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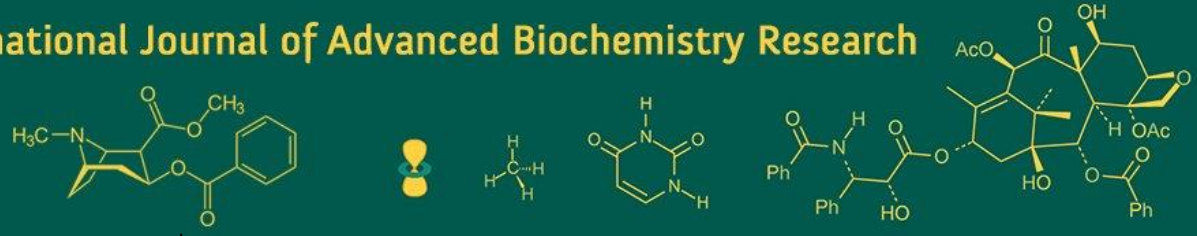

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An assessment of the oxidative stress caused by 4-nonylphenol in the eyes of adult male zebrafish

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Abstract
4-nonylphenol (4-NP) is a phenolic chemical that disrupts the endocrine system and is used in a range of industrial goods. It poses a health hazard to both humans and aquatic animals. A total of 216 adult male zebrafish (Danio rerio) were used in the research to examine the harmful effects of 4-NP on the eyes of zebrafish. The fish were exposed to 4-NP on a daily basis for a duration of 21 days. The zebrafish were placed into four groups for the experiment: control group (C_1), vehicle group (C_2), treatment 1 group (T_1), and treatment 2 group (T_2). Each group consisted of 54 zebrafish. The C_1 group was exposed to reverse osmosis water, while the C_2 group was exposed to water containing 100% ethanol as a vehicle at a concentration of 10 µl/L. The T_1 and T_2 groups were exposed to water containing 4-NP at concentrations of 100 µg/L and 200 µg/L, respectively. The T_1 and T_2 groups exhibited a notable reduction in the activity of SOD and the level of GSH in the eye, compared to the C_1 group. However, the T_2 group showed a large rise in MDA level compared to the C_1 group. The CAT activity in the T_1 and T_2 groups is significantly reduced in the eye compared to the C_1 group.

Keywords: 4-nonylphenol, eye, zebrafish, oxidative stress

Introduction
The USEPA defines endocrine-disrupting chemicals (EDCs) as exogenous substances that disrupt hormone synthesis, secretion, transportation, metabolism, binding, and elimination. Reproduction, internal equilibrium, development, and maturation need these hormones [27]. EDCs mostly damage the endocrine system by mimicking or inhibiting hormone receptors [36]. They may also affect metabolism and hormone secretion.

Some endocrine-disrupting chemicals (EDCs) are purposely produced as wastes, products, intermediates, plasticizers, and flame retardants. Butyl benzyl phthalate, bisphenol A, 4-tert-pentylphenol, 4-NP, dioxins, phenanthrene, polychlorinated biphenyls, polybrominated diphenyl ethers, and plastic packaging raw materials [26, 39]. However, numerous Endocrine-Disrupting Chemicals (EDCs) are purposely made as key components of paints, cosmetics, soaps, and plastics. Many household and commercial wastes are purposely dumped in rivers and coastal regions, while chemicals from the surrounding land are accidently released [46]. Frequent exposure to low concentrations of endocrine-disrupting chemical (EDC) combinations increases sickness risk [27].

Brain diseases and central nervous system changes may be affected by EDCs. EDCs, or neural-disrupting substances, impair neuronal transmission and network development [26]. Endocrine-disrupting chemicals (EDCs) may impair aquatic creatures' reproductive, cognitive, and developmental functioning [21]. The breakdown of nonylphenol ethoxylates produces 4-NP. Degradation of alkylphenol ethoxylates produces these compounds [23]. It is classified as an endocrine-disrupting chemical (EDC) due to its similarity to natural oestrogens, notably 17-β-estradiol, and its capacity to disrupt hormonal functioning [25]. 4-NP may influence animal reproduction by being antiandrogenic and estrogenic. Lipidophilic 4-NP accumulates in cells and organs [41].

Non-ionic detergents using nonylphenol ethoxylates are used in the cleaning, plastic, textile, and paper sectors [31]. NP and similar chemicals are released into the aquatic environment when people make cosmetics, home cleansers, paints, plastics, papers, wastewater treatment plant effluents, and sewage sludge [29].

Many studies have demonstrated that NP may cause physiological, histological, and behavioural alterations in fish [6, 5, 8]. Zebrafish are great toxicological model animals and aid other disciplines [44, 48]. Zebrafish are better toxicity models than other vertebrates due to their size, simplicity of husbandry, and early morphogenesis. Unlike trout, adult zebrafish are one to 1.5 inches long. Many companies specialise in zebrafish aquaria that may hold several thousand fish due to reduced housing and care costs. Zebrafish have been employed in labs for a long time, thus breeding and maintenance conditions are optimal [22].
Zebrafish, both larval and adult, use fewer dosing solutions (experimental chemicals, medications, contaminants) and create less waste than bigger species, reducing costs. They also need fewer labware and chemicals to treat and maintain live fish and perform tests and histological assessments (small quantities of reagents and embedding materials and microscope slides) [20].

Materials and Methods

Chemicals

4-Nonylphenol (4-NP) of analytical grade were purchased from Sigma-Aldrich Chemical Pvt. Ltd., Mumbai (Product code - 46405). Other chemical used in analysis are molecular or analytical grade.

Experimental animals and environment

The 216 adult male zebrafish (Danio rerio) employed in this study had an average body weight of 0.45±0.05 g. The fish were over three months. Mumbai-based Vikrant Aqua Culture supplied the zebrafish. An 18L by 9W by 12H, 20 L glass tank held the zebrafish. The aquarium has RO water and proper aeration. Stones enhance aquariums. Adult fish in each group were maintained in 25-28 °C water with pH 6.8-7.4, electrical conductivity 500-600 µs, and 200-250 harness. Aerators kept water oxygenated. A 14:10 light-dark cycle was maintained throughout the study. Fish were given freeze-dried blood worms (Hallofeed®, Maharashtra Aquarium, Mumbai) and fish pellets ad libitum during the study. The College of Veterinary Science and Animal Husbandry, KU, Junagadh, Institutional Animal Ethics Committee approved the experimental protocol, including fish numbers and methods (Protocol no: JAU/JVC/IAEC/SA/71/2020).

Experimental design

The study randomly divided 216 adult male zebrafish (> 3 months old) into four 54-fish groups (Table 1). The fish were exposed to 4-NP for 21 days. The Mettler Toledo MS 204S/A01 analytical weighing balance properly weighed 4-NP. Each day, fresh 4-NP water was added to maintain the level.

Table 1: Zebrafish used to evaluate different parameters during the study

Parameters	No. of Zebrafish
Oxidative stress (54 in each group; pooled samples of 9 fish and 6 replications)	216
Total	216

Collection of samples

After the experiment, all fish were mercifully killed with ice cold [51]. Each fish's eye was dissected under a stereo zoom microscope (Model CZM6, Labomad Inc., USA). Table 2 shows how eye tissue samples were taken for oxidative stress testing. After tissue homogenate was made using micro pestles, each sample was centrifuged at 12000 g for 10 minutes at 4 °C, except SOD, which was centrifuged for 40 minutes. The supernatant was stored at -80 °C before being used to measure GSH, MDA, SOD, and CAT. Two days following sample collection, oxidative stress indicators were measured.

Table 2: Collection of eye tissues for oxidative stress parameters studies

Parameters	Target	No. of fish used	Sample collection
Oxidative stress	SOD	12 pair of eye (12 fish)	Tris EDTA buffer @ 1 ml/ 100 mg tissue
	CAT/GSH	30 pair of eye (30 fish)	Phosphate buffer saline @ 1 ml/ 100 mg tissue
	MDA	12 pair of eye (12 fish)	Butylated hydroxyl toluene @ 1.5 ml/100 mg tissue

Evaluation of oxidative stress markers in eye tissue

SOD activity was easily determined by the enzyme's ability to inhibit pyrogallol from autoxidizing in Tris-EDTA at pH 7.9–10.6 [35]. Based on the reduction of dichromate in acetic acid to chromic acetate upon heating in H2O2, with the creation of perchloric acid as an unstable intermediate [43], calculated tissue CAT activity [15]. 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) oxidises GSH to create 5-thio-2-nitrobenzoic acid (TNB), a yellow complex used to measure tissue GSH levels. Thiobarbituric acid (TBA) forms an MDA-TBA combination with n-butanol to produce a layer, which is used to measure tissue MDA [34]. An ELISA plate reader (Multiscan Go, Thermofisher Scientific Cat. No. N10588) measures the absorbance of the sample and each sample combination at four wavelengths: 420 nm (SOD activity), 570-610 nm (CAT activity), 412 nm (GSH level), and 532-nm (MDA level

Data analysis: All data was statistically analysed using Graph Pad prism 9.0. Bartlett's test confirmed equal variance and Kolmogorov-Smirnov test verified data normality. Parametric one-way ANOVA and Tukey's HSD test were used to analyse homogenous variance and normal distribution data. Kruskal-Walli and Dunn tests analysed non-normal data. $p<0.05$ was judged significant, whereas $p<0.01$ and $p<0.001$ indicated extremely significant differences.

Result

Oxidative stress markers

Oxidative stress markers evaluated in eye tissue of adult male zebrafish of different groups are depicted in Figure 1. In eye, SOD and CAT activity; GSH level were significantly decreased in T_1 and T_2 groups as compared to C_1 group. However, MDA level in eye of T_1 and T_2 groups were significantly higher as compared to C_1 and C_2 groups.

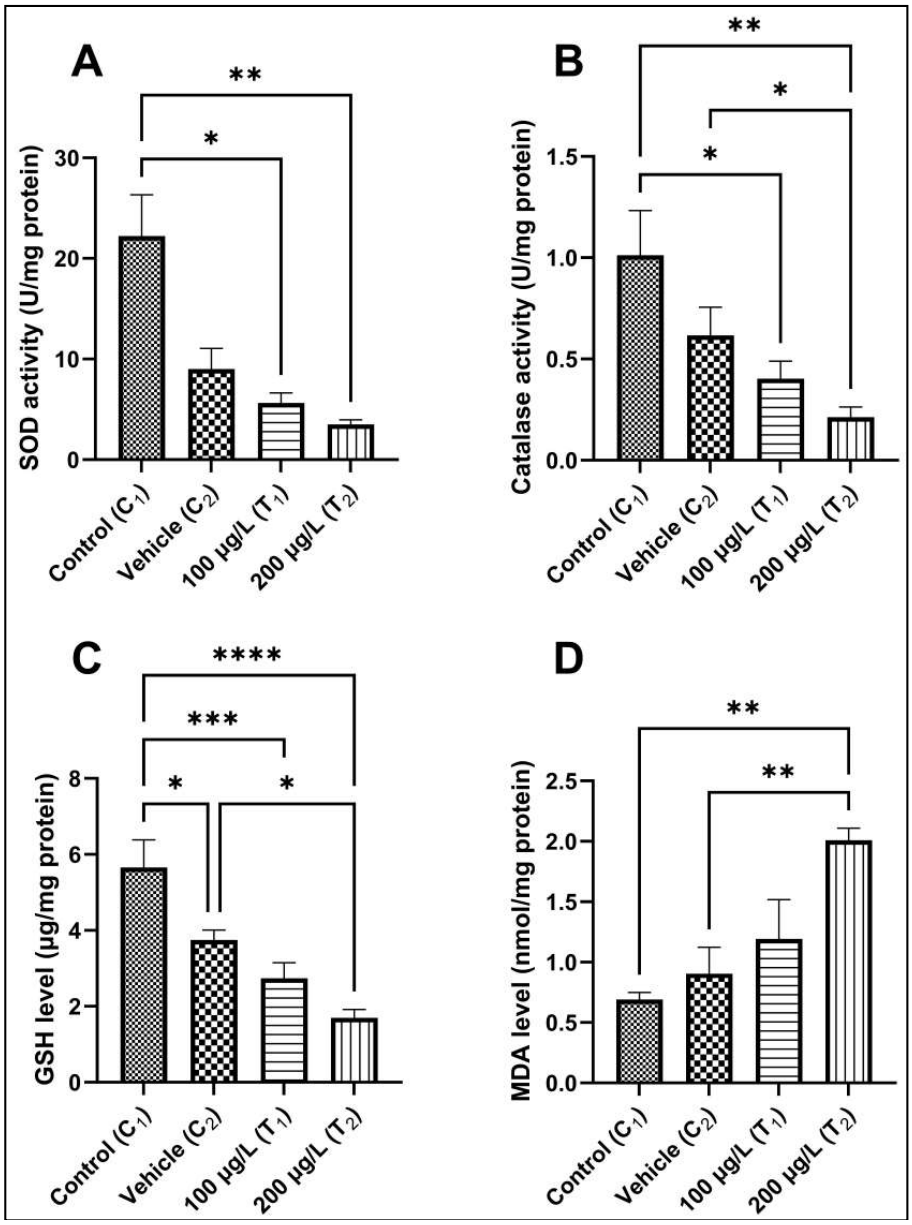

Fig 1: Oxidative stress parameters of zebrafish eye tissue at the end of experiment period; Where * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.005$, **** indicates $p<0.001$.

Discussion

All eukaryotic creatures need oxygen as fuel, and ROS may harm cells and organelles at high oxygen levels [4]. ROS dysregulation damages lipids, proteins, and DNA [13]. Organs contain enzymatic and non-enzymatic ROS neutralizers to avoid oxidative damage. When ROS production exceeds antioxidant defence or disturbs redox signalling, lipid peroxidation, protein oxidation, enzyme inactivation, and DNA breakage may cause cancer, ageing, and neurological diseases [4, 9, 32].

Since SOD, CAT, and GSH are the first line of defence against free radicals, which produce oxidative stress, variations in their activity may be valuable indicators for aquatic creatures' environmental toxicity [33, 38]. Aquatic species' antioxidant enzyme activity changes with 4-nonylphenol [11].

SOD converts superoxide into hydrogen peroxide, which CAT lowers to water and oxygen [10, 17]. SOD may convert superoxide anions to hydrogen peroxide even when its activity decreases [22]. According to Valavanidis et al. (2006), the hydroxyl radical is the most reactive biological and toxicological free radical. Water and oxygen are generated by CAT from H2O2. Oxidatively stressed tissue enzymes that convert a lot of hydrogen peroxide may limit CAT activity [30]. The inactivation of the active enzyme molecule causes CAT to inactivate fast at high hydrogen peroxide concentration [52].

Jafari (2007) implies that GSH/GSSG and GSH-related enzymes prevent oxidative damage. Non-enzymatic radical scavenger and antioxidant glutathione eliminates free radicals from oxidative metabolism that antioxidant enzymes cannot [16]. GSH's sulfhydryl group oxidises to GSSG during metabolism [12]. GR recycles GSH from GSSG and protects cells from free radicals [14]. Thus, GR and GSH recycling balance intracellular GSH-GSSG [42].

LPO damages non-enzymatic thiol antioxidants like GSH, which prevent lipid peroxidation. Several oxidative stress illnesses need GSH to scavenge free radicals [45]. 4-NP-induced neurotoxicity targets GSH inactivation [45, 54].

CAT and SOD activities in common carp brains treated with 17-β estradiol were considerably higher than in the control group, whereas NP dramatically lowered SOD and CAT

activities in tilapia liver and kidney [37]. The brains of rats administered 25 mg/kg/day NP orally for 45 days showed a significant decline in GSH and SOD activity [47].

MDA levels were higher in the treatment group than the control group. Previous research has found elevated MDA levels in zebrafish embryos (100 µg/L) exposed to 4-NP for 168 hours post-fertilization [53], adult zebrafish exposed to heavy metal for 21 days [28, 40], rats treated with NP (25 mg/kg/day, orally for 45 days [7], tilapia fish (37), and African catfish (0.1 mg/Kg body weight for 3 weeks [1]).

Various xenobiotics induce reactive species to form within the brain membrane, decreasing metabolic enzyme activity and increasing LPO [7]. SOD activity was decreased in the 4-NP group because prolonged exposure increased brain superoxide anion [47].

Conclusions

Exposure to 200 µg/L 4-nonylphenol in water for 21 days causes severe oxidative damage in zebrafish eyes.

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