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Developmental Neurotoxicity: Evaluation of Zebrafish Larvae Anxiety Behavior after Chronic Embryonic Exposure to Arsenic

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Abstract. Zebrafish has gained the attention of the scientist in the Developmental Neurotoxicity research as they are easy and cheap to maintain. Since their nervous system is highly functional, the behavior has been widely utilized in the high throughput screening system. However, a commercially available zebrafish larvae behavioral testing system is relatively expensive for a small laboratory. Thus, the purpose of this research is to set up a low cost behavior testing system for the zebrafish larvae by modifying the existed protocols. We also used arsenic as the model chemical to assess the behavioral alterations in the zebrafish larvae after chronic embryonic exposure as this toxicant is already classified as a developmental neurotoxicant. In order to accommodate the budget that we have, we have made several modifications to the overall setup and data analysis, previously established by another group. By using the system, we were able to assess the effects of chronic embryonic exposure to arsenic on the sensorimotor and basic motor response in the zebrafish larvae. In conclusion, a low cost zebrafish behavioral testing system can be developed and still feasible to produce a reliable results. Also, our results showed that chronic embryonic exposure to arsenic did not cause significant effects on the anxiety-related behavior except for the highest concentration. The right preference and the swimming speed were slightly affected in the treated groups as compared to the control.

Keywords: zebrafish, anxiety, developmental neurotoxicity, arsenic.

1. Introduction

The frequency of neurodevelopmental disorders such as autism and attention deficit hyperactive disorder (ADHD) is increasing worldwide. A large body of evidence has shown that the developing nervous system is more susceptible to any impairment resulting from the hazardous chemical exposure as compared to the adults. The degree of the adverse effects of the exposure is greatly dependent on the time and severity of the exposure during the developmental stages [1]. The effects might be subtle, but it also has the potential of being prominent, leading to perpetual damage of the nervous system. Based on a current review by [2], 12 chemicals have been listed as developmental neurotoxicants; arsenic, chlorpyrifos dichlorodiphenyltrichloroethane, ethanol, fluoride, lead, manganese, methylmercury, polybrominated diphenyl ethers, polychlorinated biphenyls, tetrachloroethylene and toluene. Nevertheless, the number of industrial chemicals that is lacking paediatric toxicity data are still high and keep on increasing due to the inefficiency of the traditional toxicity testing. The 'gap of the knowledge', together with the increasing frequency of neurodevelopmental disorders has urged the scientist in the developmental neurotoxicity research to find alternatives that can accelerate the process for Developmental Neurotoxicity Test (DNT). Consequently, utilization of alternative models such as zebrafish and cell culture for DNT has been proven to be very useful [1]. Toxicity data produced from these alternative models can be used as a 'filter' to set priorities for further evaluation using a more expensive, laborious and sophisticated models such as rodents.

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Zebrafish, an aquatic vertebrate, is easy and cheap to maintain in the laboratory, their embryos are transparent and rapidly develop *ex utero*, and able to rapidly absorb the chemicals that are added to their water, and then accumulate them in different tissues. They also produce a high number of eggs, thus make them suitable for high throughput screening to accelerate the process of understanding the toxicological effects of chemicals [3]. Behavior is proven to be useful in developmental neurotoxicity research as the detection of the changes due to neurotoxic effects ranging from the molecular to the system level [1]. Given all the characteristics listed above, and since the zebrafish also display a rich behavioral repertoire befitting them to be an alternative model for DNT study in a small laboratory. The availability of a high throughput behavioral testing system that is inexpensive to build, easy and quick to set up [4], has enabled small laboratory to conduct the DNT study.

Arsenic (As) is one of the most toxic naturally occurring contaminants in the environment and it holds the highest ranking in the current U.S. substance priority list [5]. Sources of arsenic contamination can be originated from natural deposits as well as anthropogenic sources such as mining and electronics manufacturing processes and metal smelting [6]. The source of human exposure to the inorganic form of As is mainly through drinking water, whereas through food ingestion human are subjected to be exposed to both organic and inorganic forms [7]. High-level exposure to arsenic *in utero* is associated with increased infant mortality, low birth weight, and birth defects [8], [9]. Whereas, the concern regarding the effects of *in utero* exposure to low-level of arsenic on the developing fetus is increasing. Several epidemiological studies have shown that As exposure decreased intelligence quotient, sensory and motor functions, induced different cognitive defects in the children, adolescents and adults in the Bangladesh and Mexican population [10]-[14]. In this study, with a very limited budget, we have set up a semi-high throughput behavioral testing system for the zebrafish larvae by modifying the existed protocols [4]. Arsenic was used as the model chemical to assess the anxiety behavior in the zebrafish larvae after chronic embryonic exposure.

2. Materials and Methods

2.1. Set up and modification of behavioral testing system

Based on previous studies [4], [15], [16], a system for testing the behavior of the zebrafish larvae were built with a limited budget and laboratory space (Fig. 1.A). We decreased the size of the recording cabinet to a smaller size (70.0 cm X 42.0 cm X 60.6 cm) that is suitable to be placed on a table top as compared to the previous studies (180.0 cm X 40.0 cm X 40.0 cm). The movements of the larvae were recorded by using a 18 megapixel digital camera (Canon EOS 1200D). The camera was set to time-lapse snapshot, by using the EOS Utility 2 software provided by the camera manufacturer. Camera setting used were: image quality=large, white balance=automatic, ISO-speed=1600, aperture (AV)=5.6, and exposure time (TV)=1/125s. The camera was directly connected to a 14 inch Acer Aspire 4740G laptop, to automatically transfer the captured images into the laptop's memory. Since the lens of the camera used in our study does not have the zoom function, we had to modify the system by mounting the camera on a sytrofoam. A styrofoam with a measurement of 27.0 cm length X 31.5 cm width X 27.5 cm height was used as the camera stage (Fig. 1.A).

The new set up for the recording system, lead us to do other modification where we have to use 60.0 mm petri dishes for the placement of the larvae instead of using a multiwell plates or multilane agar. Thus, we were able to record 3 groups only at the same time. The petri dishes were used instead of the multiwell plate as they provide a better optics for a short distance imaging (Fig. 1.A). Besides that, a semi-transparent plastic was used as a substitute for the plastic diffuser to minimize the moire pattern effects from affecting the images captured. Quadrants A and B were considered as the upper part and quadrants C and D were considered as the lower part of the dish (Fig. 1.C). The larva was considered to be at the edge if its distance from the midpoint exceeded 297.469 pixels. The aversive stimulus was animated at the upper section of the petri dish (at quadrants A and B) (Fig. 1.B). The aversive reaction from the larvae was evoked by using a power point presentation displayed by a netbook that was placed underneath the petri dishes. It starts with the blank white display for 5 minutes, followed by another 5 minutes of red moving disc in the upper half of the well. The red moving disc served as the aversive stimulus by moving continuously from left to right and back. The RGB values used for the red disc were 255, 0 and 0. The red color was used because the zebrafish's eyes have the tetrachromacy property by having cone cells sensitive to certain colors [17].

The movements of the larvae were captured by using the camera at 5 second intervals, for 10 minutes. It will generate around 120 pictures of the larvae's movements, with an approximate value of 600 larvae's coordinates for each replicate. A Java-based image processing program (Image J) was then used to analyze the images captured. This software can automatically measure the location and orientation of the larvae in the petri dish, by using the x and y coordinates. The first step involved the use of macro downloaded from doi:10.1016/j.bbr.2011.04.033. It was then modified in term of the particle size, so that the coordinate of the larvae's particle size can be correctly determined. The software identified the coordinates of the larvae by using the difference in the pixels of the foreground and the background. The coordinates of the larvae in each petri dish can be calculated based on the available Excel sheet provided by [4].

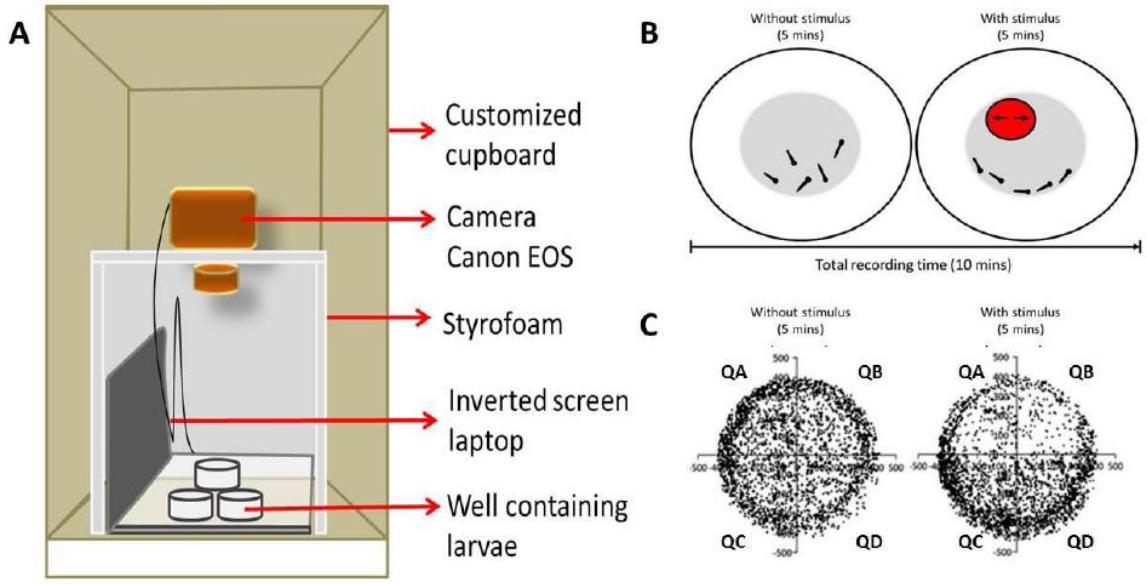

Fig. 1. Schematic diagram of the zebrafish behavioral testing system (**A**). Schematic diagram of the experimental design. Without aversive stimulus (5 mins) then followed by stimulation with aversive stimulus (5 mins). The red moving disc moved continuously from left to right and back in the upper half of the petri dish (**B**). Representative of the total larvae coordinates from 60 snapshots (**C**).

The list of coordinates generated by the ImageJ software was then analyzed based on [4], by the use of XY coordinates of the centroid and the XY coordinates of the midpoint of the bounding rectangle. The XY coordinates of the centroid represent the point of the middle of the larvae's head. Meanwhile, the XY coordinates of the midpoint of a bounding-rectangle signify the point locates at the tail region. Then, "IF" function was used to categorize the placement of the larvae, by the use of both of those coordinates. The X coordinate and the width were used to determine whether the larva was on the right or left side. On the other hand, the Y coordinate and the height denoted the up or down placement of the larva. The edge preference of the larva was measured by calculating the distance between XY coordinates of the centroid from the XY coordinates of the midpoint of the well. As for the edge preference, the larva was considered to be at the edge, if the distance from the larva to the midpoint of the petri dish was more than 297.469 pixels (approximately). If the distance is less that that, it was considered to be at the center of the well. Apart from that, the swimming speed of the larvae was measured by dividing the distance travelled by the time. The parameter measured via this analysis includes positions of the larvae, the distance between the fish, their swimming speed and the resting percentage.

2.2. Zebrafish maintenance and egg collection

The adult zebrafish were cultured at the Department of Biology, Universiti Putra Malaysia. For this experiment, the zebrafish were maintained in a circulating tank system, with 3 Female: 2 Male ratios at the temperature of 25-27 ℃. The light cycle was kept at the ratio of 14 h (light):10 h (dark) where the light was switched on from 8.00 am to 10.00 pm on the daily basis. The fish were fed alternately with *Artemia salina* or pellets for four times in a day. The breeding set-up was done a day before the experiment begins where a collection tank was inserted into the circulating tanks. The spawning process occurs as soon as the lights on.

The embryos were collected, washed and rinsed with deionized water and 0.25 mg/L methylene blue to eliminate any mold growth.

2.3. Arsenic exposure and raising the larvae

Two different concentrations of pure arsenic (2500 µM; 3500 µM) were prepared from 5 M stock solution by dissolving it in DMSO, before being diluted using the embryo media depending on the arsenic concentrations needed. Then, 30 embryos (5 hpf) were placed in the petri dishes containing different arsenic concentrations. As for the control, the embryos were kept in the embryo media. Each concentration had three replicates. The exposure media were renewed on a daily basis to avoid any bacterial or fungal growth. At the end of the exposure, the larvae were rinsed with deionized water and were maintained for behavioral assessment at 6 dpf. In the behavioral assessment, only normal 6 dpf larvae were included in the test. 6 dpf larvae were used because it was the stage where the yolk sac was still exists, so the movements of the larvae were not influenced by other factors like starvation. The larvae were acclimatized for about 5 minutes before the behavioral assessment was done. All experiments were performed in triplicates and repeated at least three times. Data is presented as mean ± SEM. Results were analyzed using Two-way ANOVA with arsenic exposure and presence of aversive stimulus as factors and followed by Duncan test. $p<0.05$ was set as the threshold for statistical significance.

3. Results

In this project, we were successfully set up a semi-high throughput system based on previous studies [4], [15], [16], with some modifications. The system was used to measure behavioral alterations in 6 dpf zebrafish larvae after chronic embryonic exposure to arsenic. The anxiety-like behavior of the larvae were analyzed based on their sensorimotor response and basic motor response. The following parameters were used to assess sensorimotor response in zebrafish; edge preference, down preference, right preference, clockwise preference, outward preference, swimming speed, resting percentage and distance between larvae. Comparison was made for each specified parameter to understand the effects of arsenic treatments on the larvae's behavior, both with or without the presence of the aversive stimulus. The following parameters did not show any alterations after treated with arsenic or stimulated with aversive stimulus; clockwise preference, outward preference, resting percentage and distance between larvae.

3.1. Edge preference of the larvae

Edge preference is one of the parameters that can be used to measure anxiety in the zebrafish [3]. The larvae tend to be at the edge of the well in a novel or stressful condition (thigmotaxis). The percentage of edge preference was calculated based on the placement of the coordinates of the zebrafish's larvae. We found that arsenic did not affect the percentage of the edge preference of the larvae. The presence of the red bouncing ball increases the percentage of the edge preference in all groups. A slight increase was observed in the control and 2500 µM arsenic, whereas, the increase was significant in the 3500 µM arsenic treated larvae (Fig. 2). This suggests that all of the larvae reacted to the presence of stimulus by moving towards the edge of the well.

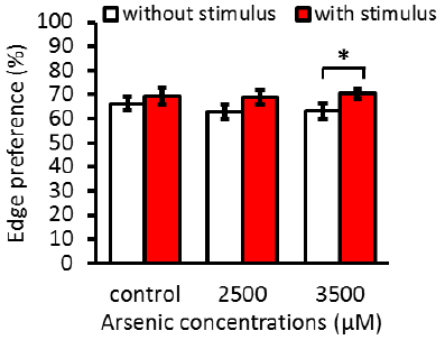

Fig. 2. The coordinates of the larvae in the petri dishes were recorded for 5 minutes (white) before stimulation with an aversive for the next 5 minutes (red). No effects on the percentage of the edge preference in the arsenic treated larvae. Stimulation with an aversive stimulus increased the percentage of the edge preference in the 3500 µM. Values are mean ±SEM. *. $p<0.05$ (Two-way ANOVA; Duncan test).

3.2. Down preference of the larvae

The percentage of down preference of the larvae can be used to investigate the avoidance behavior of the larvae after stimulation with an aversive stimulus at the upper part of the petri dish. We found that there was no significant difference of the down preference between control and arsenic treated larvae. After stimulation with the red bouncing ball, the percentage of the larvae at the down side of the dish was increased significantly in all treated groups (Fig. 3). This result suggested that all larvae showed an avoidance response to the red ball stimulus, due to the increase in the percentage of the down preference of the larvae.

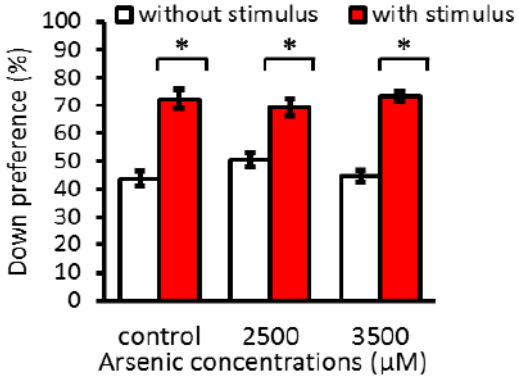

Fig. 3. The coordinates of the larvae in the petri dish were recorded for 5 minutes (white) before stimulation with an aversive for the next 5 minutes (red). No effects of arsenic treatments on the percentage of the down preference in the larvae. Stimulation with an aversive stimulus increased the percentage of the down preference in the larvae. Values are mean ±SEM. *. $p<0.05$ (Two-way ANOVA; Duncan test).

3.3. Right preference of the larvae

In a normal larvae, the right preference and the left should be in the ration of 50%:50%. This parameter can be used to visualize the behavior lateralization in the zebrafish larvae. We found a slight increase in the right preference in the control larvae. The right preference of the larvae exposed to arsenic treatments was significantly different from the control larvae, suggesting that arsenic treatments affected the right preference of the larvae. While, the introduction of aversive stimulus does not affect the right preference of all larvae (Fig. 4).

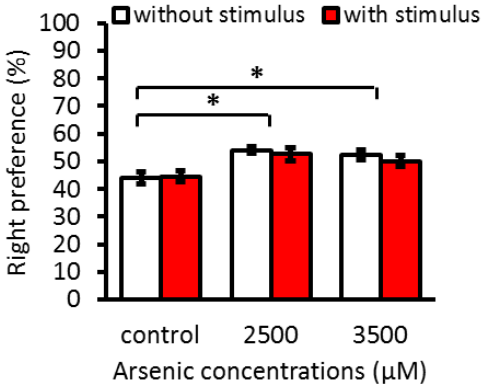

Fig. 4. The coordinates of the larvae in the petri dish were recorded for 5 minutes (white) before stimulation with an aversive stimulus for the next 5 minutes (red). Arsenic increased the percentage of the right preference in the zebrafish larvae. Values are mean ±SEM. *. $p<0.05$ (Two-way ANOVA; Duncan test).

3.4. Swimming speed of the larvae

At 6 dpf, the swimming behavior in the zebrafish larvae already developed. Generally, in a stressful condition, larvae will show slower swimming speed. There were no significant alterations even a slight increase on the swimming speed in the arsenic treated larvae as compared to the control. However, stimulation with an aversive stimulus induced a significant decrease of the average speed for the control larvae and 3500 µM arsenic treated larvae. Eventhough 2500 µM arsenic treated larvae showed a decrease in the average speed, however the difference was not significant (Fig. 5). This result showed that the presence of the red ball stimulus has induced stress in the zebrafish larvae.

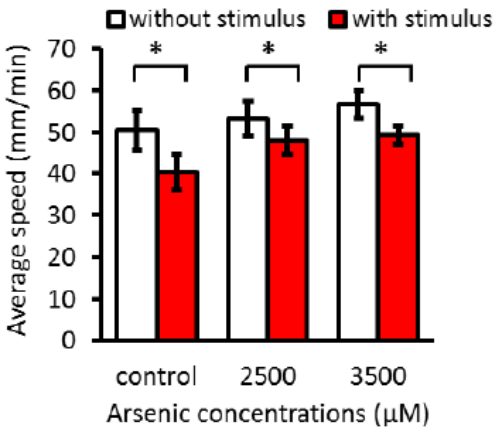

Fig. 5. The coordinates of the larvae in the petri dish were recorded for 5 minutes (white) before stimulation with an aversive for the next 5 minutes (red). The average speed of the larvae treated with different concentrations of arsenic. Arsenic induced a slight increase in the average speed of the larvae. Stimulation with an aversive stimulus decreased the average speed of the larvae in all groups. Values are mean ±SEM. *. $p<0.05$ (Two-way ANOVA; Duncan test).

4. Discussion and Conclusion

Given all the advantages offered by the zebrafish as a model organism for DNT, together with the fact that behavioral assays using zebrafish have the capability for a high throughput screening, in the present study, we set up a zebrafish behavioral testing system in our laboratory. The set up of the system was based on [4], [15], [16], with some modifications. The system is a powerful system that can be used to assess anxiety-related behavior in the zebrafish larvae. Detection of the sensorimotor impairments in the organisms can be done by evoking suitable responses to assess their visual, olfactory, auditory, or interoceptive sensory endpoints. Generally, the zebrafish larvae showed a startle response when exposed to tactile, acoustic, or visual stimuli. They also will avoid dark areas, moving objects, and open spaces. The testing system that was previously developed by [4], [15], [16], able to detect the sensorimotor impairments in the zebrafish larvae by stimulating the larvae with a red bouncing ball as an aversive stimulus. Emotional states such as fear and anxiety might be prompted when larvae are exposed to stimuli that they would ordinarily escape from or avoid. Any impairment in sensory perception or motor performance due to exposure to neurotoxicants may result in the changes to the sensory responses.

We have made several modifications to the system due to our limitations; budget constraints and small laboratory space. We decreased the size of the recording cabinet and a styrofoam was used as a holding stage for the camera. The lack of additional zoom lens for the camera and also a smaller size of the recording cabinet have decreased the imaging distance. Thus, we have to substitute the multilane agar or multiwell plates into 3 60mm petri dishes for the placement of the larvae during the behavioral recording. In addition, we have to decrease the number of the larvae to 15 larvae (5 larvae in 3 petri dishes) and substitute the plastic diffuser with a semi-transparent plastic to minimize the moire pattern effects from affecting the images captured. Also, our camera lack of direct power supply, thus we have to limit the time for recording to 10 minutes as compared to the previous studies. Typically, the larvae were imaged for 1 hour in the previous studies by [4], [16]. Besides that, we also modify the pixel size, radius of the round well and the particle size of Image J macro. However, we encountered several limitations with the system; limited number of different treatments that can be analyzed at the same time, short time for recording, deficiency of high speed camera and lack of positive control.

By using the set up, we have measured the behavioral alterations in the 6 dpf larvae after chronic embryonic exposure to arsenic. Since arsenic has been shown to be associated with different adverse effects on the developing nervous system in human [10]-[14], and animal studies [18]-[21], we interested to understand the effects of chronic embryonic exposure on the anxiety-related behavior in the zebrafish larvae. We found that the highest arsenic concentration induced a slight but significant increase in the percentage of the edge preference. Also, chronic embryonic exposure to both arsenic concentrations have significantly increased the right preference of the larvae as compared to the control. The introduction of the aversive

stimulus during behavioral testing has increased the percentage of the edge preference and the percentage of the down preference of the larvae. The speed of the larvae was decreased in the presence of the red ball stimulus. Whereas, in other parameters tested, we found no effects on the zebrafish larvae for both factors; arsenic treatment and the presence of an aversive stimulus. These findings revealed that all larvae have the ability to react to the aversive stimulus and only the highest concentration of arsenic induced impairment on the anxiety behavior in the larvae. A normal larvae will show a significant increase in the percentage at the edge as the response to aversive stimuli [4]. The increase in the percentage of the edge preference was a manifestation of the higher anxiety level of the larvae. Avoidance behavior also useful for the assessment of the visual endpoints, as it involved the detection of the stimulus by the eyes which results in the responses from the organism. [18] Reported that arsenic showed an anxiogenic effect on adult zebrafish. In this study, we found that the percentage of the right preference in the arsenic treated larvae was significantly different from the control larvae. We were unable to explain the reason for this difference. Therefore, further detailed study needs to be done to understand this finding. The larvae treated with arsenic have a relatively higher swimming speed as compared to the control larvae, although that their differences were not significant. Previous studies have shown that arsenic has the ability to cause alteration in the locomotion and anxiety in the adult zebrafish [18], induced biphasic response in locomotion after treatment with high and low concentrations in mice [21], and decreased swimming speed in the tadpoles [19].

We will continue to improve and validate the system that we have already developed. Based on the current development, there are several technical aspects can be applied in the future studies for the improvement of the system, such as prolonging the time span of the recording, substitute current camera to a high speed camera and include positive controls for chemical testing. In conclusion, a low cost zebrafish behavioral testing system can be developed and still feasible to produce a reliable results. Also, our results showed that chronic embryonic exposure to arsenic did not cause significant effects on the anxiety-related behavior except for the highest concentration. The right preference and the swimming speed were slightly affected in the treated groups as compared to the control.

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