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Research Article

HISTOPATHOLOGICAL EVALUATION OF BRAIN AND RETINA OF ADULT ZEBRAFISH EXPOSED TO SILVER NITRATE

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ABSTRACT: The purpose of the study was to assess the histological alterations in the adult zebrafish (*Danio rerio* L.) brain and retina after long-term exposure to silver nitrate ($AgNO_3$) at concentrations of 6.25, 12.5, 25, and 50 µg/L for 28 days. Although zebrafish subjected to varying concentrations of $AgNO_3$ did not die, they did demonstrate a progression in the anatomical abnormalities in their brains. Following exposure to silver nitrate at various concentrations for 28 days, mild to moderate pathological changes have been seen in the corpus cerebella, hypothalamus, lobuscaudalis cerebella, medulla, and dorsal telencephalic area of the opticum tectum in the brain, but there have been no discernible changes in the retina. It was concluded that silver nitrate has the potential to harm the nervous system.

Key words: Zebrafish, Silver nitrate, Histopathology, Brain, Retina.

INTRODUCTION

Pisciculture is becoming a very rapidly growing sector with good profits to the farmar in many parts of India. India is presently the second main aquaculture producer of the globe (Neglur *et al.* 2021). For better growth and prevention of diseases, many artificial materials are used in the inlad fisheries (Hoque *et al.* 2022). Along with them, many other synthetic chemicals are mixed with the water and so become available to the fishes.

Though silver is a rare metal in the crust of the earth (0.05-0.1 ppm), it is present in much larger proportions in ores when combined with other metals (Renner 2001). It is used to make jewellery, cutlery, and wristwatches due to its physical strength, malleability, and ductility. Silver halide crystals with light sensitivity are produced using it for consumer photography, graphic design, and radiography. Additionally, ionic silver and nanosilver's antibacterial qualities have expanded the spectrum of products in which they are used, including clothing, paints, plastics, food packaging, bandages, and wound dressings as well as household items like refrigerators and washing machines (Brumby *et al.* 2000, Benn and Westerhoff 2008).There are several applications for silver ions, which are a substantial environmental problem. There have been documented measurements of total silver concentrations of 300 g/L in steam wells, 8.9 g/L in saltwater, and 6.0 g/L in groundwater near a hazardous waste site (Howe and Dobson 2002). The current Rfd (reference dose) for oral silver exposure is 5 pg/kg/d, with a critical dose of 14 pg/kg/d for the average person.

Sulfide, chloride, and nitrate are the three forms of silver that are present in the environment. The toxicity of silver nitrate has been related to the presence of Ag^+, a free ionic silver ion. Other kinds of silver dissolved are significantly less hazardous (Leblanc *et al.* 1984). Although, there are many mechanisms and water features which reduce the toxicity of silver by reducing the production of free Ag^+, binding Ag^+, or preventing Ag^+ from adhering to reactive surfaces of organisms (Ratte 1999). Ag enters the fish and accumulates in the liver and blood plasma accompanied with the other internal anomalies including the reduction of plasma Na^+ and Cl^-

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metabolic acidosis, disruption of fluid volume regulation, hemoconcentration, and splenic constriction (Wood *et al.* 1996, Grosell *et al.* 2002).

Recently, the zebrafish (*Danio rerio* L.) has become more well-known as a reliable animal model for toxicological studies. Research on dangerous heavy metals, endocrine disruptors, and organic pollutants in the environment can be done on zebrafish. Zebrafish is a desirable model organism because of its many advantages, including its small size, short reproductive cycle, embryonic development outside of the uterus, and transparent embryos. All of these factors make zebrafish an appealing choice for quickly assessing the effects of suspect toxicants (Feitsma and Cuppen 2008, Pandya *et al.* 2021). The zebrafish genome is quite similar to the human genome (Howe *et al.* 2013). The adult zebrafish can also be utilised as a model animal for research into neurological diseases (Dai *et al.* 2014). Previous research has revealed that silver nitrate exposure produces neurotoxicity in adult zebrafish (Bilberg *et al.* 2012, Fu *et al.* 2021). However, little is known about the anatomical alterations that occur in aquatic species' brains and retina. With a focus on histopathological changes in the brain and retina, the current study was designed to evaluate the neurotoxicity potential of silver nitrate following exposure at various doses in adult zebrafish.

MATERIALS AND METHODS

Zebrafish housing

The animals were strictly maintained and treated according to guideline of Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. The experiment has been approved (Approval No: KU/JVC/IAEC/SA/81/2021) by the Institutional Animal Ethics Committee of College. Thirty healthy adult female zebrafish (4-5 months old wild type *Danio rerio*) were maintained at 5 fish/L as per standard stocking density. All fish were acclimated 15 days prior to experiment. The fish were fed with fish pellets (10 mg per fish twice a day) Tetra bits complete®. Standard water condition was maintained throughout the experiment with temperature (25 ± 3 °C) measured by water tester (WT018-1NO HiMedia Laboratories, Mumbai, India); 10:14 h (dark: light cycle) photoperiod; hardness (50-100 mg/L) measured by TDS meter (WT018-1NO HiMedia Laboratories, Mumbai, India); conductivity (500 ± 10 µS/cm) detected by digital water tester (HM Digital Inc., Hyderabad, India); and the pH of water (7 ± 0.2 pH) measured by pH meter (HI 8424 Hanna Instruments, Latina, Italy). Water was renewed every-day and monitored regularly.

Experimental design

A total of thirty zebrafish were randomly divided into five treatment groups and were exposed to different concentrations of silver nitrate in the aquarium for a maximum of 28 days. Fish from control group (group C) were kept untreated. Fish of treated groups, *viz,* T1, T2, T3 and T4 were exposed to 6.25, 12.5, 25 and 50 µg/L $AgNO_3$ in water, respectively. Silver nitrate (Purity > 99.90) (CAS No. 7761-88-8) purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India was used to induce the toxicity in fish of treatment groups. During the experiment, fresh stock was prepared by weighing 0.1 mg and dissolved in 1 mL of milli-Q water and then diluted in the tank. The accurate weighing of silver nitrate was done using a precise analytical weighing balance by Mettler Toledo (MS 204S/A01Mettler Toledo, Mumbai, India).

Histopathological examination

After the exposure period of 28 days, fish were anaesthetized using an ice-cold method, and then the dorsal, pectoral, pelvic, and caudal fins from all fish were removed. The fish were fixed in 10% neutral buffered formalin for seventy-two hours. The head was also removed from the body to allow for easier formalin penetration. The formalin-fixed fish was decalcified in a

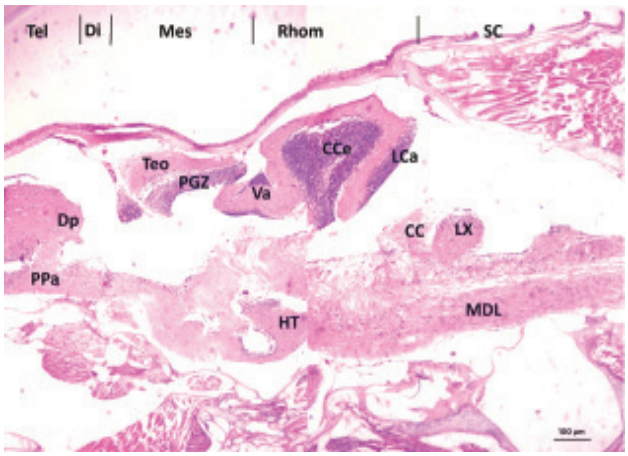

Fig. 1. Microscopic view of the brain of control group of adult female zebrafish (H&E staining).

(Different areas of the brain of normal adult zebrafish form control group; CC: Crista cerebelli; CCe: Corpus cerebelli; Di: Diencephalon; Dm: medial zone of the dorsal telencephalic area; Dp: Posterior domain of the dorsal telencephalon; HT: Hypothalamus; LCa: Lobuscaudalis cerebelli; LX: Vagal lobe; MDL: Medulla; Mes: Mesencephalon; Rhom: Rhombencephalon; OB: Olfactory bulb; Teo: Tectum opticum& PGZ: Periventricular gray zone of the optic tectum; PPa: Anterior part of the parvocellular preoptic nucleus; SC: Spinal cord; Tel: Telencephalon; Teo: Tectum opticum; Va: Valvula cerebelli).

0.35 M EDTA disodium dihydrate (pH 7.8) solution for ten days. The volume of the solution was 20 times that of the fish, and the solution was changed daily with a fresh one. The decalcified fish were embedded in paraffin and processed as per standard procedures. Embedded fish were sectioned at 5 ìm thickness with a semi-automated rotary microtome (Leica Biosystems, Germany) and were stained with hematoxylin and eosin (H & E) stain (Luna 1968). The H & E stained slides were observed under a Carl Zeiss microscope attached with an Axiocam camera (ERc5s Lab India Instruments Pvt. Ltd. India) and microscopic lesions were recorded.

RESULTS AND DISCUSSION

In this study, we examined the structural changes in the brain and retina of adult zebrafish following long-term exposure to $AgNO_3$. The principal mechanism of Ag toxicity in aquatic organisms is considered to be through oxidative phosphorylation impairment and the formation of ROS which ultimately leads to lipid peroxidation (Saleeb

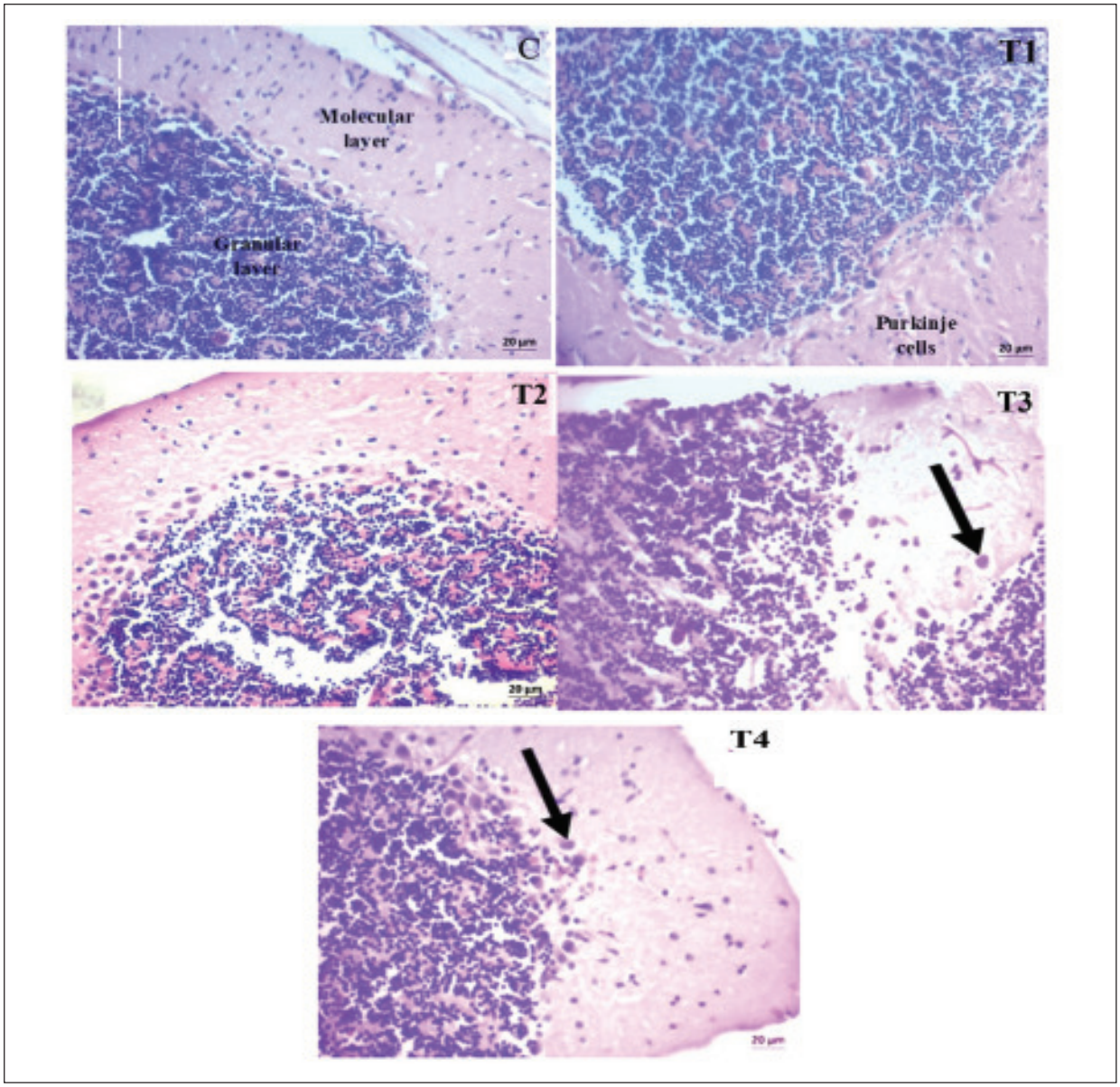

Fig. 2. Microscopic view of the corpus cerebella (CCe) of zebrafish following silver nitrate exposure (H&E staining). C) CCe of adult zebrafish from control groups for 28 days. T1) CCe of the brain of adult female zebrafish exposed to 6.25 µg/L $AgNO_3$ for 28 days. T2) CCe of the brain of adult female zebrafish exposed to 12.5 µg/L $AgNO_3$ for 28 days. T3) CCe of the brain of adult female zebrafish exposed to 25 µg/L $AgNO_3$ for 28 days. T4) CCe of the brain of adult female zebrafish exposed to 50 µg/L $AgNO_3$ for 28 days. Microscopic changes related to toxicity were mild to moderate congestion in T3 and T4 toxicity groups compared to control (depicted with arrow).

et al. 2020), an imbalance between the generation of free radicals (ROS) and their removal by antioxidant defense systems. The body has various enzymatic and non-enzymatic defense systems to counteract this damage. Overproduction of reactive oxygen species (ROS) can denature antiapoptotic proteins and trigger the development of proapoptotic proteins. As a result, apoptotic protein expression triggers the apoptosis signaling pathway (Akter *et al*. 2018). Irreversible alterations in the body progress when a cell fails to maintain it's counteract mechanism after prolonged exposure. Under a microscope, these degenerative alterations appear as microscopic lesions (Charehsaz *et al.* 2016).

The microscopic examination of the zebrafish brain of the control group showed normal architecture of different areas of the brain (Fig. 1). A microscopic view of the corpus cerebella (CCe) part of the brain of zebrafish from each group is shown in Fig. 2. Pathological changes like mild to moderate congestion were observed in the T3 and T4 toxicity groups. A microscopic view of the

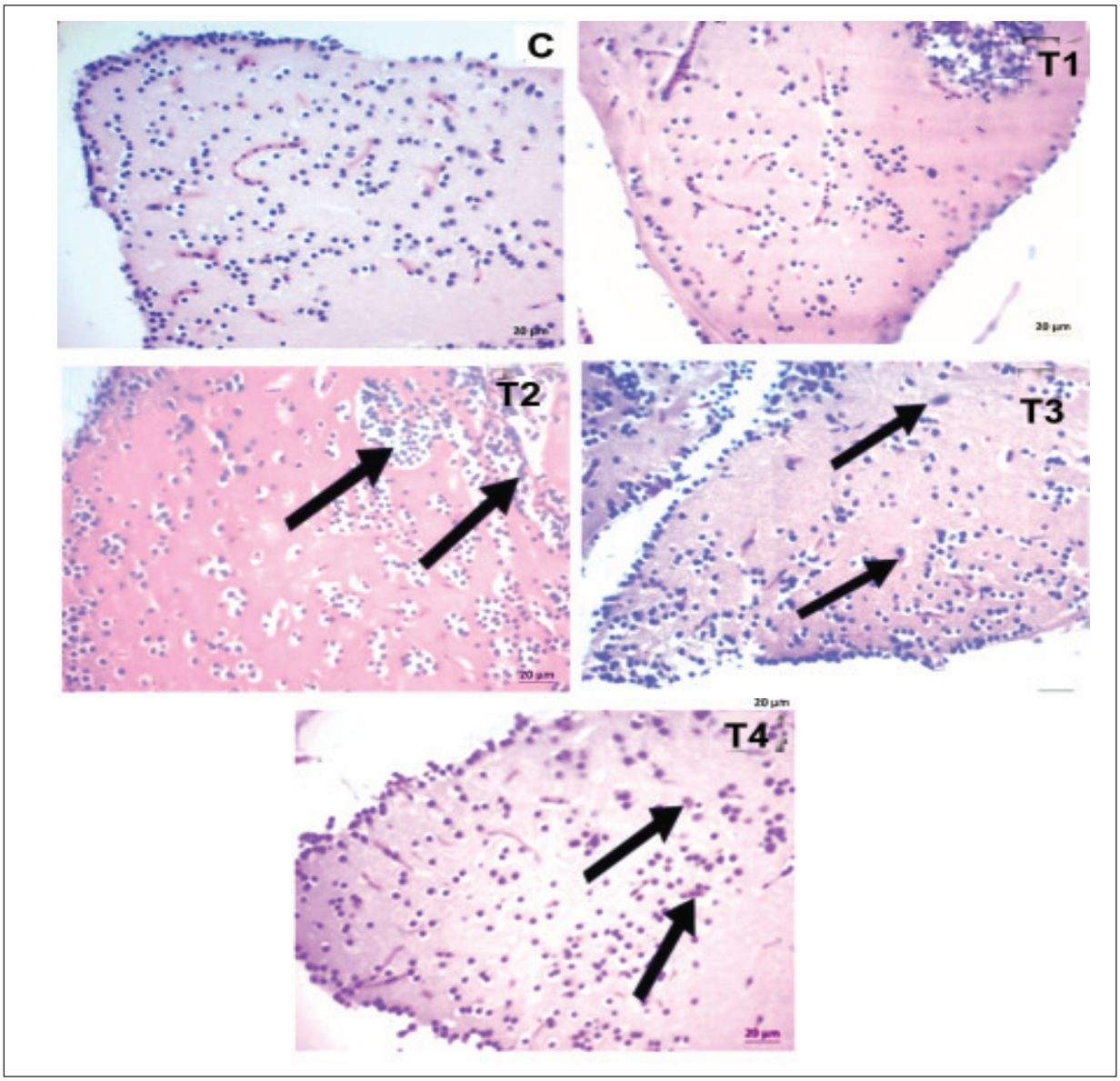

Fig. 3. Microscopic view of the hypothalamus (HT) of zebrafish following silver nitrate exposure (H&E staining). C) HT of normal adult zebrafish from control groups for 28 days. T1) HT of the brain of adult female zebrafish exposed to 6.25 µg/L $AgNO_3$ for 28 days. T2) HT of the brain of adult female zebrafish exposed to 12.5 µg/L $AgNO_3$ for 28 days. T3) HT of the brain of adult female zebrafish exposed to 25 µg/L $AgNO_3$ for 28 days. T4) HT of the brain of adult female zebrafish exposed to 50 µg/L $AgNO_3$ for 28 days. Mild to moderate degenerative changes related to toxicity were observed in toxicity groups T2, T3 and T4 (depicted with arrow).

hypothalamus (HT) of the brain of an adult female zebrafish from the normal control group and all toxicity groups is shown in Fig. 3. The hypothalamus of the brain of adult female zebrafish exposed to 12.5, 25 and 50 µg/L $AgNO_3$ showed mild to moderate degenerative changes compared to the control group. A microscopic view of lobuscaudalis cerebella (LCa) part of the brain of zebrafish in each group is shown in Fig. 4; mild degenerative changes were observed in fish from T1 group, while the T2 and T3 toxicity groups showed moderate degenerative changes in the granular cells of LCa compared to the control group. A microscopic view of the medulla (MDL) of the brain of an adult female zebrafish from the control group and toxicity groups is shown in Fig. 5. The medulla

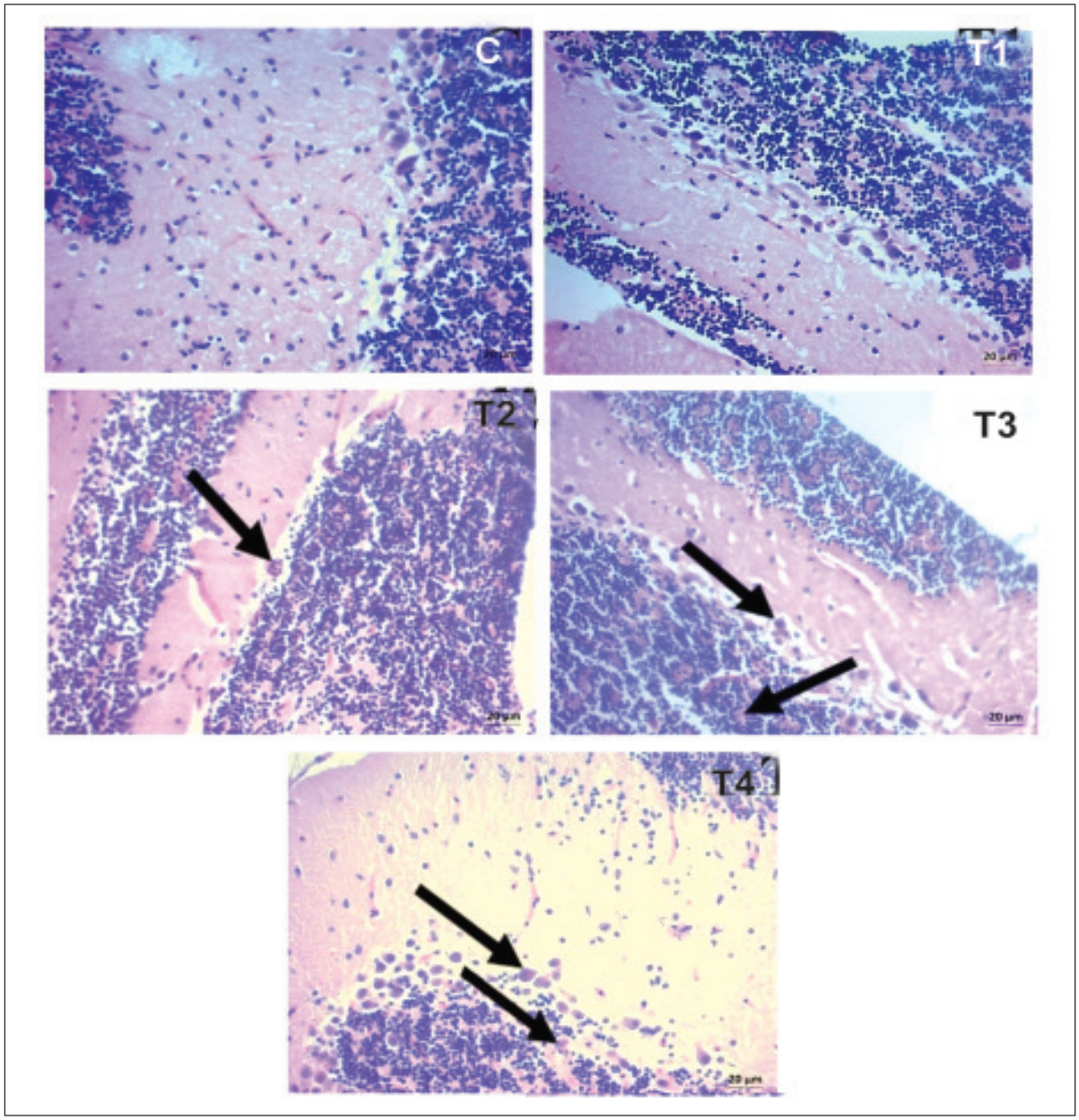

Fig. 4. Microscopic view of the lobuscaudalis cerebelli (LCa) of zebrafish following silver nitrate exposure (H&E staining). C) LCa of normal adult zebrafish from control groups for 28 days. T1) LCa of the brain of adult female zebrafish exposed to 6.25 µg/L $AgNO_3$ for 28 days. T2) LCa of the brain of adult female zebrafish exposed to 12.5 µg/L $AgNO_3$ for 28 days. T3) LCa of the brain of adult female zebrafish exposed to 25 µg/L $AgNO_3$ for 28 days. T4) LCa of the brain of adult female zebrafish exposed to 50 µg/L $AgNO_3$ for 28 days. Mild degenerative changes were observed in the T2 group while T3 and T4 groups shows moderate degenerative changes related to toxicity (depicted with arrow).

of the brain of adult female zebrafish from $AgNO_3$ treatment groups T2, T3 and T4 exhibited mild to moderate degenerative changes compared to the control group. A microscopic view of the periventricular grey zone of the optic tectum (Teo and PGZ) part of the brain of zebrafish from each group is shown in Fig. 6. The brains of adult female zebrafish from the T2, T3 and T4 groups showed mild degenerative changes in Teo and PGZ. Similar to the finding of the present study, Ag in nanoparticulate or ionic forms has been reported to cause mild to moderate pyramidal neuronal cell loss and gliosis, indicating hippocampal sclerosis in the cornuammonis sector, primarily in the CA1 sector in mice (Charehsaz *et al.* 2016). Previous report of nerve tissue bleeding and vacuolated spaces in the affected area in the brain of $AgNO_3$-treated ratsfurther supports our findings (Gueroui and Kechrid 2016).

Cross-sectional histology of the retina of control groups showed normal retinal features, which included choroid layer (CH), retinal pigmented epithelium layer (RPE), photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL),

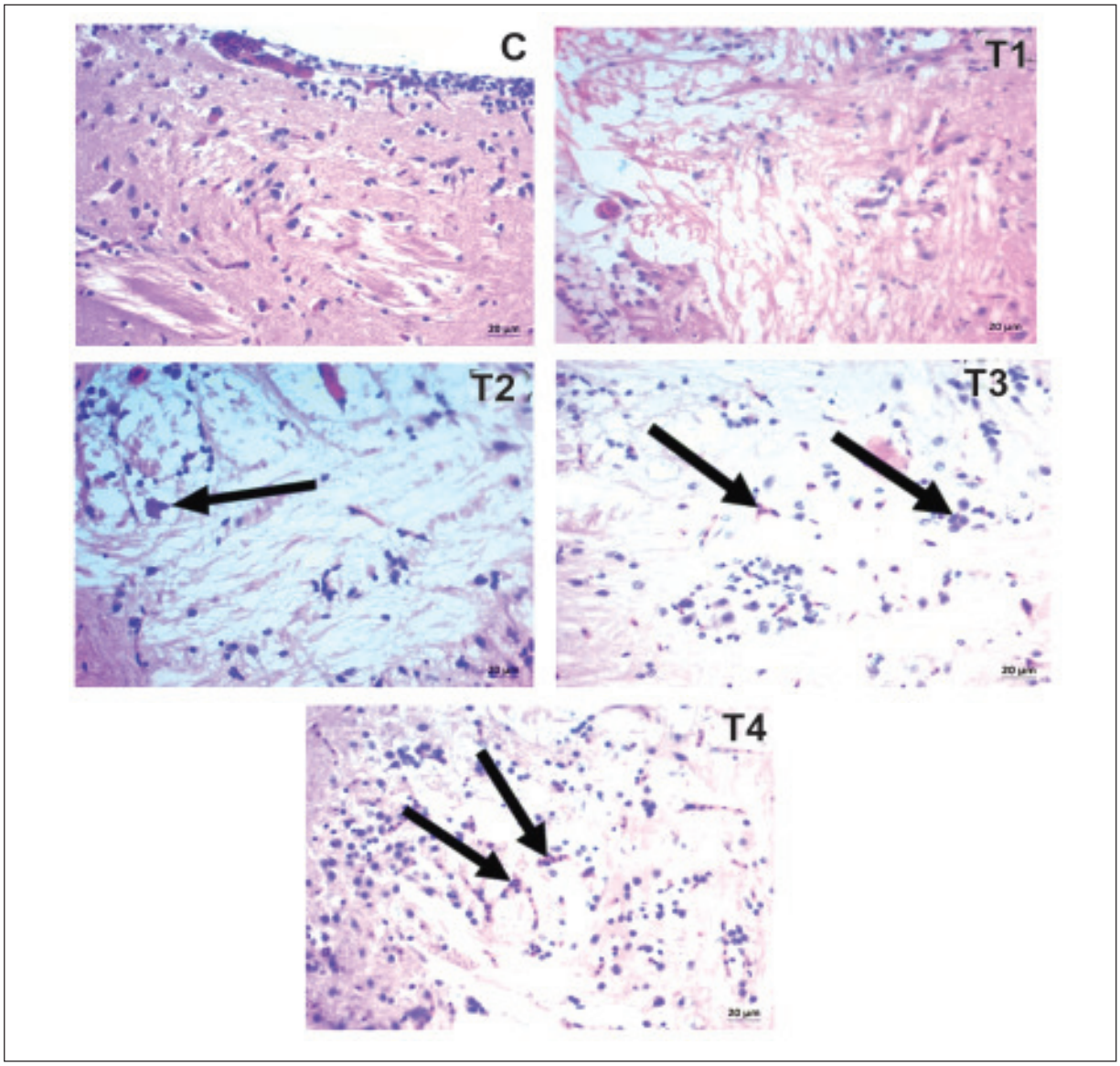

Fig. 5. Microscopic view of the medulla (MDL) of zebrafish following silver nitrate exposure (H&E staining). C) MDL of normal zebrafish from control groups for 28 days. T1) MDL of the brain of adult female zebrafish exposed to 6.25 µg/L $AgNO_3$ for 28 days. T2) MDL of the brain of adult female zebrafish exposed to 12.5 µg/L $AgNO_3$ for 28 days. T3) MDL of the brain of adult female zebrafish exposed to 25 µg/L $AgNO_3$ for 28 days. T4) MDL of the brain of adult female zebrafish exposed to 50 µg/L $AgNO_3$ for 28 days. Mild to moderate degenerative changes related to toxicity were observed in T2, T3 and T4 toxicity groups (depicted with arrow).

inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fibre layer (NFL). Upon comparison of various retinal layers from zebrafish of control group and toxicity groups, we didn't observe any histopathological changes (Fig. 7). There are no studies available on the histological changes in the retina of adult zebrafish exposed to Ag ions and other metals. However, Söderstjerna *et al.* (2014) studied the toxicity of $AgNO_3$ at 0.5, 1.0 and 5.0 µg/ml using an *in vitro* tissue culture model of the mouse retina, and reported severe insult to the retinal tissue and cells, with disrupted retinal layering, folding and rosette formation, the formation of vacuoles, as well as an induction of massive cell death revealed by the large numbers of pycnotic cells found compared to the control.

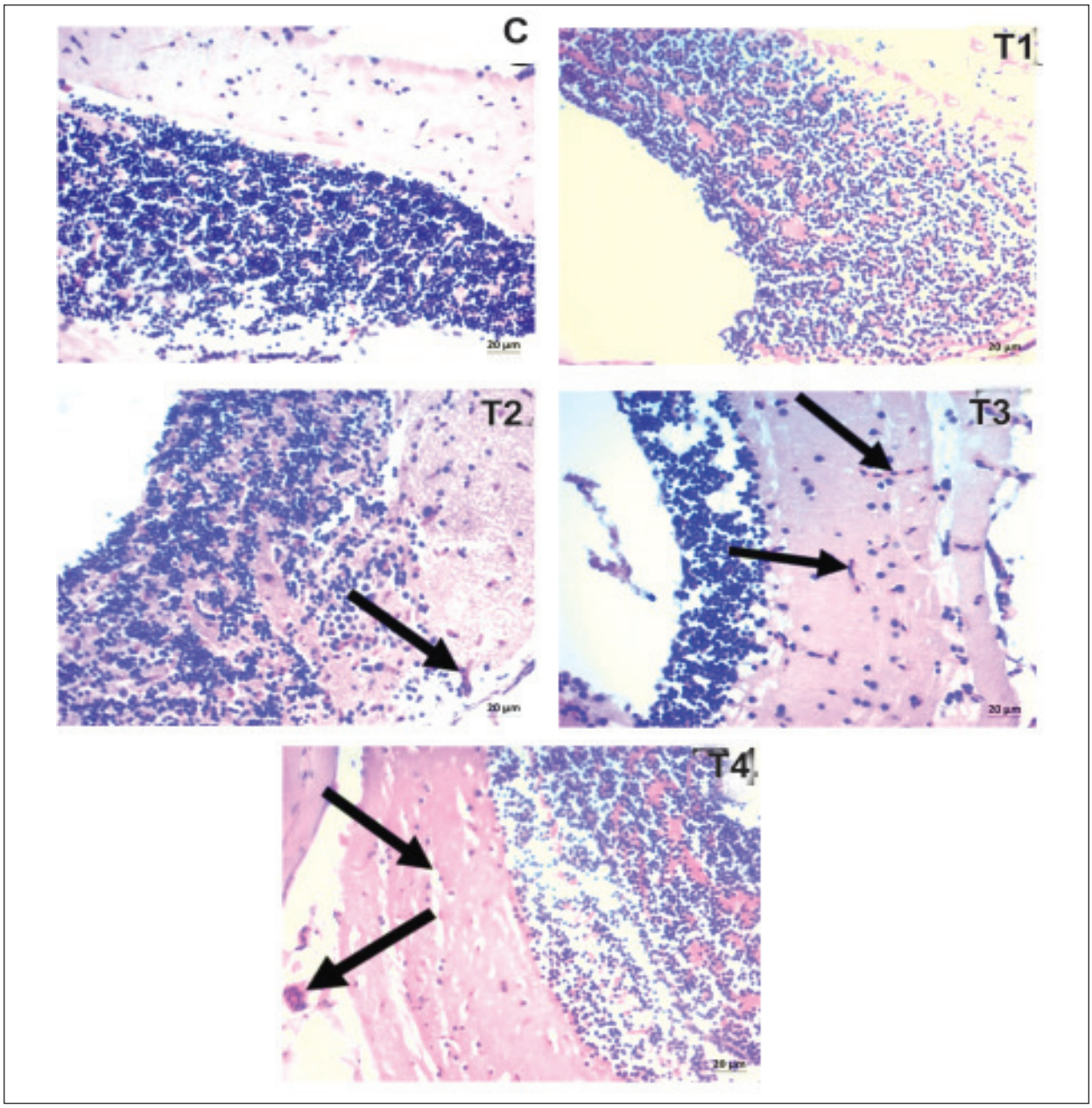

Fig. 6. Microscopic view of the periventricular grey zone (PGZ) of zebrafish following silver nitrate exposure (H&E staining).

C) PGZ of normal adult zebrafish from control groups for 28 days. T1) PGZ of the brain of adult female zebrafish exposed to 6.25 µg/L $AgNO_3$ for 28 days. T2) PGZ of the brain of adult female zebrafish exposed to 12.5 µg/L $AgNO_3$ for 28 days. T3) PGZ of the brain of adult female zebrafish exposed to 25 µg/L $AgNO_3$ for 28 days. T4) PGZ of the brain of adult female zebrafish exposed to 50 µg/L $AgNO_3$ for 28 days. Mild to moderate degenerative changes related to toxicity were observed in T2, T3 and T4 toxicity groups (depicted with arrow).

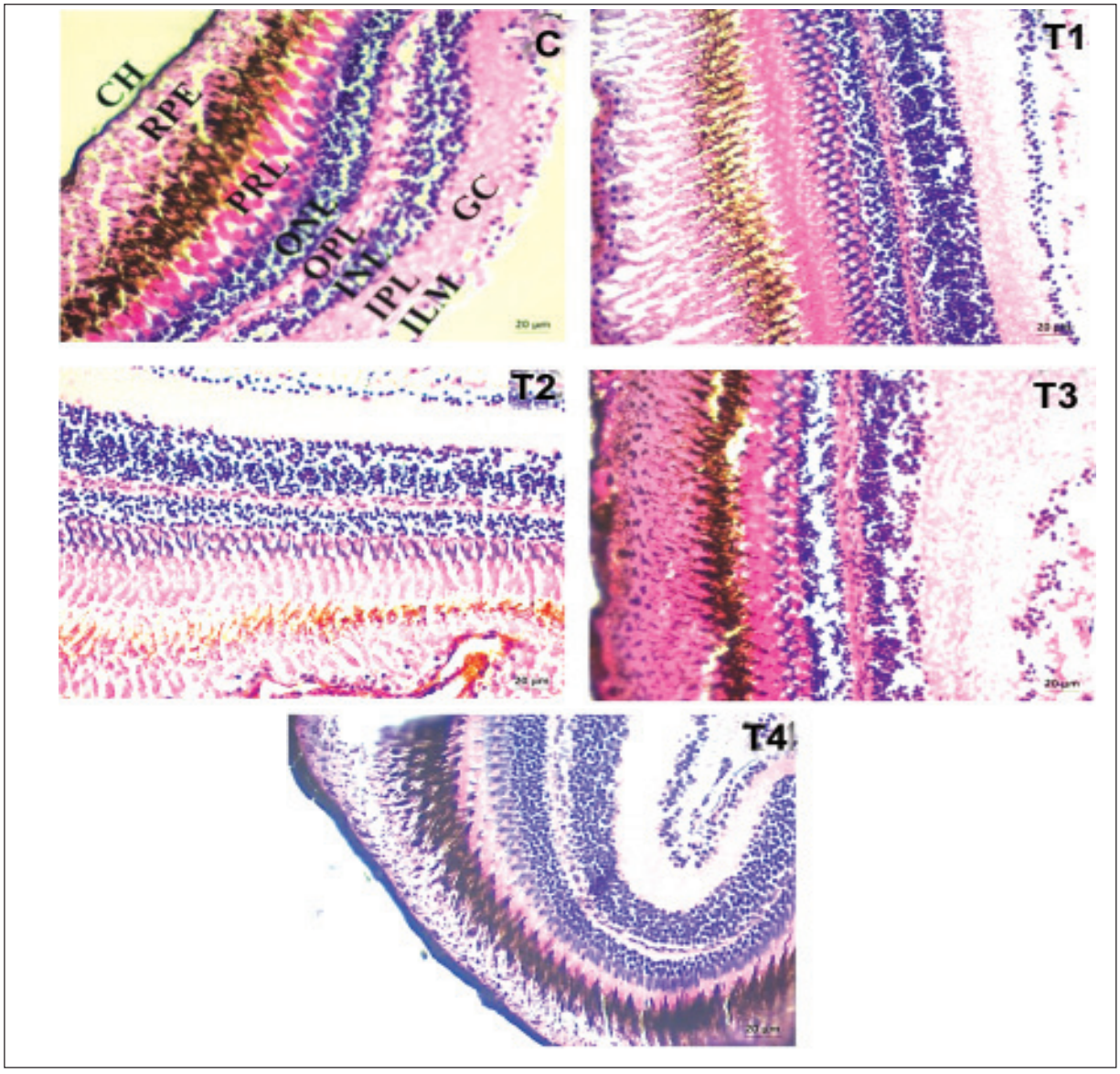

Fig. 7. Microscopic view of the retina of adult female zebrafish following silver nitrate exposure (H&E staining). C) Cross-sectional histology of neural retina of control group depicts normal retinal features which includes choroid layer (CH), retinal pigmented epithelium layer (RPE), photoreceptor layer (PRL),outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL). T1) Retina of adult female zebrafish exposed to 6.25 µg/L $AgNO_3$ for 28 days. T2) Retina of adult female zebrafish exposed to 12.5 µg/L $AgNO_3$ for 28 days. T3) Retina of adult female zebrafish exposed to 25 µg/L $AgNO_3$ for 28 days. T4) Retina of adult female zebrafish exposed to 50 µg/L $AgNO_3$ for 28 days microscopic changes related to toxicity were not observed in toxicity groups.

CONCLUSION

Silver nitrate exposure at 12.5, 25 and 50 µg/L concentrations for 28 days produces mild to moderate pathological changes in corpus cerebella, hypothalamus, lobuscaudalis cerebella, medulla, and dorsal telencephalic area of opticum tectum parts of the zebrafish brain, while no appreciable changes were observed in the retina, indicating the neurotoxic potential of silver nitrate.

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