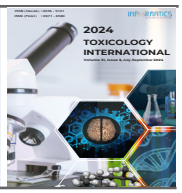
Toxicology International, *31(3) : 477-487; 2024. DOI: 10.18311/ti/2024/v31i3/43008*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



Evaluation of Respiratory Toxicity Potential of Acrylamide in Zebrafish: Oxidative Stress Parameters, mRNAs Expression, and Histomorphological Changes in Gills

Harsh R. Patel, Harshad B. Patel*, Bhulesh V. Paida, Pavan M. Patel, Divya M. Ramchandani, Urvesh D. Patel and Chirag M. Modi

Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh - 362001, Gujarat, India; harshadvet@kamdhenuuni.edu.in

Abstract

Anthropogenic contamination of surface water by chemicals is a worldwide concern, which affects human health and aquatic organisms including fish. Acrylamide (ACR), a Neo-Formed Contaminants (NFC) leads to serious toxic effects on the respiratory system of marine inhabitants. The mechanisms underlying ACR-induced oxidative stress and altered mRNA expressions in zebrafish are unclear. The present investigation aimed to evaluate the effects of ACR exposure for 28 days on the activity or level of antioxidant response elements (ARE), mRNA expressions of antioxidant genes and histomorphological changes in the gills of adult zebrafish. The 270 adult zebrafish were randomly allocated into 03 experimental groups viz. control group, T1 group (ACR: 8.5 mg/L) and T2 group (ACR: 17 mg/L). After 28 days of ACR exposure, Superoxide Dismutase (SOD) activity was significantly ($p<0.01$) decreased in the gills of zebrafish of the T2 group; and no change in Catalase (CAT) activity was observed in both the treatment groups. The levels of reduced Glutathione (GSH) were significantly ($p<0.001$) decreased and Malondialdehyde (MDA) were significantly ($p<0.001$) increased in a concentration-dependent manner. The *sod* and nuclear factor erythroid 2-related factor 2 (*nrf2)* mRNA expressions were significantly ($p<0.001$) downregulated in the gills of zebrafish of both treatment groups. Noticeable histomorphological alterations were recorded in the gills of zebrafish of the T2 group. Alterations in ARE activity, mRNA expressions of antioxidant genes and histopathological findings suggest ACR exposure has been shown to produce oxidative stress-mediated damage in the gills of adult zebrafish.

Keywords: Acrylamide, Gills, Histopathological Changes, mRNA Expression, Oxidative Stress Parameters, Zebrafish

1. Introduction

Acrylamide (ACR) is a hydrophilic, unsaturated alkene compound with crystalline properties. It serves as a fundamental building block for synthesizing polymers and copolymers[1]. ACR-derived polymers and copolymers find widespread applications across multiple industries, including wastewater treatment, paper and textile manufacturing, soil conditioning, ore processing, and cosmetics[2]. In the food industry, heating food produces

*Author for correspondence

Article Received on: 22.03.2024 Revised on: 05.06.2024 Accepted on: 11.06.2024

thermal process contaminants, notably ACR, formed during the cooking of starchy foods or cereal products above 120°C. Human exposure to ACR occurs through foods like potato chips, French fries, and cigarette smoke, posing health risks[3].

The ACR exists in monomeric and polymeric forms, with monomers being more toxic, affecting aquatic environments and raising environmental concerns[4]. Acrylamide (ACR) levels were detected at <5 µg/L in water, with higher concentrations linked to polyacrylamide water treatment. Public wells in West Virginia contained 0.024-0.041 µg ACR/L[5]. Swedish women's mean daily intake of ACR is 25.9 µg/day, associated with cancer[6]. Potato chips contain up to 3-4 mg/kg of ACR in India[7].

Aquatic animals including fish are highly susceptible to waterborne exposure of environmental contaminants. The toxicity of environmental contaminants is commonly studied using rodent and non-rodent animal models. Recently, the zebrafish has emerged as a promising model organism for toxicological research. Smaller body size, high egg production, transparent embryonic development, and genetic similarity to humans are unique features of zebrafish which enables it as a model organism for investigating the effects of xenobiotics exposure on genetics, developmental biology, environmental toxicology, DNA repair process, carcinogenesis, and disease progression[8].

In fish, the gills act as the primary respiratory organ. The gills with their extensive surface area and unique morphology, serve as crucial entry points for pollutants in the body and typically encounter chemicals dissolved in water before other organs[9]. Their wide surface area and smaller water-blood diffusion distance make them particularly more susceptible to xenobiotic exposure in freshwater environments. A significant water volume flows through the gill lamellae for oxygen uptake. Additionally, gills contribute to osmotic regulation, acid-base balance, and excretion of nitrogen, and are highly susceptible to waterborne chemicals[10].

The extensive use of Acrylamide (ACR) in industries poses a significant occupational exposure risk[11]. ACR disrupts cellular redox balance, leading to reactive oxygen species (ROS) generation. Increased ACR exposure alters the antioxidant enzymes[12]. The SOD, CAT, and glutathione peroxidase (GPx) are primary antioxidant enzymes, while glutathione s-transferase (GST) plays an important role in Phase II xenobiotic metabolism. Glutathione (GSH) serves as a crucial cellular antioxidant, supporting GPx and GST activities[13,14]. The ROS imbalance can cause mitochondrial and lysosomal damage, affecting energy production and triggering inflammation through mtDNA damage and lipid peroxidation. This deregulation results in severe phenotypic and genotypic consequences. ACR-induced oxidative stress can lead to apoptosis and respiratory toxicity[1].

Acrylamide exposure (10, 30, 100, or 300 mg/L) for 114 hours has induced developmental toxicity in zebrafish larvae[15]. Similarly, severe oxidative stress-induced alterations in the gills of zebrafish after 40 days exposure of to ACR (18.36 mg/L) were observed[16]. Furthermore, it was concluded that ACR exposure at 1 mM for 3 days led to oxidative injury and downregulated the expression of *sod-1*, *sod-2*, and *catalase* genes involved in oxidative insult in zebrafish larvae[17]. Similarly, it was documented significant pathological abnormalities in the gill filaments and cells of zebrafish after 40 days of exposure to ACR concentrations of 2.04, 6.12, and 18.36 mg/L[16].

Previous research has documented the harmful effects of ACR on the neurological and ocular systems of aquatic organisms[18]. It is crucial to prioritize understanding the impact of ACR on the respiratory system of aquatic species. Furthermore, there is a dearth of literature regarding the toxic effects of prolonged ACR exposure in the gills of zebrafish. Hence, this study was designed to investigate the effects of sub-acute exposure to ACR on oxidative stress-related factors, mRNA expressions of antioxidant elements, and histopathological alterations in the gills of adult zebrafish.

2. Materials and Methods

2.1 Chemicals

Acrylamide and all other required chemicals used during the study of molecular or analytical grade and purchased from standard manufacturers/suppliers.

2.2 Experimental Conditions

The study was performed on adult male zebrafish (*Danio rerio*) of 4-6 months of age. The following laboratory conditions were maintained during the whole experiment viz. room temperature (25 to 28°C), pH (6.8-7.4), electrical conductivity (500-600 µs/cm), hardness of water (200-250 ppm) and the light: dark cycle (14 hours: 10 hours). All fish fed with standard fish diet @ 12-14 mg/fish two times a day. All experimental procedures

approved wide experiment no: KU-JVC-IAEC-SA-85-21; Date: 19/12/2020). The experimental study was carried out at the Laboratory Animal House Facility, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh.

2.3 Experimental Design (Table 1)

The 270 adult zebrafish were randomly allocated in 03 experimental groups. The exposure levels of ACR and duration were selected based on previous reports[19]. Zebrafish exposed to 8.5 and 17 mg/L of ACR which was around 1/10th and 1/5th of reported 72 h LC_{50} values, respectively[19]. The Group 1 zebrafish were reared in RO water without ACR exposure (control group - C). Zebrafish of Group 2 (Treatment 1-T1) and Group 3 (Treatment 2-T2) were exposed at 8.5 and 17 mg/L of ACR, respectively. The exposure levels of ACR in tanks were maintained by daily replacement with fresh water containing a particular concentration of ACR.

2.4 HPLC Protocol for Detection of ACR

2.4.1 Apparatus

The HPLC system (LC-2010 CHT, Schimadzu), consisting gradient pump and ultraviolet detector was used for estimation of ACR. Chromatographic separation was carried out with a C_{18} analytical column (0.46 cm x 25 cm; ID 5 µm) at 30°C of column temperature.

2.4.2 Chromatographic Condition

The mobile phase was HPLC water (100%). The mobile phase was filtered and degassed before delivery in the column (flow rate of 0.5 mL/min). The effluents were monitored at 195 nm wavelength in a UV detector.

Table 1. Experimental groups and types of different treatments given to zebrafish

Groups (90 fish in each group)	Treatments
Control group (C1)	Fish in normal RO water
Toxicity group (T1)	Fish in RO water containing Acrylamide (8.5 mg/L of water); [1/10 of LC50(72h)]; (LC_{50} Value – 86.71 mg/L)
Toxicity group (T2)	Fish in RO water containing Acrylamide (17 mg/L of water); [1/5 of LC50(72h)]; (LC_{50} Value - 86.71 mg/L)

2.4.3 Procedure for Water Sample Preparation

To measure the ACR concentration, the representative water samples were collected daily from exposure tanks. After 10 times dilution with milli-q water, samples were centrifuged at 12000 g for 15 min to separate the clear supernatant. The 10 µL of supernatant was inserted into the HPLC system through an autosampler.

2.4.4 Preparation of Standard Curve

The exposed concentrations of ACR in the tanks were 0.12 mM (8.5 µg/mL) and 0.24 mM (17 µg/mL) in the T1 and T2 groups, respectively. For standardization and validation of the method, known standards of ACR (0.042, 0.084, 0.169, 0.338, 0.676, 1.353, 2.706, 5.412, 10.825, 21.650, and 43.350 µg/mL) were prepared in HPLC water. The 10 µL of each was injected into the system for further processing.

2.4.5 Calibration

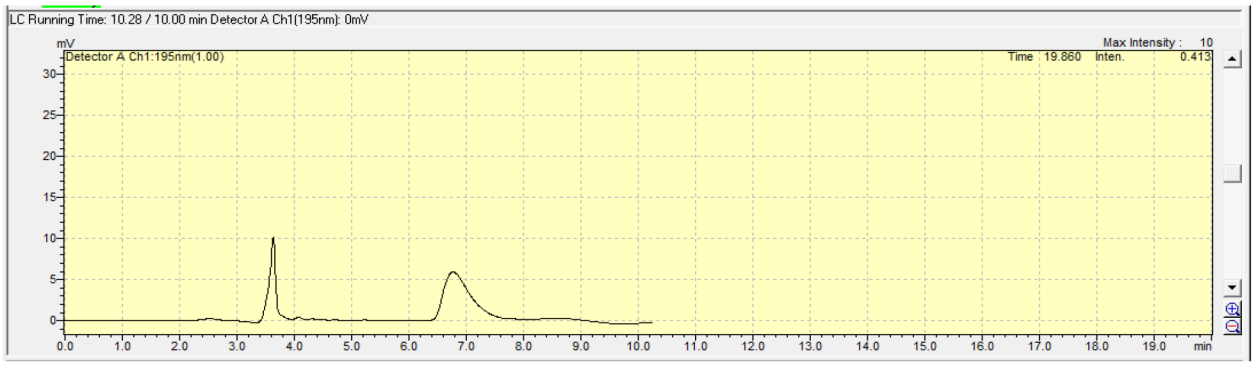
Standard curve prepared using concentration and the respective area of peak of each known standard. The method was highly sensitive and replicable. The assay linearity was observed between 0.042 to 43.350 µg/mL standard concentrations with correlation coefficient value (r)>0.9998. The lower Limit Of Detection (LOD) and lower Limit Of Quantification (LOQ) were 0.023 and 0.042 µg/mL, respectively with C.V.<6.21%. The chromatograms of ACR in collected water samples are depicted in Figure 1.

2.5 Evaluation of Oxidative Stress Parameters in Gills

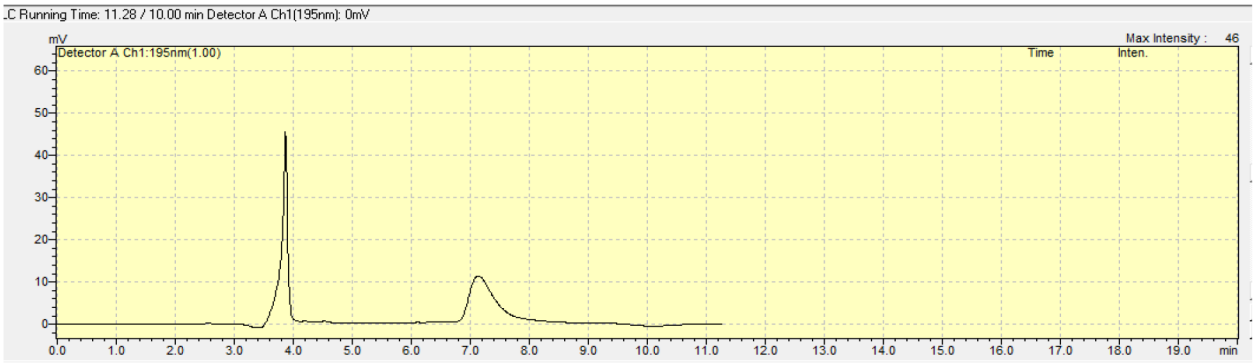
On the 29th day of the experiment, zebrafish were sacrificed by keeping them on ice for 10 minutes[20]. Pooled gill samples were collected in Tris-EDTA buffer (100 mg/mL) for estimation of SOD activity, in phosphate buffer saline (100 mg/mL) for evaluation of CAT activity and reduced GSH level, and in butylated hydroxyl toluene buffer (100 mg/1.5 mL) for evaluation of Malondialdehyde (MDA) level. The homogenates were centrifuged at 12000 g at 4°C for 10-12 min except for SOD and Supernatants were collected and stored in cryovials at -86°C until used for further analysis as per standard described methods earlier[21-24].

2.6 Evaluation of mRNA Expressions in Gills

The gill samples (pooled from 5 fish) were collected in a DNase & RNase free storage vial in RNA later solution to study the mRNA expression levels of *sod*, *cat* and *nrf2* genes. The high-quality total RNA was isolated as per the standard protocol[25]. The RNA samples having 260/280 ratio ~ 2.0 were treated with DNA-free™ Kit for removal of genomic DNA contamination and cDNA was synthesized using first strand cDNA synthesis kit. The gene-specific primers used in the study are mentioned (Table 2)[26-29] and amplified in a real-time PCR system using the Maxima SYBR Green/ROX qPCR master mix. The PCR Confirmation with a specific set of primers for *β-actin, sod, cat* and *nrf2* is shown in Figure 2. The fold expression changes were studied by $2^{-\Delta\Delta Ct}$ method[30].



(A)



(B)

Figure 1. Chromatogram of ACR in 10 times diluted water sample from tank.
(A) T1 group (0.85 µg/mL, *i.e.* 8.5 µg/mL)
(B) T2 group (1.7 µg/mL, *i.e.* 17 µg/mL)

Table 2. Sequences of primers of targeted genes used for real-time PCR amplification

Target gene	Primer sequence (5'-3')	Annealing temp. (°C)	Product size (bp)	References
SOD	FP 5'-CAACACAAACGGCTGCATCA-3'	60	132	26
	RP 5'-TTTGCAACACCACTGGCATC-3'			
CAT	FP 5'-AGTTCCCTCTGATTCCTGTG-3'	60	173	27
	RP 5'-ATGGCGATGTGTGTCTGG-3'			
Nrf_2	FP 5'-TGTTGGTTCGGAGGCTCTTAA-3'	60	62	28
	RP 5'-AGGCCATGTCCACACGTACA-3'			
β-actin	FP 5'-GCTGTTTTCCCCTCCATTGTT-3'	60	60	29
	RP 5'- TCCCATGCCAACCATCACT -3'			

SOD: Superoxide Dismutase, CAT: Catalase, Nrf_2: NF-E2-related factor 2, β-actin: Housekeeping Gene

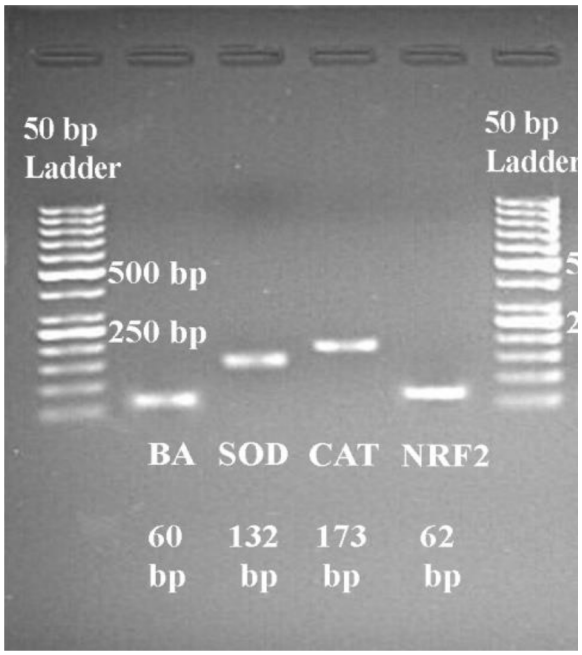


Figure 2. PCR Confirmation. with specific primers for β-actin, SOD, CAT and Nrf$_2$ gene in gills of zebrafish.

2.7 Histopathological Examination

Eighteen zebrafish (n = 6) were used for histopathological examination of gills. After formalin fixation for 2 days, fish were decalcified in EDTA solution for another 7 days. After adequate decalcification, histomorphological changes in gills were recorded as per the method described earlier[31].

2.8 Data Analysis

Statistical analyses of all data were carried out using Graph-Pad Prism software (Version 9.0.; Graph Stats Technologies Private Limited, India) for Windows. Data with normal distribution was analyzed by one-way Analysis Of Variance (ANOVA) followed by Tukey's high significant difference test. Whereas, data without normal distribution was analyzed by non-parametric Kruskal-Wallis test followed by Dunn's test. Where $p<0.05$, $p<0.01$ and $p<0.001$ were considered as statistically significant differences among treatment groups.

3. Results

3.1 Oxidative Stress Parameters

Alterations in oxidative stress parameters are presented in Figure 3. The SOD enzyme activity was significantly

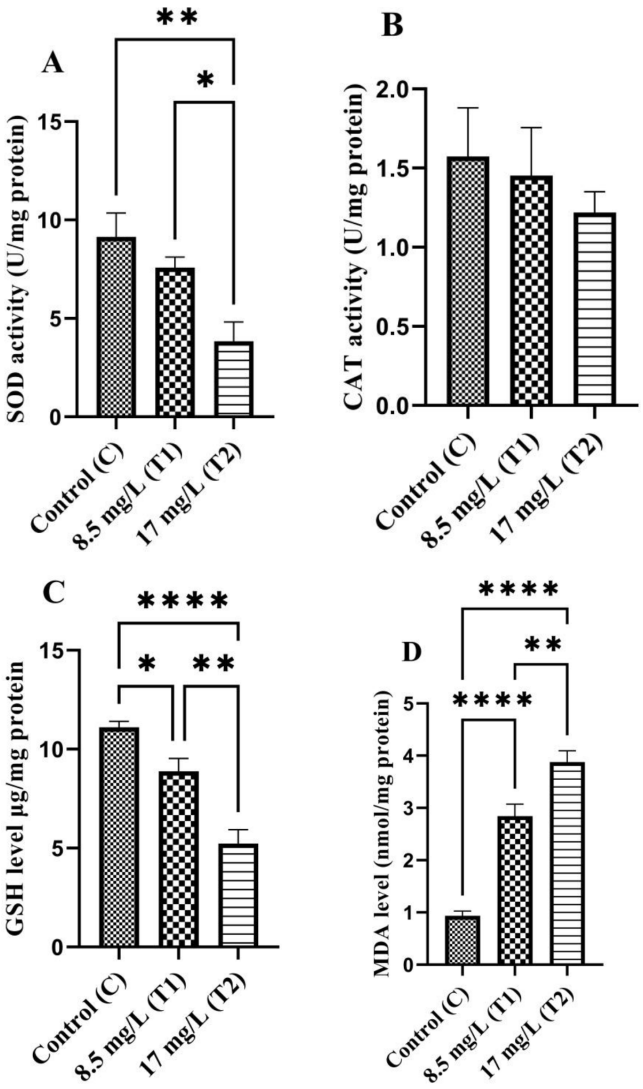


Figure 3. Oxidative stress parameters of zebrafish gills at the end of the experiment (n = 162, 54 from each group, pooled samples = 9, replications = 6). **(A)** SOD activity in gills (U/mg protein); **(B)** CAT activity in gills (U/mg protein.); **(C)** GSH level in gills (µg/mg protein); **(D)** MDA level in gills (nmol/mg protein). Where * indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.001$.

($p<0.01$) decreased in the gills of zebrafish of the T2 (17 mg/L) group in comparison to the control group. The levels of GSH and MDA were significantly ($p<0.001$) decreased and increased, respectively in a dose-dependent manner.

3.2 Gene Expression Analysis

Box and whisker plot analysis for *sod*, *cat* and *nrf2* mRNA expression levels is depicted in Figure 4.

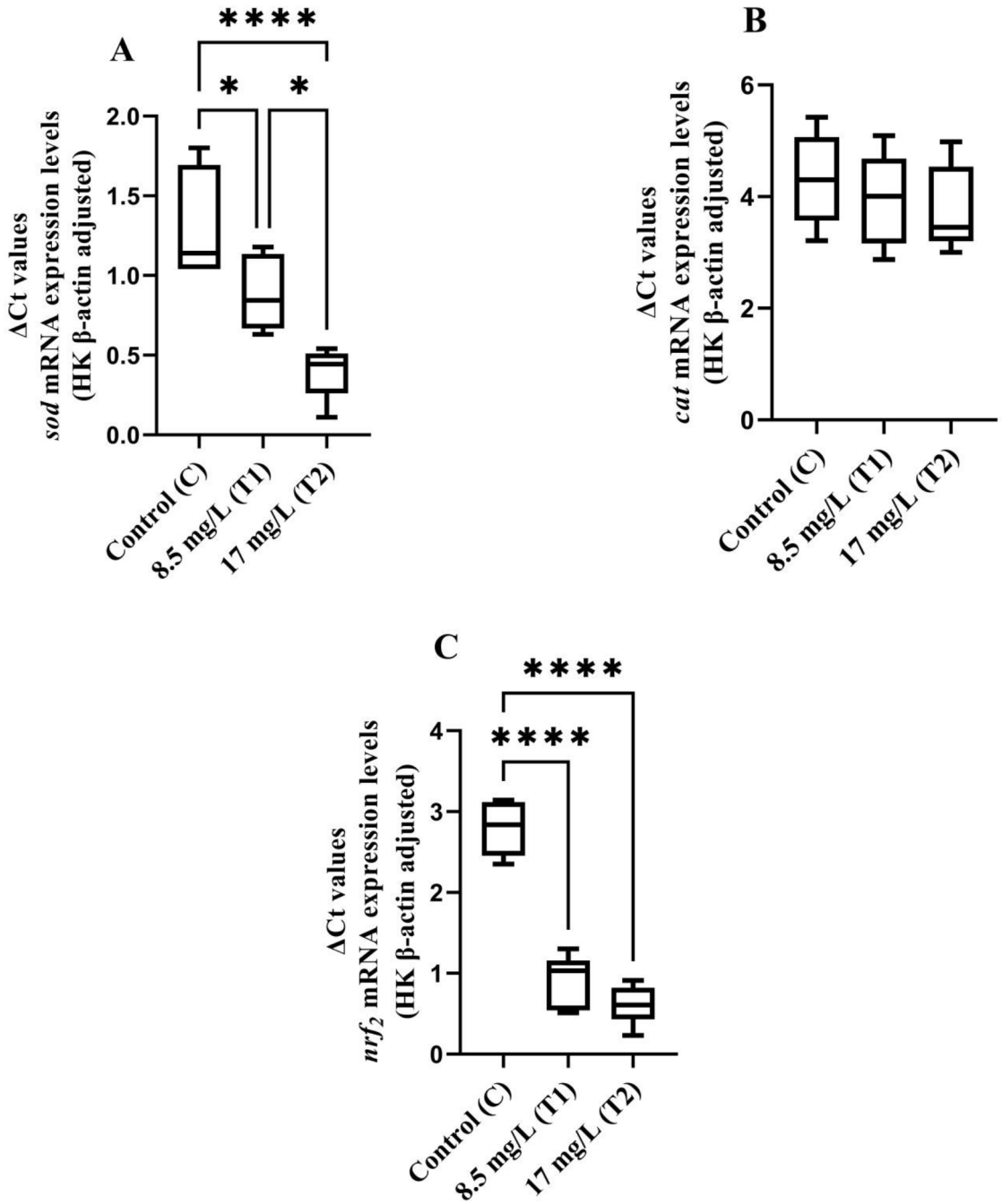


Figure 4. mRNA expression profile of *sod, cat* and *nrf2* genes in zebrafish gills (n = 90, 30 from each group, pooled samples = 5, replications = 6). **(A)** *sod* mRNA expression levels ($2^{-\Delta\Delta Ct}$); **(B)** *cat* mRNA expression levels ($2^{-\Delta\Delta Ct}$); **(C)** nrf_2 mRNA expression levels ($2^{-\Delta\Delta Ct}$). Where * indicates $p<0.05$, **** indicates $p<0.001$.

The *sod* and *nrf2* mRNA expressions in the gills of zebrafish of both groups were significantly ($p<0.001$) downregulated as compared to the control group. However, no significant alterations were recorded in mRNA expression levels of the *cat gene* among different treatment groups.

3.3 Histopathological Examination of Gills

Histopathological examination revealed the normal appearance of gills with well-arranged gill arch and racker, primary and secondary lamellae, and chondrocytes in the control group. An almost normal

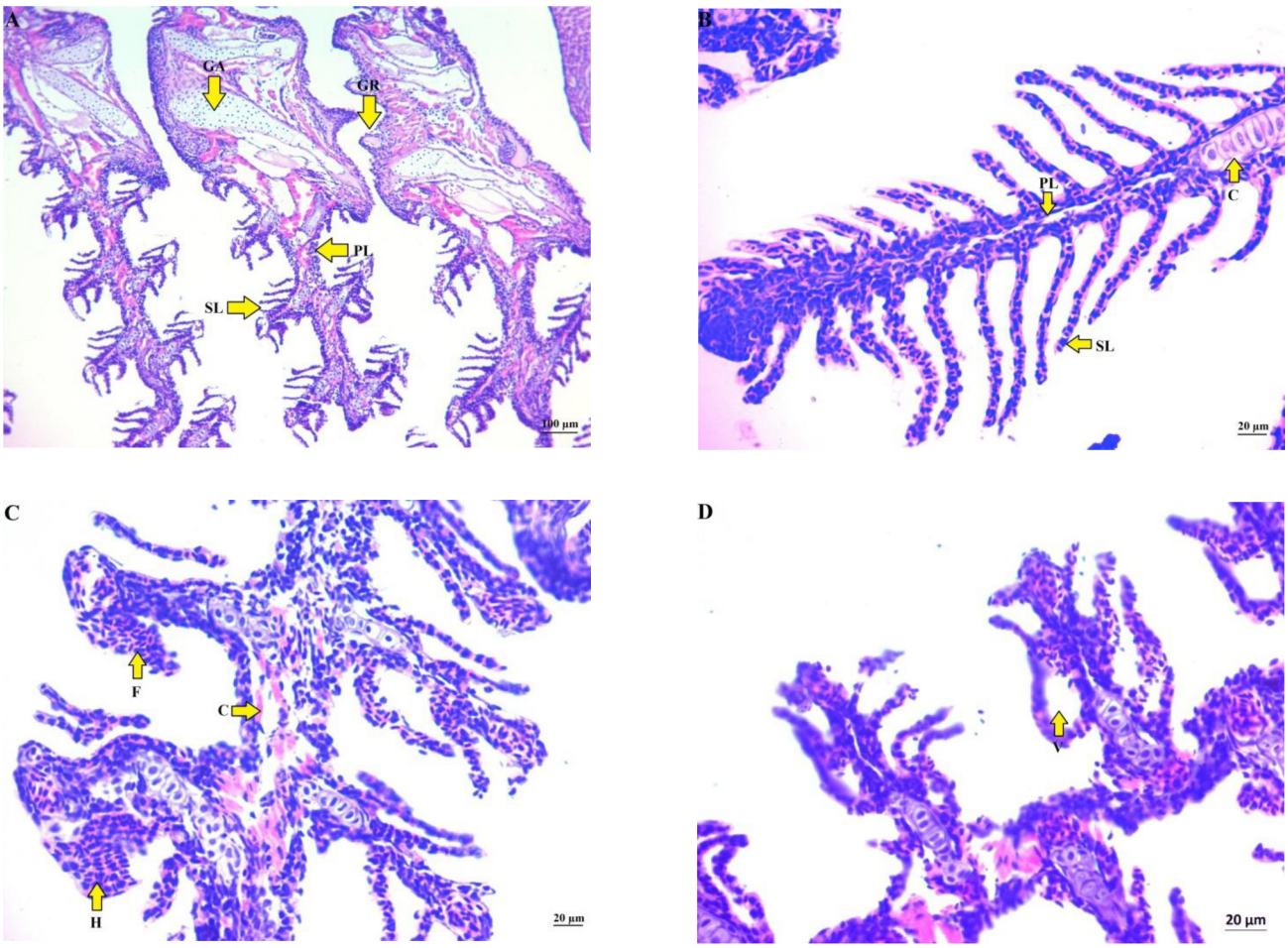


Figure 5. Microscopic view of zebrafish gills **(A)** control group showed normal structure of Gill Arch (GA), Gill Racker (GR), Primary Lamellae (PL) and Secondary Lamellae (SL) (x 100, H and E staining); **(B)** ACR (8.5 mg/L, for 28 days) exposure group showed almost normal architecture of Chondrocytes (C), primary lamellae (PL) and secondary lamellae (SL) (x 400, H and E staining); **(C)** ACR (17 mg/L, for 28 days) exposure group showed Congestion (C) in primary lamellae along with epithelial hyperplasia (H) at the end of secondary lamellae, as well as Fusion (F) of secondary lamellae (x 400, H and E staining); **(D)** ACR (17 mg/L, for 28 days) exposure group showed Vacuolization (V) formation of secondary lamellae (x 400, H and E staining).

appearance of gills was noticed in the T1 (8.5 mg/L) group. However, congestion in primary lamellae, fusion of secondary lamellae, epithelial hyperplasia and vacuolization in secondary lamellae were noticed in the T2 (17 mg/L) group (Figure 5).

4. Discussions

Limited data exists regarding the respiratory toxicity of ACR in laboratory animals, particularly zebrafish. This study represents the inaugural investigation revealing the deleterious effects on gills after prolonged exposure to ACR in adult zebrafish. The primary objective was to reveal the oxidative changes and alterations in expressions of mRNAs of antioxidant genes in the gills of zebrafish. Extensive histological alterations were observed in the gills of zebrafish subjected to prolonged ACR exposure. These findings offer valuable molecular insights involved in ACR-induced oxidative insult in the respiratory system of zebrafish.

Zebrafish share similar xenobiotic metabolism pathways with higher vertebrates, which are crucial for mitigating xenobiotic-induced stress[32]. The generation of ROS is a natural occurrence in cell metabolism. Nevertheless, heightened ROS production can result in oxidative stress, potentially compromising cellular antioxidant defences. Changes in cellular redox balance can consequently impact gene expression patterns and the activities of antioxidant enzymes[33].

Numerous antioxidant genes have been pinpointed in zebrafish, aiming to mitigate oxidative stress[34]. Fish possess various enzymatic and non-enzymatic defence mechanisms to counteract heightened oxidative stress levels[35]. Principal antioxidant enzymes including SOD, CAT, GPx, and GST serve as crucial indicators of oxidative stress levels. Additionally, the balance between reduced Glutathione (GSH) and oxidized Glutathione disulfide (GSSG) plays a pivotal role in non-enzymatic antioxidant defence pathways.

SOD serves as the primary defence mechanism against oxidative stress, catalyzing the dismutation of superoxide free radicals (O^{2-}) into water (H_2O) and hydrogen peroxide (H_2O_2). CAT and GPx play pivotal roles in converting hydrogen peroxide (H_2O_2) into water and oxygen, thus shielding the organism from oxidative stress. GST, functioning as a Phase II detoxification enzyme, facilitates the binding of electrophilic metal groups with the sulfhydryl groups of GSH, thereby enhancing their water solubility. Glutathione Reductase (GR) is responsible for regulating the cellular equilibrium between reduced glutathione (GSH) and oxidized glutathione (GSSG), thus maintaining the cell's redox balance[36]. The increased ratio of GSSG to GSH is indicative of greater oxidative insult. The GSH serves as a crucial reducing agent, playing a vital role in the cellular antioxidant system[37]. GSH participates in non-enzymatic reactions with metals, similar to other thiols, acting as a scavenger for superoxide free radicals and serving as a substrate for GST and GPx[32,38]. In the current investigation, the activities of SOD, CAT, and GSH levels were reduced, while the MDA level was elevated in both treatment groups (T1 and T2). Similarly, a significantly ($p<0.05$) increased level of MDA was observed in *Mytilus galloprovincialis* exposed to ACR (1-10 mg/L) for 21 days[39]. Snails (*Theba pisana*) exposed to ACR (2.28 µg/g) for 2 weeks demonstrated an increase in MDA levels[40]. Zebrafish embryos treated with ACR (1 and 2 mM) for 6 hours post-fertilization (hpf) exhibited a reduction in SOD and CAT activity, reduced GSH levels and elevated MDA levels[41]. A marine bivalve mollusk *(Mactra stultorum)* exposed to ACR (5, 10, and 20 mg/L) for 5 days demonstrated elevated levels of MDA in gills[42]. Their findings suggest that ACR could potentially exert harmful effects on gills by inducing oxidative stress. ACR exposure had also significantly altered MDA and GSH levels in the lungs of rat[43]. Rat treated with ACR (50 mg/kg, i.p.) for 4 weeks showed ACR-induced oxidative stress in the nervous system and naringin protected against ACR toxicity due to its antiapoptotic and antioxidant potential[44]. Adult male zebrafish treated with ACR (43.35 mg/L) for 7 days resulted in alterations of oxidative stress markers (SOD and CAT activity, and the level of GSH and MDA) in the brain and eye[18].

The oxidative stress and inflammation induced by ACR were mitigated, and tissue damage was alleviated by inhibiting the consumption of antioxidant enzymes and preventing lipid peroxidation[39]. Malondialdehyde (MDA) is a byproduct of Lipid Peroxidation (LPO)[45]. Elevated levels of MDA indicate oxidative stress triggered by different factors, including xenobiotics[46]. The findings of the present investigation related to ACR-induced lipid peroxidation are supported by other studies[47-50]. According to our results, increased MDA levels may result from excessive production of ROS, causing membrane damage and lipid peroxidation in gills tissue.

Reactive species serve as crucial triggers for activating nrf_2, a pivotal controller of the cellular antioxidant response. This activation occurs through nrf_2 interaction with Antioxidant Response Elements (AREs) which initiates antioxidant enzymes gene expression[51]. Normally, nrf_2 remains in an inactive state in the cytoplasm, bound to its regulator Keap1. The Keap1 is a suppressor, tethering with nrf_2 and curtailing its transcriptional activity[52]. However, upon stimulation factors, nrf_2 disassociates from Keap1 and stimulates the transcription of genes[53]. Remarkably, the nrf_2-Keap1 system isn't exclusive to mammals but extends to fish, including zebrafish, underscoring its evolutionary conservation across vertebrates and its pivotal role in defending cells against oxidative stress[54,55].

In the current investigation, the mRNA expression levels of *sod*, *cat* and nrf_2 in the gills were down-regulated in both treatment groups (T1 and T2), suggesting oxidative stress in the treated zebrafish. Similarly, zebrafish embryos exposed to ACR at concentrations of 1, 2, 3, 4, and 5 mM for 6 hours post-fertilization (hpf) resulted in significant, dose-dependent down-regulation of mRNA expression levels of *sod1*, *sod2*, *catalase*, *gpx1*, and nrf_2 in the treated zebrafish embryos[41]. Another investigation on zebrafish larvae found that ACR exposure at dose rates of 1 mM for 3 days significantly regulates the amount of *sod*-1, *sod*-2, *catalase*, *gr* and *prx* mRNA expression[17]. In agreement with our findings, adult male zebrafish exposed to ACR (43.35 mg/L) for 7 and 21 days had shown to produce downregulation of *sod*, *cat*, and *nrf2* genes in the brain and eye[18].

In the present investigation, the gills of zebrafish of the T2 (17 mg/L) group exhibited noticeable histopathological changes. In support of our observation, metaplasia and hyperplasia of and fusion of secondary lamellae have been observed in rainbow trout exposed to ACR (from 12.5 to 50 mg/L) for 15 days[56]. ACR exposure (2.04, 6.12 and 18.36 mg/L) for 40 days damaged the gill filaments and gill cells[15]. Mild edema (10 mg/L); vacuolization and edema (20 mg/L) and irregular structure of secondary lamellae (30 mg/L) observed in gills after exposure of ACR for 4 days in *Capoeta capoeta* fish[57]. These findings emphasize the detrimental impact of ACR on fish gill health and the importance of addressing ACR contamination in aquatic environments.

5. Conclusions

Exposure to ACR induced oxidative stress in the gills of zebrafish, as evidenced by the down-regulation of key antioxidant enzyme genes including *sod*, *cat*, and nrf_2. These findings unequivocally suggest the toxicological impact of ACR on the gills of zebrafish. Moreover, notable histopathological alterations were observed in the gill structures.

6. Acknowledgements

Authors are highly thankful to Dr. B. B. Javia, Dr. A. R. Bhadaniya and Dr. A. R. Ahlawat, Associate Professor, COVSAH, Junagadh for allowing laboratory facilities for the research work.

7. References

1. Zamani E, Shokrzadeh M, Fallah M, Shaki F. A review of acrylamide toxicity and its mechanism. Pharm Biomed Res. 2017; 3(1):1-7. http://pbr.mazums.ac.ir/article-1-146-en.html
2. Friedman M. Chemistry, biochemistry, and safety of acrylamide: A review. J Agric Food Chem. 2003; 51(16):4504-26. https://doi.org/10.1021/jf030204
3. Duan X, Wang QC, Chen KL, Zhu CC, Liu J, Sun SC. Acrylamide toxic effects on mouse oocyte quality and fertility in vivo. Sci Rep. 2015; 5(1):1-11. https://doi.org/10.1038/srep11562
4. Weston DP, Lentz RD, Cahn MD, Ogle RS, Rothert AK, Lydy MJ. Toxicity of anionic polyacrylamide formulations when used for erosion control in agriculture. J Environ Qual. 2009; 38(1):238-47. https://doi.org/10.2134/jeq2008.0109
5. Brown L, Rhead M. Liquid chromatographic determination of acrylamide monomer in natural and polluted aqueous environments. Analyst. 1979; 104(1238):391-99. https://doi.org/10.1039/AN9790400391
6. Mucci LA, Wilson KM. Acrylamide intake through diet and human cancer risk. J Agric Food Chem. 2008; 56(15):6013-19. https://doi.org/10.1021/jf703747b
7. Shamla L, Nisha P. Acrylamide in deep-fried snacks of India. Food Addit Contam. 2014; 7(3):220-25. https://doi.org/10.1080/19393210.2014.894141
8. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013; 496(7446):498-503.
9. Evans DH, Piermarini PM, Choe KP. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol Rev. 2005; 85:97-177. https://doi.org/10.1152/physrev.00050.2003
10. Wendelaar-Bonga SE. The stress response in fish. Physiol Rev. 1997; 77:591-625. https://doi.org/10.1152/physrev.1997.77.3.591
11. Dybing E, Farmer PB, Andersen M, Fennell TR, Lalljie SPD, Müller DJG, *et al.* Human exposure and internal dose assessments of acrylamide in food. Food Chem Toxicol. 2005; 43(3):365-410. https://doi.org/10.1016/j.fct.2004.11.004
12. Chen W, Shen Y, Su H, Zheng X. Hispidin derived from Phellinuslinteus affords protection against acrylamide-induced oxidative stress in Caco-2 cells. Chem-Biol Interact. 2014; 219:83-9. https://doi.org/10.1016/j.cbi.2014.05.010
13. Hellou J, Ross NW, Moon TW. Glutathione, glutathione S-transferase, and glutathione conjugates, complementary markers of oxidative stress in aquatic biota. Environ Sci Pollut Res. 2012; 19:2007-23. https://doi.org/10.1007/s11356-012-0909-x
14. Ighodaro OM, Akinloye OA. First line defense antioxidants-Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPX): Their fundamental role in the entire antioxidant defense grid. Alex J Med. 2018; 54:287-93. https://doi.org/10.1016/j.ajme.2017.09.001
15. Park JS, Samanta P, Lee S, Lee J, Cho JW, Chun HS, *et al.* Developmental and neurotoxicity of acrylamide to zebrafish. Int J Mol Sci. 2021; 22(7):3518. https://doi.org/10.3390/ijms22073518
16. Xiuming C, Fei L, Yu F. Toxic effects of acrylamide on zebrafish organs and DNA damage of germ cell. Asian J Chem. 2016; 10(1):382-88.
17. Sharma C, Kang SC. Garcinol pacifies acrylamide induced cognitive impairments, neuroinflammation and neuronal apoptosis by modulating GSK signaling and activation of pCREB by regulating cathepsin B in the brain of zebrafish

larvae. Food Chem Toxicol. 2020; 138:111246. https://doi.org/10.1016/j.fct.2020.111246
18. Kachot RL, Patel UD, Patel HB, Modi CM, Chauhan R, Kariya MH, *et al*. Neurotoxicity of acrylamide in adult zebrafish following short-term and long-term exposure: Evaluation of behavior alterations, oxidative stress markers, expression of antioxidant genes, and histological examination of the brain and eyes. Environ Sci Pollut Res. 2023; 30(14):40116-31. https://doi.org/10.1007/s11356-022-25112-z
19. Faria M, Ziv T, Gomez-Canela C, Ben-Lulu S, Prats E, Novoa-Luna KA, *et al*. Acrylamide acute neurotoxicity in adult zebrafish. Scient Rep. 2018; 8(1):1-14. https://doi.org/10.1038/s41598-018-26343-2
20. Wilson JM, Bunte RM, Carty AJ. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). J Am Assoc Lab Anim Sci. 2009; 48(6):785-89.
21. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974; 47(3):469-74. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x
22. Sinha AK. Colorimetric assay of catalase. Anal Biochem. 1972; 47(2):389-94. https://doi.org/10.1016/0003-2697(72)90132-7
23. Ellman G. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959; 82(1):70-7. https://doi.org/10.1016/0003-9861(59)90090-6
24. Lykkesfeldt J. Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: comparison with ultraviolet-visible spectrophotometry. Clin Chem. 2001; 47(9):1725-27. https://doi.org/10.1093/clinchem/47.9.1725
25. Sambrook L, Russell RW. Molecular cloning: A laboratory, 3rd ed. Cold Spring Laboratory Press. Cold spring harbor, New York; 2001.
26. Sarkar S, Mukherjee S, Chattopadhyay A, Bhattacharya S. Low dose of arsenic trioxide triggers oxidative stress in zebrafish brain: Expression of antioxidant genes. Ecotoxicol Environ Saf. 2014; 107:1-8. https://doi.org/10.1016/j.ecoenv.2014.05.012
27. Jaramillo ML, Pereira AG, Davico CE, Nezzi L, Ammar D, Müller YMR, *et al*. Evaluation of reference genes for reverse transcription-quantitative PCR assays in organs of zebrafish exposed to glyphosate-based herbicide, Roundup. Animal. 2018; 12(7):1424-34. https://doi.org/10.1017/S1751731117003111
28. Velasques RR, Sandrini JZ, da Rosa CE. Roundup® in zebrafish: Effects on oxidative status and gene expression. Zebrafish. 2016; 13(5):432-41. https://doi.org/10.1089/zeb.2016.1259
29. Bautista FEA, Junior ASV, Corcini CD, Acosta IB, Caldas SS, Primel EG, *et al*. The herbicide atrazine affects sperm quality and the expression of antioxidant and spermatogenesis genes in zebrafish testes. Comp Biochem Physiol C Toxicol Pharmacol. 2018; 206:17-22. https://doi.org/10.1016/j.cbpc.2018.02.003
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2-δδct method. Methods. 2001; 25(4):402-8. https://doi.org/10.1006/meth.2001.1262
31. Luna LG. Routine staining procedures: Hematoxylin and eosin stains. Manual of histologic staining methods of the armed forces institute of pathology, 3rd ed. McGraw-Hill, New York; 1968. p. 32-9.
32. Bhattacharya S, Bhattacharya A, Roy S. Arsenic induced responses in fresh water teleosts. Fish Physiol Biochem. 2007; 33(4):463-73. https://doi.org/10.1007/s10695-007-9173-2
33. Cao C, Leng Y, Liu X, Yi YP, Li P, Kufe D. Catalase is regulated by ubiquitination and proteosomal degradation. Role of the c-Abl and Arg tyrosine kinases. Biochem. 2003; 42(35):10348-53. https://doi.org/10.1021/bi035023f
34. Liu Y, Wang J, Wei Y, Zhang H, Xu M, Dai J. Induction of time-dependent oxidative stress and related transcriptional effects of perfluorododecanoic acid in zebrafish liver. Aquat Toxicol. 2008; 89(4):242-50. https://doi.org/10.1016/j.aquatox.2008.07.009
35. Di Giulio R, Meyer J. Reactive oxygen species and oxidative stress. The Toxicology of Fishes; 2008. p. 273-324.
36. Cong B, Liu C, Wang L, Chai Y. The impact on antioxidant enzyme activity and related gene expression following adult zebrafish *(Danio rerio)* exposure to dimethyl phthalate. Animals. 2020; 10(717):1-12. https://doi.org/10.3390/ani10040717
37. Bhattacharya A, Bhattacharya S. Induction of oxidative stress by arsenic in *Clarias batrachus*: Involvement of peroxisomes. Ecotoxicol Environ Saf. 2007; 66(2):178-87. https://doi.org/10.1016/j.ecoenv.2005.11.002
38. Saez GT, Bannister WH, Bannister JV. Free radicals and thiol compounds. The role of glutathione against free radical toxicity. In: Vina J, editors, Glutathione: Metabolism and physiological functions. CRC Press, Boca Raton, Fl, USA; 1990. p. 237-54.
39. Larguinho M, Cordeiro A, Diniz MS, Costa PM, Baptista PV. Metabolic and histopathological alterations in the marine bivalve *Mytilus galloprovincialis* induced by chronic exposure to acrylamide. Environ Res. 2014; 135:55-62. https://doi.org/10.1016/j.envres.2014.09.004
40. Radwan MA, El-Gendy KS, Gad AF, Khamis AE, Eshra ESH. Ecotoxicological biomarkers as investigating tools to evaluate the impact of acrylamide on Thebapisana snails. Environ Sci Pollut Res. 2019; 26(14):14184-93. https://doi.org/10.1007/s11356-019-04784-0
41. Albalawi A, Alhasani RHA, Biswas L, Reilly J, Akhtar S, Shu X. Carnosic acid attenuates ACR-induced retinal toxicity in zebrafish embryos. Exp Eye Res. 2018; 175:103-14. https://doi.org/10.1016/j.exer.2018.06.018

42. Trabelsi W, Chetoui I, Fouzai C, Bejaoui S, Rabeh I, Telahigue K, *et al*. Redox status and fatty acid composition of Mactracorallina digestive gland following exposure to acrylamide. Environ Sci Pollut Res. 2019; 26(22):22197-208. https://doi.org/10.1007/s11356-019-05492-5
43. Acaroz U, Ince S, Arslan-Acaroz D, Gurler Z, Kucukkurt I, Demirel HH, *et al*. The ameliorative effects of boron against acrylamide-induced oxidative stress, inflammatory response, and metabolic changes in rats. Food Chem Toxicol. 2018; 118:745-52. https://doi.org/10.1016/j.fct.2018.06.029
44. Santhrani T, Vineela S, Devi CM. Neuroprotective effect of naringin on acrylamide induced cytotoxicity in U87MG cells and Wistar rats: An *in-vitro* and *in-vivo* study. Toxicol Int. 2021; 28(1):49-56.
45. Morrow JD, Frei B, Longmire AW, Gaziano JM, Lynch SM, Shyr Y, *et al*. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers-smoking as a cause of oxidative damage. New Engl J Med. 1995; 332(18):1198-203. https://www.nejm.org/doi/full/10.1056/ NEJM199505043321804
46. Banerjee BD, Seth V, Bhattacharya A, Pasha ST, Chakraborty AK. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicol Lett. 1999; 107(1-3):33-47. https://doi.org/10.1016/S0378-4274(99)00029-6
47. Abd-Elkareem M, Khalil NSA, Sayed AH. Hepatotoxic responses of 4-nonylphenol on African catfish (*Clarias gariepinus*): Antioxidant and histochemical biomarkers. Fish Physiol Biochem 2018; 44(3):969-81. https://doi.org/10.1007/s10695-018-0485-1
48. Aydogan M, Korkmaz A, Barlas N, Kolankaya D. The effect of Vitamin C on bisphenol A, nonylphenol and octylphenol induced brain damages of male rats. Toxicology. 2008; 249(1):35-9. https://doi.org/10.1016/j.tox.2008.04.002
49. Mohamed WA, El-Houseiny W, Ibrahim RE, Abd-Elhakim YM. Palliative effects of zinc sulfate against the immunosuppressive, hepato-and nephrotoxic impacts of nonylphenol in Nile tilapia (*Oreochromis niloticus*). Aquaculture. 2019; 504:227-38. https://doi.org/10.1016/j.aquaculture.2019.02.004
50. Wu M, Xu H, Shen Y, Qiu W, Yang M. Oxidative stress in zebrafish embryos induced by short-term exposure to bisphenol A, nonylphenol, and their mixture. Environ Toxicol Chem. 2011; 30(10):2335-41. https://doi.org/10.1002/etc.634
51. Osburn WO, Kensler TW. nrf2 signaling: An adaptive response pathway for protection against environmental toxic insults. Mutat Res-Rev Mutat Res. 2008; 659(1-2):31-9. https://doi.org/10.1016/j.mrrev.2007.11.006
52. Sporn MB, Liby KT. nrf2 and cancer: The good, the bad and the importance of context. Nat Rev Cancer. 2012; 12(8):564-71. https://doi.org/10.1038/nrc3278
53. Dai G, Vaughn S, Zhang Y, Wang ET, Garcia-Cardena G, Gimbrone Jr MA. Biomechanical forces in atherosclerosis-resistant vascular regions regulate endothelial redox balance via phosphoinositol 3-kinase/Akt-dependent activation of Nrf2. Circ Res. 2007; 101(7):723-33. https://doi.org/10.1161/CIRCRESAHA.107.152942
54. Kobayashi S, Ishimaru M, Hiraoka K, Honda C. Myb-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta. 2002; 215(6):924-33. https://doi.org/10.1007/s00425-002-0830-5
55. Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. Antioxid Redox Signal. 2005; 7(3-4):385-94. https://doi.org/10.1089/ars.2005.7.385
56. Petersen DW, Cooper KR, Friedman MA, Lech JJ. Behavioral and histological effects of acrylamide in rainbow trout. Toxicol Appl Pharmacol. 1987; 87(1):177-84. https://doi.org/10.1016/0041-008X(87)90095-0
57. Kilicle PA, Evren KOÇ, Dogan A, Süleyman GUL, Doğan ANC, Ersan Y. Investigation of the effect of acrylamide on *Capoeta Capoeta* (Guldensttead 1773) by histopathological, electrophoretic and biochemical methods. Caucasian J Sci. 2020; 7(2):153-66. https://doi.org/10.48138/cjo.836674