Pharmacogn. Res., 2023; 15(4):785-795.
https://www.phcogres.com

Original Article

Ameliorative Effect of Rutin against Zirconium Oxide (ZrO_2) Nanoparticle induced Behavioural, Biochemical and Tissue Morphological Changes in *Danio rerio*

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ABSTRACT

Background: Zirconium Oxide (ZrO_2) is one of the most widely used metal oxide nanoparticles with unique features that permit its usage in various medical applications, including drug delivery, targeting, labelling, and loading. Rutin is a bioflavonoid found in various natural sources and has diverse biological activities and pharmaceutical applications. Some studies have evaluated the impacts of ZrO_2 NPs on aquatic creatures, but little is known about their ability to recover after exposure. Since the toxicity of ZrO_2 nanoparticles is not known, it would be crucial to investigate their toxicity using zebrafish (*Danio rerio*) as a model organism. **Objectives:** In the present work, the toxicity of ZrO_2 was investigated in *Danio rerio* using behavioural alterations, biomarkers of oxidative stress and cellular damage. The morphology of the gill tissues, as well as the optimal amount of rutin for mitigating deleterious effects was evaluated. **Materials and Methods:** Fish were treated for 14 days, and seven study groups were examined: control, ZrO_2 exposure alone at three distinct concentrations (5 mg/L, 10 mg/L, and 20 mg/L), and combined with rutin (100 mg/L). **Results:** Compared to control groups, *Danio rerio* treated with ZrO_2 alone or in combination with rutin produced worse outcomes. However, rutin-supplemented groups exhibited greater improvement than ZrO_2 alone groups. ZrO_2 affects cells by causing oxidative stress and decreasing the antioxidants SOD, CAT, GPx, GSH, and Vitamin C. Enhanced oxidative stress induces behavioural and morphological modifications. The structural examination of the gill tissues revealed hyperplasia, lamellar fusion, filament erosion, and a dilated marginal channel or epithelial lifting. **Conclusion:** According to our data, the sub-lethal concentration of ZrO_2 NPs for *Danio rerio* is 10 mg/L. Although ZrO_2 was detrimental to the groups exposed to it, supplementing 100 mg/L of rutin was able to protect against its toxicity.

Keywords: Antioxidants, Behavioural changes, Histology, Nanoparticles, Oxidative stress, Zebrafish, ZrO_2 - NPs.

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Received: 16-06-2023;
Revised: 05-07-2023;
Accepted: 04-08-2023.

INTRODUCTION

Nanostructured crystalline particles or Nanoparticles (NPs) have attracted the interest of researchers due to their size-dependent features and extensive applications in biomedical and nano-biotechnological fields, including medical diagnostics, bio-sensing and bioelectronics, gene therapy, drug delivery, *in vivo* imaging, tracking, etc. In the past decade, nanotechnology has garnered tremendous interest in various research fields and applications.[1,2]

Zirconium Oxide (ZrO_2) is one of the most often utilized metal oxide nanoparticles. ZrO_2, also called zirconia, is a technologically significant material with fine natural colour, high stability, toughness, high chemical strength, and desirable corrosion, chemical, and microbiological resistance. The crystal structure of zirconia is monoclinic, tetragonal, and cubic.[3,4]

Zirconium oxide, or ceramic steel, has been used in the production of cutting tools, engine components, and furnaces.[5] ZrO_2 has been utilized recently in the medical industry for drug delivery, targeting, labelling, and loading.[6] Small blocks of ZrO_2 particles, such as micro/nano powders, have been extensively explored and created in recent years. Nanoscale ZrO_2, which exhibits enhanced mechanical properties and superior biocompatibility, is typically included in various dental applications, such as dental fillings for the fabrication of dental crowns, dental implants and other therapeutic applications, and tissue engineering.[7,8] In addition, it is frequently employed in manufacturing gas sensors, developing metal oxide semiconductors, ceramic devices and as a catalyst support material.[9]

DOI: 10.5530/pres.15.4.083

The combination of ZrO_2 NPs like zirconia composite is employed as a carrier to transport the medication to the desired spot.[10] Since ZrO_2 serves as a medication carrier, it is crucial to examine its harmful consequences.[11]

Rutin (3, 3′, 4′, 5, 7- pentahydroxyflavone-3-rhamnoglucoside) is a bioflavonoid found in various natural sources, including fruits and vegetables, particularly in tea, passion flower, buckwheat and apple.[12] It has diverse biological activities and pharmaceutical applications such as antioxidant, anti-proliferative, anti-inflammatory, anti-viral, and anti-carcinogenic properties. It inhibits platelet aggregation while decreasing hyperlipidemia. Additionally, it has low toxicity and thereby is a good candidate for possible clinical uses.[13-15]

Currently, numerous studies examine the biological impact of nanoparticles.[16] Wistar mice exposed to ZrO_2 NPs demonstrated that this nanoparticle could boost the production of Reactive Oxygen Species (ROS) within the cell.[17] Since little is known about the toxicity of ZrO_2 nanoparticles, it is crucial to investigate their toxicity using Zebrafish as a model organism (*Danio rerio*). The Zebrafish is a model organism commonly used in scientific studies due to its appealing characteristics, such as its high resemblance to the human genome, small size, low cost, good repeatability, quick embryonic development, and ease of use in a laboratory setting. In addition, the biochemical, molecular, and behavioural responses of this species have been utilized as a benchmark for assessing the toxicity of various NPs.[18]

This work was planned to identify the sub-lethal concentration of ZrO_2 and the ameliorative impact of rutin against the damage induced by ZrO_2 NPs exposure in zebrafish (*Danio rerio*) by assessing their behavioural responses, biochemical parameters, and morphological alterations.

MATERIALS AND METHODS

Zirconium Oxide (ZrO_2) synthesis and characterization

The ZrO_2 was produced using the precipitation technique. In a 250 mL glass beaker, 0.2 M $ZrOCl_2.8H_2O$ and 2 N NaOH were combined and agitated with a magnetic stirrer. The resulting precipitate was placed in three glass beakers and heated in an oven at 150°C for 24 hr, after which it was washed and filtered to remove all traces of Chlorine Ions (Cl-). Then, the material was dried at 100°C for 24 hr and calcined at 600°C for 4 hr.[19] UV-visible absorption spectroscopy, Fourier Transform Infra-Red (FTIR) spectroscopy, X-ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray (EDX) were used to characterize the synthesized ZrO_2.

Fish and experimental conditions

A commercial breeding center in Chennai, Tamil Nadu, India, provided adult zebrafish (*Danio rerio*). Fish were acclimatized for one week at the aquatic facility using dechlorinated tap water prior to the trial. The photoperiod was sustained at 12L:12D, and the water temperature remained constant at 26.0±1.0°C. The physico-chemical parameters of water such as the pH and dissolved O_2 levels were measured daily. Fish were fed commercial feed twice a day.

Experimental design

Fish were randomly classified into 7 groups. Each group comprised of 6 animals in 3 L of dechlorinated tap water (2 fish/L). Each group were treated as below:

Group 1: Control, Group 2: ZrO_2 (5 mg/L), Group 3: ZrO_2 (10 mg/L), Group 4: ZrO_2 (20 mg/L), Group 5: ZrO_2 (5 mg/L) + Rutin (100 mg/L), Group 6: ZrO_2 (10 mg/L) + Rutin (100 mg/L), Group 7: ZrO_2 (20 mg/L) + Rutin (100 mg/L).

The water, ZrO_2 NPs and Rutin of both control and treated groups were rehabilitated daily. All the above treatments were given for 14 consecutive days, with each group receiving the same stuff.

Behavioural analysis

The T-maze is a multi-species operative task used to assess memory and explore zebrafish learning and memory activities. The Novel Tank Test (NTT) was performed in order to analyze the anxiety-like behaviour and alterations in exploratory activity in zebrafish. The Light/Dark Preference Test (LDT), investigates zebrafish anxiety and exploratory behaviour in the presence of a motivational conflict between light and dark sleeves. The T-maze, NTT and LDT were carried out, according to the procedure stated by Devaraj *et al.*, 2021.[20]

Sample collection

After 14 days, the fish were immersed in cold water and sacrificed. The liver was dissected and cleaned twice with PBS. The tissue was stored at -80°C for later examination. The liver was rinsed with ice-cold saline and then homogenized using a chilled mortar and pestle with 1 mL of homogenization buffer, pH 7.4 (50 mM Tris–HCl buffer with 1 mM EDTA and 0.25 mM sucrose). The homogenate was centrifuged (10,000 rpm) for 15 min at 4°C, and the supernatant was collected for further analysis.

Analyses of biochemical parameters

The supernatant collected from the tissue homogenate was used to evaluate the biochemical parameters: The specific activity of Acetylcholinesterase (AChE) was estimated by the method of Ellman *et al.*, 1961.[21] Oxidative stress markers, specifically Reactive Oxygen Species (ROS) was measured according to Beauchamp and Fridovich., 1971.[22] Lipid Peroxidation (LPO) was estimated according to Devasagayam and Tarachand., 1987.[23] Superoxide Dismutase (SOD) was estimated according to the protocol of Marklund and Marklund., 1974.[24] Catalase (CAT) was determined using the method of Aebi., 1984.[25]

Glutathione Peroxidase (GPx) was estimated by the method of Rotruck *et al.*, 1973.[26] reduced Glutathione (GSH) was analyzed using the protocol of Ellman., 1959.[27] Vitamin C was examined using the procedure described by Roe and Kuether., 1943.[28]

Histological examination

The adult fish's gill was removed and cleaned thoroughly with 0.9% saline, immediately preserved in 10% neutral buffered formalin, embedded in low melting point paraffin (56°C), and sectioned (5 µm). Sections were deparaffinized, dehydrated in graded ethanol for several hours, stained with H&E and inspected under a microscope (Nikon Eclipse 50i).

Statistical analysis

The mean ± Standard Error of Means (SEM) was used to denote all the data. Comparing the outcomes of the different groups was done using a one-way Analysis of Variance (ANOVA) followed by a Duncan's Multiple Range Test (DMRT). The data were evaluated using a statistical program SPSS version 20.0 software package. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Characterization of ZrO_2

UV-visible spectroscopy

The UV-vis absorption spectrum was used to evaluate the optical properties of nanoparticles. The absorption spectrum was recorded between 200 and 800 nm. The absorption peak of ZrO_2 was detected at 263 nm, as illustrated in Figure 1.

X-ray Diffraction (XRD)

The average crystallite size of ZrO_2 nanoparticles was analyzed with the XRD technique. The XRD pattern of synthesized ZrO_2 nanoparticles is illustrated in Figure 2. The main strong peaks were observed at 30.2°, 35.1°, 50.3°, 59.9°, 62.8°, 74.3° and 82.0° which correspond to the planes (111), (200), (220), (311), (222), (400) and (331) respectively. It matched the peaks described by Sigwadi *et al.*, 2019[29] and so the targeted nanoparticle was confirmed. The broader peaks indicate that the particles' size is in the nano range. The particle size was estimated from the width of XRD peaks using Scherrer's formula in the following equation, $D = (0.94\lambda)/(\beta\cos\theta)$. The calculated crystal size was found to be 10.46 nm.

Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier transform infrared spectroscopy is used to recognize the existing functional groups in the targeted nanoparticles. FTIR plays a dynamic role in the stretching or bending vibrations. Because of these alterations, one can effortlessly conclude which groups play a precise part in the overall mechanism. The absorption of infrared light energy with wavelengths ranging from 4000 to 400 cm^{-1} was observed using the FTIR spectra. The FTIR spectra of ZrO_2 are shown in Figure 3. The prominent peaks were observed at 3404 cm^{-1}, 1610 cm^{-1}, 1343 cm^{-1}, 1124 cm^{-1} and 486 cm^{-1}. The peak at 3404 cm^{-1} corresponds to O-H stretching, while a peak in the region 1610 cm^{-1} may be due to adsorbed moisture. The peak at 1343 cm^{-1} is attributed to O-H bending, and then the peak at 1124 cm^{-1} corresponds to Zr-OH bending vibration. Finally, the peak at 486 cm^{-1} is attributed to Zr-O vibration, which predicts the targeted nanoparticle.

SEM with EDX

The morphology of the synthesized ZrO_2 NPs was examined by SEM technique. The nature and structure of the attained products depend upon the experimental conditions and preparation method. The surface of the material was significantly magnified in the micrographs produced by Scanning Electron Microscopy (SEM). Figure 4 (a and b) shows the micrographs of ZrO_2 NPs with different magnifications. ZrO_2 NPs were spherical with uniform size and monodispersed. Nevertheless, the particles

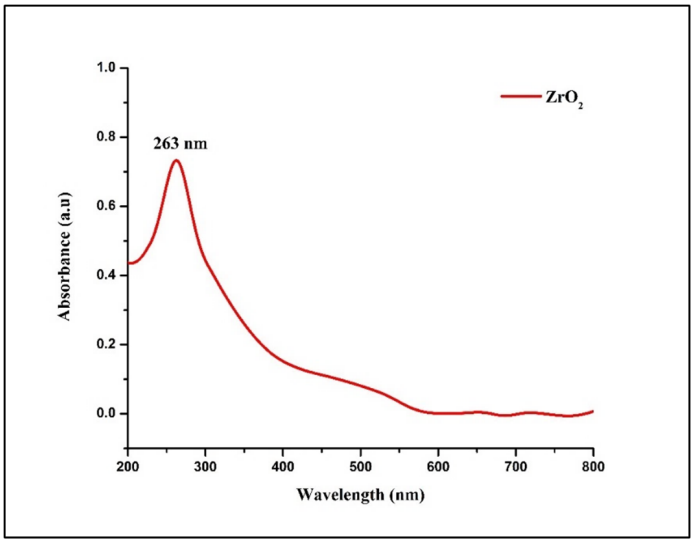


Figure 1: UV-visible spectrum of ZrO_2.

Table 1: EDX measurements of ZrO_2.

Element	Line	Mass%	Atom%
O	K	30.02±0.27	70.98±0.65
Zr	L	69.98±0.32	29.02±0.13
Total		100.00	100.00
			Fitting ratio 0.3192

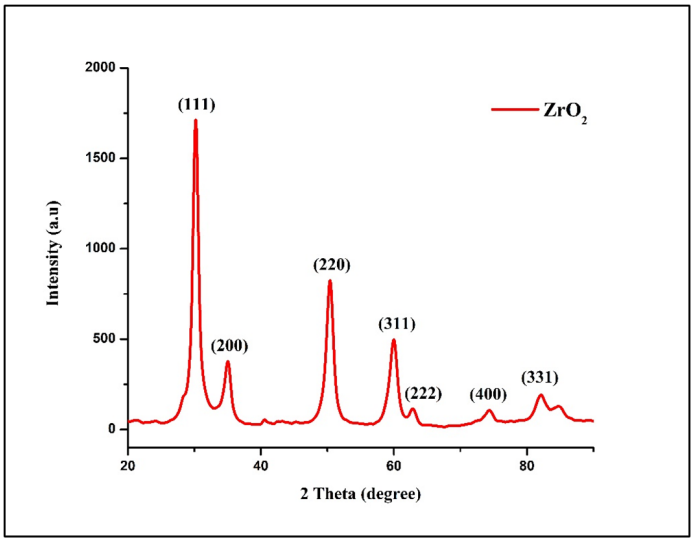


Figure 2: XRD pattern of ZrO_2.

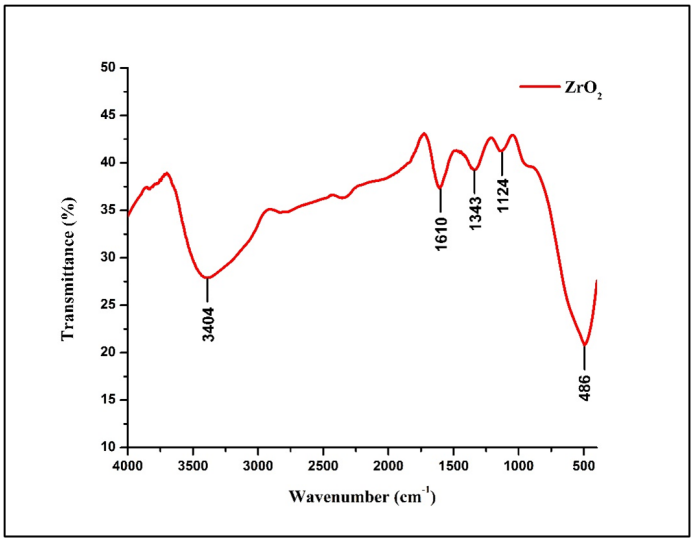


Figure 3: FTIR spectra of ZrO_2.

aggregate may be due to the binding of the reducing agent used to synthesize NPs.

The EDX analysis indicates the synthesized NPs containing elemental mass % Zr (69.98 ± 0.32) and O (30.02 ± 0.27), which specifies its higher purity without any impurities. The EDX analysis confirms that the synthesized NPs is ZrO_2. Figure 5 represents the EDX analysis and the details which are shown in Table 1.

Behavioural analysis of *Danio rerio*

The learning, memory, anxiety and exploratory behaviour of the adult zebrafish were analyzed through behavioural tests including T-maze, Novel Tank Test (NTT) and Light and Dark test (LDT). The fish in our study subjected to ZrO_2 showed significantly dull behaviour when compared to the control and ZrO_2 + Rutin groups. However, the betterment in the behaviour of the fish were observed in the ZrO_2 + Rutin treated groups while compared to the ZrO_2 alone treated groups.

T-maze for learning and memory

Time spent in the green and red arm of the T-maze: The total time spent on the green and red arm of the T-maze by the control, ZrO_2, ZrO_2 + Rutin treated groups have been graphically represented in Figure 6 (a).

The number of entries in the green and red arm as a parameter: The number of entries to the green and red arm of the T-maze by the control, ZrO_2, ZrO_2 + Rutin treated groups is graphically represented in Figure 6 (b).

Novel Tank Test (NTT) for checking anxiety-induced behaviour changes

Time spent in the top and bottom zones of the tank:

a) b)

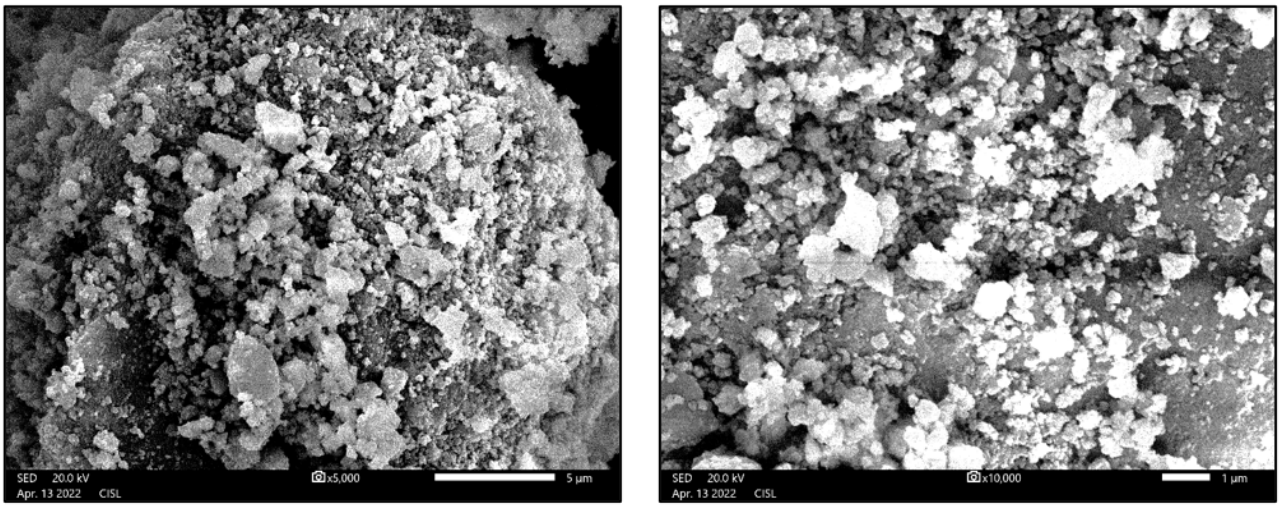


Figure 4: a) and b) Micrographs of ZrO_2 NPs with different magnifications.

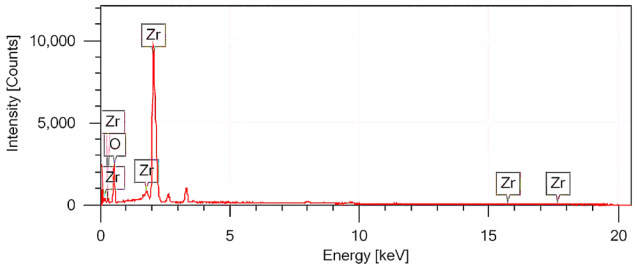


Figure 5: Energy-dispersive X-ray for ZrO_2.

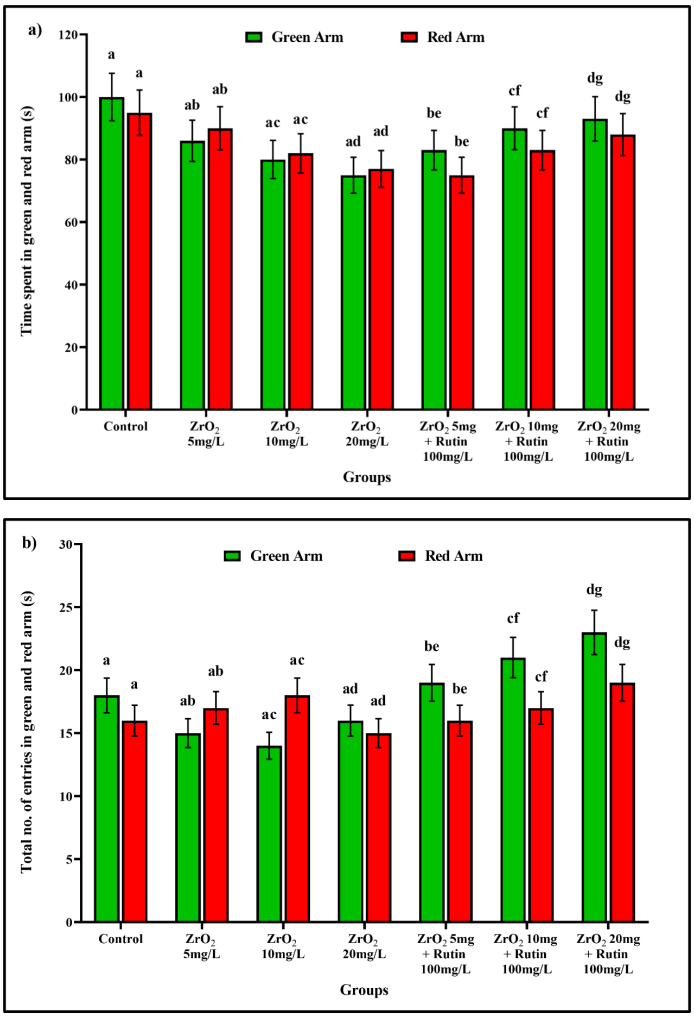
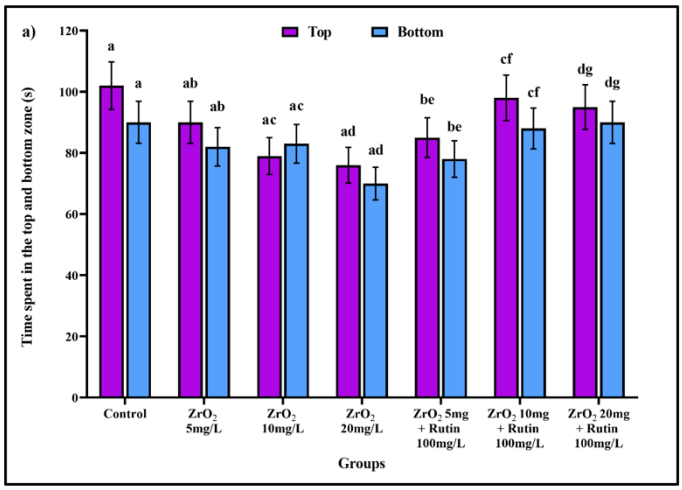


Figure 6: T-maze (a) Graphs depicting the time spent in the red and green arm of the T-maze by the control, ZrO_2 exposure and ZrO_2 and rutin-supplemented groups. (b) Total average no. of entries in the green and red arm of the T-maze by the control, ZrO_2 exposure and ZrO_2 and rutin-supplemented groups. Data are stated as mean ± SEM. Distinct letters denote significant variances between the groups ($p< 0.05$).

The total time spent in the top and bottom zones of the tank by the control, ZrO_2, ZrO_2 + Rutin treated groups is graphically represented in Figure 7 (a).

The number of entries to each zone as a parameter: The number of entries to the top and bottom zones by the control, ZrO_2, ZrO_2 + Rutin treated groups is graphically represented in Figure 7 (b).



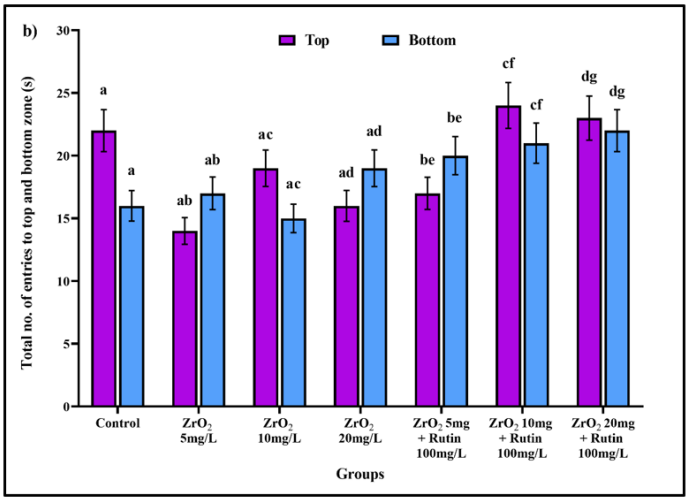


Figure 7: Novel Tank Test (NTT) (a) Graphs depicting the time spent in the top and bottom zone of the tank by the control, ZrO_2, ZrO_2 + Rutin supplemented groups. (b) The total average number of entries in the top and bottom zone of the tank by the control, ZrO2, ZrO_2 + Rutin supplemented groups. Data are stated as mean ± SEM. Distinct letters denote the significant variances between the groups ($p< 0.05$)

Light and Dark Test (LDT) for assessing anxiety-like behavioural changes

Time spent in the light and dark zone of the tank: The total time spent in the light and dark compartments by the control, ZrO_2, ZrO_2 + Rutin treated groups is graphically represented in Figure 8 (a).

The total number of crossings between the compartment as a parameter: The total number of entries to the light and dark compartment by the control, ZrO_2, ZrO_2 + Rutin supplemented groups is graphically represented in Figure 8 (b).

Acetyl Cholinesterase (AChE) activity

AChE activity (Figure 9) in the brain tissues decreased in the 5mg/L, 10mg/L and 20mg/L ZrO_2 treated groups as compared to the control, whereas the groups subjected to rutin treatment

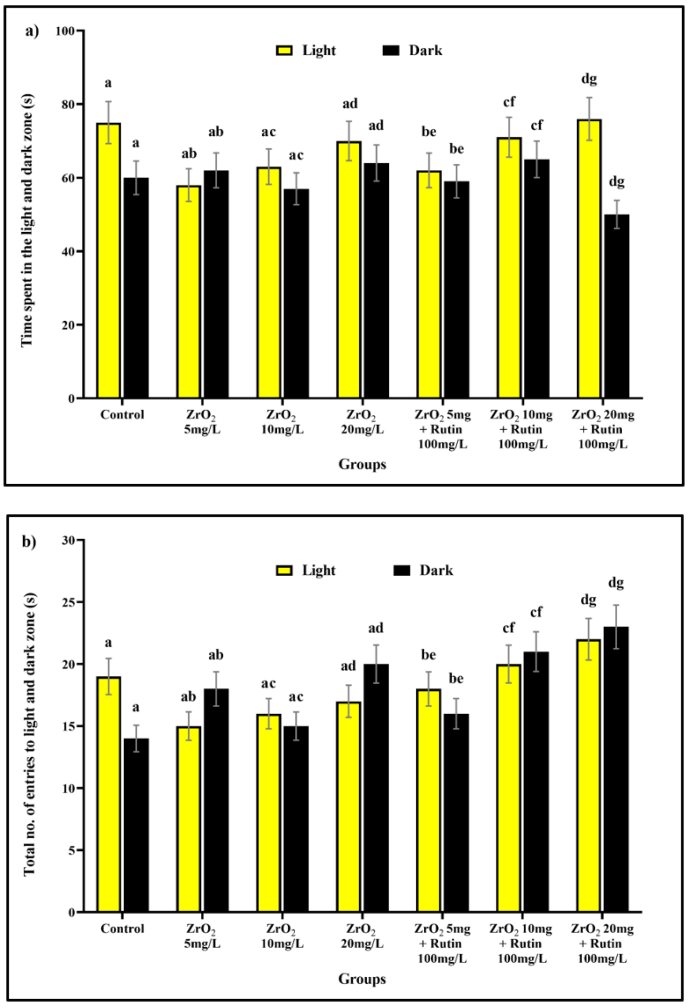


Figure 8: Light and Dark test (LDT) (a) Graphs depicting the time spent in the light and the dark zone by the control, ZrO_2, ZrO_2 + Rutin supplemented groups. (b) The total average number of entries in the light and dark compartments of the LDT tank by the control, ZrO_2, ZrO_2 + Rutin supplemented fish groups. Data are stated as mean ± SEM. Distinct letters denote significant variances between the groups ($p< 0.05$).

at 100mg/L along with ZrO_2 (5, 10, 20 mg/L) showed persistent elevation in the AChE activity in a dose-dependent manner.

Oxidative stress markers

The activities of oxidative stress markers such as SOD (Figure 10a), CAT (Figure 10b), GPx (Figure 10c), GSH (Figure 10d), Vit C (Figure 10e), ROS (Figure 10f) and LPO (Figure 10g) in the liver tissues of normal control and experimental adult zebrafish were evaluated. SOD, CAT, GPx, GSH and Vit C activity were markedly reduced in the groups exposed to ZrO_2 (5mg/L, 10mg/L and 20mg/L) as compared to the control. However, in the groups treated with ZrO_2 combined with the optimum dose of rutin, the antioxidant levels were elevated as compared to the ZrO_2 alone groups and in fact, the antioxidant levels were near to those of the control. In addition, when compared to the control, the groups exposed to ZrO_2 showed a steady rise in the oxidant's

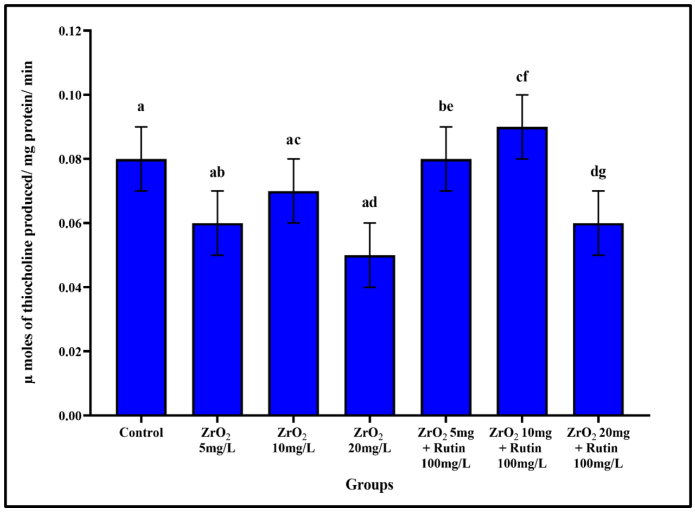


Figure 9: AChE activity in the brain tissue of control, ZrO_2, ZrO_2 + Rutin supplemented groups. Data are stated as mean ± SEM. Distinct letters denote significant variances between the groups ($p< 0.05$).

levels, such as ROS and LPO, in the zebrafish. The ROS and LPO markers were considerably reduced in a dose-dependent manner on supplementation with rutin to ZrO2-treated fish. However, the antioxidant levels in the ZrO_2 + rutin treated groups were elevated as compared to ZrO_2 alone treated fish.

Histology

Histological observations of the adult zebrafish gill tissue from both control and treated groups were analyzed and illustrated in Figure 11 (a-g). Control specimens of the gill of zebrafish showed normal histological structure without any pathological lesions. In contrast, gill tissues exposed to 5 mg, 10 mg and 20 mg of ZrO_2 showed toxicity as evident by hyperplasia, lamellar fusion, erosion of the filament and dilated marginal channel or epithelial lifting. However, rutin supplementation along with ZrO_2 showed markedly reduced changes in the dilation of marginal channel or epithelial lifting, lamellar fusion and shortening of secondary lamellae.

DISCUSSION

Nanomaterials are inescapable in human and environmental exposures, making them a part of our everyday lives. There is an increasing interest in using metal oxide NPs such as zirconia in biomedical tissue engineering and implants, particularly orthopedic and dental implants.[30] Due to the extensive use and production of NPs, the amount of these particles in the environment is rising over time.[18]

ZrO_2 NPs evaluated on Wistar rats demonstrated that these nanoparticles can induce red blood cell damage.[31] Exposure to ZrO_2 NPs in a *Drosophila melanogaster* model affected phenotypic, neuronal development, and function.[32] In addition, nano-ZrO_2 exposure had the most damaging effects on the growth and reproduction of aquatic crustaceans, such

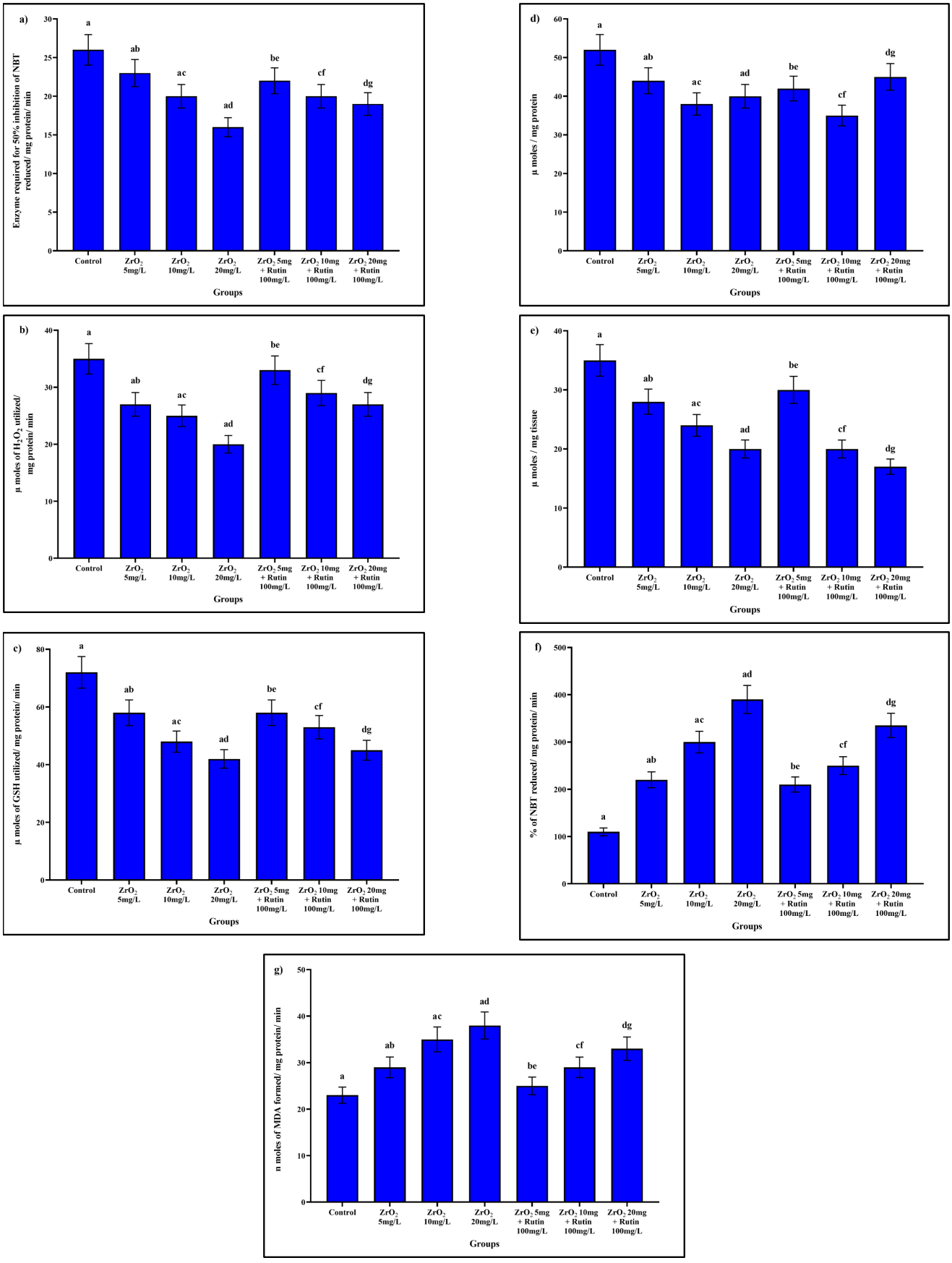


Figure 10: The oxidative stress markers such as SOD (a), CAT (b), GPx (c), GSH (d), Vit C (e), ROS (f) and LPO (g) in the liver tissue of control, ZrO_2, ZrO_2 + Rutin supplemented groups. Data are stated as mean± SEM. Distinct letters denote significant variances between the groups ($p< 0.05$).

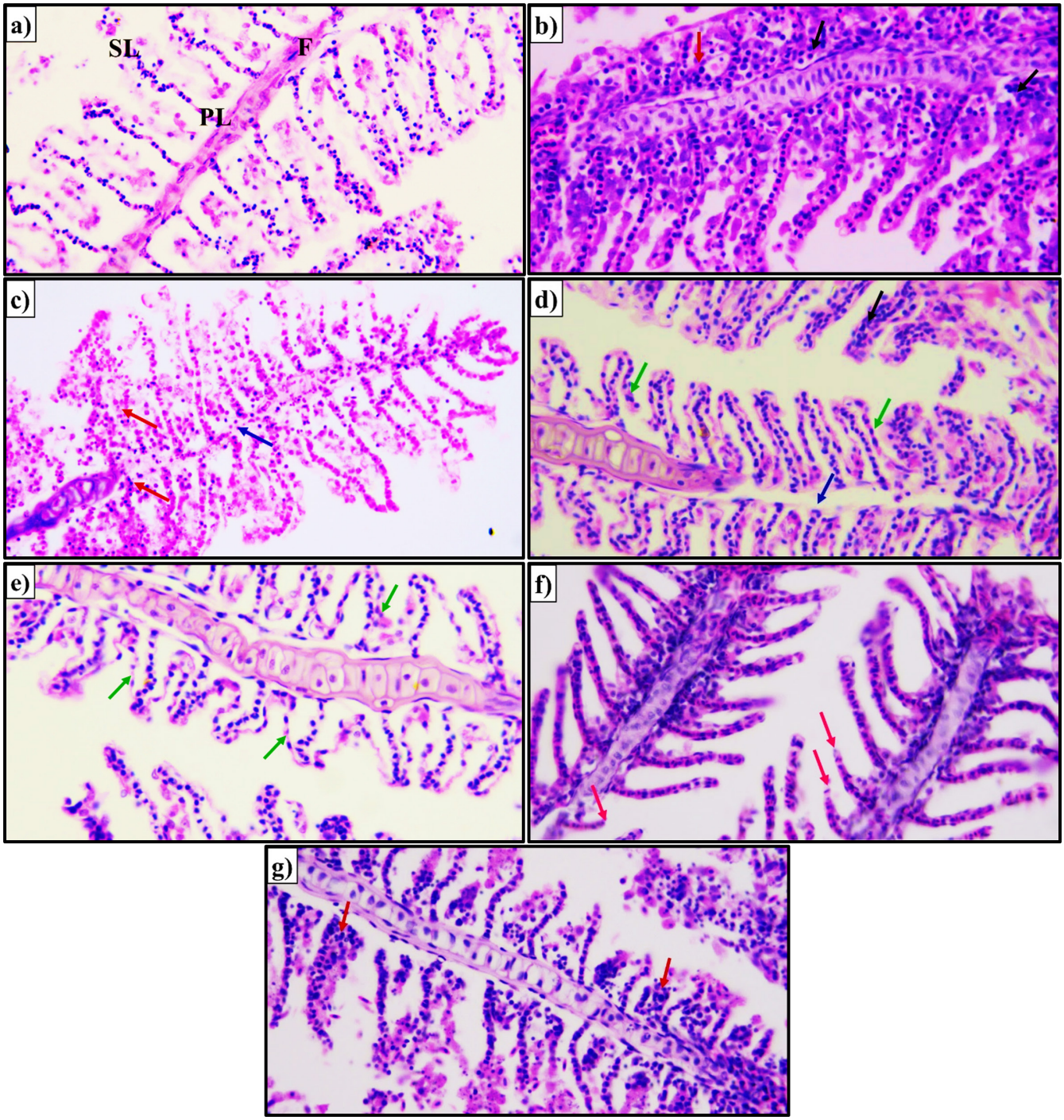


Figure 11: Histological alterations in the gill sections of Zebrafish (*Danio rerio*) after 14 days of ZrO_2 and Rutin exposure. (a) control group revealing normal histology, including gill Filaments (F), Primary Lamellae (PL) and Secondary Lamellae (SL). (b, c and d) cells exposed to 5 mg\L, 10 mg\L and 20 mg\L of ZrO_2 exhibited hyperplasia (black arrow), lamellar fusion (red arrow), erosion of the filament (blue arrow) and dilated marginal channel or epithelial lifting (green arrow). (e, f and g) cells exposed to ZrO_2 (distinct doses mentioned above) along with 100 mg/L of Rutin show reduced changes such as dilated marginal channel or epithelial lifting (green arrow), lamellar fusion (red arrow) and shortening of secondary lamellae (pink arrow). (H&E staining, Scale bar: 10 µm).

as *Thamnocephalus platyurus* and *Daphnia magna*.[33] The effect of zirconia nanoparticles (15–20 nm in size) was examined in the early embryonic phases of the Zebrafish model as it has been used as an appropriate *in vivo* model for nanomaterial toxicity research.[34] Other investigations on various NPs have demonstrated their toxicity to adult zebrafish but, this is the first study to document the harmful effects of ZrO_2 nanoparticles on adult zebrafish.

The current study evaluated the potential ameliorative effects of rutin on zebrafish ZrO_2-induced toxicity. ZrO_2 was supplied to zebrafish for 14 days to test the toxicity while they were exposed to rutin at different doses. After the study period, several

behavioural, biochemical, and histological examinations were conducted on the fish.

Generally, the nanoparticles elicit anxiety behaviour, inhibit exploratory behaviour and disturb working memory ability in zebrafish in a concentration-dependent manner.[35] However, in this study, we have revealed that rutin lowers ZrO_2-induced oxidative stress, restores zebrafish memory ability, and recovers scototaxis and bottom-dwelling behaviour. The bottom-dwelling behavior is a characteristic of fish under oxidative stress or fear. This reveals that the fish exposed to ZrO_2 NPs were under stress, staying at the bottom of the tank rather than floating at the top. However, administration of rutin caused free movement of the fish in the tank and reduced bottom dwelling behaviour, which can be attributed to reduced stress. In addition, rutin treatment at 100mg/L groups significantly reversed the scototaxis behavioural alterations and memory disabilities caused by ZrO_2 NPs administration, as evidenced by a substantial rise in the number of entries to the lighted zone in LDT and green arm in T-maze and the amount of time spent in the light zone in LDT and green arm in T-maze.

Earlier research demonstrated that brain Acetylcholinesterase (AChE) activity is a useful diagnostic tool for organophosphates, and carbamate pollution and describes the inhibition of AChE enzyme as the reason for the decreased locomotor activity in fish.[36,37] The buildup of Acetylcholine (ACh) disrupts the synchronization between the neuronal and muscle junctions, resulting in altered fish behaviour. Therefore, in the present investigation, the brain AChE activity of fish treated with ZrO_2 NPs was evaluated and found to be reduced. The subsequent recovery of enzyme activity in rutin-supplemented groups demonstrates that fish can withstand the stress of the toxicant. Similarly, we found a marked correlation between AChE activity and behavioural changes in the fish.

Next, we studied the activities of several antioxidants in zebrafish to demonstrate whether the altered behavioural response generated by ZrO_2 NP is related to oxidative stress and to determine whether rutin can protect against ZrO_2 NP-induced oxidative stress. NPs display toxicity by inducing oxidative stress via several cellular responses.[38] When metal ions are liberated from NPs, Reactive Oxygen Species (ROS) production elevates, forming free radicals that induce cytotoxicity in zebrafish. Inactivation of the antioxidant response is typically noticed upon exposure to NPs, as the antioxidant levels decrease.[39] ROS levels increased in the ZrO_2 NP-treated groups, showing that ZrO_2 NPs induce oxidative stress. The resulting large increase in ROS caused the biomolecules, including proteins and lipids, to be highly reactive.

LPO is known to disrupt cellular membrane integrity and is implicated in the pathophysiology of numerous liver lesions. Increased levels of Malondialdehyde (MDA), a lipid peroxidation product produced in the liver, may be attributed to an increase in the formation of reactive oxygen metabolites, particularly hydroxyl radicals, and a modification of the antioxidant defense system.[40] LPO is also regarded as one of the NP-induced toxicity pathways.[41] Metal ions are recognized to magnify LPO by Fenton's reaction of lipid hydroperoxyl radical, as indicated by the dose-dependent increase in the LPO levels of the treated groups. Similar elevated LPO levels were detected in carp (*Cyprinus carpio*) and catfish (*Ictalurus nebulosus*) treated with the pesticide dichlorvos.[42] However, in this study, ZrO_2 NPs + rutin treated groups showed decreased ROS levels, which may be due to the rapid and efficient consumption of ROS generation by rutin.

The antioxidant defense mechanism in zebrafish is triggered in response to ROS, comparable to the mammalian system.[43] SOD is often regarded as the principal antioxidant enzyme against ROS and LPO. Because SOD is the first enzyme to deal with O_2 radicals, and since CAT aids in converting H_2O_2 into H_2O, oxidative stress is probable to alter their activity.[18] In our study, the SOD and CAT activities were significantly reduced in the ZrO_2 NPs exposed groups. When SOD and CAT activity is suppressed, oxyradicals aggregate and oxidative damage occurs. This would imply that the body's antioxidant defense mechanism failed to shield it from the dangers of nanoparticle exposure. A similar finding was made in Zebrafish embryos treated with ZrO_2 NPs.[34] However, in our study, rutin supplementation along with ZrO_2 NPs improved SOD and CAT activity compared to ZrO_2 alone treated zebrafish.

To eliminate the toxic H_2O_2, CAT collaborates with the GPx enzyme.[44] GPx also collaborates with glutathione to remove H_2O_2 via glutathione oxidation. GSH is a critical non-enzymatic antioxidant that uses its thiol group to quench oxyradicals.[45] It participates in numerous cellular processes, including ROS scavenging, electrophile detoxification, thiol-disulfide status maintenance and signal transduction in oxidant and antioxidant pathways.[46,47] GSH levels can be reduced by NPs.[48] GSH depletion was observed to be severe in the previous studies by Ahamed *et al.*, 2010[49] and Akhtar *et al.*, 2012.[50] Our results also correlate with the above finding showing depleted GPx and GSH activity in fish treated by ZrO_2 NPs, which may be due to the toxicity of the NPs. In the presence of rutin, GPx and GSH levels were restored near to control levels emphasizing the protective beneficial effects of rutin.

Vitamin C, a non-enzymatic antioxidant, suppresses oxidative stress by multiple methods, such as ROS scavenging, by trapping radicals in the aqueous phase and stopping peroxidation, thus defending the membrane from oxidative damage.[51] Vitamin C was significantly reduced in ZrO_2 NPs exposed fish as compared to controls. This could be correlated with the NP-induced increase in the formation of free radicals over time and subsequent reduction in the antioxidant. However, rutin administration

had a protective beneficial positive effect in altering the levels of Vitamin C.

Fish gills perform vital metabolic processes, including gas exchange, osmoregulation, ion regulation, and excretion of nitrogenous waste. The surface of the gill is in direct contact with the contaminants and toxins. Gills are crucial indicators for measuring water-borne exposure to environmental toxins due to their significant wide variety of functions and exist as the primary interactive area with xenobiotics.[52] Several NPs are known to damage the gill tissue. After acute exposure, it could cause lesions in the gills of zebrafish due to physical irritation and tissue blockage on the surface of the gills. Investigators found that rainbow trout had edema, altered mucocytes and hyperplasia on exposure to NPs.[18] In the present study, the gill tissues of zebrafish exhibited significant histopathological changes such as hyperplasia, lamellar fusion, erosion of the filament and dilated marginal channel or epithelial lifting on ZrO_2 NPs exposure. These modifications are not chemical-specific and have been described in distinct studies with certain fish species in response to other chemicals similar to NPs. Nevertheless, the groups supplemented with rutin showed reduced damage to the zebrafish gills.

CONCLUSION

This is the first publication detailing the harmful effects of ZrO_2 NPs on adult zebrafish. This study indicates that chemically generated ZrO_2 NPs are hazardous. According to this study, the sub-lethal dose of ZrO_2 NP for *Danio rerio* was found to be 10 mg/L. ZrO_2 NPs treatment led to alterations in the zebrafish behaviour, damage to the gills, elevated liver LPO and reduced liver antioxidants. However, all these changes were significantly reduced in the groups treated with the rutin ZrO_2 NP composite. These data underline the marked protective effects of the presence of the flavonoid rutin in the ZrO_2 NP composite. In future, it is essential to gain a deeper understanding on the physiological mechanisms involved in the detoxification process following ZrO_2 exposure, in addition to the molecular mechanism and epigenetic alterations caused by ZrO_2 NP exposure. This would throw light on the mechanism of action of rutin in suppressing ZrO_2 NP toxicity.

ACKNOWLEDGMENT

The authors would like to acknowledge RUSA 2.0 for providing financial support and the Department of Biochemistry and Biotechnology, Annamalai University for providing the laboratory facilities and all other required consumables and equipment.

CONFLICT OF INTEREST

The authors declare no conflict of interest to disclose.

ABBREVIATIONS

FTIR: Fourier Transform Infra-Red; **XRD:** X-ray Diffraction; **SEM:** Scanning Electron Microscopy; **EDX:** Energy Dispersive X-ray; **NTT:** Novel Tank Test; **LDT:** Light/Dark Preference Test; **AChE:** Acetylcholinesterase; **LPO:** Lipid Peroxidation; **ROS:** Reactive Oxygen Species; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GPx:** Glutathione Peroxidase; **GSH:** reduced Glutathione.

SUMMARY

- Zirconium Oxide (ZrO_2) nanoparticles are used in various medical applications.
- Rutin is a bioflavonoid with diverse biological and pharmaceutical applications.
- The study aimed to investigate the toxicity of ZrO_2 in zebrafish (*Danio rerio*).
- Fish were treated with different concentrations of ZrO_2 for 14 days, which led to observable alterations in behavior, changes in oxidative stress markers, and cellular damage, but rutin supplementation improved the outcomes.
- Based on the study's findings, the sub-lethal concentration of ZrO_2 for *Danio rerio* was determined to be 10 mg/L and Rutin at 100 mg/L could protect against ZrO_2 toxicity.

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Cite this article: Premnath BJ, Pratima BJ, Ravichandiran R, Srinivasan MK, Nalini N. Ameliorative Effect of Rutin against Zirconium Oxide (ZrO_2) Nanoparticle induced Behavioural, Biochemical and Tissue Morphological Changes in *Danio rerio*. Pharmacog Res. 2023;15(4):785-95.