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Evaluating the role of waterborne silver in Zebrafish (Danio Rerio) development

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ABSTRACT Silver concentrations in the aquatic ecosystem may elevate as a consequence of anthropogenic action along with natural leaching from silver sites. Salts of Ag, like AgNO3, are soluble and separate into free ions Ag+ that are famed to be harmful to adult fish's ion-regulatory systems. Less is known about the impacts on fish embryos and their progression. The study's target was to first examine how dissolved silver affected zebrafish Survival rate and hatchability, also the study comprehend the sublethal reactions linked to osmoregulatory disruption (Na+K+-ATPase, electrolytes level), the oxidative stress parameter total glutathione (GSH), cardiac development (nkx2.5 gene), and defence opposed to toxic metal (evaluated the metallothionein Mt2 expression). Embryos with age less than 1 hpf have been subjected to (0) Ag (without adding silver), 2.5, 5, 7.5, 10, and 15 µg/L-1 AgNO3 for up to 72 hr. Even though embryo's survival rate wasn't significantly influences by mounting concentrations of total Ag, a fall off hatching rate and increment in heartbeat were detected ($p<0.05$ by ANOVA test). Living and dead embryos have been collected at both 24 & 72 hpf to estimate the concentrations of silver, metal and biochemistry. Using ANOVA analysis, it was also detected a significant under probability 0.05 increment in embryonic Ag in living embryo at both 24 as well 72 hpf, along with 24 h dead embryos. Besides, using two way ANOVA analysis, it had been detected more significant ($p<0.05$) accumulation in 24 hpf living embryos. Dead embryo as well living one at 72h exposing to Ag possessed significantly ($p<0.05$) lower Sodium and Potassium levels. Also, live embryos exhibited a transient significantly ($p<0.05$) increment in Calcium concentrations at 24 h. Although, there were non-significant impact of Ag on Na+K+-ATPase efficacy, Mt2, total GSH concentrations in 24h and 72h embryos, but 4, 4, and 4 folds significantly ($p<0.05$) increment respectively were showed in the non-exposed 72h aged embryos versus non-exposed 24h aged embryos. Conversely, nkx 2.5 gene expression significantly ($P<0.05$) reduced by 1-fold at 24h aged embryos as compare with control group. While, nkx2.5 reduce 2-fold in 72h non-exposed embryos in comparison with 24h non-exposed embryos.

KEYWORDS zebra fish (Danio rerio) embryos, early embryonic development, heavy metals, silver toxicity, Na+K+-ATPase, glutathione, cardiac genes nkx2.5, Mt2 biomarker

1. INTRODUCTION

Silver is normally found in the natural environment and levels can be enhanced in some anthropogenic operations, such as mining and the production of photographic foils. Silver nanoparticles (Ag-NPs) are increasingly incorporated into numerous retail products worldwide for their antibacterial and antifungal capacity which may allow for the liberation of free silver ions (Ag^+) [1], [2]. The silver's ionic form (Ag^+) is bioavailable and excessively poisonous to aquatic organism [3], [4]; yet, the complexes of Ag^+ with chloride ion had a considerable less toxicity. As a result, freshwater organisms are more vulnerable to the toxicity of silver than are those that live in saltier habitats such as seawater and estuaries or in water with greater concentrations of other Ag^+ complex agents such as dissolved organic matter (DOM) and sulphides [3], which binding with Ag^+ and remove its availability as well as toxicity [5].

Fish at the early stages of phase history, being sensitize to silver versus those older ones [6]. Rising mortality as well delayed/lessen hatching rate were often apprised although the severity of these impacts differ in accord to species of fish and silver concentrations. Embryos of Rainbow trout exhibited increased mortality (about 56%) at day 32 post-fertilization in the course of long-term silver exposure [7], whilst, in acute exposure the mortality rate had reached to 100% from 8 to 17 day's post-fertilization [8]. Detain hatching and diminished survival rate were liked to water's Ag concentrations [9].

In the course of the embryonic development of vertebrate, the firstly organ that developing and functioning heart is [10], [11], thus it is being a target organ to examine the metal poisoning [12]–[14]. Nkx2.5 is a key gene that required to development of heart, as well it seems to be engaged with trig-

gering myocardial cells differentiation at early phase of *Danio rerio* embryonic development [15]. It's well-established that metals impede the leverage of Na^+ K^+-ATPase in mature aquatic fauna, disrupting the balance of osmoregulatory function and electrolyte. This disruption leads to cardiac complications and, eventually, mortality of those aquatic creatures [16]–[18].

Furthermore, Na^+ pump seems to be required for cardiac laterality and cardiomyocytes variation at earliest stages of embryonic evolution [19]. Given the information gap on impacts of metals, especially Ag, on early life phase of zebrafish, the present investigation aims to inspect this potentially fruitful research zone to better understanding for the influence of Ag on the development of heart of *Danio rerio*. The study's objectives are to: (1) estimate the toxic effects of exposure to waterborne silver ions on zebrafish survival, hatch, and cardiac functions in their earliest life stages; (2) examine whether early exposure to silver ions is correlated with osmoregulatory system weakness (Na^+K^+-ATPase & electrolytes balance); and (3) ascertain effects of Ag ions on *mt2* stimulation and *nkx2.5* expression, two important genes that are crucial for cardiac development.

2. METHODOLOGY

2.1. Experimental animal model

Adult zebrafish their age ranged between 0.5-1.5 year have been taken from the zebrafish's research facility/University of Plymouth and let to form embryos for conduction our experiment. Facility's environment are 28 ^{o}C and 12h photoperiod, the adult fish have been held in a glass aquarium filled with re-circulating (10% daily replacement of water) and Plymouth's de-chlorinated water supply. The characteristics of aquarium water including pH, temperature, and oxygen content have been detected employing HACH HQ40d multi reader and the values were (mean±S.E.M, n = 6) pH, 8.36 ± 0.11; temperature, 28.17 ± 0.27 °C; and dissolved oxygen (D.O.), 7.44 ± 0.03 mg L^{-1}. Trace metals concentrations of the aquarium water were measured by inductively coupled plasma mass spectrometry (ICP-MS) and concentrations (mean mg/L^{-1}±SE, n= 6) of metal ion were Ag^+, 0.127 ± 0.070; Ca^{2+}, 15.333 ± 0.073; K^+, 0.819 ± 0.017; Na^+, 7.767 ± 0.093. Adult fish have been provided with *ad libitum* feeding of flake food (Tetramin) twise dialy, and once daily on *Artemia sp.* nauplii. Those fish initiated spawning, according to standard method referenced by Hill et al. [20], then all embryo were collected, cleanse from the debris, and utilized in the current investigation within 2h of fertilization.

2.2. Experimental design

The current study included conducting three experiments to determine the impacts of $AgNO_3$ on zebrafish's early life phases. The trials applied in a similar design with all silver exposures (0, 2.5, 5, 7.5, 10, and 15 µg/L^{-1}) replicated in three distinct beakers (400mL glass beaker possess 300mL of exposures water) and every one stock with 170 embryos. In every trial, exposure has been stopped at 72h post-fertilization (hpf) [exposures were from 2-72 h hpf]. 3% HNO_3 has been used to wash all glass wares before to start each trial. Stock solution (1g/L^{-1} $AgNO_3$) preparation includes dissolving about 1.577g of $AgNO_3$ (from the company of Sigma-Aldrich), in 1L of deionized H_2O. The nominal concentrations 0 (without adding $AgNO_3$), 2.5, 5, 7.5, 10, and 15 µg/L^{-1} Ag have been prepared from the stock solution before start to work. The exposure water has been refreshed every 24 hours. The actual (ICP-MS) concentrations (mean ± SE, N = 6 beaker) of the nominal silver concentrations in exposure at 1 h, 24 h and 72 h as follows: at 1st h were 0.03 ± 0.00, 2.43 ± 0.03, 4.71 ± 0.04, 7.06 ± 0.10, 9.07 ± 0.14, 14.24 ± 0.11, at 24th h 0.07 ± 0.00, 1.34 ± 0.04, 2.67± 0.13, 3.97 ± 0.15, 5.20 ± 0.09, 8.50 ± 0.12 and at 72nd h 0.05 ± 0.02, 0.80 ± 0.02, 1.53 ± 0.02, 2.45 ± 0.31, 3.51 ± 0.09, 5.98 ± 0.78 µg L^{-1}.

TABLE 1. Measured silver concentrations at time of exposure

Time (h)	Nominal Ag (µg,L^{-1})					
	0	2.5	5	7.5	10	15
1st h	0.03 ± 0.001^a	2.43 ± 0.03^a	4.71 ± 0.04^a	7.06 ± 0.10^a	9.07 ± 0.14^a	14.24 ± 0.11^a
24th h	0.07 ± 0.00^a	1.34 ± 0.04^b	2.67 ± 0.13^b	3.97 ± 0.15^b	5.20 ± 0.09^b	8.50 ± 0.12^b
72nd h	0.05 ± 0.02^a	0.80 ± 0.02^c	1.53 ± 0.02^c	2.45 ± 0.31^c	3.51 ± 0.09^c	5.98 ± 0.78^c

2.3. Trace metal determination

Trace metals determination in both living and dead zebrafish embryos has been applied in accord to method that references by Sheir and Handy [21]. 20 living embryos (with a typical wet weight of 20 embryos was 35 mg) along with 20 dead ones (typical wet weight was 31mg) had been collected in 1.5mL Eppendorf tubes washed by EDTA solution (0.2 mmol/L^{-1}). Specimens had been dehydrated at 70°C for 24h using a particular oven (Gallenkamp Model OV-160). Following drying the batches of 20 living and dead embryos were typically around 35 and 30mg, respectively. Whilst, averages of typical wet-weight and dry-weight of 10 living hatch embryos were 32 and 31mg, respectively. Each dried sample was digested in 0.5ml concentrated nitric acid (69% analytical grad, Fisher Scientific) for 2h at 60°C in a water bath, after that cooling and diluting to 3ml using Milli-Q H_2O. Specimens of embryos have been examined to determine Ag^+, Ca^{2+}, K^+, and Na^+ concentrations using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific Series 2, Hemel Hempstead, UK). This instrument has been calibrated employing mixed matrix-matched standards between (100-100000 µg/L^{-1}), which have been prepared from Arista ® plasma emission grade solutions. Throughout time of the analysis, the precision has been checked subsequent to each ten specimens by running a blank and standard. Where, the Nitric acid (25%) without adding any metal was utilized as a blank for instrument's calibration.

2.4. Biochemistry

Regarding to biochemical examination, specimens of embryos have been collected according to experimental design, they washed twice using deionized water, then weighed, and homogenized to readiness for the evaluation of GSH, total protein concentrations, in addition to Na^+-K^+-ATPase.

Briefly, 400µL of ice-cold buffer [20 mmol L^{-1} 4-(2-hydroxyl methyl) piperizine-1-ethane sulfonic acid (HEPES), 300mmol/L^{-1} sucrose and 0.1mmol/L^{-1} EDTA, pH 7.8] was added to each specimen, and the specimen has been sonicated for 10s (Misonix, Microson, Ultrasonic Cell Disrupture, XL, 20 levels) has been carried out to break down and homogenize the embryos. Specimens have been centrifuged [2 min 8000xg (10000 rpm)] to pellet the solid materials, and aliquots of the supernatant were distributed for subsequent glutathione, Na^+-K^+-ATPase, and total protein assays.

Total GSH has been determined in 20 µL of embryo homogenates in triplicate reactions (96-well plates), and each reaction contained 300µL buffer with final concentration of 76.5 mmol/L^{-1} phosphate buffer (pH 7.5), 3.8 mmol/L^{-1} EDTA, GSH reductase (0.12 U/mL), 20µL of buffered 10mmol/L^{-1} DTNB (5-5'-ditbiobis-(2-nitrobenzoic acid). After equilibration for one minute, 20µL of 3.63mmol/L^{-1} NADPH has been add up to every well to start the reaction, and alterations of absorbance rate have been read at 412nm through a time of fifteen minutes at thirty second intervals in a plate reader (Molecular Devices, USA).

The assay of Na^+K^+-ATPase has been done in accord to the modification of Bonting et al. [22] assay by Silva et al. [23]. This assay basically bases on the releasing of inorganic phosphate from ATP in 40µL of embryonic homogenate after 20 min incubation with/without 1mmol/L^{-1} ouabain (a Na^+ K^+-ATPase inhibitor). Forty µl of the embryonic homogeneous sample was used and the reaction has been terminated by adding of 1mL of cold trichloroacetic acid (8.6% w/v of trichloroacetic acid/100ml deionized H_2O) to every tube, after that adding 1mL of coloured reagent (9.6% w/v $FeSO_4.6H_2O$, 1.15% w/v ammonium heptamolybdate dissolved in 0.66M sulfuric acid). Absorbance at 660 nm (JENWAY, 7315 Spectrophotometer) of samples relative to standards was used to determine Na^+K^+-ATPase activity.

2.5. Gene expression analysis

Each sample consisted of 100 embryos collected at 16 hpf were used for gene expression analyses. Briefly, total RNA of zebrafish embryos have been extracted according to the manufacturer's instructions (mini kit for animal tissue by Qiagen), and frozen embryos were sonicated (Misonix, Microson XL, 20 levels) in 350 µL RLT buffer prior to pipetting onto a QiaShredder column and receiving a 15 min DNase treatment to remove DNA. The extracted RNA has been eluted into 30µL of RNase-free water and the spectrophotometer (Nano Drop, ND-1000 spectrophotometer) has been used to detect quantity as well quality of these extracted RNA. For cDNA synthesis, the specimens have been diluted to 100ng/µL^{-1} total RNA, and 800ng were utilized to synthesize the cDNA by following the protocol of manufacturer of ImProm-llTM Reverse Transcription system (Promega), with Hexanucleotide primers and deoxynucleotide mix (Sigma-Aldrich). All the cDNA samples were stored at -80°C prior to relative quantification of transcript numbers by q-PCR.

2.6. Quantitative reverse transcriptase PCR (qRT-PCR)

Primer Blast (NCBI) was used to assist the selection of the primers for *nkx2.5*, and primers (Table 1) for zebrafish metallothionein (*mt2*) and the reference gene β actin (β actin) were depended on our previous study [24]. Lyophilised primers (Eurofins MWG Operon, Ebensburg, Germany) have been reconstituted using RNase-free water to 100µmol, then combined with SYBR Green JumpStart Taq Ready Mix to obtain the total volume (20 µL) final reaction concentration of 375 nmol. Step One Real-Time PCR System, Applied Biosystems was used for the detection of fluorescence over 40 cycles with 94°C for denaturing, 55-60°C for primer-specific annealing, and 72°C for extension. The cycle threshold 25,000 had set for analysis of all q-PCR runs, and a standard curve generated from dilution of cDNA template was run on each plate for the normalisation among different q-PCR runs. Relative fold variations of genes expression have been calculated according to delta-delta CT (2-$\Delta\Delta$Ct) method with the delta CT obtained from the difference in CT of the target gene and the reference gene in the unexposed control embryos [24].

2.7. Statistical analysis

The results were analysed using STAGRAPHICS 5.1 (Statistical Graphic Corp, USA). Dependent variables have been modelled in accord to independent variables Ag concentrations (continuous variables), data followed the normal distribution and variance homogeneous, two-way ANOVA analysis was applied to calculate the differences between the independent variables with inclusive of the relevant interaction term, which if it is being significant, simple impacts of levels of single independent variables have been checked out using one-way ANOVA analysis. P values of < 0.05 have been considered as significant. The data are presented as mean standard error (SE). To calculate the fold-changes of nkx2.5 and mt2 with normalization to β- actin, a comparative quantification (2-$\Delta\Delta Ct$) had applied [24].

3. RESULTS

3.1. Impacts of silver on embryonic survival

The exposure 24h to silver revealed non-significant ($p > 0.05$) impacts on the mortality rate of embryos as compared with unexposed controls, as shown in Figure: 1A.

And when the experiment is over (72 hpf) the successful hatching of all studied groups have been determined (Figure: 1B). And after 72 hpf of exposure the embryos to silver (1 – 72 hpf) the hatching rate decreased in conjunction with the increase in silver concentrations. Embryos that were subject to 10 and 15μg/L^{-1} silver showed a significant ($p < 0.05$) decreased hatching rate as compared with the others (Figure: 1 B). Regarding to heart rates, they were estimated at 36 hpf when the heart has typically developed. Using 10 and 15μg/L^{-1} of silver led to a significant ($p<0.05$) increment in the rate of heart at 36 hpf as compared with unexposed controls and all other groups, (Figure. 1C).

TABLE 2. Specific primers for the studied genes in zebrafish with reference sequence number from NCBI and product length (bp)

Genes	Reference seq. No.	Forward	Reverse	Product (bp)	Annealing (°C)
nkx2.5	NM_131421.1	AGTTCTCTTCTCTCAGGCGCAG	TGGCACAGAGATGCGTCTCGGA	223	58
mt2	NM_001131053.2	CTGCGAATGTGCCAAGACTGGAAC	GCGATGCAAAACGCAGACGT	243	59
β-actin	NM_131031.1	ACACAGCCATGGATGAGGAAATCG	TCACTCCCTGATGTCTGGGTCGT	138	55

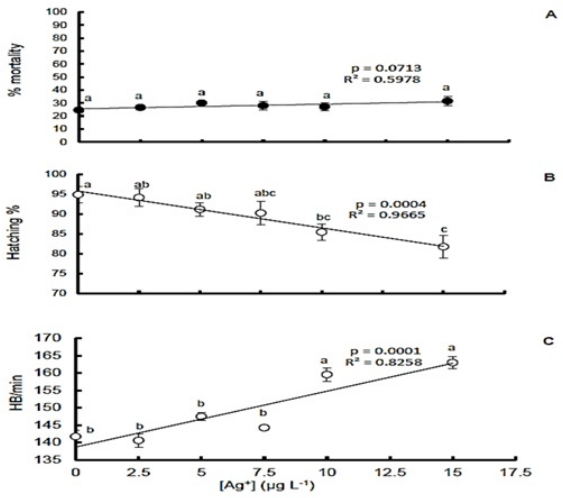

FIGURE 1. Silver is applied to embryos as $AgNO_3$. 170 embryos were exposed in a glass container for the controls and each treatment, with six replicates for each condition. At 24 hpf, the mean mortality (SE) of the embryos (A) in each container was measured. The number of hatched embryos (B) at 72 hpf was calculated as a percentage of the embryos that survived to that point. While at 36 hpf, the mean heart rate (SE) of nine embryos (C) from each container was measured. For each treatment, there are six beakers, and the data are means ± S.E.M. Statistical differences between concentrations are indicated by different letters (ANOVA, $p < 0.05$). Statistically, non-significant impact is shown by similar letters (ANOVA, $p > 0.05$)

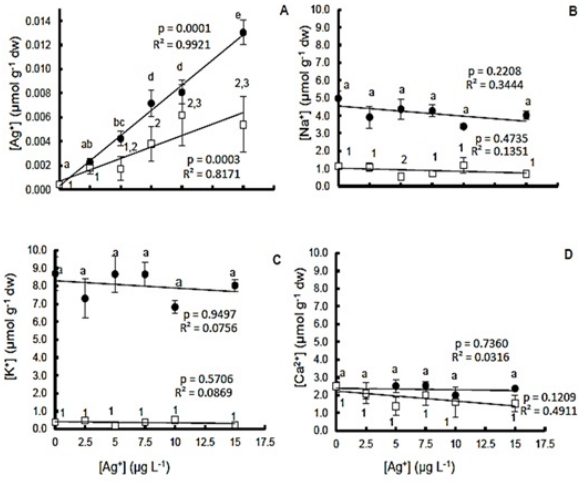

FIGURE 2. Metal levels [μmol metal g^{-1} dry weight for embryo (±SE)]. Live embryos have the circle symbol, while dead embryos have the square symbol. Zebrafish embryos at 24 hpf relative to the nominal total Ag exposure concentrations. Measured silver (A), sodium (B), potassium (C), and calcium (D) concentrations were assessed in embryos exposed to $AgNO_3$. Each exposure condition had six independent repeats, and each one contained 170 embryos at the beginning of the exposure. The total number of embryos analysed for each condition was (20). Different letters and numbers refer to statistical differences between concentrations (ANOVA, $p < 0.05$). Similar letters and numbers represent non-statistically significant differences between concentrations (ANOVA, $p > 0.05$)

3.2. Embryonic metal levels

Metals in embryos were assessed using ICP-MS. A significant ($p<0.05$) raise in embryonic Ag concentrations in both 24h living & dead as well as 72h living embryos was observed as the Ag concentration increased. These increments were more obvious in 24h living embryos versus 24h dead embryos as well as 72h living ones (Figure: 2A, and Figure: 3A), respectively. A significant ($p < 0.05$) reduction of Na^+, K^+, and Ca^{2+} levels was observed in 24h dead and 72h living embryos. This reduction was more evident in dead embryos (Figure: 2B, C, D, and Figure: 3B, C, and D). This reduction was greatly obvious in dead embryos compared to 72h living hatch ones.

3.3. Biochemistry

Despite embryos having increased their Ag concentrations in 24h and 72h groups (Figure 2A), exposure to varied silver levels demonstrated non-significant impacts on Na^+ K^+-ATPase efficacy in 24h and 72h groups (Figure: 4A). As well, GSH has been evaluated in the groups of embryos, but the results of statistical analysis revealed non-significant ($P>0.05$) impacts of Ag on GSH in 24h and 72h groups (Figure: 4B). Nevertheless, the raised Ag concentrations revealed no considerable impact on Na^+ K^+-ATPase and GSH in both 24h and 72h groups (Figure: 5 A&B). The considerable ($p<0.05$) impact of $AgNO_3$ on Na^+ K^+-ATPase and glutathione was found in 72h (Figure: 4 A and B) compared with the 24h group.

3.4. Gene expression

mt2 expression significantly ($p<0.05$) reduced by escalating Ag^+ in 24h embryos (Figure: 5A), while mt2 expressions showed non-significant ($p>0.05$) differences (Figure: 5A) in 72h hatched embryos with raised silver concentrations. Conversely, nkx2.5 expression reduced significantly ($p<0.05$, Figure: 5B) in 24h embryos as the silver concentrations increased, while no impact was found in 72h embryos. However, a significant ($p<0.05$) increment in the expressions of mt2 (4-fold) as well as a reduction in the expression of nkx2.5 (3-fold) was recorded in unexposed 72h hatched embryos compared to unexposed 24h ones (Figure: 5A and B).

4. DISCUSSION

4.1. Mortalities and embryonic Ag concentration

Embryonic subjection to Ag levels for 24h didn't demonstrated any significance regarding impacts on mortality, in

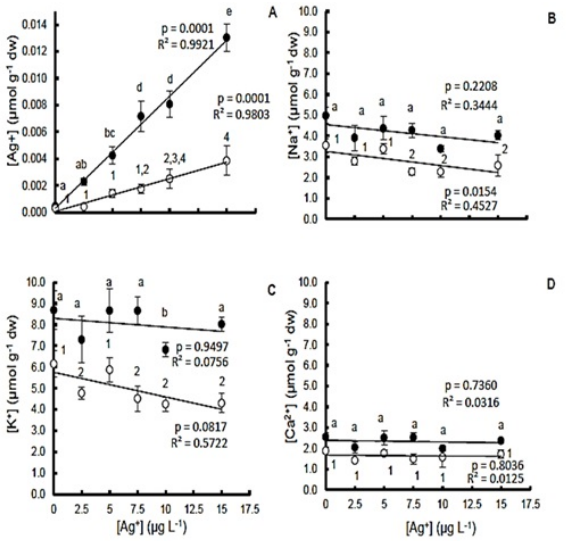

FIGURE 3. **Metal concentrations in living embryos at 24 hpf pointed by a solid circle symbol and 72 hpf with an open circle. In relation to Ag^+ exposure concentrations are expressed as [μmol metal g^{-1} dry weight of the embryo ($\pm$SE)]. Embryos exposed to Ag^+ had their concentrations of Ag (A), Na^+ (B), K^+ (C), and Ca^+ (D) measured. There were six separate repeats for each exposure condition, and at the start of the exposure, each one had 170 embryos. Twenty live embryos were examined for each condition. Statistical differences between concentrations are indicated by different letters and numbers (ANOVA, $p < 0.05$). No statistically significant changes between concentrations are indicated by similar letters and numbers (ANOVA, $p > 0.05$)**

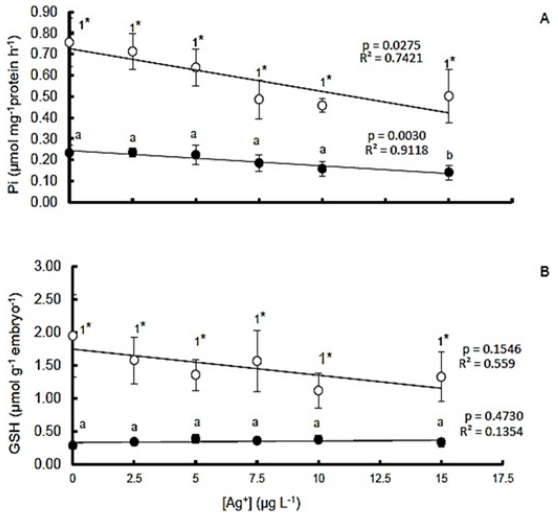

FIGURE 4. **Average Na^+ K^+-ATPase (A), GSH (B) in living embryos at 24h and 72 hours post-exposure to silver (solid and open circles, respectively). Each replication has 170 embryos, and the results are means$\pm$SE (n=6). However, the ANOVA table test revealed that Ag had no statistically significant influence on total GSH levels or Na^+ K^+-ATPase activity (p>0.05). LSD significantly affected Na^+ K^+-ATPase at 10μg/L^{-1} versus control. *Mean that embryos aged 24 and 72 hours differ significantly (p<0.05)**

spite of the deady embryos manifested an augment in Ag concentrations compared with controls. Initial phases of embry-

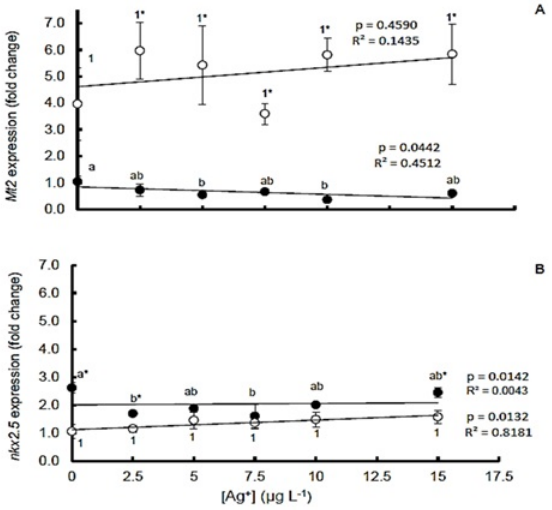

FIGURE 5. **Relation to silver concentrations: (A) the expression of mt2; (B) nkx2.5. Solid circle embryos were sampled at 24 hpf, open circle embryos were sampled at 72 hpf. The 2-$^{\prime\prime}Ct$ technique was utilized to calculate fold changes in gene expression, using β-actin as a housekeeping gene. Each treatment has three samples, and the data are means $\pm$ S.E.M. ANOVA under p<0.05 shows that there is a significant effect between the concentrations when different letters appear. A non-considerable impact (p>0.05) is indicated by the same numbers. * Explains how 24- and 72-hour-aged embryos differ from one another**

onic development, especially blastula and gastrula were deem susceptible to innumerable stress and representing phases when solidify of chorion happens [25]. Although, chorion's hardness executes crucial roles in the embryonic shielding from toxicants, involving of dissolved metals [26], resultant chorion swelling may help in transferring of toxicant via changing permeability of vitelline membranes to the poisonous metal causing disruption of cations exchanging between peri-vitelline fluid and H_2O [27]. The disruption of ions regulation and electrolytes disparity are potential causes of embryo death as in mature fish [3], [8].

Severe exposures of rainbows trout embryos to 0.11, 1.55 up to 14.15 $\mu g/L^{-1}$ Ag for five days showed non-significantly impacts on embryo's death-rate [8]. Chronic exposures of rainbows trout embryos to silver con. of 0.117, 1.22, for 32 days revealed no impacts on embryo's death-rate, while, this rate attained 56% by day 32 postfertilization in embryos when subjected to 13.51$\mu g/L^{-1}$ for the alike time [7].

The increment of embryonic Ag is more distinct in 24h living embryos versus 24h deady and 72h living hatched embryos (Figure: 2A and 3A). This increment revealed that 24h living embryos were adept to adjust the silver accumulation more than others. The death of embryos as a consequence of directly passively ions binding or as a result of absorbing higher levels of Ag when alive. Silver's gathering within the tissues maybe as result of water absorbing by peri-vitelline spaces brings about swelling of chorion that enhance influx of H_2O and ion during the initial period postfertilization [28], [29]. Nevertheless, existence of $-SH$ groups as a component

of chorionic protein enabling it to be binding to metals cation like Ag^+, Cu^+ and Hg^+ as Na^+ antagonist and aid the entry of Ag and others ion into the embryo [30]–[32].

4.2. Hatching

Embryos subjected to 10 as well 15 $\mu g/L^{-1}$ of silver for 72h revealed more noticeable delays hatching versus others group. These delays hatching was reported in a previous investigation on embryos of *Danio rerio* that subjected to 1μM of silver for five dpf [9]. It is generally established that hatches mechanism is a series of enzymes and mechanical impacts that started by secreting of chorionase, which break apart the zona interna of chorion over the hatches [33]–[35]. The decline and retardation of growth, along with the disruption of synthesis and secretions of this enzyme leading to decompose the zona interna of chorion, and lead to lessen and delay hatching [33], [34]. Also, retardation of embryonic development as a result to metals subjection was proposed as a prime cause to delay hatching [33], [36]. Decelerate progression of embryo would as well prolonged the period at which embryo being moving, where embryo's motion (e.g., twisting) are needed to break down and open chorionic's zona externa [37].

4.3. Heart rate

Embryos subjected to Ag for 72h demonstrated an augment in heart rates, which are obvious in embryos subjected to 10 & 15$\mu g/L^{-1}$ (Figure: 1C). This augment may be due to poisonous impacts of metals. Fish are adept to endure with disturbances and stressor like the physical and chemical stressors to conserve its homeostatic status (Barton, 2002) [38]. The augment may be linked to inhibit of Na^+ K^+-ATPase guiding to change in K^+: Na^+ ratio, which identifies by K^+ leak to extracellular fluids as well escalated intracellular Na^+. The alterations associated with a subsequent Ca^{2+} influx on the Na^+/Ca^{2+} exchangers, which guide to boost sarcolemma's depolarisations [39].

4.4. Electrolytes concentrations of embryos

Decline in Ca2, K, and Na was showed in lifeless embryos 24h as well 72h living hatch ones (Figure: 2 and 3, respectively). This decline may be because disruption of osmoregulatory. It is probable due to the impacts of silver on ions regulation in living 72h hatch embryo, and due to passively ions exchange in 24h lifeless ones. Nevertheless, electrolytes leakage post-mortem is usually happened and come about as a passive losing by spreading to Sweetwater compared the passive entry of silver by spreading into deady embryos. Conversely, Ca reduction may be take place as a result of releasing of calcium from calcium release channel and Ca^{2+}-ATPase, which were impeded by reaction of silver with−SH group [40].

4.5. Impacts of Ag on Sodium-Potassium-ATPase, GSH and mt2

Despite the increment of Ag levels in embryo, non-significantly impairs of Sodium-Potassium-ATPase has been observed in embryo subjected to Ag for 24h, and 72h (Figure: 4 A). Proteomics analysis of embryonic homogenate for those subjected to 5 and 15 $\mu g/L^{-1}$ Ag for 24h had been revealed inducing of ATP synthase subunit alpha and beta with escalated silver levels to help in synthesis or replacement the deteriorate Sodium-Potassium Pump. In mature fish the deterioration of the Sodium-Potassium Pump is a principle pathway of Ag toxicity as well for other poisonous metal [41]–[44].

The numbers, locations, and well-developing ionocyte that are abundant with Sodium-Potassium-ATPase execute key roles in controlling of osmoregulation. The existences of ionocyte (chloride cell) present on yolk sac membranes & skin tegument deem as an alternative to the Non-fully formed osmo-regulatory organs as gill and kidney [45]–[48]. Ag as the other transition metals ions had ability to attach with −SH group of alpha subunit of Na^+K^+-ATPase molecules. The contestant between Ag^+ & Mg^{2+} on binding sites on alpha subunit of Sodium-Potassium-ATPase molecules block the hydrolysis of ATP as substantial step in Na^+K^+-ATPase activation [5], [16], [49]. The non-significantly impairs of Sodium-Potassium-ATPase activity in the course of developing of 24h embryos maybe justified on the base of the presence of the low and unwell developing ionocytes, though, there is an incremental maturing of ions transportance epithelia related with Na^+ pump [45], [50]. On the other hand, though there was non-significant impacts of Ag on Na^+K^+-ATPase activity in living 72h hatches embryos, the existence of higher number and well-developing ionocytes as a principle site for the osmoregulation justify the significant increment in Na^+K^+-ATPase activity as compared with living 24h embryos (Figure: 4 A).

Although, increment embryonic Ag concentrations (Figure: 2 A) at 24h and 72h embryos, it was observed non-significant induction for GSH (Figure 4B). Non-significant impacts on Na^+ pump and without considerable alterations in mt2 suggested there was no indication of oxidative damages in the current study. Conversely, boosted induction of GSH levels in 72h hatched versus 24hrs embryos likely to be evidence to well-developing enzymatic system for GSH synthesis. Yet, the unalterable in GSH levels maybe because the exposed to small concentrations of Ag, which being down threshold to prompt the inducement of GSH & mt2 [51].

4.6. Mt2 expression

Mt2 expression has been reduced in 24h embryos as compared with controls (Figure: 5 A), despite the increment of Ag accumulation in embryos (Figure: 2 A). The expressions maybe interfered with existence of higher mother's Mt2 that interacted with accumulating of embryonic Ag. The exposures to silver for 72h didn't show significant inducing in the expression of mt2 despite the increment of embryonic Ag accumulation and decrease quantity of maternal Mt2 at this

phase of embryonic development [52]. The nominal concentrations and increment embryonic Ag maybe not adequate to stimulate mt2 expressions. It is well established that threshold of metal levels in aquatic organisms should be enough to stimulate the induction of Mt2 and GSH [51].

4.7. nkx2.5 expression

Up to 24h exposing to Ag was demonstrated a significant reduce in nkx2.5 expressions, whereas, non-significant impact revealed in embryos subjected to Ag up to 72h (Figure: 5 B). The increment embryonic Ag ions concentrations may execute as an inhibitor to RNA-binding protein, which deem as a critical determinant in RNA transcription. As Ag ions have ability to bind with −SH groups of several enzymes, leading to hindrance the proteins functions [53], [54]. Conversely, cysteine, histidine (C2H2) zinc finger protein act as a principle cofactor to enhance efficacy of RNA polymerase and adept to change genes expressions via linking by competing with zinc in their site on transcription factors [55]. Escalated embryonic Ag accumulation might compete with zinc at the binding site, or interacting with thiol group of cysteine, histidine (C2H2) zinc finger protein, leading to inhibit the catalytic function of zinc, thus impede genes expressions. Reduction of nkx2.5 expression in 72h or hatch embryos versus to 24h aged embryos (Figure: 5B), assured that gene activity at this age isn't necessary as a result of complete the differentiation of cardiac myocytes.

5. CONCLUSION

Results of the present work revealed that earliest life phases of zebrafishes are sensitized to Ag found in water. 24h living embryos accrue more silver than 72h hatched and 24h dead embryos, which begin to display Ag saturation. Living embryos with 24h age also possess higher K^+ and Na^+ cons. compared with lifeless and 72h living hatch embryos. However, this could be an indication of greater amounts of these ions in the yolk rather than enhanced ionoregulatory ability of 24 hour-old embryos, and indicates that osmoregulatory toxicity was unlikely. The present study demonstrates that silver has no or little effect on mt2 mRNA expression. The expressions of nkx2.5 genes were affected by silver exposure during the 24 hpf (early stage of embryonic development), whereas, not affected at 72 hpf (hatched embryos), which indicate the importance of nkx2.5 gene for forming and developing of heart throughout the earliest phase of embryonic development. Proteomics analysis is a valuable tool to investigate the environmental risks that are bring about by heavy metal or other pollutants.

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