Arsenic hampered embryonic development: An in vivo study using local Bangladeshi Danio rerio model

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\textbf{ABSTRACT}

Zebrafish (Danio rerio) has appeared as a valuable and popular model species to study the developmental and toxicological impact of environmental pollutants. To get insights on the toxicological effect of arsenic on early embryonic development, a controlled breeding of local Bangladeshi zebrafish followed by comprehensive microscopic analysis was conducted to study the embryonic development after exposure to different concentrations of arsenic ranges from 4 – 120 μM post-fertilization. Zebrafish embryos exposed to 2 mM of arsenic displayed distinguishable developmental delay compared to control. At three days post-fertilization, a distinct phenotype appears in arsenic-treated embryos, which can be characterized by dechorionated embryos, larger egg mass, pericardial edema, abnormal heart rate, and abnormal head development. Remarkably, the death rate of the arsenic-treated embryos was significantly higher compared to control. Collectively, these findings indicate that exposure to arsenic may result in abnormal embryonic development. These results suggest for proper management of the pregnant mother in the arsenic-exposed area, and may also explain the incidence of increased miscarriage/abortion rate in arsenic water drinking pregnant mother.

1. Introduction

Heavy metals are the metal or metalloid compound that has a higher density than water, usually having density more than 5 g/cm\textsuperscript{3} [1]. Naturally, these heavy metals are essential at minute concentrations to keep different biochemical as well as physiological homeostasis in the living organisms; however, become poisonous when they surpassed particular threshold concentration [2,3]. Arsenic, the 20\textsuperscript{th} most plentiful element of the world, marked as most toxicant among the heavy metals and emerged as a signifi-
cant menace for the environment as well [4]. Globally, around 20 crore people are directly or indirectly incorporated with excessive levels of arsenic. The scenario is even worsened in Bangladesh; with about two crore persons are regularly using arsenic-contaminated ground-water, and approximately 43,000 persons are dying every year due to arsenic poisoning [13–15]. In Bangladesh, hand-pumped tube well is used as the key source for drinking and household working water. Unfortunately, more than half of them (~ 10 million around the country) were found to supply groundwater with arsenic load over 50 μg/L (5x higher than EPA and WHO recommendation) [16–18]. Furthermore, rice, which is the main food of water is ten μg/L, which is recommended by the Environmental Protection Agency (EPA) and the World Health Organization (WHO) [12]. Chronic excessive arsenic contamination through the drinking water found to be associated with several human health issues.

Abbreviations: mpf, minutes post-fertilization; hpf, hours post-fertilization; dpf, days post-fertilization

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about 16 crore persons of the country, is grown on over 70 thousand hectares of land (which constitutes ∼7% of country’s total rice cultivated area) where the average groundwater arsenic load was reported to over 50 μg/L [19,20]. Subsequently, arsenic entered and accumulated in the food chain, and a considerable proportion of the Bangladeshi people are affected directly or indirectly by consuming those foods.

Epidemiological investigations have affirmed that both the intense and continuous exposure to arsenic are harmful to human health; can cause many diseases including cancer (in lung, bladder, and skin), cardiovascular diseases, diabetes, developmental, reproductive and neurological disorders [21–31]. Most importantly, arsenic is well

Fig. 1. Different stages of embryogenesis of local Bangladeshi zebrafish under a microscope (40X). Some key diagnostic features at the following stages are indicated with arrows; 1K cell: yolk syncytial layer (YSL) nuclei. Germ ring: germ ring. Shield: embryonic shield. 75 % epiboly: Brachet’s cleft. Three somites: third somite. 21 somite: lens primordium.
found to be contaminated with heavy metals including arsenic [36]. Thus, it is urgent to assess the toxic effect of arsenic on human health, particularly during the early embryonic development.

Danio rerio, commonly known as zebrafish, has arisen as a vital model animal for toxicology related research in early developmental stages [37–39]. Besides high sequence homology with human genes, other advantages include rapid external development, optical transparency during early embryonic development, permeability to low molecular weight molecules which make them ideal for high-throughput screening against teratogenic, cardiotoxic and neurotoxic drugs [37–45]. Their small size, easy maintenance, and high fecundity make them highly favorable for the experiments demanding a large number of replicates [46]. The current study utilized wild-caught zebrafish embryos from Bangladesh to gain insights into the impact of arsenic during the initial embryonic growth.

2. Methodology

2.1. Collection of zebrafish and optimization of culture condition

Adult zebrafish were collected after being adopted from Mymensingh freshwater. Fishes were housed and cultured at the zebrafish facility of Shahjalal University of Science and Technology (SUST) under standard laboratory environments. A strict light cycle of 14 h light following a dark period of 10 h, was maintained. The water quality parameters temperature and pH were recorded once daily. Tanks were cleaned every three days and refreshed with clean water and sterilized every two weeks. Embryos are reared in an E3 medium (containing methylthioninium chloride) incubated at 28 °C [46] up to 9dpf for normal growth. Dead embryos were removed, and the E3 medium is refreshed every day by replacing half of the fresh E3 medium. All procedure for animal use were reviewed and approved by the SUST Research Center (authorized institutional body for research care).

2.2. Feeding

Adult fish were fed with pellet food (TetraBits® Complete, Tetra GmbH, Germany) twice a day. On the contrary, embryos from the beginning of the 6th day were fed with 50 μM solid dry granules once a day and Artemia spore once a day. Artemia decapsulation was done by the removal of the hard shell by exposure of Artemia cyst to a sodium chloride solution with aeration for 24 h in light.

2.3. Zebrafish breeding

Breeding tanks of 1.5-liter capacity (locally made) were used for breeding. On the day before breeding, during the late evening, two females and two males were kept separated by a transparent separation sheet. Breeding tanks were then filled with about 2/3 of tap water. In the next morning, the separator was removed, refreshed with clean water and checked time to time for successful breeding. Fertilized eggs were deposited through a perforated net on the floor of the breeding tank and were then collected in E3 medium. The breeding technique was repeated for fishes at 8–10 days intervals.

2.4. Mathematical analysis

The breeding performance indicators including the spawning success, fecundity, fertilization rate, hatching rate, and larvae’s survival rate up to 20 days post-fertilization (dpf) were calculated by using standard formula.

2.5. Preparation of arsenic solution

Serial dilution of sodium arsenate (CAS NO-10048-95-0, Loba Chemie, Mumbai, India; MW 312.01 g/l) ranges from 0.2 to 1.5 mM

Table 1

| Developmental stage | Time (hpf) |
|---------------------|------------|
| Zygote              | 0-0.75     |
| Cleavage            | 0.75-2.42  |
| Blastula            | 2.42-5.42  |
| Gastrula            | 5.42-10    |
| Segmentation        | 10-24      |
| Pharyngula          | 24-48      |
| Hatching            | 48-72      |
| Early Larva         | 72 hpf-29dpf |

Table 2

| Breeding performance indicator | Mean ± S.D | Sample size |
|--------------------------------|------------|-------------|
| Spawning Success (%)           | 80 % ± 16.33 | 20          |
| Fertilization rate (%)         | 75 % ± 8.164 | 20          |
| Hatching rate (%)              | 95 % ± 4.08  | 20          |
| Survival rate (%)              | 81.67 % ± 6.236 | 20          |

Fig. 2. Hatching rate (%) of As treated embryos compared to control after 48hpf. As exposed embryos exhibit decreased hatched rate proportion to increase As concentration. Here, the sample size for each treatment is 30, and they were replicated three times at (28.5 °C). *$p \leq 0.001$.

Fig. 3. Mortality rate (% of embryos died) of arsenic-exposed embryos at different time point. Arsenic-treated embryos showed, higher mortality compared to control. Mortality rate increased with increasing As concentration and time point. Sample size = 30, for each treatment, and they were replicated three times at $28.5 \, ^{\circ} \text{C}$.

*p ≤ 0.05; *p ≤ 0.001.

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was tested to get the minimum morphological effect. Further, sodium arsenate concentration of 1.0 mM, 1.5 mM, and 2.0 mM was tested to observe their effect on embryonic development.

2.6. Evaluation of arsenic concentration

The effective level of arsenic was evaluated as described by Haley and Turner (2016), and Chakraborty et al. (2012) with minor modification [48,49]. Sodium arsenate solution (Cat: A6756; Merck KGaA, Darmstadt, Germany; MW 312.01 g/l) was used as a standard for...
2.8. Growth measuring and survival rate counting of zebrafish to arrest pigmentation. Merck KGaA, Darmstadt, Germany) was supplemented to each petri dish. Also, to observe the internal organ formation clearly under a light microscope, arsenic-free and arsenic-free medium arsenate + E3 medium was treated, while control remained arsenic-free. For arsenic exposure respective concentration of so- dium arsenite was used for hpf. No signs of dorsal curvature were seen in the control at any time point. Statistical significance was calculated comparing against the control. \( p \leq 0.05; \) \( p \leq 0.005; \) \( p \leq 0.001. \)

Table 3

| Features | Normal/AB line | Local BD/wild |
|----------|----------------|---------------|
| Embryogenesis | Cleavage | 45 min to 2 h [47] | 40 min to 1 h 47 min |
| Blastula | 2 h 15 min to 4 h | 1 h 55 min to 4 h |
| Gastrula | 5h 40 min [47] | 6h |
| Germ-ring | 2 h [47] | 1 h 47 min |
| Shield | 6h [47] | 6h 20 min |
| Concentration of arsenic used in the experiment | 0.5–10 mM [52] | 0.2–2 mM |
| Minimum concentration of phenotype/ effect | 1.0 mM [52] | 1 mM |
| Hormesis phenotype | Found at 2 mM after 48hpf [52] | Not found |
| Hatching rate (1 mM arsenic at 48hpf) | 1.10 ± 0.03 % [52] | 26.67 % |
| Mortality rate (1 mM arsenic at 48hpf) | −9 % [52] | −15 % |
| Mortality rate (1 mM arsenic at 48hpf) | −22 % [52] | −44 % |

To get insights into the breeding performance, the key indicators, including spawning, fecundity, fertilization rate, and hatching rate, were checked in a cohort of 30 embryos (Table 2). Untreated control embryos showed 100 % hatching rate.

3. Results

3.1. Embryogenesis of local Bangladeshi zebrafish

Zebrafish embryogenesis was observed under a microscope (40X) from fertilization to adult stage (Fig. 1). Different time points were documented for some specific developmental stages and found that zygote (4 cells) forms 40 min post-fertilization (pf), and it takes 48 hpf for hatching (Table 1).

3.2. Breeding performance

To get insights into the breeding performance, the key indicators, including spawning, fecundity, fertilization rate, and hatching rate, were checked in a cohort of 30 embryos (Table 2). Untreated control embryos showed 100 % hatching rate.

3.3. Arsenic exposure decreases hatching rate

Arsenic-treated (1 mM, 1.5 mM, 2 mM) embryos showed an extended time (approximately 96–120 hpf) to hatch from the chorion compared to control. Arsenic exposure revealed that hatching rate decreased with increased arsenic concentration; at 72 hpf hatching rate of 27 %, 13 %, and 3 % embryos were found in 1 mM, 1.5 mM, 2 mM arsenic concentration respectively (Fig. 2). Untreated control embryos died at 72 hpf.

3.4. Arsenic exposure causes increased mortality rate

The rate of mortality was found significantly and proportionally higher with concentration in the arsenic-exposed embryos compared to control until 56 hpf (Fig. 3). All of the embryos (100 %) treated at 1.5 mM or 2.0 mM of arsenic died at 72 hpf (Fig. 3).

3.5. Arsenic exposure persuades irregular morphology and impaired development

Embryos exposed to 1.0 mM and higher concentrations of arsenic results morphological and developmental abnormalities, which can be characterized as deformation in the midbrain, tail bud, egg mass, otic vesicle, and hatching gland cell (Fig. 4A, B). These anomalies were found in the arsenic-exposed embryos compared to control until 56 hpf (Fig. 3). All of the embryos (100 %) treated at 1.5 mM or 2.0 mM of arsenic died at 72 hpf (Fig. 3).

4. Discussion

This study has determined the impacts of arsenic on the early embryonic development of local zebrafish. Embryonic exposure to higher levels of arsenic increased mortality and induced physiological abnormalities in early development, including prolonged hatching time, abnormalities in cardiovascular systems, and lowered growth. Previously reported findings in rodents also showed that arsenic treatment results decreased reproduction rate and development. Furthermore, it was also reported that oral treatment of arsenic during evaluation of arsenic solutions.

2.7. Experimental setup

Three replicates, each constituting of 30 embryos, were used for every treatment. For arsenic exposure respective concentration of sodium arsenite + E3 medium was treated, while control remained arsenic-free. Also, to observe the internal organ formation clearly under a light microscope, 0.003 % 1-phenyl-2-thiourea (PTU; CAS NO- 103-85-5, Merck KGaA, Darmstadt, Germany) was supplemented to each petri dish to arrest pigmentation.

2.8. Growth measuring and survival rate counting of zebrafish embryos

Rate (%) of embryos come out from the chorion at a given time point was enumerated to analyze the hatching rate. The embryos were analyzed under an inverted and dissecting microscope at different time points to observe any phenotypic deformities as well as the rate of survivability.

2.9. Irregular heart pump measuring

To estimate the effect of arsenic in cardiovascular activity, heart pump was counted per minute in a cohort of arsenic-treated embryos and compared with control at 48 hpf. Also, the heart shape was observed under a light microscope.
the pregnancy period contributes to the fetus neural tube defects (NTDs) [50,51]. In zebrafish, arsenic was also reported to cause abnormal neural development, including impairment of patterning in the central nervous system (CNS) [52]. Toxicology experiments of some chemicals revealed that neural deficiencies have crosstalk with cardiac dysfunction [53]. Besides cardiac dysfunction, arsenic exposure could induce neural defects during embryogenesis [54]. Our result certainly showed that arsenic exposure led to cardiac dysfunction, which may also have contributed to the abnormal heart rate [55,56]. Another study suggested that cleft spine (spina bifida), sticking-ribs, skeletal malformation and a stress fracture in one of the vertebrae (Spondyloysis) are among the noteworthy abnormalities caused by sodium arsenate in human [50,57,58]. The present study has also shown that arsenic-treated embryos are affected by the development of lordosis (dorsal curvature). Ahmad et al. (2001) showed that the rates of miscarriages, stillbirth and preterm delivery in pregnant women, who were exposed to arsenic, were remarkably higher than the pregnant women who were unexposed [59]. Overall, these results indicate the impacts of pre-eminent levels of arsenic in drinking water and its relationship with pregnancy and fetal complications in human. Arsenic has been reported to associated with hatching delay, increase mortality and fish demor-mities including anomaly in spine in both natural and laboratory population [60]. Comparing to our findings on impacts of arsenic on local Bangladesh/wild zebrafish with normal/AB line revealed that minimum concentration to recapitulate phenotype in both group is similar, no hornsess phenotype found in wild embryos, hatching rate and mortality rate are much higher compare to the normal embryos (Table 3). Interestingly, embryogenesis process was found slower in the loca embryos compared to the normal embryos (Table 3).

5. Conclusion

The overall results suggest that chronic higher level of arsenic exposure causes abnormal embryonic development, including severe heart defects in embryos. These results essence the proper management of pregnant mothers in the arsenic-exposed area. This study stressed the essence of molecular analysis to obtain further insights into the effects of arsenic on embryonic growth.

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CRediT authorship contribution statement

Tamanna Kabir: Investigation, Writing - original draft. Saeed Anwar: Investigation, Validation, Writing - original draft. Javin Taslem Mourousi: Validation, Writing - original draft. Jakir Hossain: Resources. Md. Golam Rabbane: Resources. Md. Masuder Rahman: Formal analysis. Tohura Tahsin: Writing - review & editing. Md. Nazmul Hasan: Formal analysis. Manik Chandra Shill: Validation. Mohammad Jakir Hosen: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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