

EFFECTS OF LEAD ON THE DEVELOPMENT OF ZEBRAFISH EMBRYO

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ABSTRACT

This study aimed to assess the effects of lead treatment on zebrafish embryo development. Embryos were treated with lead in various concentration at 0.1 µg/l, 1 µg/l, 10 µg/l, 20 µg/l, 100 µg/l. The results demonstrated that there was no difference in ratio of live embryo between control group and lead treatment group for 24 h. However, the live ratio of embryo was reduced in the high concentration of lead treatment from 72 h to 168 h. Lead treatment induced an increase heart rate in zebrafish embryos for 48 h and 72 h. In addition, the down-regulation of GADD45A (Growth arrest and DNA damage-inducible protein 45 alpha), GADD45G (growth arrest and DNA-damage-inducible 45 gamma), SOD1 (superoxide dismutase 1) and SOD2 (superoxide dismutase 2) was observed in zebrafish embryos for 168 h of lead treatment.

Keywords: growth arrest and DNA damage GADD45 proteins, lead, superoxide dismutase, zebrafish embryo development.

INTRODUCTION

The mining industry increases the concentration of toxins and toxicants in the soil and aquatic environment. These increases include both man-made compounds, such as chemicals and pharmaceuticals, and toxicants, naturally occurring molecules, such as metals and metalloids [1]. Moreover, water pollution not only affects the survival and reproduction of aquatic organisms but also adversely impacts human health through bioconcentration. Zebrafish (*Danio rerio*) is an ideal model for studying eco-environmental monitoring and multitudinous pollutant evaluations, such as toxic heavy metals, endocrine disruptors, and organic pollutants [2]. Cadmium are reported to be maternally transferred to oocytes in zebrafish which alters gene expression, retards

development, and increases incidences of pericardial edema in larval zebrafish [3,4]. A decrease of P2X7R expression was observed in the zebrafish larvae treated with HgCl₂ [5]. Nickel decreased each morphological parameter and reduced spontaneous movement of zebrafish [6]. Arsenic exposure is able to promote significant decrease in the locomotor activity as evaluated by the number of line crossings in zebrafish [7]. In this study, we evaluate the effects of lead treatment on zebrafish embryo development, by assessing the ratio of live embryo, heart rate, and expression of several gene relating to embryo development.

MATERIAL AND METHODS

Embryo collection and lead treatment

Zebrafish were bred in a 14 h:10 h Light:Dark cycle. The fish were mated with a male/female ratio of 1:2 in a baffle tank which separated males and females. In the next day, the septum is removed for fish mating. The embryos were collected after 30 mins of mating. Embryos were bred in 250 ml Erlenmeyer flask and treated with lead in different concentration, including 0.1 µg/l, 1 µg/l, 10 µg/l, 20 µg/l, 100 µg/l.

Evaluation of live embryos

The live embryo showed a transparency, round shape, normal chorion, and uniformly yolk. The dead embryos were removed from experiments. The ratio of live embryo was estimated at 24 h, 48 h, 72 h, and 168 h. The heart rate of embryos was recorded and counted under microscope.

RNA isolation

Tissue samples were eluted with 250µl of RLT lysate and vortex for 30 sec. 350 µl of 70% Ethanol was added and the solution was suspended. 700 µl of the sample were transferred to the RNeasy column and placed in 2 ml eppendorf. Samples were centrifuged at 11,000 rpm for 1.5 min at 4°C. The flow-through was discarded and 700 µl of RW1 solution was added to the RNeasy column. Samples were centrifuged at 11,000 rpm for 1 minute at 4°C. 500 µl of RPE solution was continuing added to the RNeasy column after the flow-through was discarded. Samples were centrifuged at 11,000 rpm for 1.5 min at 4°C. The RNeasy column was moved to the new eppendorf. 50 µl of RNase-free water was transferred to the RNeasy column. Samples were centrifuged at 11,000 rpm for 1.5 min at 4°C. The RNeasy column was removed and the total RNA sample was stored at -80°C.

Real-time RT-PCR

Gene expression was quantified by Real-time qRT-PCR using PCR BIO 1-Step RT-PCR Kit (PCR Biosystems). The thermal cycle of the reaction was as follows: 45°C for 10 min, 95°C for 2 min, 40 cycles include: 95°C for 10 sec, 58°C for 15 sec, 62°C for 15 sec. Melting curve analysis was carried out from 60°C to 95°C with each reading step of 0.5°C for 30 sec. The primers included *GADD45A* (FWD: 5'- AAC GTG GTC TTG TGT CTG CT -3'; REV: 5'- AGG TCC ATC GAC TCT CCT CC -3'), *GADD45G* (FWD: 5'-CGC CTT GGA TAC GTC CG-3'; REV: 5'-CTC TTG ACA CGC GAC CAG TA-3'), *SOD1* (FWD: 5'- TGA GAC ACG TCG GAG ACC -3'; REV: 5'- TGC CGA TCA CTC CAC AGG -3'), *SOD2* (FWD: 5'- TTC AGG GCT CAG GCT GG -3'; REV: 5'- ATG GCT TTA ACA TAG TCC GGT -3'), *ETEF*

(FWD: 5'- GTA CTA CTC TTC TTG ATG CCC -3'; REV: 5'- GTA CAG TTC CAA TAC CTC CA -3'). The relative quantitative method of gene expression was conducted according to the $2^{-\Delta\Delta Ct}$ method. The expression of the target gene in each experimental group was determined by normalizing the Ct value of the target gene with the reference gene (*ETEF*) and the control group.

Statistical analysis

The data were analyzed for statistical significance by one-way ANOVA where $P \leq 0.05$ was considered statistically significant.

RESULTS

Figure 1 showed that there was no difference in the ratio of live embryo from control group and other groups for 24 h lead treatment. After 48 h, an increase of lead treatment began to reduce the number of live embryo. A remarkable decrease of live embryos was observed at 72 h treatment with lead. The live ratio of embryo in control group was 95.83%. This ratio was decreased in groups of 0.1 $\mu\text{g/l}$ and 1 $\mu\text{g/l}$ treatment (90.83 % and 91.67 %, respectively). The group of 10 $\mu\text{g/l}$ showed the lower ratio of live embryo (82.50 %) than control group and groups of 0.1 $\mu\text{g/l}$ and 1 $\mu\text{g/l}$ lead treatment. The groups of 20 $\mu\text{g/l}$ and 100 $\mu\text{g/l}$ lead treatment exposed the lowest ratio of live embryo than other group for 72 h (74.17 % and 52.50 %, respectively).

After 168 h treatment of lead, the control group still showed a high ratio of live embryo (90.83%), while groups of 0.1 $\mu\text{g/l}$ and 1 $\mu\text{g/l}$ treatment exhibited the reduced live ratio (77.50 % and 70.00 %, respectively). The extreme decrease was observed in groups of 10 $\mu\text{g/l}$, 20 $\mu\text{g/l}$ and 100 $\mu\text{g/l}$ (6.67 %, 5.83 % and 4.17 %, respectively).

In this study, we also assess the effects of lead treatment on heart rate of zebra fish embryo. As seen in the Figure 2, an increase of lead induced the heart rate for 48 h and 72 h treatment. After 48 h of treatment, the heart rate of control group was 113.3 bpm. The group of 0.1 $\mu\text{g/l}$ lead treatment showed a higher heart rate (121.3 bpm) than control group. Groups of 1 $\mu\text{g/l}$, 10 $\mu\text{g/l}$ and 20 $\mu\text{g/l}$ lead treatment showed an increase of heart rate (127.3 bpm, 128.0 bpm, and 130.7 bpm, respectively). However, there was no difference in heart rate of these groups. The highest heart rate was observed in group of 100 $\mu\text{g/l}$ lead treatment (148.0 bpm).

After 72 h of lead treatment, each group showed a higher heart rate of embryo than themselves from 48 h treatment. The heart rate of embryo from control group was 122.0 bpm. Groups of 0.1 $\mu\text{g/l}$ and 1 $\mu\text{g/l}$ lead treatment exposed an increased heart rate than control group (126.7 bpm and 133.3 bpm vs. 122.0 bpm). Groups of 10 $\mu\text{g/l}$ and 20 $\mu\text{g/l}$ lead treatment showed the higher heart rate than above groups (140.3 bpm and 143.3 bpm, respectively). The highest heart rate of embryo was exhibited in group of 10 $\mu\text{g/l}$ lead treatment.

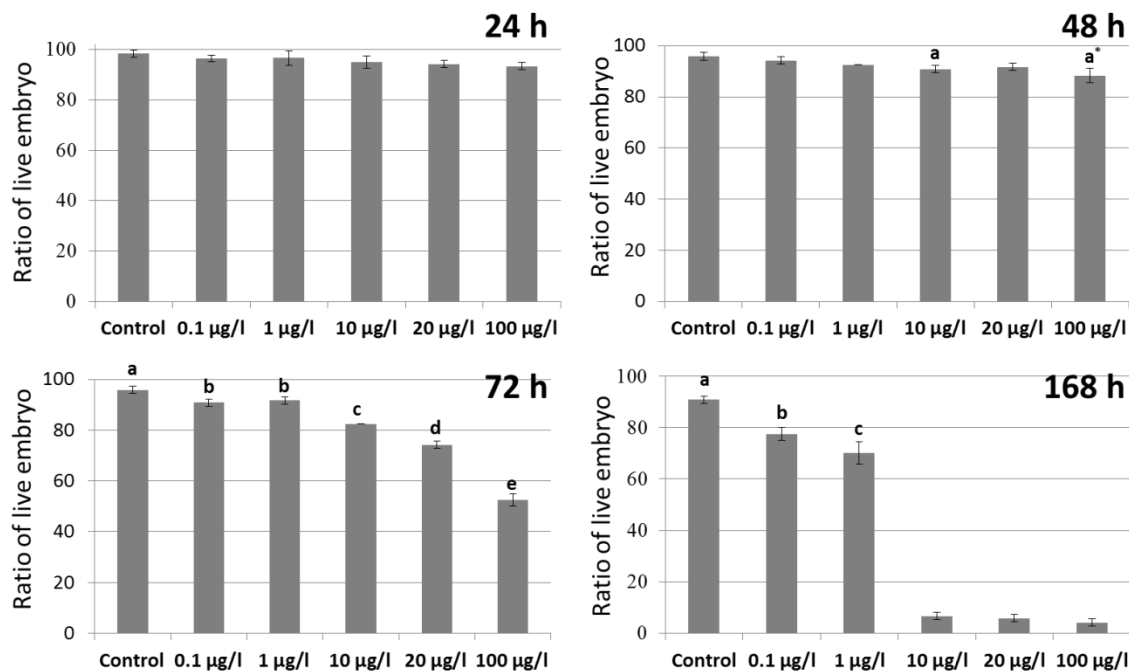


Figure 1. The effect of lead treatment on the ratio of live embryo. $P \leq 0.05$ was considered statistically significant

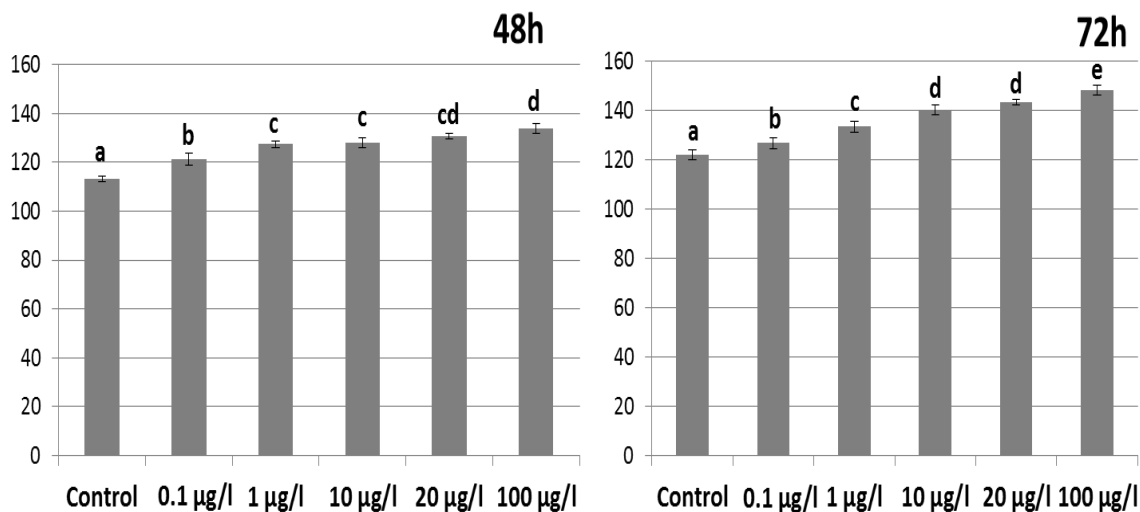


Figure 2. The effect of lead treatment on heart rate of zebrafish embryo. bpm (beat per minute). $P < 0.05$ was considered statistically significant.

In the present study, we also estimate the transcript expression of embryo development-related genes. Figure 3 demonstrated that lead treatment reduced the transcript expression of *GADD45A*. The lead treatment also induced a reduction of *GADD45G* transcript expression. The down-regulation of *SOD1* and *SOD2* was exhibited in groups with lead treatment.

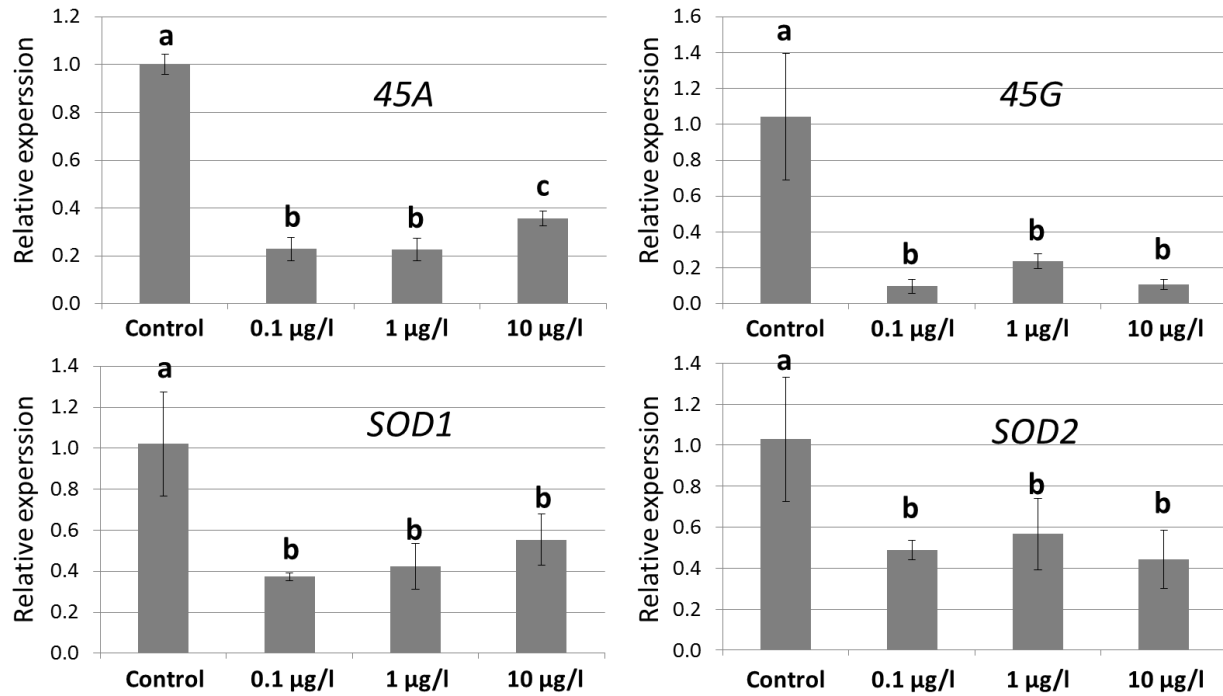


Figure 3. Quantification of mRNA expression in larva at 168 h. The Ct values were normalized using *etef* as the internal control. $P \leq 0.05$ was considered statistically significant.

DISCUSSION

The previous studies reported the toxicity of heavy metals on fish such as oxidative stress [8], respiratory problems [9] and morphological deformations of the fish [10,11]. Moreover, the heavy metals (such as Al and Cd) showed the significant effects on the rate of development and swimming parameters of zebrafish [12]. In the present work, an increase of lead treatment showed the reduction of zebrafish embryo development. Lead treatment not only affects embryo development, but also increases heart rate of zebrafish.

The growth arrest and DNA damage-inducible (GADD) gene GADD45 is a member of a group of genes induced by agents that damage DNA and/or cause growth arrest. Up-regulation of GADD45 gene is determined in many cell types and has been implicated in terminal differentiation, growth suppression, and apoptosis [13-17]. Superoxide dismutases (SODs) belong to the family of isoenzymes which related to the scavenging of O_2 radicals. There are three isoforms of superoxide dismutase enzymes including SOD1, SOD2 [18], and SOD3 [19]. In this study, the expression of GADD45A, GADD45G, SOD1, and SOD2 was reduced in all group induced by lead treatment, leading to abnormality of zebrafish embryos during development and resulting in the reduction of live embryos.

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