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Huyen Nguyen Thi Thuong,
Quan Ke Thai,
Vi Le Thi Tuong,
Tri Truong Van,
Dung Tran Thi Phuong

EXAMINING THE EFFECTS OF LEAD ON THE LIFE OF LARVAL ZEBRAFISH (1-7 DAYS OLD)

Abstract

Lead (Pb) is a toxic metal and and can cause variety of disorders and effect on neu-ronal function and neurodevelopment. Using zebrafish as a model, the aim of this study was to evaluate the effects of concentrations of Pb2+ on the life of zebrafish larvae (from 1 to 7 days old). About 3-5 minutes after mating, collecting embryos and embryos were continuously exposed to Pb2+ at the different concentrations: 0 µg/L, 20 µg/L, 40 µg/L, 60 µg/L, 80 µg/L, 100 µg/L, 120 µg/L, 140 µg/L in embryo Hank medium. After hatching, larvae were transferred to larval Hank medium supplemented corresponding concentrations of Pb2+. The results show: (i) in the different examined concentrations of Pb2+, the minimum concentration of Pb2+ affected the survival rate of larval zebrafish is 40µg/l; (ii) at every examined concentrations of Pb2+, the survival rate of larval zebrafish was affected significantly on the 6th and 7th days. Based on the results obtained, we set up a equation to predict the survival rate of zebrafish larvae using two factors: concentrations of Pb2+ and time of culture. The lethal concentration and time to larval zebrafish stage is 7 cultured days in 68.9 µg/l concentraion of Pb2+.

Key words: lead, zebrafish or Dianio rerio, larvea, heavy metal.

The development of the industry and agriculture leads to the increasing of environ-mental pollution. One of the cause of the pollution is heavy metals pollution which is considered an urgent problem, especially for the aquatic ecosystem. When heavy metals such as mercury (Hg), cadmium (Cd), arsenic (As), and lead (Pb)... accumulate in water, they can lead to the dangers in aquatic animals, hence the humans' health [4]. Lead is a toxic metal and and can cause a variety of disorders and effect neuronal function and neurodevelopment (Neal et al., 2011 [10]; Rice et al., 2011 [12]). In experimental animals, acute lead exposure can result in neurotoxic effects such as, behavioral abnormalities, learning impairment, hearing loss, and impaired cognitive functions [3].

The use of fish as bioindicators in order to evaluate the heavy metal pollution in aquatic environments have been reported (Ebrahimi et al., 2010 [4]). The study of the effects of Pb2+ on the living organisms using zebrafish (*Danio rerio*) as model organism have been implemented by scientists all over the world. In Vietnam, the influences of Pb2+ was evaluated primarily by the chemical or physical methods, there is not an accurate evaluation on the growth of aquatic animals, especially on vertebrate animals. Fishes at embryo and larval stage have the highest sensitivity in their life [1, 6]. The accumulation of heavy metal in the body or organs of embryos affect to the growth of fishes. This study used infected zebrafish embryos to examine the affect of lead on larvae.

Material and method

Zebrafish maintenance and mating

The Zebrafish (about 2 months old) were maintained in light controlled room (14-hours light and 10-hours dark cycle) at room tem-

perature [5, 13, 16] until getting sexual maturity. Males and females were kept separately. All experimental procedures were carried out in the Laboratory of Anatomy - Human and Animal physiology, the University of Education in Ho Chi Minh city.

The glass mating tanks (60 mm diameter) were filled two-thirds full, with marbles at the bottom to make racks for embryo clinging and to prevent embryo cannibalism. Males and females at a ratio of 1:2 are separated by a transparent bulkhead in a tank with a light-dark cycle condition right before mating. [14, 15]

Collect embryo and treat with different concentrations of Pb^{2+}

Embryo and larval Hank media (pH 7-7.5) were used in embryo and larval zebrafish experiments, respectively. $Pb(NO_3)_2$ was dissolved in Hank medium at stock concentrations of 10 mg/l, and then diluted to final concentrations in embryo Hank media at the stages indicated [14].

About 3-5 minutes after mating, fishes were transferred to a new tank. Bottom of the mating tank would be checked quickly for the present of embryos. After the removal of marbles, embryos from mating tanks would transferred to glass beaker by siphon. Only morphological good-quality embryos (morphological life, homogeneous cytoplasm, not mis-shapen) were used for experiments. All embryos were incubated at room temperature, pH 7-7.5. Embryos were continuously exposed to Pb^{2+} at the following examined concentrations: 0 µg/L, 20 µg/L, 40 µg/L, 60 µg/L, 80 µg/L, 100 µg/L, 120 µg/L, 140 µg/L in the embryo Hank medium. Four replicates (n = 4), each of which containing 20 embryos in a 60-mm diameter glass Petri dishes, were cultured in glass beaker with a volume of 200ml embryo Hank medium with supplementation of Pb^{2+} at examined concentrations (totally 80 embryos for every concentration). After hatching, larvae were transferred to larval Hank medium with supplementation of Pb^{2+} at corresponding concentrations [2, 3, 5, 12].

Evaluate the survival of larvae

Concentrations of Pb^{2+} and pH of the larval Hank medium were kept constantly. The survial/death rate of larvae was calculated every 24 hours, to evaluate the survival rate of larval zebrafish depending on the correlation between time and concentration of Pb^{2+}. Based on the collected data, an equation was set up to predict 50% lethal threshold of zebrafish larvae using these factors.

Statistical Analysis

All data obtained from this study were calculated by Minitab 16, R software. Data are given as the mean ± SE. For all statistical tests, differences were considered statistically different at $p < 0.05$. Use of logistic regression analysis method with Poission regression model to analyze the correlation between the survival rate of larval zebrafish and examined factors. This model is a function: $\log\left(\frac{\mu_i}{N_i}\right) = \alpha + \beta x_i$; this means log of the survival rate of larval zebrafish is a function depend on x factor.

When the parameter α and β was estimated by maximum likelihood-based method:

$$\begin{cases} \sum_{i=1}^{n} y_i = \sum_{i=1}^{n} (e^{(\hat{\alpha}+\hat{\beta}x_i)}) \\ \sum_{i=1}^{n} x_i y_i = \sum_{i=1}^{n} x_i (e^{(\hat{\alpha}+\hat{\beta}x_i)}) \end{cases}$$ and

$$\begin{cases} \hat{p}(y \mid x) = e^{\hat{\alpha}+\hat{\beta}x} \\ RR(x_i \mid x_0) = \frac{\hat{p}_i}{\hat{p}_0} = \frac{e^{\hat{\alpha}+\hat{\beta}x_i}}{e^{\hat{\alpha}+\hat{\beta}x_0}} = e^{\hat{\beta}(x_i - x_0)} \end{cases}$$

$\hat{p}(y \mid x)$: Predicted the survival rate follow x

$RR(x_i \mid x_0)$: Risk ratio of the survival rate with x_i versus x_o

Results and discussion

The survival of larval zebrafish at the examined time and concentrations of Pb^{2+}

The zebrafish larvae were cultured in embryo Hank medium with the 7 examined concentrations of Pb^{2+} and control group. The survival rate of larvae by cultured every day shown in table 1.

Table 1

The survival rate of larval zebrafish by days (%)

Pb concentration (µg/l)	1	2	3	4	5	6	7
0	62.50± 5.41 (50/80)	61.25± 5.45 (49/80)	61.25± 5.45 (49/80)	55.00± 5.56 (44/80)	53.75± 5.57 (43/80)	53.75± 5.57 (43/80)	51.25± 5.59 (41/80)
20	70.00± 5.12 (56/80)	70.00± 5.12 (56/80)	70.00± 5.12 (56/80)	66.25± 5.29 (53/80)	66.25± 5.29 (53/80)	62.50± 5.41 (50/80)	60.00± 5.48 (48/80)
40	63.75± 5.37 (51/80)	57.50± 5.53 (46/80)	33.75± 5.29 (27/80)	27.50± 4.99 (22/80)	26.25± 4.92 (21/80)	22.50± 4.67 (18/80)	21.25± 4.57 (17/80)
60	77.50± 4.67 (62/80)	58.75± 5.50 (47/80)	47.50± 5.58 (38/80)	38.75± 5.45 (31/80)	37.50± 5.41 (30/80)	26.5± 4.92 (21/80)	25.00± 4.84 (20/80)
80	61.25± 5.45 (49/80)	52.50± 5.58 (42/80)	51.25± 5.59 (41/80)	45.00± 5.56 (36/80)	37.50± 5.41 (30/80)	37.50± 5.41 (30/80)	37.50± 5.41 (30/80)
100	62.50± 5.41 (50/80)	58.75± 5.50 (47/80)	57.50± 5.53 (46/80)	55.00± 5.56 (44/80)	30.00± 5.12 (24/80)	30.00± 5.12 (24/80)	30.00± 5.12 (24/80)
120	75.00± 4.84 (60/80)	71.25± 5.06 (57/80)	71.25± 5.06 (57/80)	68.75± 5.18 (55/80)	57.50± 5.53 (46/80)	51.25± 5.59 (41/80)	43.75± 5.55 (35/80)
140	76.25± 4.76 (61/80)	73.75± 4.92 (59/80)	58.75± 5.50 (47/80)	53.75± 5.57 (43/80)	35.00± 5.33 (28/80)	31.25± 5.18 (25/80)	30.00± 5.12 (24/80)

Examining the fluctuations of the survival rate of larvae by days in medium without Pb^{2+}

Based on the results of table 1, we examined the fluctuations about the survival rate of larvae by days in medium without Pb (control group). The results shown in table 2.

Table 2

Effect of time on the survival rate of larvae in medium without Pb^{2+}

Predictor	The estimated coefficient	p-value	Risk Ratio	95% CI
Constant - α	-0.424	0.0005	0.965	0.914 - 1.020
Day - β	-0.035	0.2080		

As shown in table 2, the survival rate of larvae in control group had varied during cultured days, but the difference is not statistically significant (p = 0.2080). Specifically, the survival rate of larvae was decreased 0.965 times (equivalent 3.5%) every day (from 62.50% on the first day to 51.25% on the 7th day, see table 1). It could be infered that this variation occurred randomly (p = 0.2080); after hatching, the fish had been developing from embryo to larvae, and not yet adapted to new conditions; in the mean time, larvae subsisted largely on yolk-sac reserves until the onset of exogenous feeding. After 5-6 days post fertilization (dpf), duration for the completely development of the functional digestive system, the yolk-sac would gradually exhausted (Kimmel et al., 1995 [8]), and disappeared on the 7th day (Jardine and Litvak, 2003 [7]). From this point on (and preferably before), the larvae must be self-feeding. In the life time of fish, larval stage (especially, the first larval stage) is often the more sensitive than adult stage (Arufe et al, 2004 [1], Hwang et al. 1995 [6]).

Thus, our experimental results showed that the survival rate of larvae in control group after cultured days decreased slightly. Based on the results obtained, we plotted a chart to predict the survival rate of larvae in cultured medium without Pb^{2+} (Fig 1).

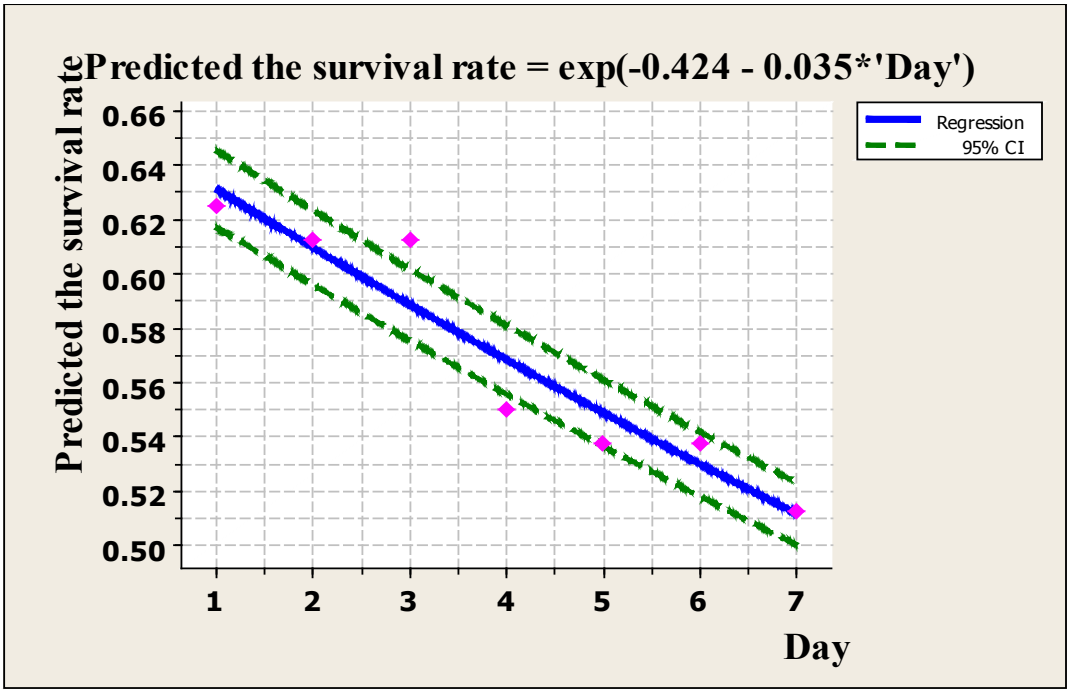

Fig 1. A chart for prediction of the survival rate of larvae in medium without Pb^{2+}

Examining of fluctuation of the survival of larvae by cultured days in medium with Pb^{2+}

Based on the results of table 1, we evaluated of fluctuation of the survival of larval zebrafish by cultured days in medium with the examined concentrations of Pb^{2+}. The results shown in table 3.

Table 3

Effect of time on the survival rate of larvae in medium with the examined concentrations of Pb^{2+}

Pb concentration (µg/l)	Predictor	The estimated coefficient	p-value	Risk Ratio	95% CI
20	Constant - α	-0.306	0.0071	0.974	0.926-1.025
	Day - β	-0.026	0.3121		
40	Constant - α	-0.265	0.0541	0.810	0.753-0.871
	Day - β	-0.210	1.46e-08		

As shown in table 2, at lead concentration of 20 µg/l, the survival rate of larval zebrafish decreased after three days cultured. Specifically, the survival rate of larvae was decreased 0.974 times (equivalent 2.6%) every day cultured (from 70.00% on the first, second and third days to 60.00% on the 7th day, see table 1). And, this variation is due to random (p = 0.3121). In other words, the 20 µg/l concentration of Pb^{2+} is not lethal threshold of larvae zebrafish.

Meanwhile, at lead concentration of 40 µg/l, the survival rate of larval zebrafish has statistically significant difference by cultured days (p = 1.46 e-08). Specifically, the survival rate of larvae was decreased 0.810 times (equivalent 19%, 95% confidence interval, change in the range from 12.9% to 24.7%) by cultured everyday (from 63.75% on the first day to 21.25% on the 7th day, see table 1). The survival rate of larvae in concentrations of Pb^{2+} (60, 80, 100, 120 and 140 µg/l) also give similar results. This meant that the minimum concentration of Pb^{2+} in the tests effecting on the survival rate of larval zebrafish is 40 µg/l.

Our analysis showed the same results reported by Duo et al. (2011) [3] and Rice et al. (2011) [12]. According that, when embryo stages were exposed to low concentrations of Pb^{2+}, they were not affected the activites and behaviors of fish later. Based on the results obtained, we can plot a chart predict the survival rate of larvae by cultured days in Hank medium with 20 µg/l Pb^{2+} (Fig 2) and 40 µg/l Pb^{2+} (Fig 3).

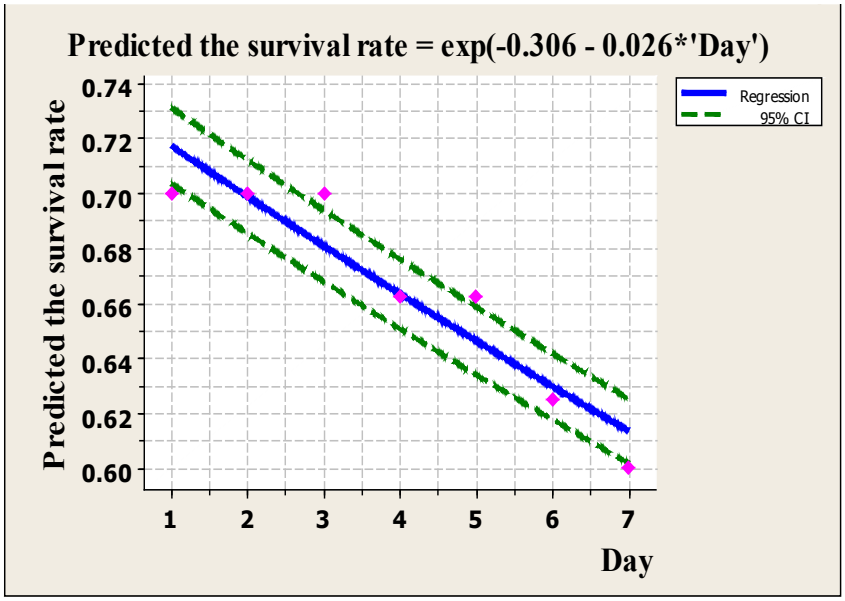

Fig 2. A chart predict the survival rate of larvae in medium with 20 µg/l Pb^{2+}

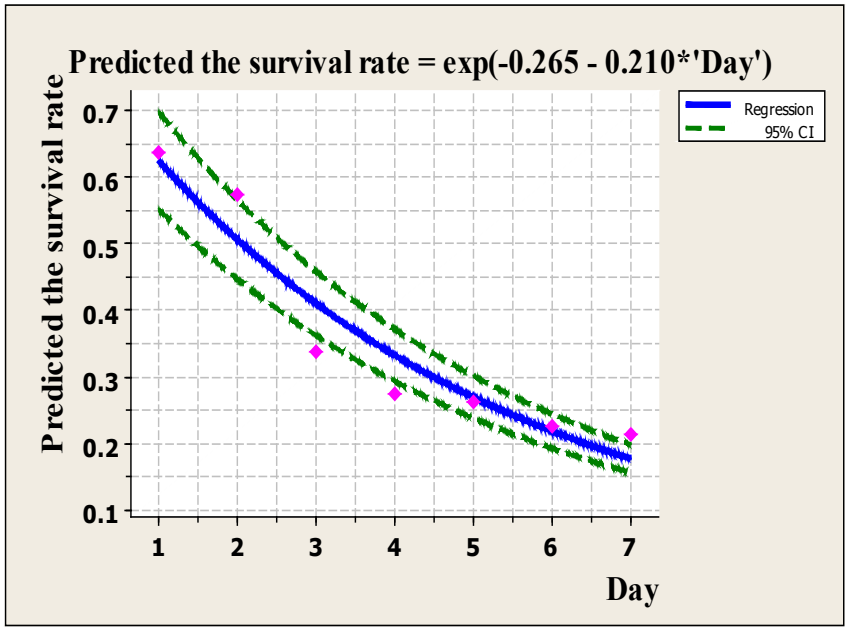

Fig 3. A chart predict the survival rate of larvae in medium with 40 µg/l Pb^{2+}

Examining of the effect of lead concentrations on the survival rate of larvae by every cultured day

Base on the result of table 1, we fixed the timelines by every day (from the first day to the 7th day, 7 timelines, respectively), then evaluated to the fluctuation of the survival rate of larval zebrafish following the increasing the concentration of Pb. The results shown in table 4.

The results in table 4 shown that the increasing the concentration of Pb^{2+} didn't affect the fluctuation of the survival rate of larval zebrafish on the first day (p = 0.399). As mentioned above (section 3.2), on the first day after hatching, larvae subsist largely on yolk-sac, so the embryo viability remained. Thus, when the larvae were exposed to Pb^{2+} (20, 40, 60, 80, 100, 140 µg/l) in larval Hank medium, Pb^{2+} could not penetrate into the fish's body in order to adversely impact in them [9].

Table 4

Effect of lead concentrations on the survival rate of larvae at the examined time

Day	Predictor	The estimated coefficient	p-value	Risk Ratio	95% CI
1	Constant - α	-0.439	7.5e-07	1.001	0.999 - 1.003
	Day - β	0.001	0.399		
5	Constant - α	-0.691	4.97e-11	0.998	0.995 - 1.000
	Day - β	-0.002	0.085		
6	Constant - α	-0.749	6.1e-12	0.997	0.995 - 0.999
	Day - β	-0.003	0.048		
7	Constant - α	-0.781	2.12e-12	0.997	0.994 - 0.999
	Day - β	-0.003	0.031		

Analyzing the results in table 1 on the days (2nd, 3rd, 4th and 5th), we also get similar results on the first day. This show the increasing the concentration of Pb^{2+} didn't affect the fluctuation of the survival rate of larval zebrafish on the cultured days (from the 1st to the 5th day). Thus, on the early days (from the 1st to the 5th day), the fluctuation of the survival rate of larval zebrafish is not affected clearly by the increasing the concentration of Pb^{2+}.

On the 6th and the 7th day, the survival rate of larval zebrafish decreased when the concentration of Pb^{2+} increased, the difference is statistically significant ($p < 0.05$). Specifically, the survival rate of larvae was decreased 0.997 times when the concentration of Pb^{2+} increased 1µg/l (equivalent 0.3%, 95% confidence interval, change in the range from 0.1% to 0.6%) on the 7th day (from 60.00% in 20 µg/l concentration to 30.00% in 140 µg/l concentration, see table 1). This can be explained in the way that: (i) on the 6th and the 7th days, Pb^{2+} penetrated into the fish's body via gills, skin and mouth to accumulate and affect to the survival rate of larval zebrafish. According to Peterson et al., 2010 [11], there are 30 genes involving to the development of the fish's body which were mutated during the Pb^{2+} exposure time; (ii) Moreover, the zebrafish cultured conditions have a lots of disadvantageous factors such as light, noise, the concentration of O_2 or CO_2 which can stress zebrafish. Our results of experiments consistent with publish of Hwang et al. 1995 [6], in which they have demonstrated the larvae are the most sensitive stage for the fish life cycle, the sensitiveness of early larval stage increase when the environment is pollution by heavy metal (such as lead). The lavae usually do not have gills, their skin have permeability to respise and transfer ions. Thus, the toxic substances can penetrate into fish's body through their skin which causing to change disadvantage for fish, especially, larval stage [9, 13]. During the experiment, we saw a few malformations (edema in the head or heart) of the larval zebrafish (Fig 5).

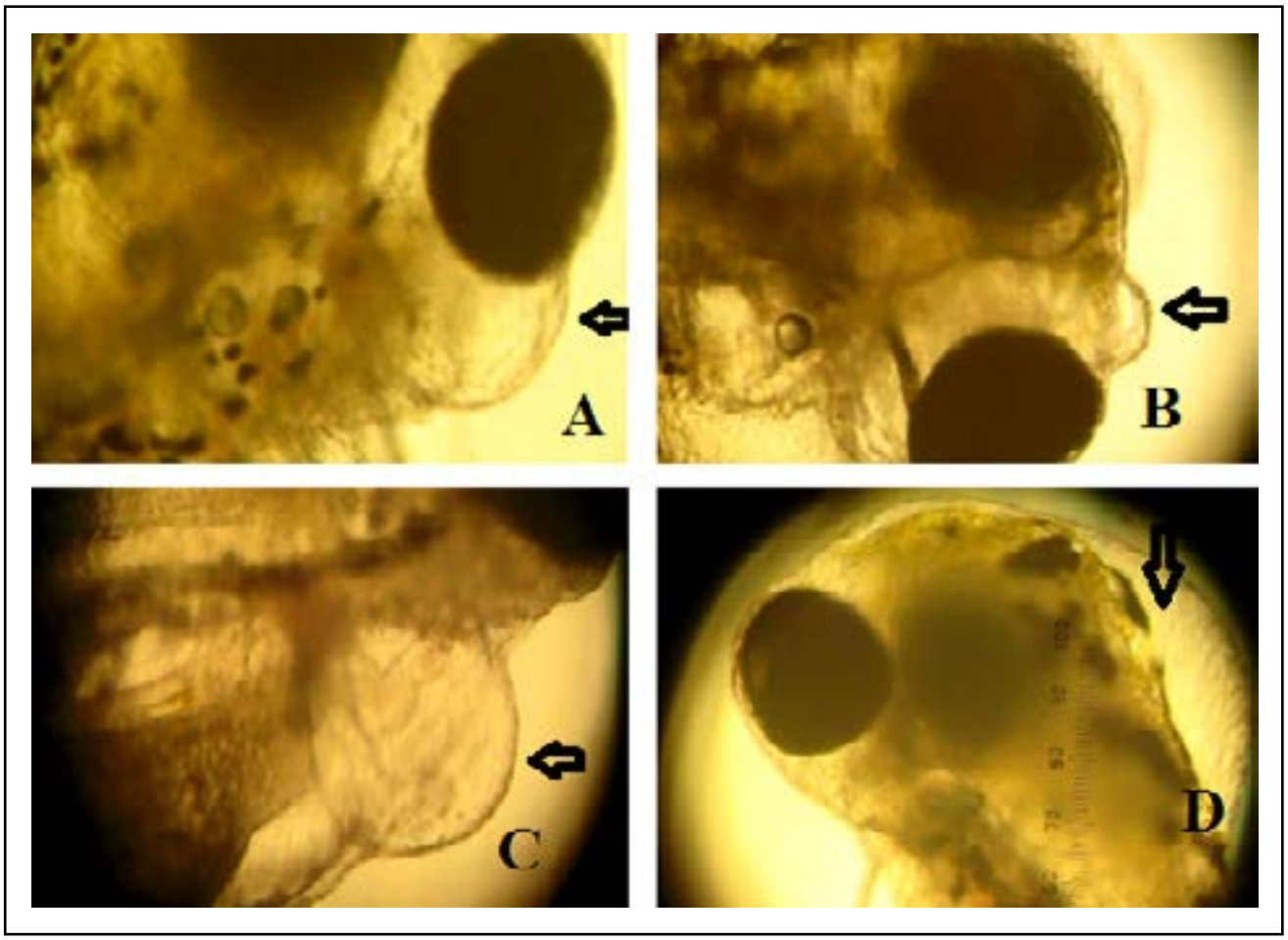

Fig 5. A few malformations of the larval zebrafish (X40)
The larvae was edema in the head (A, B, D) or heart (C)
⇨ - The position of larvae was edema - measure: 500µm

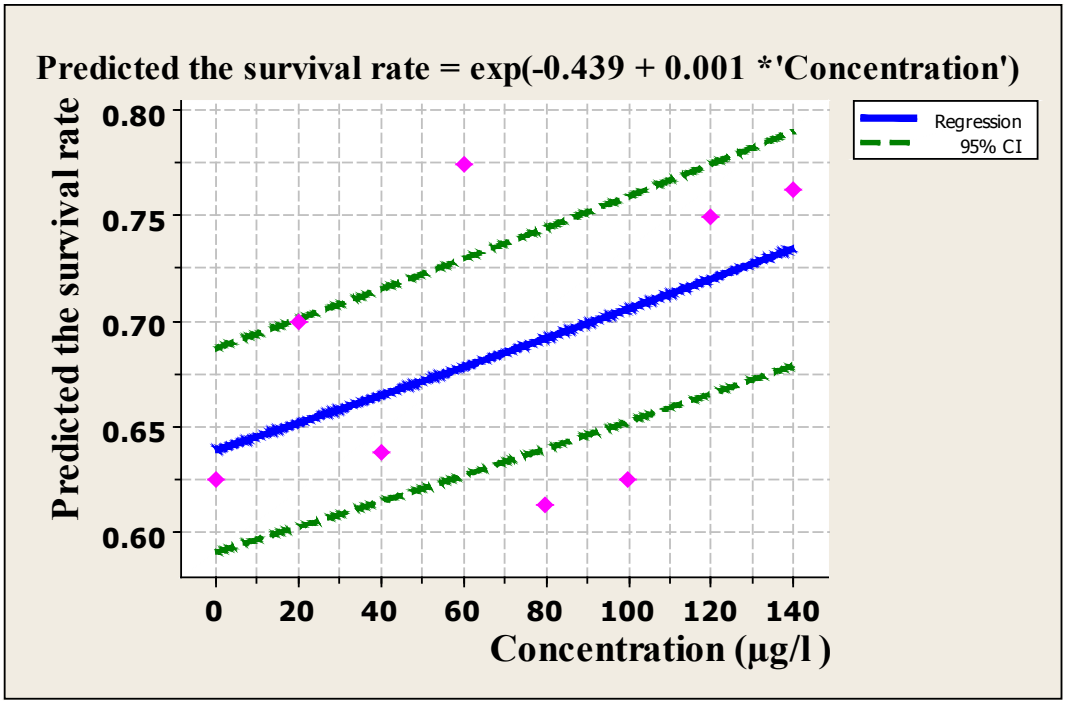

Fig 4. A chart for prediction of the survival rate of larvae following concentration of Pb^{2+} (on the 1st day)

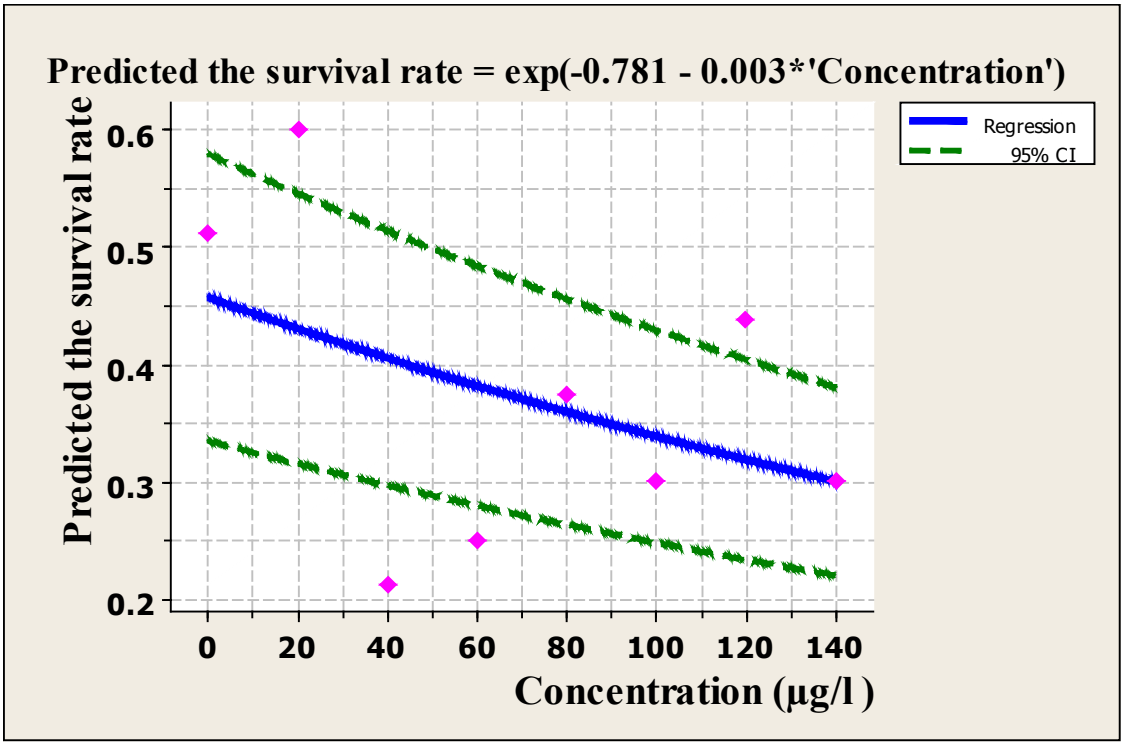

Fig 5. A chart for prediction of the survival rate of larvae following concentration of Pb^{2+} (on the 7^{th} day)

Based on the results obtained, it can be concluded that the time and the concentration of Pb^{2+} factors interacted each other to affect the fluctuation of the survival/death rate of zebrafish larvae.

Examining the effect of the interaction between time and concentration of Pb^{2+} on the survival rate of larval zebrafish

Based on the resluts of table 1, we evaluated the fluctuation of the survival rate of zebrafish larvae by affecting the interaction of time and concentration of Pb^{2+} factors according to the Poission regression model which is stated as following:

$Log(\mu_i/N_i) \sim (Time * Concentration)$

Or: $Log(\mu_i/N_i) = \alpha + \beta_1*Time + \beta_2*Concentration + \beta_3*Time* Concentration$

The results shown in table 5.

Table 5

Effect of interaction between time and concentration of Pb^{2+} on the survival rate of zebrafish larvae

Predictor	The estimated coefficient	p-value	Risk Ratio	95% CI
Constant - α	-0.419	1.5e-07	-	-
Day - β_1	-0.058	0.0025	0.944	0.909 - 0.980
Concentration - β_2	0.002	0.0136	1.002	1.000 - 1.004
interaction - β_3	-0.001	0.0014	0.999	0.998 - 0.999

The results in table 5 shown the fluctuation of the survival rate of zebrafish larvae are affected during the time, concentration and interaction between the time and the concentration factor, all the differences are statistically significant ($p < 0.05$). Consequently, we set up a equation to predict the survival rate of larval zebrafish follow effect of factors:

Predicted the survival rate = $\exp^{(\alpha + \beta_1 * \text{Time} + \beta_2 * \text{Concentration} + \beta_3 * \text{Time} * \text{Concentration})}$

Apply the equation to the data collected from our analysis, in the case of $\alpha = -0.419$; $\beta_1 = -0.058$; $\beta_2 = 0.002$; $\beta_3 = -0.001$, we have a prediction equation:

Predicted the survival rate = $\exp^{(-0.419 - 0.058 * \text{Time} + 0.002 * \text{Concentration} - 0.001 * \text{Time} * \text{Concentration})}$ (1)

Using the prediction equation (1), we can plot a chart to predict the survival rate of larvae following interaction between Pb^{2+} concentration and culture time (Fig 6); and can calculate LCt_{50} (lethal concentration and time) of Pb^{2+} to the larval zebrafish stage (7 days) as follows:

The survival rate estimates on the first day in Hank medium without Pb^{2+} is:

Pr_0 (time = 1, Pb^{2+} = 0) = $\exp^{(-0.419 - 0.058 * 1)}$ = 0.621

The survival rate estimates on the 7th day in Hank medium without Pb^{2+} is:

Pr_x (time = 7, Pb^{2+} = X) = $\exp^{(-0.419 - 0.058 * 7 + 0.002 * X - 0.001 * 7 * X)}$ = $\exp^{(-0.825 - 0.005 * X)}$

LCt_{50} means: $Pr_x / Pr_0 = 0.5 \rightarrow \exp^{(-0.825 - 0.005 * X)} / 0.621 = 0.5$

$\rightarrow -0.005 * X = \text{Ln}(0.3105) + 0.825 \rightarrow \mathbf{X = 68.9\ \mu g/l\ Pb^{2+}}$

So, at 68.9 µg/l concentraion of Pb^{2+}, the survival rate of larval zebrafish will decrease 50% by cultured 7 days. This means the lethal concentration and time to larval zebrafish stage is 7 cultured days in 68.9 µg/l concentraion of Pb^{2+}.

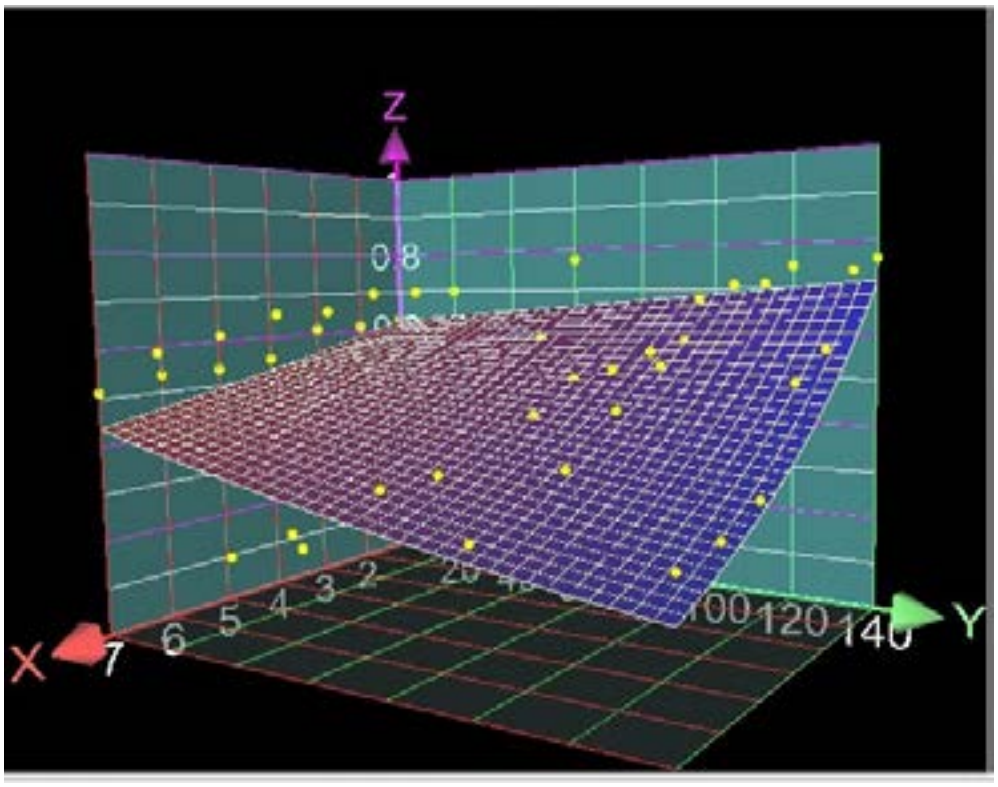

Fig 6. A chart predict the survival rate of larvae following interaction between concentrations of Pb^{2+} and culture time

Conclusion

Among the examined concentrations of Pb^{2+}, the minimum concentration of Pb^{2+} affected to the survival rate of larval zebrafish is 40µg/l.

The survival rate of larval zebrafish was affected significant on the 6th and 7th cultured days at a certain concentration of Pb^{2+}.

An equation for prediction of the survival rate of zebrafish larvae was set using two factors: concentrations of Pb^{2+} and culture time.

The lethal concentration and time to larval zebrafish stage is 7 cultured days in 68.9 µg/l concentraion of Pb^{2+}.

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DATA ABOUT THE AUTHOR:

Huyen Nguyen Thi Thuong[1]*, Quan Ke Thai[2], Vi Le Thi Tuong[1], Tri Truong Van[1], Dung Tran Thi Phuong[1]

[1]University of Education, Ho Chi Minh city, Vietnam

[2]Saigon University, Ho Chi Minh city, Vietnam

*Corresponding author: Huyen Nguyen Thi Thuong.

Email: *huyenntth@hcmup.edu.vn*