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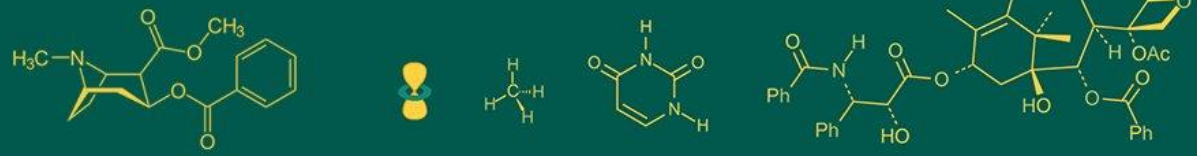

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Effect of tributyltin exposure on the eye of zebrafish

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Abstract
Tributyltin (TBT), as an organotin substance, is recognized as one of the important xenobiotic pollutants. The present study was carried out to investigate the toxicological effects of TBT on the sensory organ eye of adult female zebrafish. Adult zebrafish were exposed to three concentrations of TBT for 28 days (125, 250, and 500 ng/L) to evaluate the oxidative stress markers and histological changes in the eye. Mortality was not observed in any toxicity group. TBT exposure at 250 and 500 ng/L significantly decreased the CAT activity and total antioxidant capacity (TAC) of eye tissue. The higher levels of TBT exposure (250 and 500 ng/L) significantly increased lipid peroxidation as indicated by increased levels of MDA. Histopathological evaluation of the retina of adult zebrafish following TBT exposure at the highest concentration revealed mild depigmentation of the retinal pigmented epithelium layer.

Keywords: Tributyltin, oxidative stress, histological changes, eye

Introduction
Various chemicals present in the ecosystem affect the health of aquatic animals by altering various physiological and biochemical alterations. Endocrine-disrupting chemicals have a damaging effect on the nervous system including the cortex, hippocampus, hypothalamus, and other brain regions. Such chemicals affect the nerve membrane permeability, various channels, and other organelles which is due to oxidative stress (Agarwal *et al*., 2015; Zhang *et al*., 2017) [1, 29]. Tributyltin (TBT) is a hazardous endocrine disruptor that was widely used in various industries including plastic industries, also as a fungicide in the wood sector, and as a component of antifouling paints used on ship hulls and docks (Hoch, 2001) [12].
The TBT has been restricted to be used due to severe toxicity in different species of aquatic animals (Fent, 1996) [9]. As it is a very stable compound, it is still detected in water resources which may be due to its use in certain developing countries (Sousa *et al*., 2009; Garg *et al*., 2011; Cao *et al*., 2009) [25, 10, 3]. Antifouling paints containing TBT are still manufactured and sold in certain countries (Uc-Peraza *et al*., 2022) [27]. Total organotin concentrations range values of 1.76–486.62 ng Sn/g (dry weight) in Asian harbors (Wang *et al*., 2019) [28]
The visual system has a high sensitivity to TBT (Guo *et al*., 2022) [11]. TBT targets the retina and causes apoptosis in developing zebrafish which may be related to oxidative stress (Dong *et al.*, 2006) [6]. The retina is a key component of circadian rhythm input, and its photoreceptors convert light signals into electrical signals via phototransduction cascades, which are projected to different parts of the brain via nerve fiber (Falcon *et al*., 2010) [7]. TBT has been shown to induce the apoptosis of retinal nerve cells in zebrafish (Dong *et al*., 2006) [6]. Shi *et al*. (2021) [23] examined the effects of oxidative stress on behavior in the brains and eyes of TBT-exposed medaka and observed that superoxide dismutase (SOD) and catalase (CAT) activity were adversely affected by decreased locomotor activity and social interaction. Following TBT exposure to the tropical guppy (*Poecilia vivipara*) de Paulo *et al*. (2020) [5] found significant changes in retinal and corneal structure and organization.
Various studies have been conducted related to TBT exposure in aquatic animals, but there is a need to evaluate its effect on the eyes following long-term exposure at various concentrations in zebrafish, which is considered an aquatic animal model that has retinal layers similar to humans. Thus, the present study was designed to evaluate the effect of long-term TBT exposure on oxidative stress markers of the eye and histology of the retina of adult zebrafish.

Materials and Methods

Chemicals

Analytical grade tributyltin chloride was purchased from Sigma-Aldrich (CAS number-1461-22-9). Other chemicals used in experiments were of molecular or analytical grade.

Experimental animals and the environment

Adult female zebrafish (Wild-type) above 3 months of age were maintained in an aquarium having a 20 L water capacity. Before the start of the study, a 2-week acclimatization period was observed and fish pellets (Tetra bits complete®, Tetra GmbH-Germany) were used to feed the fish. The standard light-dark cycle of 14:10 h was maintained during the entire study.

Experimental design

A total of 144 adult female zebrafish were randomly divided into four groups (36 fish in each group). Control group fish were kept untreated whereas other groups were treated with TBT (Sigma–Aldrich, CAS number-1461-22-9) at concentrations of 125 ng/L, 250 ng/L, and 500 ng/L for 28 days. In the present study, concentrations of TBT exposure were selected based on previous literature (Sousa *et al.*, 2009; Garg *et al.*, 2011) [25, 10]. The concentration of TBT in each tank was maintained by changing the water daily with fresh water containing a particular strength of TBT. After an exposure period of 28 days, oxidative stress markers in eyes were evaluated using 120 zebrafish (30 in each group and 6 replications). A total of 24 zebrafish were used to evaluate histological changes (n=6) from each group. All fish were humanely sacrificed by the ice-cold method on the 29th day of the experiment. The eyes of each fish were dissected under stereozoom microscopes (Model CZM6, Labomed Inc., USA). Eyes from each zebrafish were collected in phosphate buffer saline for estimation of total antioxidant capacity (samples from 6 fish/group). Samples of eyes from zebrafish were also collected in tris–EDTA buffer (8.5 pH) to evaluate the activity of SOD (samples from 6 fish/group). For evaluation of CAT activity and protein level, samples from 24 fish (6 fish/ group) were collected in a phosphate buffer (7.5 pH; PB). Samples of eyes from 48 fish (12 fish/group; a pooled sample from 2 zebrafish) were collected in a butylated hydroxytoluene buffer to estimate the malondialdehyde (MDA) level. For histopathological examination, 6 fish from each group were kept in 10% neutral buffered formalin (NBF).

Evaluation of oxidative stress markers in eyes

SOD activity was determined by previously published methods (Marklund and Marklund, 1974) [17]. CAT activity was determined as per the method described by Beers and Sizer (1952) [2]. The level of MDA was determined as thiobarbituric acid (TBA) forms the MDA-TBA complex (Lykkesfeldt, 2001) [16]. Total antioxidant capacity was determined by using the kit (EZassay antioxidant activity estimation kit, Himedia).

Histopathological evaluation

Twenty-four zebrafish (6 from each group) were used for the eye histopathology. After sacrifice, the whole fish was fixed in 10% neutral buffered formalin for 72 hours followed by decalcification using sodium EDTA solution for 10 days. Standard methodology was followed to prepare a paraffin block and sections of tissue (5 µm) were cut using a semi-automated rotary microtome (Leica Biosystems, Germany). Tissue sections were stained with haematoxylin and eosin (H & E) stains (Luna, 1968) [14]. The stained slides were observed for microscopic pathological changes of the eye using an optical microscope (Zeiss primo star) and histological photographs were captured.

Statistical analysis

Statistical analyses of all data were carried out using GraphPad Prism 9.4.1. Kolmogorov-Smirnov test was used to evaluate the normality of data along with Bartlett's test to confirm the equal variance. All data didn't have either normal distribution or equal variances and were analyzed by the Kruskal-Wallis test followed by Dunn's test. The value of $p<0.05$ (*) was considered as statistically significant and $p<0.01$ (**), $p<0.005$ (***) and $p<0.001$ (****) was considered as highly statistically significant.

Results

TBT exposure did not produce mortality in zebrafish of all toxicity groups during the study. No significant changes were found in SOD activity in the zebrafish eye of all groups (Figure 1). However, CAT activity (Figure 2) was significantly lower in the TBT-250 and TBT-500 ng/L groups as compared to that of the control group. TAC was significantly lower in the TBT-250 and TBT-500 ng/L groups as compared to that of the control group (Figure 3). However, the MDA level was found significantly higher in TBT-500 ng/L groups as compared to that of the control group (Figure 4). 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), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL) as shown in figure 5. The retina of zebrafish exposed to 125 ng/L and 250 ng/L of TBT did not exhibit histopathological changes. However, TBT exposure at 500 ng/L caused mild depigmentation of the retinal pigmented epithelium layer.

Discussion

There are a set of antioxidant enzyme systems in fish, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) to deal with ROS-induced damage, which can reflect the body's anti-stress ability (Lushchak, 2011) [15]. A decrease in SOD enzyme activity causes oxygen intolerance and initiates cellular degeneration. ROS acts as a cytotoxic substance, accumulates, and causes cellular damage (Rajakrishnan and Menon, 2001) [22]. The CAT enzyme is found in all aerobic cells. Still, it is most prevalent in hepatocytes, renal cells, and erythrocytes, and it is found intracellularly in the cytoplasm, endoplasmic reticulum, and peroxisomes (Percy, 1984) [21]. A decrease in the CAT enzyme results in degenerative alterations caused by oxidative stress (Nandi *et al.*, 2019) [20]. MDA is a significant metabolite generated by lipid peroxidation. As a result, MDA functions as an oxidative stress biomarker (Singh *et al.*, 2014) [24]. TBT exposure significantly enhanced MDA content in the eyes of adult female zebrafish. This could be because of the attack of non-detoxified radicals on cellular macromolecules when the amount of ROS and other radicals exceeds the capability of antioxidant enzymes. Excess radical generation might

increase the ROS level and ultimately cause lipid peroxidation. Although the precise mechanism of TBT-induced dysfunction is unknown, TBT toxicity could be attributed to its propensity to promote cellular oxidative stress in tissues (Merlo *et al*., 2016) [18]. Similar to our observation, TBT has been reported to cause lipid peroxidation in trout following exposure for 30 days. TBT-induced lipid peroxidation may be due to increased ROS generation via the caspase-dependent death process mediated by Ca^{2+} (Nakatsu *et al*., 2007) [19]. Kim *et al*. (2019) [13] also reported that exposure to trimethyltin resulted in ROS-mediated apoptosis in zebrafish retinal cells. Alteration in either SOD or CAT activity in the eye of zebrafish following TBT exposure demonstrates the potency of TBT to cause oxidative stress-mediated changes in these organs. The higher MDA levels in rainbow trout may potentially be related to dibutyltin (DBT), a consequence of TBT breakdown. A mechanistic study determined that DBT can lead to oxidative stress, which raises intracellular ROS, mitochondrial mass, and mitochondrial ROS (Chantong *et al*., 2014) [4].

Similar to our observation, TBT exposure has been reported to cause degenerative changes in the corneal epithelium, lens, pigment layer of the retina, and choroid of minnow *Phoxinus* larvae, eventually resulting in the opaque color of the eyes (Fent and Meier, 1992) [8]. When Tropical Guppy (*Poecilia vivipara*) was exposed to TBT (15.8–818 ng Sn/L) for 7 days, the retinal pigment epithelium was altered in pigment positioning, the photoreceptor layer was disorganized, and iris melanin was hyperpigmented (de Paulo *et al*., 2020) [5]. Mild histological changes in the eye of zebrafish in the present study might be attributed to a lower concentration of exposure.

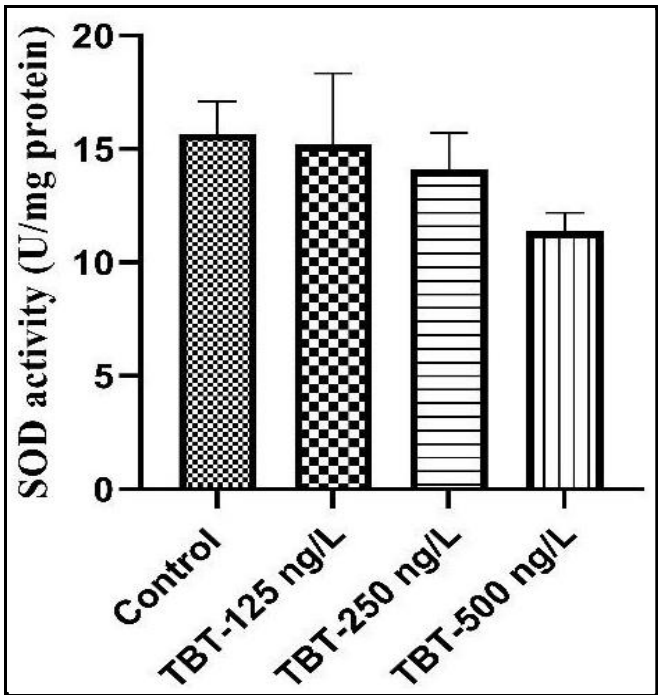

Fig 1: SOD activity in eyes of zebrafish following TBT exposure for 28 days

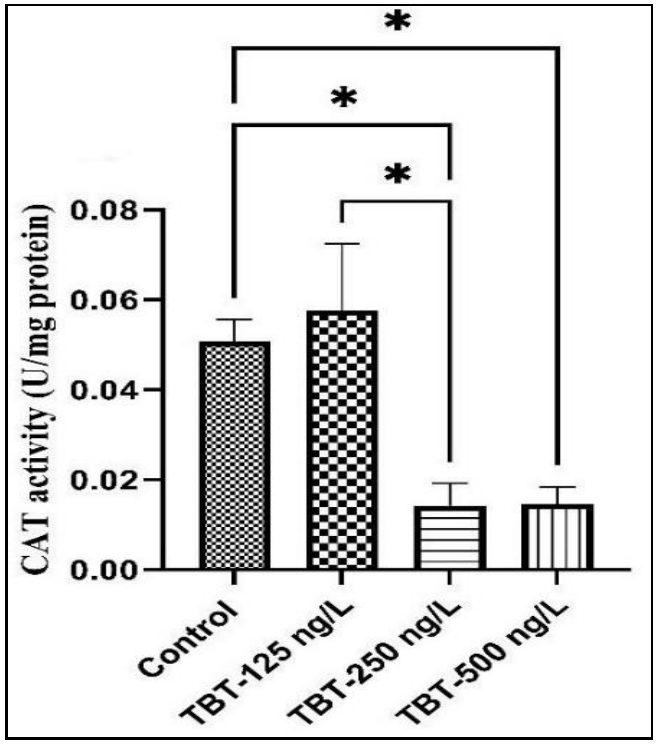

Fig 2: CAT activity in eyes of zebrafish following TBT exposure for 28 days.

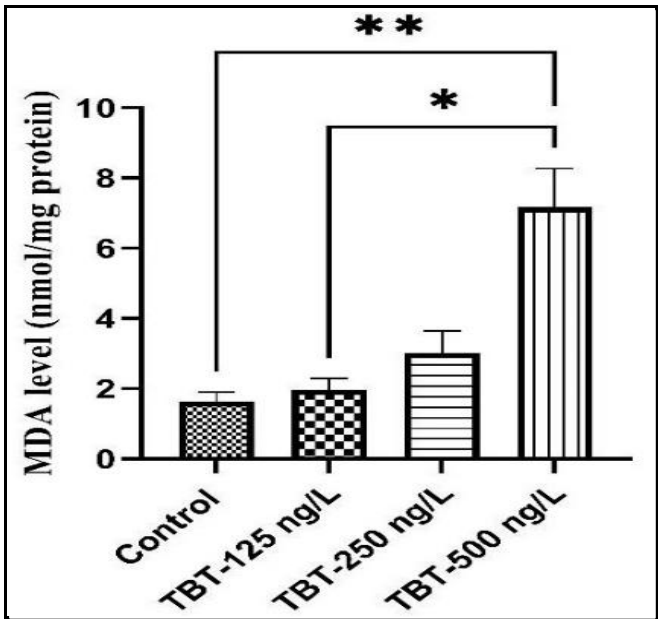

Fig 3: MDA levels in eyes of zebrafish following TBT exposure for 28 days.

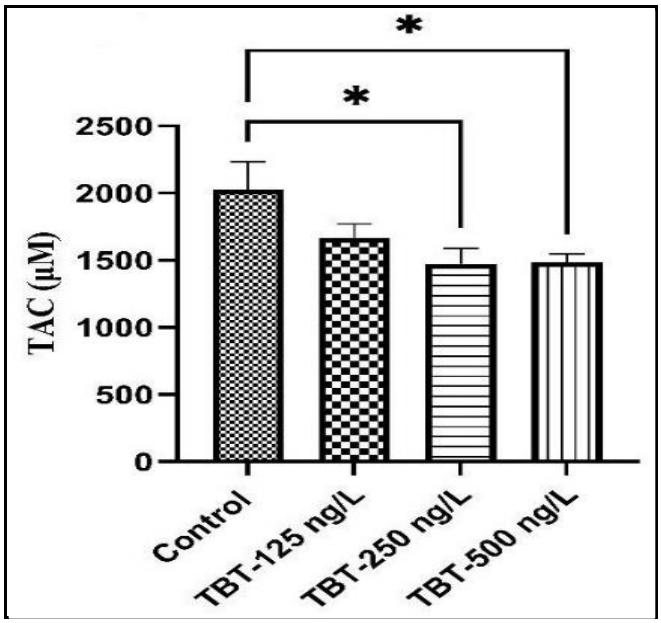

Fig 4: Total antioxidant capacity (TAC) of eyes of zebrafish following TBT exposure for 28 days.

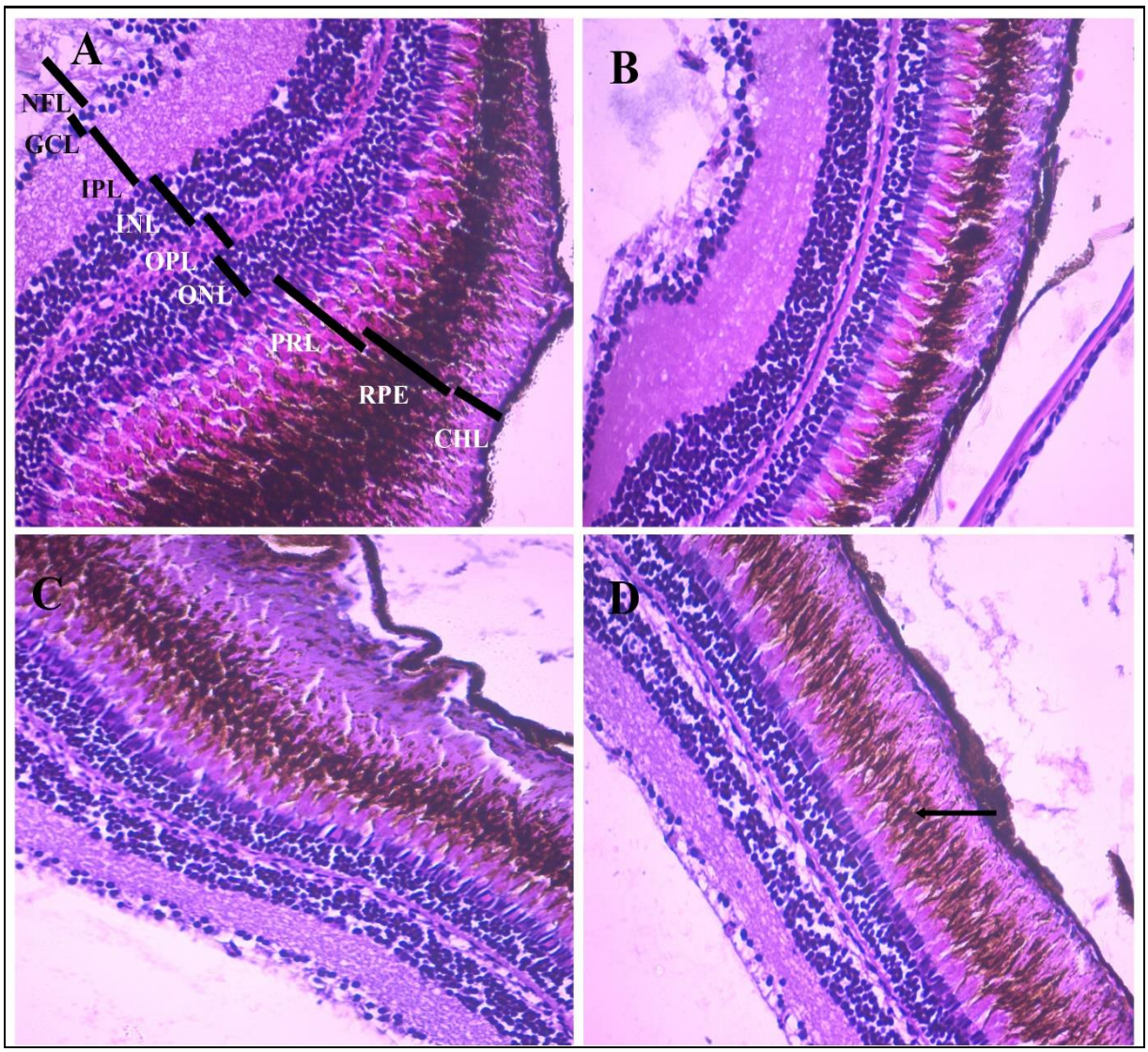

Fig 5: Microscopic changes in the retina of zebrafish of different groups (H & E, 400 ×). A: Cross-sectional histology of the retina of control group showing normal choroid layer (CHL), retinal pigmented epithelium layer (RPE), photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and nerve fiber layer (NFL). B and C: The retina of zebrafish exposed to TBT at 125 ng/L and 250 ng/L showed the normal structure of all layers of the retina. D: The retina of zebrafish exposed to TBT at 500 ng/L showed mild depigmentation of the RPE layer (Thin arrow).

Conclusions

Long-term tributyltin exposure in adult zebrafish altered oxidative stress markers and higher levels of TBT caused lipid peroxidation in the eyes of fish. TBT exposure did not produce remarkable histological changes in the zebrafish retina of all toxicity groups. However, the highest dose of TBT caused mild depigmentation of the retinal pigmented epithelium layer.

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

Consent for publication: All the authors approved the manuscript for publication.

Competing interests: The authors declare no competing interests.

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