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Evaluation of behavioural alterations in zebrafish following exposure to silver nitrate

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Abstract
Increasing in the concentration of silver in the environment affects the health of ecosystem including human and animal health. The present study was carried out to evaluate the effect of silver nitrate on behavior of zebrafish following exposure to various concentrations for 28 days. Total 30 zebrafish were divided into 5 groups. Animals of four toxicity groups were exposed to silver nitrate at various concentrations (6.25, 12.5, 25 and 50 µg/L). No mortality was found in animals after exposure of silver nitrate. Fish exposed to a higher concentration exhibited altered motility with slow speed of swimming, freezing and operculum movement followed by fast movement. In adult zebrafish, higher silver nitrate concentrations (25 and 50 µg/L) caused neurobehavioural changes such as hypolocomotion, anxiety-like behaviour, diminished social preferences, and decreased social recognition.

Keywords: Silver nitrate, zebrafish, behavioural alterations

Introduction

One of the pollutants dispersed in wastewater is silver. Many freshwater and marine invertebrates, including fish, are persistently poisoned by these metals. The type of silver ions affects toxicity of silver, particularly free silver ions, which have the highest potential for toxicity (Ratte, 1999) [14]. It is well known that aquatic organisms are severely harmed by silver in its ionic state. But according to a previous report, colloidal silver, which is a kind of nanosilver, has been used for more than a century and is recognized as a biocidal substance (Nowack *et al.* 2011) [9]. When Ag is released into the environment, there is a risk to both the environment and human health. Ag is also included in cosmetics, cleaning goods, detergents, and personal care items, and when these products are used or disposed of, these compounds can immediately goes into the environment. Since soluble silver compounds are more easily absorbed than metallic or insoluble silver, they may have a negative impact on a person's health. Humans who are overexposed to silver ions experience convulsions, gastrointestinal damage, stomach pain, and diarrhoea as acute symptoms. The most frequent side effects of continuous exposure to silver in people include pigmentation of the skin and mucous membranes, which permanently turn grey or bluish-gray, and eye discoloration (Fung and Bowen, 1996) [3].

The connection between an animal's internal physiology and its external environment results in altered animal behaviour. Fish survival, growth, and reproduction are all impacted by ecological risks, and toxicant-induced behavioural impairments, which frequently lead to underlying physiological abnormalities that can be used to evaluate these risks (Pyle and Ford, 2017) [11]. Adult fish display more sophisticated activities (such as learning, memory, and cognition) compared to embryonic stage, which may reveal additional details about neurotoxicity in aquatic animals. Ag can also harm tissue and accumulated in the liver tissue, which can impair fish's resistance to low oxygen levels and induce oxidative stress. To assess how different contaminants affect aquatic organisms' behavioural paradigms, behavioural tests have been employed extensively.

The greater ecotoxicological objective of appreciating the impact of eco-toxicants on fish population is supported by knowledge of the effects of toxicants on fish. It is not well understood how silver exposure, both short-term and long-term, affects aquatic species' behaviour patterns. In order to assess the behavioural changes in zebrafish after exposure to silver nitrate, the current investigation was carried out.

Materials and Methods
Chemicals
Analytical grade silver nitrate ($AgNO_3$) was purchased from Himedia laboratory Pvt. Ltd., Mumbai.

Experimental animals and the environment
Adult female zebrafish (3-months old wild type) were procured from Vikrant Aquaculture Mumbai, India. The zebrafish were maintained as per standard stocking density of 5 fish/L of water. The fish were fed with fish pellets (Tetra bits complete®) 10 mg/fish twice a day. Before starting the experiment, acclimatization period of 2 weeks was observed and water quality parameters in each group were evaluated daily. The water temperature (25±3 °C) and photoperiod 10:14 h (dark: light cycle) were maintained in standard range throughout the experiment. Water used in the experiment has TDS of 50–100 mg/L; conductivity of 500±10 S/cm) and pH of water 7.0±0.2. Water was renewed in each tank daily.

Experimental design
Total of 30 zebrafish were randomly divided into 5 groups. Zebrafish of the control group were kept in normal RO water. Zebrafish of 2nd, 3rd, 4thand 5thgroup were exposed to 6.25, 12.5, 25 and 50 µg/L of silver nitrate, respectively for 28 days. During the experiment, fresh stock solution of silver nitrate was prepared by dissolving 0.1 mg of $AgNO_3$ in 1 mL milli-Q water and then diluted in the tank as per required concentration. Accurate weighing of silver nitrate was done using precise analytical weighing balance Mettler Toledo (MS 204S/A01 Mettler Toledo, Mumbai, India). The concentration of silver nitrate in tank was maintained by changing the water daily with fresh water containing silver nitrate. Behaviour changes in adult zebrafish were evaluated on7th, 14th, 21st and 28th daysof the experiment.

Evaluation of behavioural alterations
The light-dark preference test, novel tank test, social preference test, and social recognition test were utilized to assess the behavioural changes (Tran and Gerlai, 2016, Ogi *et al.* 2021, and Madeira and Oliveira, 2017) [13, 10, 7].

Statistical Analysis
Statistical analyses of all data were carried out using GraphPad prism 9.0. Kolmogorov-Smirnov test was used to evaluate the normality of data along with Bartlett's test to confirm the equal variance. Data with normal distribution and homogeneous variance were analyzed by parametric one-way analysis of variance (ANOVA) followed by Tukey's HSD test. The data that didn't have either normal distribution or equal variances were analyzed by Kruskal-Wallis test followed by Dunn's test. Where *$p<0.05$ was considered as statistically significant and **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ were considered for highly statistical significant difference.

Results and Discussion
Mortality was not observed in fish exposed to different levels of $AgNO_3$. Fish exposed to a higher concentration displayed altered motility, including slower swimming. Comparing the higher concentration group to the low dose and control groups, freezing was seen with operculum movement followed by fast movement.

In novel tank test, the mean values of time spent (seconds) in the upper zone by zebrafish at 25 and 50 µg/L were significantly lower than those of the control group at 7th, 14th, 21st day (Figure 1 to 3). On 28th days, the mean values of time spent (seconds) in the upper zone by zebrafish at 50 µg/L were significantly lower followed by 6.25 µg/L compared to all other groups (Figure 4). While the mean number of entries in the upper zone by zebrafish exposed to 50 µg/L was much lower than that of the other groups, it was followed by 25 µg/L. Fish staying in the lower zone suggests that they are not being as adventurous.

In light dark preference test, the mean value of time spent on the dark side by zebrafish of 50 µg/L group was significantly higher than to all other groups. The mean value of the total no. of entries towards the dark side by zebrafish exposed to 25 and 50 µg/L was also significantly higher compared to all other groups at 7th, 14th, 21stand 28th day of experiment (Figure 5 to 8) which indicates anxiety-like behaviour and hypo-locomotory activity at higher exposure level.

In social preference test, the mean value of time spent on the conspecific zone by zebrafish of the 25 and 50 µg/L groups was significantly lower compared to all other groups. The mean value of the total no. of entries towards the conspecific zone by zebrafish exposed to 50 µg/L was significantly decreased compared to all the groups at 7th, 14th, 21st and 28thday of experiment (Figure 9 to 12).

In social recognition test, the mean values of time spent in the familiar zone by zebrafish of 50µg/L was significantly lower followed by 25 µg/L exposed group and other groups. The mean value of the total no. of entries in the familiar zone by zebrafish exposed to 50 µg/L was significantly lower followed by 25 µg/L compared to all the groups at 7th, 14th, 21stand 28thday of experiment (Figure 13 to 16) which indicates impairment of social recognition ability.

The potential for exposure to both ionic silver (Ag^+) and nanosilver in aquatic environments has increased as a result of the growing industrial and consumer use of nanosilver. A novel and promising method, neurobehavioral phenomics of zebrafish, blends behavioural phenotypes with numerous genetic and environmental variables (Kalueff et al. 2016) [5]. Animal behaviours are the result of the interaction between an animal's internal physiology and its external environment. Fish locomotor activity was shown to be affected and their physical fitness decreased at concentrations of 25 and 50 µg/L. Therefore, behavioural analysis was done, and the outcomes showed that $AgNO_3$ at 25 and 50 µg/L concentration had a neurotoxic effect on the social behaviours of adult zebrafish. In agreement with our findings, abnormal behaviour including agitation, fast, sudden movements, gulping of air, rolling motions, and swimming on the back were also observed at concentrations of 50 mg/L and 75 mg/L Ag-NPs in African catfish (*Clarias gariepinus*) (Sayed, 2016) [12]. Following exposure to 10 and 100 µg/L of silver for 15 days, Naguib et al. (2020) [8] reported anomalous behaviour such as hypo-locomotion and remained stationary in place. However, at 0.3, 1 and 3 ppm Ag-NPS, considerable hyperactivity was seen in larvae of zebrafish at 3 days post-fertilization (dpf) (Gonzalez *et al.* 2018) [4]. Similar neurobehavioral effects were seen in female mice after receiving silver nanoparticles at a level of 3 mg/kg body weight. NP-treated offspring spent more time engaging in stereotypical head-dipping behaviours and travelled farther with considerably less latency, and they were less likely to remain motionless throughout the testing period (Lyu et al. 2021) [6].

Zebrafish have a natural tendency to initially remain at the bottom of a novel environment (e.g., a test tank) and then gradually explore the higher portions of the test tank have proposed that height in the tank may be a useful measure of anxiety. One of the tests that is most frequently used to measure anxiety in preclinical investigations is the light-dark preference test. This test evaluates an animal's innate or spontaneous propensity to explore or avoid areas of a given environment. Previous research has found that this significantly altered locomotion and increased anxiety. There was a reduction in locomotor activity, and animals given the treatment spent more time in the bottom of the tank and less time in the top (Baldissarelli *et al.* 2012) [1]. Our findings demonstrated that silver nitrate has produced anxiety-inducing effect on zebrafish because the animals spent more time near the tank's bottom.

Zebrafish exhibit a phenotype that is quite sociable. The social preference test was used to determine whether exposure to $AgNO_3$ had any negative effects on the zebrafish's ability to interact with one another. The test is helpful in efforts to establish a reliable zebrafish model for neuropsychiatric and neurodevelopmental disorders in humans that are defined by social deficits as well as how a person reacts to or engages with social stimuli. Preference was affected in the fish exposed to $AgNO_3$ treatment. A social recognition is necessary to assess a person's capacity to recognise and remember specifics about other people. Fu et al. (2021) [21] carried out a similar experiment and found that control fish spent more time in the tank's familiar section than in the new one, while fish treated with $AgNO_3$ spent about the same amount of time in both the familiar and novel areas. These results suggest that exposure to 10 ppb of $AgNO_3$ results in impaired social preferences and social recognition.

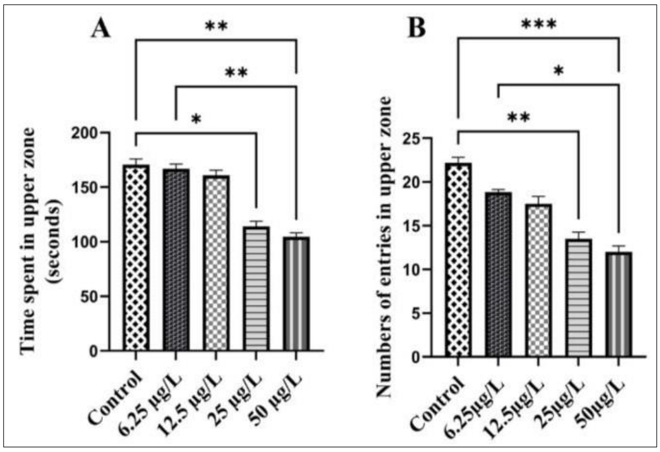

Fig 1: Effect of $AgNO_3$ on exploratory behaviour on day 7. (A) Time spent in upper zone (B) Number of entries in upper zone

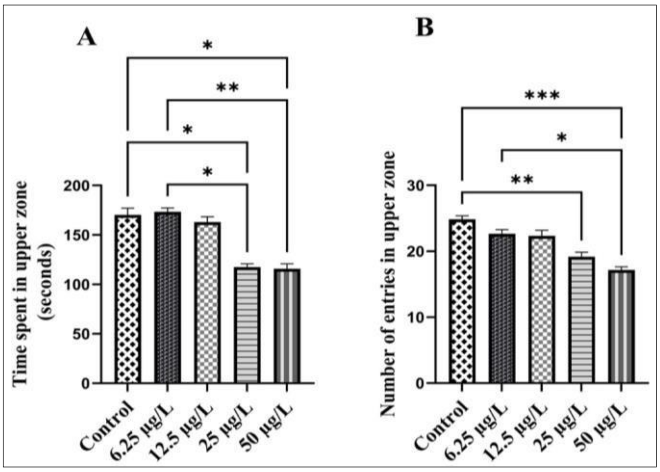

Fig 2: Effect of $AgNO_3$ on exploratory behaviour on day 14 (A) Time spent in upper zone (B) Number of entries in upper zone

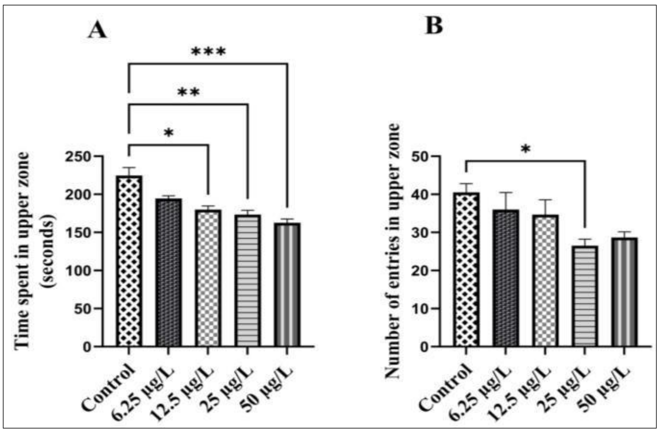

Fig 3: Effect of $AgNO_3$ on exploratory behaviour on day 21 (A)Time spent in upper zone(B) Number of entries in upper zone

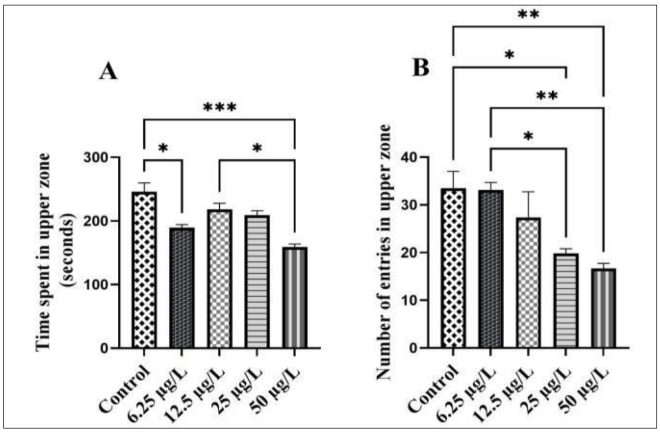

Fig 4: Effect of $AgNO_3$ on exploratory behaviour on day 28 (A)Time spent in upper zone (B) Number of entries in upper zone

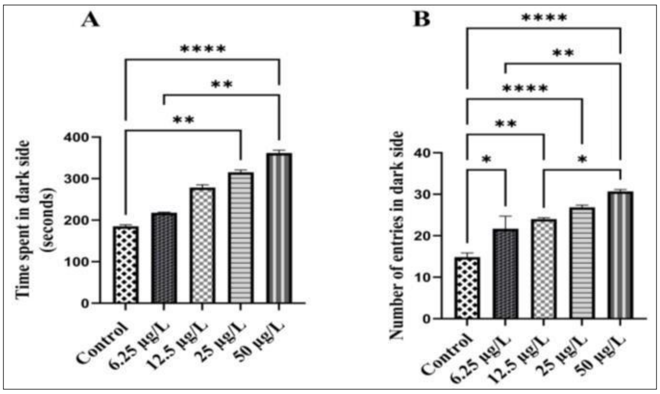

Fig 5: Effect of $AgNO_3$ on light dark preference on day 7 (A) Time spent in dark side (B) Number of entries in dark side

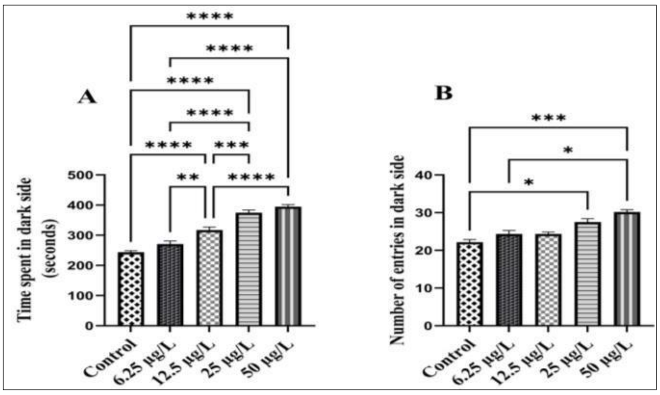

Fig 6: Effect of $AgNO_3$ on light dark preference on day 14 (A) Time spent in dark side (B) Number of entries in dark side.

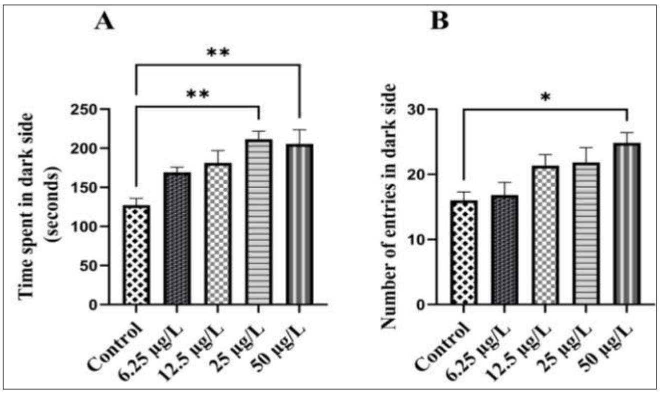

Fig 7: Effect of $AgNO_3$on light dark preference on day 21 (A) Time spent in dark side (B) Number of entries in dark side

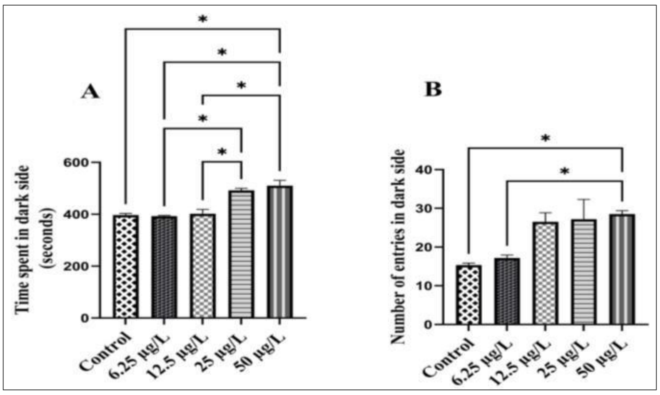

Fig 8: Effect of $AgNO_3$on light dark preference on day 28 (A) Time spent in dark side (B) Number of entries in dark side

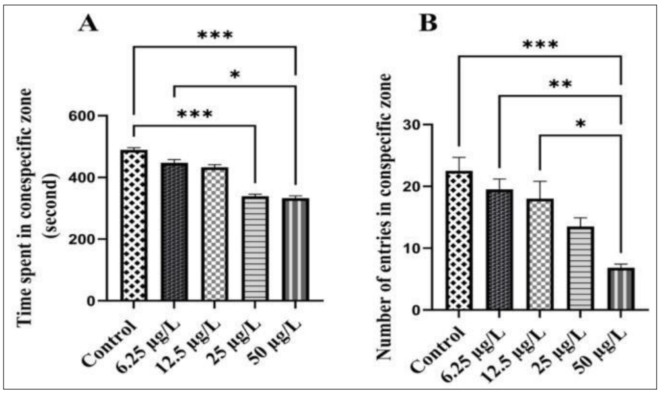

Fig 9: Effect of $AgNO_3$ on social preference on day 7 (A) Time spent in conspecific zone (B) Number of entries in conspecific zone

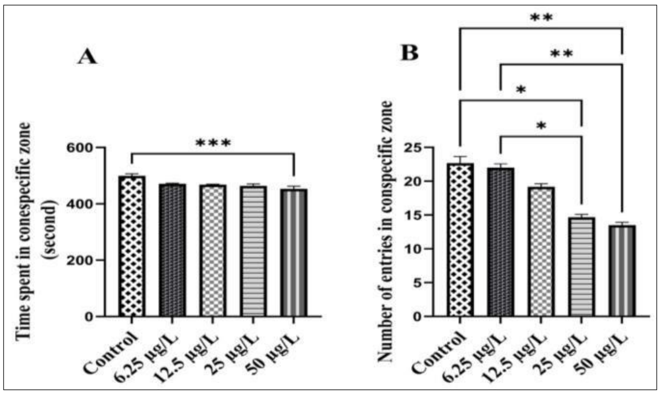

Fig 10: Effect of $AgNO_3$ on social preference on day 14 (A) Time spent in conspecific zone (B) Number of entries in conspecific zone

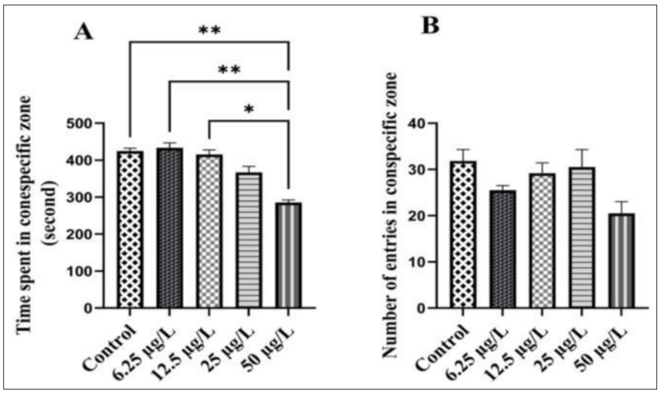

Fig 11: Effect of $AgNO_3$on social preference test on day 21 (A) Time spent in conspecific zone (B) Number of entries in conspecific zone

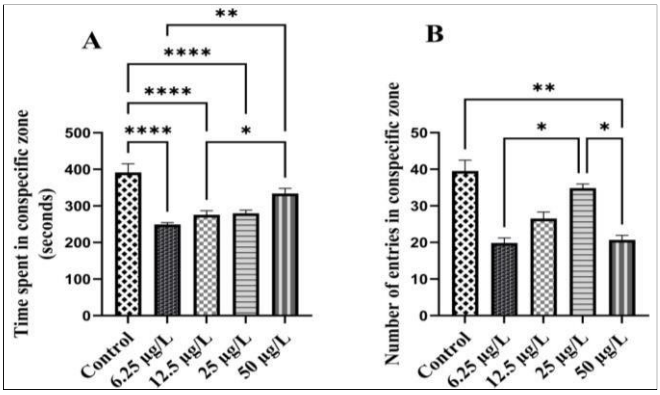

Fig 12: Effect of $AgNO_3$on social preference on day 28 (A) Time spent in conspecific zone (B) Number of entries in conspecific zone

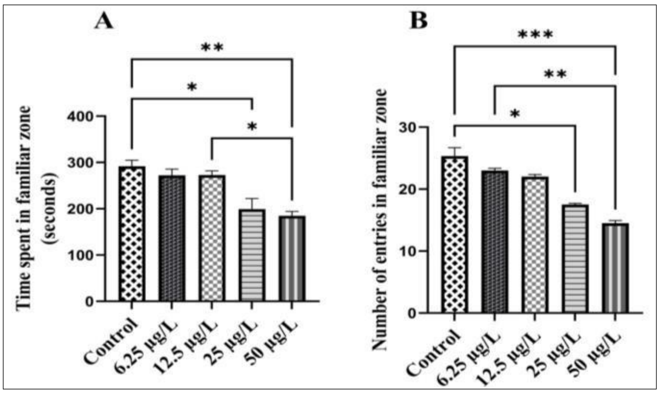

Fig 13: Effect of $AgNO_3$ on social recognition ability on day 7 (A) Time spent in familiar zone (B) Number of entries in familiar zone

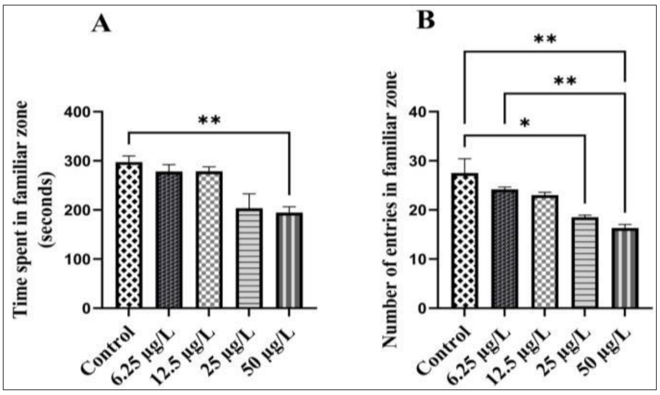

Fig 14: Effect of $AgNO_3$ on social recognition ability on day 14(A) Time spent in familiar zone (B) Number of entries in familiar zone

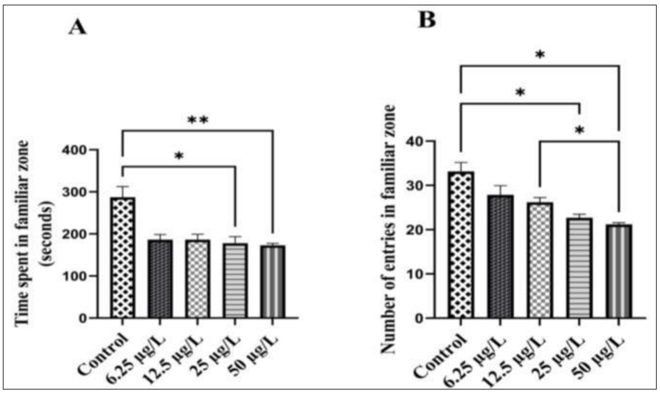

Fig 15: Effect of $AgNO_3$ on social recognition ability on day 21 (A) Time spent in familiar zone (B) Number of entries in familiar zone

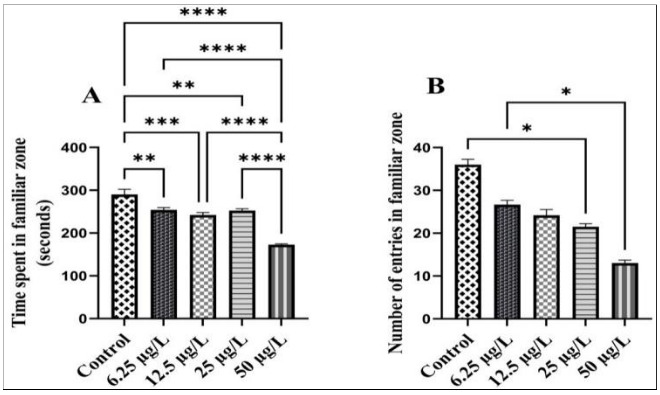

Fig 16: Effect of $AgNO_3$ on social recognition ability on day 28 (A) Time spent in familiar zone (B) Number of entries in familiar zone

Conclusion

Higher concentrations of silver nitrate led to neurobehavioural alterations in adult zebrafish, including hypo-locomotion, anxiety-like behaviour, reduced social preferences, and social recognition.

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Ethics approval and consent to participate: Experimental procedure was approved by the Institutional Animal Ethics Committee of the college.

Consent for publication: All the authors approved the manuscript.

Competing interests: The authors declare no competing interests.

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