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Arsenic Trioxide Induced Neurotoxicity and Cardiotoxicity in Zebrafish (*Danio rerio*): Assessment of 8-hydroxy-2′deoxyguanosine Activity and Histopathological Alteration

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ABSTRACT:
Arsenic trioxide is extensively utilized for its industrial, agricultural, and medicinal applications. However, its widespread use has led to significant arsenic pollution in aquatic ecosystems, raising global concerns. Despite its medicinal benefits, its effectiveness is hindered by its harmful effects on the human organ system. Therefore, this study aimed to investigate the neurotoxic and cardiotoxic effects of waterborne arsenic trioxide at concentrations of 10 ppb, 50 ppb, and 500 ppb over 90 days, using zebrafish (*Danio rerio*) as the animal model. Neurotoxicity and cardiotoxicity were assessed through histopathological alterations in the optic tectum and periventricular gray zone (PGZ) of the midbrain, as well as in the ventricular myocardium of the heart in adult zebrafish, serving as indicators of morphological changes. The immunoreactivity of the 8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody was also assessed using an immunofluorescence assay to indicate oxidative DNA damage. The results revealed that arsenic trioxide exposure caused severe histopathological alterations, including disorganization of the optic tectum layers, clumping of mononuclear cells, pyknosis, spongiosis, and neuronal degeneration in the brain. Histopathological analysis of the heart showed structural damage to the myocardium, characterized by myofilament dissociation and infiltration of inflammatory cells between cardiomyocytes. Furthermore, immunofluorescence assays demonstrated a strong 8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody reactivity in neural and cardiac tissues of arsenic trioxide-exposed groups, with a significant dose-dependent increase ($P<0.05$). These findings suggest that prolonged exposure to arsenic trioxide induces neurotoxicity and cardiotoxicity in zebrafish, highlighting its toxic effects on aquatic organisms and reinforcing the utility of zebrafish as a valuable model for assessing toxicity relevant to human health.
Keywords: Arsenic Trioxide, Neurotoxicity, Cardiotoxicity, Histopathology, Immunofluorescence, 8-OHdG

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INTRODUCTION

According to the Agency for Toxic Substances and Disease Registry, arsenic is ranked among the most hazardous environmental pollutants, posing a significant threat to human health (ATSDR, 2013). Arsenic trioxide (ATO) has historically been used to treat various diseases and is widespread in the environment, with human exposure occurring through both natural and human-made sources, as well as intentional acts such as suicide and homicide attempts. Millions of people consume arsenic-contaminated groundwater used for drinking (Dong et al., 2018; Klancko, 2003; Kuivenhoven et al., 2025; Liu et al., 2024). Alarmingly, around 200 million people worldwide suffer from chronic arsenicosis due to prolonged exposure to arsenic-contaminated drinking water (Flora, 2011). The World Health Organization (WHO) and the U.S. Environmental Protection Agency (EPA) have set a recommended limit of ≤10 ppb (µg/L) for arsenic in drinking water (WHO, 2011; EPA US, 2001). However, in several developing countries, including India, the permissible limit remains at 50 ppb (Sarkar et al., 2017; BIS, 2010). In some areas, arsenic concentrations in drinking water have been reported as high as 800 ppb to 1980 ppb, which is highly concerning given arsenic's severe toxicity (Dipp et al., 2018). Long-term exposure to arsenic is linked to serious health conditions, including cancer, skin lesions, cardiovascular diseases, and developmental disorders. Chronic arsenic exposure through drinking water has been shown to increase blood-brain barrier (BBB) permeability, allowing arsenic to accumulate in various regions of the brain (Rodríguez et al., 2010; Manthari et al., 2018). The central nervous system (CNS) is particularly susceptible to its toxic effects, which can manifest as cognitive disorders, reduced intelligence, learning deficits, and impaired synaptic plasticity (Escudero-Lourdes, 2016; Garg & Bandyopadhyay, 2025). Additionally, chronic exposure to arsenic at concentrations of ≥500 µg/L has been associated with various cardiac diseases, including coronary artery disease, carotid atherosclerosis, myocardial injuries, and other cardiovascular and neural disorders documented in multiple reviews (Vineetha et al., 2019; Alamolhodaei et al., 2015; Mahadik et al., 2024)

The principal toxicological mechanism involved in arsenic-induced neurotoxicity and cardiotoxicity is the generation of reactive oxygen species (ROS), leading to oxidative stress. Arsenic-induced oxidative stress disrupts the balance between antioxidants and pro-oxidants by impairing antioxidant enzymes. This disruption damages key cellular macromolecules, including deoxyribonucleic acid (DNA), proteins, and lipids, resulting in molecular, physiological, and histopathological alterations (Zhao et al., 2019; Garza-Lombó et al., 2019; Ganie et al., 2023). Arsenic is a potent inducer of oxidative stress in various organ systems in fish, including the brain (Sarkar, 2017). The excessive production of ROS leads to oxidative DNA damage and apoptosis in neural cells. Given its crucial role in regulating fish physiology, the brain is a key target organ in fish toxicology studies. Similarly, cardiac tissue is highly susceptible to oxidative stress due to its low levels of enzymatic and non-enzymatic free radical detoxifying agents. As a result, the heart is considered one of the primary target organs of arsenic toxicity (Mishra et al., 2014; Bhattacharya et al., 2014).

The zebrafish (*Danio rerio*) has emerged as an excellent and widely used model organism in toxicological research. It is an increasingly valuable vertebrate model for neurotoxicology studies and central nervous system (CNS) drug discovery. Furthermore, the molecular mechanisms governing cardiac development in zebrafish closely resemble those found in higher vertebrates, making it a promising model for studying toxicology. Additionally, zebrafish serve as a cost-effective model for studying the effects of environmental chemical exposure on various biological processes relevant to human health. Nearly 87% of the human genome is believed to have homologous counterparts in the zebrafish genome (Cheng et al., 2020; Poon et al., 2013).

This study aimed to investigate the neurotoxic and cardiotoxic effects of arsenic trioxide through histopathological analysis, a direct and

effective tool for physiological assessment, specifically chosen to evaluate arsenic's harmful impact (Lam et al., 2006). Additionally, 8-hydroxy-2′-deoxyguanosine (8-OHdG) activity analysis, a widely used biomarker of oxidative DNA damage, was employed to assess arsenic-induced genotoxicity because arsenic bioaccumulation can lead to various adverse effects in aquatic organisms (Faita et al., 2013; Byeon et al., 2021).

MATERIAL AND METHODS

Zebrafish Maintenance

Healthy adult wild-type zebrafish (2–4 months old) of both sexes were obtained from an authorized fish supplier. Prior to the experiment, the fish were acclimated in a zebrafish housing tank in the laboratory for 15 days. The experiments were conducted under controlled conditions, maintaining a water temperature of 27 ± 1.5 °C, a 14-hour light/10-hour dark photoperiod, a pH of 7.0 ± 0.5, and a dissolved oxygen concentration of 7.0 ± 0.5 mg/L. Fish were fed commercial food twice daily, and wastewater was renewed daily. All animal procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, and the study was approved by the Institutional Animal Ethics Committee (IAEC No. 379/S/01/CPCSEA/IAEC) at Dr. Harisingh Gour Vishwavidyalaya, Sagar (M.P.), India.

Chemical Exposure Protocol

After acclimatization, zebrafish were randomly assigned to four groups: a control group and three arsenic trioxide exposure groups (10 ppb, 50 ppb, and 500 ppb). Arsenic trioxide (CAS No: A1010, purity 99%, Sigma Aldrich) was dissolved in 1N NaOH to prepare a stock solution, which was heated until fully dissolved. To maintain consistent exposure levels, a freshly prepared stock solution was added to the respective experimental groups throughout the study. Following 90 days of exposure, fish were randomly sampled from each tank and anesthetized using ice-cold water. The brain and heart were then dissected for further examination. The treatment regimen was adapted from a previously published research article (Sarkar et al., 2014, 2017; Dipp et al., 2018).

Histopathological Examination

After exposure, the brain and heart were carefully excised from both control and arsenic-exposed zebrafish and fixed overnight in freshly prepared 4% paraformaldehyde. The tissues were then dehydrated in a graded series of alcohol (30% to 100%), cleared in xylene, embedded in paraffin, and sectioned at a thickness of 6 µm using a microtome. The sections were subsequently mounted on gelatin-coated glass slides. Following deparaffinization in xylene, tissue sections were rehydrated through a graded series of alcohol (100% to 30%) and stained with Hematoxylin and Eosin (H&E) according to the manufacturer's instructions. Images of the stained brain and heart sections were captured at 200x magnification using an inverted trinocular microscope (Magnus INVI).

Immunohistochemical Examination

The immunohistochemical examination followed the same procedure as previously described (Maurya & Mishra R, 2017). Brain and Heart tissue sections (6 µm thick) were deparaffinized in xylene, rehydrated through a graded series of alcohol solutions, and rinsed in water. Antigen retrieval was performed using a preheated Trypsin solution, and nonspecific labeling was blocked with bovine serum albumin (BSA). The primary antibody, 8-OHdG (Sc-66036, Santa Cruz Biotechnology), was applied at a 1:500 dilution and incubated overnight at 4°C in a humidified chamber. The following day, sections were incubated with the secondary antibody, fluorescein isothiocyanate (FITC), at a 1:500 dilution for 120 minutes at 4°C. To label nuclei, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Image and Statistical Analysis

Immunofluorescence images were captured using a confocal laser scanning microscope (CLSM MEA53100, Nikon Corporation) with the FITC channel. Immunofluorescence analysis was performed by comparing fluorescence intensity using ImageJ software. The intensity was measured in pixels within a defined area and expressed as mean intensity per mm^2.

Experimental data are presented as the mean ± standard deviation (S.D.) and analyzed using a one-way analysis of variance (ANOVA), followed by a post hoc test for multiple comparisons. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

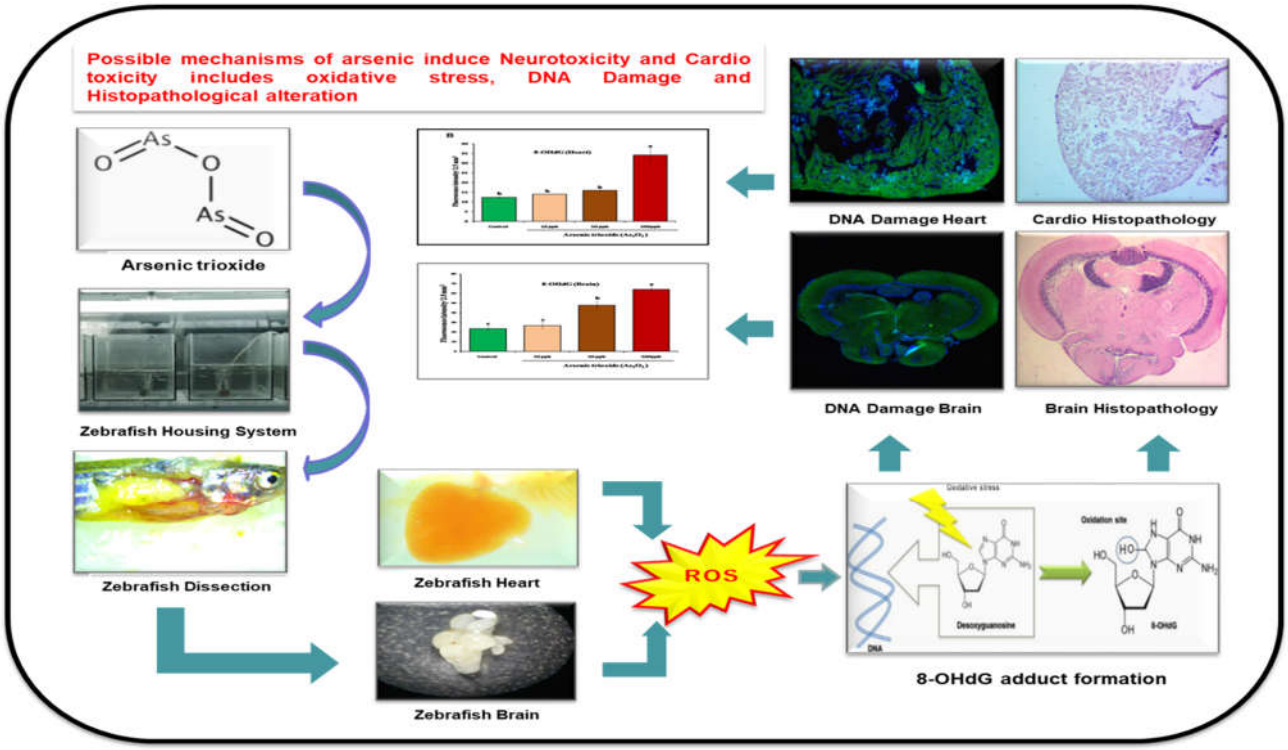

Figure 1: Graphical abstract of arsenic trioxide induced Neurotoxicity and Cardio toxicity

RESULTS

Histopathological Analysis of Brain

In this study, the zebrafish brain was sectioned in the coronal plane, focusing on the periventricular hypothalamic zone. Histopathological examination of the optic tectum (TeO), including the periventricular grey zone (PGZ), after H&E staining, revealed a significant increase in the clumping of mononuclear cells in the PGZ and the presence of spongiosis in the exposed groups (50 ppb and 500 ppb As_2O_3) compared to the control group (Figure 2).

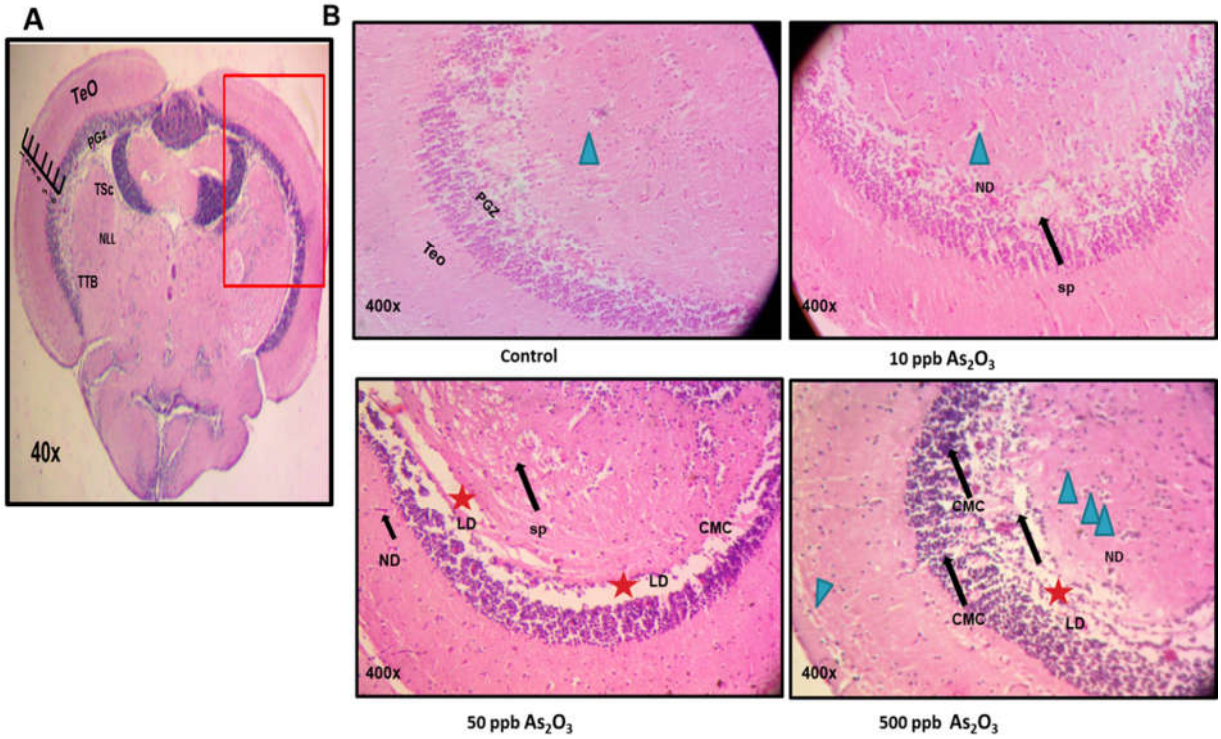

Figure 2: Histology of adult zebrafish brain. Representative images of the coronal sectional view of zebrafish brain following H&E staining. The whole adult zebrafish brain (A) shows intact structural morphology, and red box indicates selected region. The 6 layers of optic tectum are shown in number (1-6), 1-stratum marginale (SM), 2- stratum opticum (SO), 3- Stratum fibrosum et grisium superficiale (SFGS), 4- Stratum griseum centrale (SGC), 5- Stratum album centrale (SAC) and 6- Stratum periventriculare (SPV). optic tectum (TeO), periventricular gray zone (PGZ) central nucleus of torus semicircularis (TSc), tectobulbular tract (TTB), nucleus of the lateral lemniscus (NLL) (40x). Image B showed different As_2O_3 exposure group Vs control (400x). Arrows depict deformities, clumping of mononuclear cell (CMC) pyknotic cells (PY) spongiosis (SP), and neuronal degeneration (ND) and star depict disownments of different optic tectum layers (LD).

Immunohistochemical Analysis of Brain

8-OHdG immunoreactivity was detected in the optic tectum (TeO) and the periventricular grey zone (PGZ) of the zebrafish midbrain using an immunofluorescence assay with an 8-OHdG monoclonal antibody (Figure 3 A & B). The immunofluorescence reaction was observed in the brain tissues of zebrafish, with the control group showing minimal expression of 8-OHdG immunoreactivity (23.48±2.7). Following exposure to 10 ppb As_2O_3, mild immunoreactivity was observed (26.73±3.5); at 50 ppb exposure, moderate immunoreactivity was noted (47.73±4), and at 500 ppb exposure, severe immunoreactivity was detected (64.16±2.9) (Figure 4.1.3). The results showed that exposure to 10 ppb, 50 ppb, and 500 ppb As_2O_3 significantly increased 8-OHdG immunoreactivity by 13.85%, 103.27%, and 173.25%, respectively, compared to the control group (Figure 3).

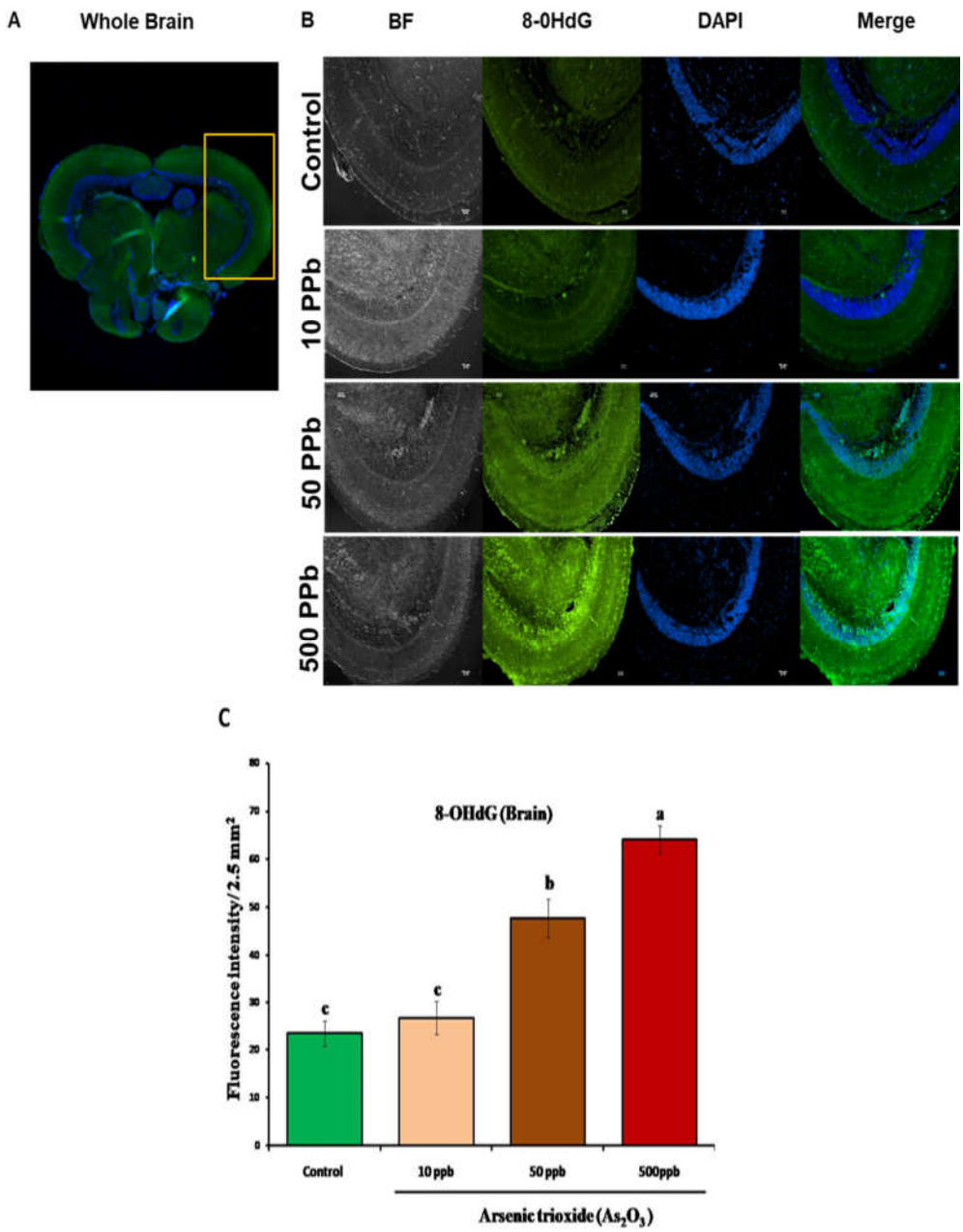

Figure 3: Immunofluorescence shows levels of oxidized DNA in the zebrafish brain following 8-hydroxy-2' -deoxyguanosine (8-OHdG) antibody staining (A) Representative immunohistochemical localization of selected area (yellow box) in whole brain (B) Representative immunohistochemical localization of 8-OHdG (green), DAPI (Blue) and BF (Bright field) in optic tectum of zebrafish brain after different concentration of As_2O_3 exposure for 90 day Vs control. Scale bar: 20µm. (C) Graph of 8-OHdG immunofluroroscence intensity/2.5 mm² area in the brain. Values are expressed as mean ± SEM (N = 3). Different letters indicate significant differences between treatments (one-way ANOVA, Tukey's pairwise comparison, $p<0.05$).

Histopathological Analysis of Heart

Histopathological examination of the ventricular myocardial tissue in the control group revealed normal histoarchitecture, with well-arranged cardiomyocytes and an ellipsoidal nucleus. The 10 ppb As_2O_3 exposure group exhibited no significant alterations compared to the control. In contrast, the 50 ppb As_2O_3 group displayed minor histopathological changes, including dense dark nuclei and mild inflammatory cell infiltration. The 500 ppb As_2O_3 exposure group showed extensive myocardial degeneration, characterized by necrosis, dissociation of myofilaments, and pronounced inflammatory cell infiltration (Figure 4).

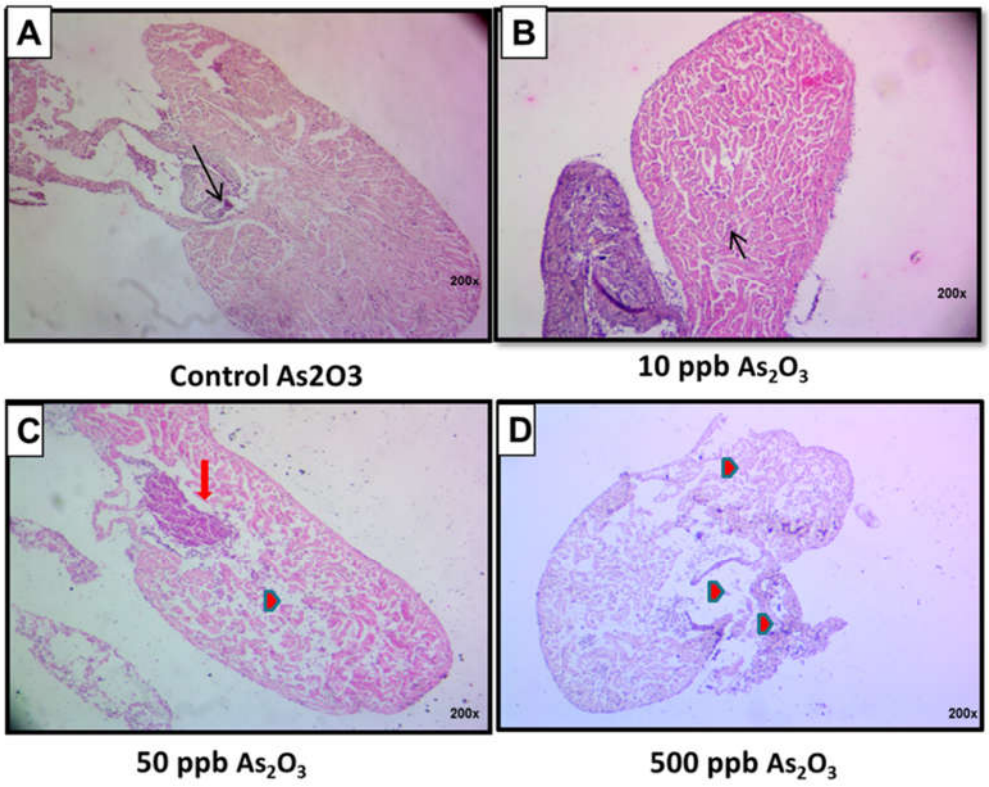

Figure 4: Represent histopathological study of zebrafish Heart stained with Haematoxylin and Eosin. The arrow indicates (↑) some fibres show small dark nuclei; Red Arrow indicates inflammatory cells infiltration; Arrowhead indicates: widely separated cardiac muscle fibres and myocardial degeneration.

Immunohistochemical Analysis of Heart

The immunofluorescence study revealed a statistically significant difference in fluorescence intensity in the ventricular myocardial tissue of zebrafish hearts (Figure 5). In the control group, only slight immunoreactivity (12.41 ± 0.05) was observed. However, 8-OHdG antibody immunoreactivity increased in a dose-dependent manner with As_2O_3 exposure. In the 50 ppb As_2O_3 exposure group, a mild increase in immunofluorescence intensity (16 ± 0.28), corresponding to a 28% rise, was detected in ventricular myocardial tissue compared to the control. In contrast, zebrafish exposed to 500 ppb As_2O_3 exhibited a severe 175% increase in immunoreactivity (34.2 ± 3.4) relative to the control group (Figure 5).

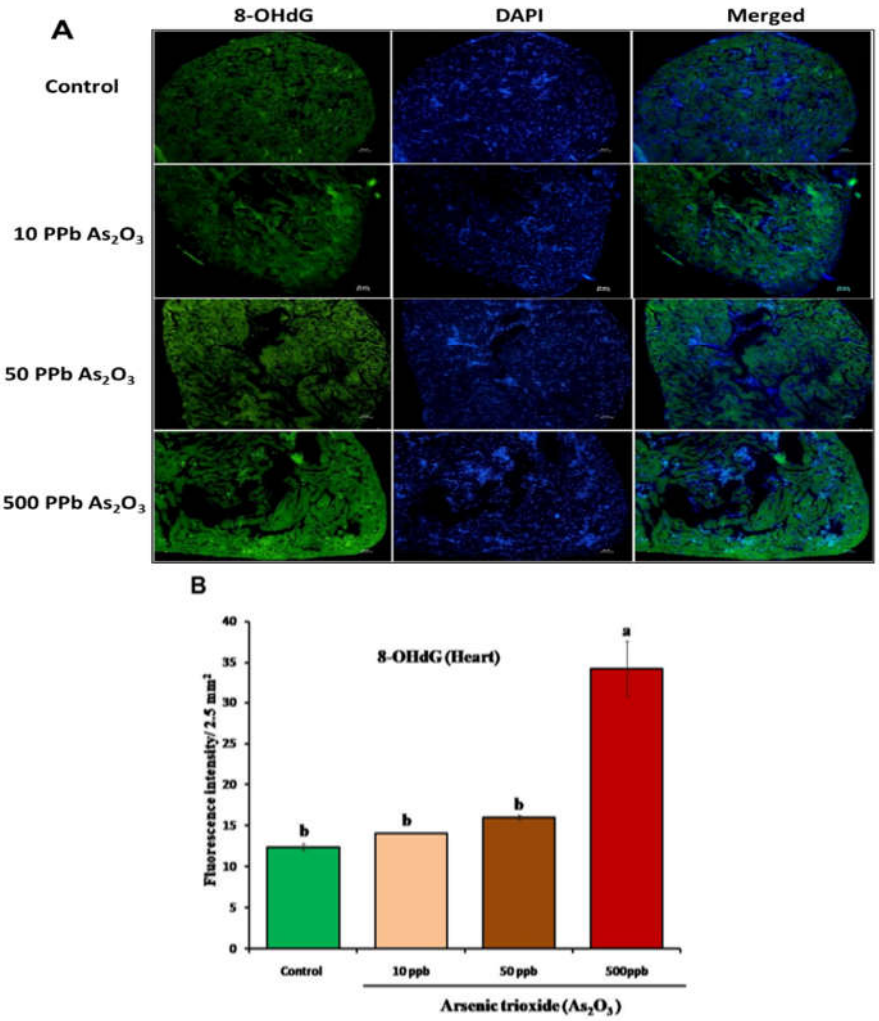

Figure 5: A; Representative confocal images of the Heart showing Immunofluorescence stains with 8-OHdG (green), DAPI (blue), and Merged (8-OHdG + DAPI) (Scale bar = 20 µm). B; Bar charts showing the statistical analysis of the 8-OHdG immunofluorescence intensity/mm² area in heart tissue calculated through image J software. The relative fluorescence intensity was expressed as the mean ± SEM with significant differences between treatments (one-way ANOVA, Tukey's pairwise comparison, $p<0.05$

DISCUSSION

Arsenic trioxide is a widespread metalloid pollutant found in various environmental media, including aquatic ecosystems, where it accumulates through both anthropogenic and natural sources. Exposure to arsenic and its bioaccumulation can have detrimental effects on aquatic organisms and the environment (Sun et al., 2020; Byeon et al., 2021). Therefore, assessing the impact of pollutants and their environmental risks in aquatic systems is crucial for human well-being.

In the present study, the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was examined as an indicator of oxidative DNA damage in the brain and heart of zebrafish. The results demonstrated a dose-dependent increase in 8-OHdG immunoreactivity, with a

significantly higher response observed at elevated arsenic concentrations (Figure 3 & 5). 8-hydroxyguanine, commonly referred to as 8-hydroxy-2′-deoxyguanosine (8-OHdG), is a key biomarker of reactive oxygen species (ROS)-induced DNA damage (Qin et al., 2008). When ROS production exceeds the capacity of the antioxidant defense system, oxidative stress occurs. This oxidative stress is a well-documented response to arsenic-induced toxicity. Several studies have reported that a 90-day exposure to arsenic trioxide induces significant oxidative stress and alters antioxidant status in the brain, liver, kidney, and intestine of zebrafish (Sarkar et al., 2014, 2017; Thakur et al., 2021). Previous research has demonstrated that ROS can cause DNA breakage, leading to the formation of 8-OHdG residues. In particular, studies have confirmed increased 8-OHdG immunoreactivity and degenerative changes in cerebral cortex neurons and Purkinje cells of mice exposed to low levels of arsenic (Piao et al., 2005). DNA damage is widely recognized as a key mechanism underlying the carcinogenic effects of arsenic. Excessive ROS production disrupts the body's antioxidant balance, leading to macromolecular damage, including DNA fragmentation (Biswas et al., 2010). Cells possess multiple mechanisms to repair DNA damage; however, arsenic exposure compromises these repair pathways (Tam et al., 2020; Flora, 2011; Karri et al., 2016). For example, arsenite can inhibit the function of PARP-1, a crucial DNA repair protein, even at low concentrations, thereby exacerbating oxidative DNA damage (Qin et al., 2008). Arsenite is a potent gene and chromosomal mutagen, with oxy-radicals playing a significant role in its mutagenic process. Evidence suggests that arsenic induces oxygen radicals, contributing to genotoxicity in mammals (Kessel et al., 2002). Similar findings have been observed across species. For instance, arsenic trioxide exposure has been shown to induce 8-OHdG generation in rat cardiac tissue (Varghese et al., 2017). Studies on arsenic-intoxicated rats revealed increased DNA fragmentation in myocardial tissues (Bhattacharya et al., 2014). Additionally, arsenic exposure disrupts the ionic balance of macromineral contents and triggers ROS bursts, negatively impacting the antioxidant defense system and leading to oxidative stress—a major contributor to cardiotoxicity (Vineetha et al., 2019; Zhao et al., 2020). Arsenic-induced cardiotoxicity involves oxidative stress, depletion of antioxidant enzymes, apoptosis, and autophagy through the PI3K/Akt/mTOR pathway, as observed in the heart tissues of common carp (*Cyprinus carpio* (Zhao et al., 2020). Furthermore, arsenite may interfere with DNA repair mechanisms, leading to persistent DNA damage through ROS production (Seok et al., 2007).

To further evaluate arsenic toxicity, we employed histopathological analysis as a key indicator of arsenic-induced damage in fish. Histopathology is a well-established and widely used technique for assessing tissue alterations in response to toxicants and has broad applications in various life sciences domains (Costa, 2018). Numerous studies have reviewed histopathological parameters as biomarkers for aquatic animals exposed to xenobiotics (Yancheva et al., 2016; Au, 2004). The potential of histopathological assessments in evaluating neurotoxic injury in fish has been highlighted by Simpson and Waterman (1989). Our study confirmed histopathological changes in the optic tectum region of zebrafish brains after 90 days of exposure to different concentrations of arsenic trioxide. These histopathological alterations clearly indicate that arsenic trioxide disrupts the normal structural and functional integrity of the zebrafish brain, initiating neurodegeneration. Similarly, a previous histopathological study on *Catla catla* revealed vacuolization in the deep layers of the optic tectum and the formation of clear spaces between layers and around the nucleus following exposure to high concentrations of copper ions, indicative of neurodegeneration (Patel and Bahadur, 2011). Additionally, prior studies have shown that the periventricular grey zone (PGZ) of the zebrafish optic tectum regulates scototaxis (anxiety-like) behavior (Mohanti et al., 2016). In a previous study, histopathological examinations of arsenic-exposed fish revealed aggregated, disorganized, and necrotic cells with irregular outlines in the various layers of the optic tectum (Roy et al., 2006). Comparable histological responses have been documented in *Channa punctatus*, where significant neurodegenerative changes were observed in the deep layers of the

optic tectum following high-concentration exposure to Chlorpyrifos (Mishra and Devi, 2014). Similarly, sublethal exposure to copper has been associated with neurodegenerative changes in the brain of *Catla catla*, a freshwater fish (Patel and Bahadur, 2011).

The present histopathological study of the ventricular myocardial tissue of the zebrafish heart revealed severe pathological changes in arsenic trioxide-exposed groups. The severity of these changes increased progressively with higher concentrations of arsenic trioxide. Maximum arsenic accumulation was observed on day 90 in zebrafish exposed to high arsenic concentrations (Liu et al., 2006). Similar histopathological changes were previously reported in rats, where arsenic exposure led to cardiac muscle separation, vacuolated cardiomyocytes, and cellular infiltration (Zahran and El-Sekily, 2015). A separate study on mice also demonstrated structural abnormalities, including myofibrillar loss and cardiomyocyte necrosis, following arsenic trioxide exposure (Wang et al., 2019). These results align with prior findings, where sodium arsenite ($NaAsO_2$) exposure resulted in significant arsenic accumulation in cardiac tissues, reduced cardiac antioxidant enzyme activity, and disrupted the normal radiating pattern of myocardial cell plates (Manna et al., 2008).Several studies have established that arsenic is highly toxic to freshwater fish, with toxicity increasing in response to both higher concentrations and prolonged exposure. For instance, *Oreochromis mossambicus* (tilapia) exhibited a dose- and time-dependent toxic response to arsenic exposure (Ahmed et al., 2013). Another study reported that 90-day arsenic exposure induced cardiotoxicity in the chicken heart (*Gallus gallus*), leading to severe histopathological alterations and disruptions in cardiac enzyme levels, including aspartate transaminase (AST), creatine kinase (CK), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and α-hydroxybutyrate dehydrogenase (α-HBDH). These enzymes serve as biomarkers for assessing myocardial damage, ventricular failure, and acute myocardial infarction (Li et al., 2017). Histopathological analysis of the arsenic trioxide-exposed zebrafish hearts revealed extensive necrosis, inflammatory cell infiltration, and cytoplasmic vacuolization. These findings confirm that arsenic trioxide significantly exacerbates myocardial morphological damage (Zhao et al., 2020).

CONCLUSION

In this study, we investigated the effects of arsenic trioxide on zebrafish, revealing dose-dependent pathological remodeling of neural and cardiac tissues, characterized by intense 8-OHdG immunofluorescence and structural alterations. *Danio rerio* has emerged as a valuable model species for assessing heavy metal toxicity in aquatic environments by analyzing genotoxicity and histopathological changes to elucidate the mechanisms of toxicity. However, further research is needed to better understand the long-term effects of arsenic exposure on neural and cardiac tissues, as well as the molecular pathways involved in arsenic-induced neurotoxicity and cardiotoxicity.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this research.

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