride had a significant effect (P < 0.001) on the increase in mass during tadpole development (Tables II, III). At 20% of larval development (hindlimb bud development), tadpoles grown in 0.5 mg L⁻¹ were smaller than those in all other treatments (P < 0.001). 0.1 mg L⁻¹ tadpoles were also smaller than those in the control and 0.02 mg L⁻¹ groups, but larger than 0.5 mg L⁻¹ tadpoles (P < 0.01). At 60% of larval development time (completion of toe differentiation), tadpoles in the 0.5 mg L⁻¹ group still had significantly lower masses (0.338 ± 0.020 g) than tadpoles from all other treatments. Tadpoles from the control and 0.5 mg L⁻¹ treatments were smaller than 0.1 mg L⁻¹ and 0.02 mg L⁻¹ tadpoles. Paired-samples t-tests revealed no differences in relative mass gain (mass gain as a percentage of body mass) during development for any treatment group, including tadpoles in the 0.5 mg L⁻¹ group, which could be observed to be slow, with all tadpoles rapidly gaining mass over periods of 20-60% and 60-75% of larval development (P < 0.01).

The differences between the different test groups were subsequently compared using the chi-square goodness-of-fit test (Chi-square). One-way analysis of variance (ANOVA) was used to test the effect of treatment on the duration of metamorphosis. These three periods were chosen to study the effect of salinity: (1) early in development, (2) when toe differentiation was about to occur, and (3) when metamorphosis was approaching. All statistical analyses were performed with the SPSS ver. 12.0 for Windows statistical package (SPSS, Chicago, IL, USA) near the time of metamorphosis. All statistical analyses were performed with the SPSS ver. 12.0 for Windows statistical package (SPSS, Chicago, IL, USA) and survival data were analyzed using MS Excel 2020 (Microsoft, Rochester, NY, USA). Results are expressed as mean ± standard error.

IV. RESULT ANALYSIS

Before death, tadpoles were observed to be less mobile, less active, preferring to stay at the bottom of the container, and unresponsive, with abdominal edema and slow growth. Therefore, the death of tadpoles may be due to a series of pathological changes such as renal failure or stagnation of blood flow due to vascular stasis in the tadpoles of black-spotted frogs caused by the high osmolarity of high concentration of cerium chloride solution. Blood smears were prepared and placed under a microscope, and 500 erythrocytes were randomly selected from each smear for observation. It was found that the nuclei of erythrocytes in the high-concentration group of dead tadpoles showed abnormalities such as double nuclei, broken nuclei, and no nuclei, indicating that the high concentration of cerium chloride solution had a teratogenic effect on erythrocytes.

In addition, abnormal behavior and deformities were observed in tadpoles: tadpoles in the 0.5 mg L⁻¹ treatment group were sluggish and inactive throughout larval development and frequently remained at the bottom of the container. In contrast, tadpoles in the other treatment groups were active and swimming during this time. Qualitative observations revealed several clearly identifiable abnormalities and deformities in the tadpoles in the 0.1 mg L⁻¹ and 0.5 mg L⁻¹ treatments. Within a week of the start of the experiment, some tadpoles in the 0.1 mg L⁻¹ and 0.5 mg L⁻¹ treatments showed swollen abdomens (Fig. B),

![Fig. 1. Four types of tadpole erythrocytes. A) normal erythrocytes; B) binucleated erythrocytes; C) fragmented erythrocytes; D) anucleated erythrocytes.](image_url)
and others showed kinked tails (Fig. C).

![Three forms of tadpoles](image)

**Fig. 2. Three forms of tadpoles.**
A) Individuals with normal abdomen; B) Individuals with swollen abdomen; C) Individuals with tail deformation.

Individuals with swollen abdomens swam with their ventral sides above them and usually did not survive. Twenty percent of the tadpoles in the high concentration group during the test had a deformed body shape and became sunken in appearance; these individuals also died shortly after the onset of these symptoms. No deformities were observed in tadpoles from the blank control group or in tadpoles with salt concentrations of 0.004 mg L\(^{-1}\) and 0.02 mg L\(^{-1}\). Several studies investigating the effects of environmental contaminants (e.g., nitrates and metals, including rare earth elements) on larval development and metamorphosis size have shown that tadpoles exposed to intermediate levels of contaminants had a greater mass than lower levels of contaminants or control treatments (Hecnar, 1996). Studies investigating the effects of nitrite on bullfrog tadpoles (Smith et al. 2004) suggest that responses of this nature may be an example of a counter-dose response or a hormonal effect where an increase in stressor concentration leads to an increase in the stress response of the individual, thereby overcompensating for the effects of the stressor. Many other examples of hormonal effects have been reported in biological, pharmacological, and toxicological studies, where the performance of the organism is stimulated at low levels of toxin and inhibited at high levels (Stebbing, 1982). After exposure to such chemicals, the development of Müllerian ducts will be affected, thus affecting reproductive development. The Larval Amphibian Growth and Development Assay (LAGDA) is a globally harmonized test guideline developed by the U.S. Environmental Protection Agency in collaboration with the Japanese Ministry of the Environment. LAGDA was designed to assess the apical effects of chronic chemical exposure on growth, müllerian-mediated metamorphosis, and reproductive development in amphibians. Based on the LAGDA test, it is known that exposure to cerium chloride causes a decrease in thyroxine and hypertrophy and hyperplasia of thyroid follicular cells in tadpoles, which in turn affects the hypothalamic-pituitary-thyroid axis.

V. DISCUSSION

According to the data obtained from the experiment, it is found that cerium chloride can both promote and inhibit the growth and metamorphosis of black-spotted frog tadpoles within a certain concentration range, and the specific effect depends on the specific concentration of cerium chloride solution. For example, at low concentrations of cerium chloride solution, the growth and metamorphosis of *C. melanogaster* tadpoles showed the effect of promotion; beyond a certain range, it showed the effect of inhibition; and as the concentration increased, the effect of inhibition gradually increased, and beyond its threshold value, i.e., lethal concentration (24h LC\(_{50}\)), lethal inactivation occurred. As the concentration of cerium chloride solution is related to the osmotic pressure of the solution, when the concentration of cerium chloride solution is high, the osmotic pressure of the solution is large, leading to the convexity of the cell membrane, coupled with the effect on the endocrine system, the relevant hormone secretion changes, together leading to the abdomen showing the phenomenon of convexity, which can cause death in serious cases. On the contrary, when the concentration of cerium chloride salt solution is low, as mild stress, it will promote the growth and metamorphosis of tadpoles. Combined with the experimental results, it can be inferred that when tadpoles were treated with low concentrations of cerium chloride, this change promoted tadpole feeding and enhanced tadpole vigor, thus increasing the rate of tadpole growth and metamorphosis development. However, as the concentration of cerium chloride increases, especially when the concentration exceeds a certain range, the growth and metamorphosis of tadpoles are inhibited, and feeding and other vital activities are also inhibited. The activity of tadpoles will change with the concentration of cerium chloride, and the activity will gradually become stronger and then start to weaken as the concentration of cerium chloride increases until the activity reaches zero at the lethal dose.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

REFERENCES


