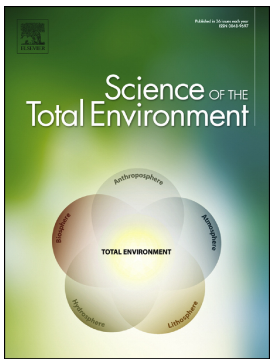

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Transcriptional, Biochemical and Histological Alterations in Adult Zebrafish (*Danio rerio*) Exposed to Benzotriazole Ultraviolet Stabilizer- 328

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ABSTRACT

The occurrence of Benzotriazole Ultraviolet Stabilizer-328 (BUV-328) in different environmental and biological matrices is of immediate environmental concern. In the present study, we evaluated the toxicity of BUV-328 in zebrafish liver tissues to understand the role of oxidative damage in hepatotoxicity. Adult zebrafish were exposed to 0.01, 0.1 and 1 mg/L

of BUV-328. At the end of 14, 28 and 42 days, liver tissues were examined for the responses of antioxidant enzymes, gene expression and histopathological alterations. The results indicated that superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were elevated at concentrations of 0.1 and 1 mg/L on 14th and 28th day. Glutathione S-transferase (GST) activity and malondialdehyde (MDA) levels were elevated in all the treated groups. The transcriptional levels of genes encoding *sod, cat, gpx* and *gst* enzymes were increased at 14th day and then declined (except *sod* on 28th day). Moreover, transcription of *cyp1a,* and *hsp70* were up-regulated throughout the study period. Histopathological lesions such as hypertrophy, cellular and nuclear enlargement, cytoplasmic and nuclear degeneration, necrosis with pyknotic nuclei, lipid and cytoplasmic vacuolization and nuclear displacement to the periphery were found to be increased with the dose and exposure duration. In brief, our findings indicate that even a low dose of BUV-328 is toxic to induce oxidative stress and liver damage in zebrafish over a long period of exposure.

Keywords**:** Oxidative stress; Gene expression; Histopathology; Cytochrome P450; Heat shock proteins

1. Introduction

2-(2H-Benzotriazol-2-yl)-4,6-di-*tert*-pentyl phenol [benzotriazole UV stabilizer - 328] is one of the widely used benzotriazole type ultraviolet light (UV) absorbents which imparts good light stability to plastics and other organic polymers (Carpinteiro *et al*., 2012; Denghel *et al*., 2019). Most of the (2- hydroxyphenyl) benzotriazole derivatives are quite stable and highly lipophilic in nature, with predicted octanol-water partition coefficient (Kow) value of > 6, resulting in significant resistance to hydrolysis and biodegradation (Wick et al., 2016). Owing to its photostability and high hydrophobic nature, BUV-328 has been widely used in personal hygiene products, building materials, automobile components, sports equipment, films, varnishes and aeroplane defogging fluids to prevent yellowing and light-induced

degradation (Hart et al., 2004; Giokas et al., 2007; Diaz-Cruz and Barcelo, 2009; Denghel et al., 2019). Because of the high production volume and widespread applications, BUV-328 has become ubiquitously dispersed in the environment via the manufacturing process and the effluent discharged from wastewater treatment plants (Cuderman and Heath, 2007). The European Chemicals Agency (ECHA) has classified BUV-328 as a persistent, bioaccumulative and toxic substance of very high concern (Annex XV report, 2014; ECHA, 2014, 2018).

Even though only trace amounts of BUV-328 enters the aquatic environment, its high lipophilicity (log Kow = 7.25) and poor degradability can lead to relatively high levels of the compound in various environmental as well as biota samples (Nakata et al., 2010; Kim et al., 2011a; Kim et al., 2011b; Zhang et al., 2011; Kim et al., 2012; Nakata et al., 2012; Kim et al., 2015; Asimakopoulos et al., 2013a; Asimakopoulos et al., 2013b; Lai et al., 2014; Liu et al., 2014; Langford et al., 2015; Lee et al., 2015; Apel et al., 2018; Vimalkumar et al., 2018; Kim et al., 2019; Lu et al., 2019; Shi et al., 2019; Peng et al., 2020). The concentration ranges from ng/L to sub-µg/L in water and µg/g in aquatic organisms (Carpinteiro et al., 2010; Montesdeoca-Esponda et al., 2013, 2019). Despite these findings, limited information is available on the toxicological and ecotoxicological implications of BUV-328 exposure.

Acute toxicity of BUV-328 (48-h LC_{50} >10 mg/L) has previously been reported by Kim et al. (2011c) in the freshwater crustacean *Daphnia pulex*. The authors have suggested that BUV-328 is a highly persistent, bioaccumulative and moderately toxic substance. However, it has been reported that BUV-328 to exert adverse effects on the antioxidant defense system in the freshwater green algae *Chlamydomonas reinhardtii* after chronic exposure (Giraudo *et al*., 2017). The combined toxicities of BUV-328 and BUV-234 have been studied by Giraudo et al. (2020) in juvenile rainbow trout (*Oncorhynchus mykiss*). Furthermore, Denghel et al. (2019) have investigated the oxidative phase I metabolism of

BUV-328 in an *in vitro* model involving human liver microsomes. Although some studies have asserted the toxic effects of BUV-328 on organisms, the chronic effects of the substance on zebrafish (*Danio rerio*) have not been thoroughly investigated. Hence, to assess if and how fish are affected by BUV-328, a multi-biomarker approach was used in this study to address the adverse biological responses associated with different concentrations of BUV-328 contamination.

It is well known that exposure to a broad range of environmental contaminants can stimulate the production of reactive oxygen species (ROS) and increase the level of cellular oxidative stress in aquatic organisms (Livingstone, 2003). Teleosts possess a complex defense system to protect themselves from oxidative damage. Specifically, fish antioxidant enzymes such as SOD, CAT, GPx and GST are known to work as the first line enzymatic defense against ROS production (Yu, 1994; Roch, 1999). These enzymes are sensitive to environmental xenobiotics and are frequently used as a tool in ecological risk assessment (Burgos-Aceves et al., 2018). Furthermore, an overwhelming generation of ROS can react with cellular macromolecules, particularly through lipid peroxidation (LPO) (Larose et al., 2008). Malondialdehyde (MDA), an end product of LPO, can be used to evaluate the degree of damage (Wahsha et al., 2012). Additionally, the production of ROS is also responsible for inducing the expression of many genes (Chen et al., 2011). Transcriptomic responses, which occur upon pollutant exposure, are rapid and thus considered to be sensitive indicators for investigating the adverse effects of environmental contaminants, including their molecular mode of action (Lettieri, 2006; Oggier et al., 2011). Cytochrome P450s production is associated with ROS. Therefore, changes in the amount of cytochrome P450 1A (*cyp1a*) mRNA induced by xenobiotics could be considered as a stress biomarker that signifies the pollution of aquatic environment (Dong et al., 2009). Likewise, heat-shock protein 70

(*hsp70*), a molecular chaperone which has been used as stress indicator in many toxicity tests (Scheil et al., 2010).

Hence, the present study was aimed at exploring the toxic effects of BUV-328 exposure on the oxidative stress mechanism (LPO), antioxidant response machinery (such as SOD, CAT, GPx and GST) and gene expression levels related to stress response (such as *sod, cat, gpx, gst, cyp1a,* and *hsp70*) in zebrafish. In addition, the histopathological alterations were also investigated in the liver tissues to identify potential hepatic lesions induced by BUV- 328 exposure. Adult zebrafish (*Danio rerio*) was used as a model organism in this study owing to its desirable features such as short life span, high fecundity, high homology to mammalian species, etc. (Segner 2009). To our knowledge, the above said parameters have not been thoroughly studied so far with regard to BUV- 328 toxicity in fish.

2. Materials and methods

2.1. Ethics statement

All experiments and animal handling were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2. Chemicals

Benzotriazole UV stabilizer 328 (BUV-328; CAS 25973-55-1; 98% purity) was obtained from Sigma-Aldrich, USA. Dimethyl sulphoxide (DMSO; CAS No 67-68-5) supplied by Sigma-Aldrich, USA was utilized to prepare the stock solutions of BUV-328. The other chemicals employed in the present investigation were of analytical grade and used without further purification.

2.3. Experimental set-up

Adult wild-type (AB strain) zebrafish (*Danio rerio*), with a mean body weight of 0.28 ± 0.04 g and a body length of 2.46 ± 0.04 cm, were procured from a local fish dealer. The organisms were acclimatized to the laboratory conditions for two weeks in glass aquarium prior to the experiments according to the guidelines of the Organization for Economic Co-operation and Development (OECD, 1996). The fish were reared in re-circulating aerated freshwater maintained at 26 ± 1°C, with a photoperiod of 12:12 h (light/dark) regimen. During the acclimatization period, they were fed with commercial fish food at *ad libitum*, and water renewal was done once a day.

After acclimation, fish (750 numbers) were randomly divided into five experimental groups such as water control group, solvent control group and three BUV-328 exposure groups at concentrations of 0.01, 0.1 and 1 mg/L. Each group was maintained in three replicates and each replicate contains 50 fish in 25 L test solution. Stock solutions of BUV-328 were prepared freshly in DMSO and exposed at concentrations of 0.01, 0.1 and 1 mg/L for 42 days. The test solutions in the aquarium were renewed every 24 h to maintain the appropriate concentration of BUV-328 and the water quality. During exposure, the fish were fed once a day with commercial fish food before one hour of renewal period. They were starved for 24 h prior to experimentation. On days 14, 28 and 42, fish were randomly selected from exposure and control tanks (n = 15/replicate) and liver samples were collected and divided into three aliquots. Five individual fish per replicate of each treatment group were taken for one aliquot. The first aliquot was used immediately for biochemical analysis. The second aliquot was preserved in TRIzol reagent for transcriptional expression analysis and the third aliquot was fixed in 10% formalin for histological observation.

2.4. Biochemical analysis

The liver tissues were rinsed, homogenized with 50 mM ice-cold potassium phosphate buffer (pH 7.0), centrifuged for 10 min (10,000 rpm) and the clear supernatant was collected to measure the protein content, enzyme activities (SOD, CAT, GPx and GST) and MDA level. Each assay was performed in triplicates. SOD activity was estimated (Marklund and Marklund, 1974) by measuring the inhibition of pyrogallol autooxidation at 420 nm, and the enzyme activity was expressed as Units/mg protein. CAT activity was estimated by measuring the absorbance of hydrogen peroxide at 590 nm and expressed as µmol H_2O_2 consumed/min/mg protein (Aebi, 1984). GST activity was determined (Habig et al., 1974) after the complexation of glutathione (GSH) with 1-chloro-2, 4-dinitrobenzene CDNB) at 340 nm, and the result was given in µmol of CDNB conjugate formed/min/mg protein. GPx activity was estimated (Rotruck et al., 1973) after the oxidation of glutathione (GSH) in the presence of H_2O_2 at 412 nm and the data was expressed as µg GSH formed/min/mg protein. MDA content was estimated according to Devasagayam et al (2003) at 532 nm, which is based on 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA) reactivity, and the result was expressed as nmol/mg protein. The protein concentration was obtained using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.5. Quantitative real-time PCR experiments (RT-qPCR)

TRIzol reagent was used to extract the total RNA from frozen liver tissues. The quantity and quality of the extracted total RNA were examined spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies, USA) at 260 nm. Only RNA samples with OD_{260nm}/OD_{280nm} between 1.8 and 2.0 were selected for subsequent tests. Complementary DNA (cDNA) was synthesized from 2 µg total RNA using the cDNA synthesis kit (iScript, Bio-Rad, USA). Quantitative real-time PCR (qRT-PCR) amplification was carried out using CFX96™ Real-Time PCR Detection System (Bio-rad, USA) and SYBR Green kit (Kapa

Biosystems, USA), with *β-actin* gene as the endogenous control. Primer sequences were selected from literature and have been reported in Table (S1). The PCR conditions were as follows: an initial denaturation step at 95°C for 10 min, 40 cycles of amplification at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min. All sample reactions were performed in triplicates. The amplifications were subjected to melting curve analysis to confirm the reaction specificity. Fold changes were calculated according to the $2^{-\Delta\Delta Ct}$ method with the formula $F=2^{-\Delta\Delta Ct}$, $^{\Delta\Delta}C_t = (C_{t,\text{ target gene}}-C_{t,\text{ reference gene}})-(C_{t,\text{ target gene}} - C_{t,\text{ reference gene}})_{\text{control}}$ (Livak and Schmittgen, 2001).

2.6. Histopathological observation

Liver samples were initially fixed in 10% neutral buffered formalin. The fixed tissue samples were dehydrated in a series of graded ethanol, embedded in paraffin wax, sectioned at 4 µm thickness, and stained with hematoxylin and eosin (H&E) for histopathological analysis (Pearse, 1968; Roberts, 1978; Humason, 1979). The sections were examined and photographed using a light microscope (Leica DME light microscope).

2.7. Statistical analysis

Statistical analysis was carried out by using GraphPad Prism 5.0 software package (GraphPad Software Inc., San Diego, CA). The results obtained from each experimental group were subjected to one-way analysis of variance (ANOVA), followed by Bonferroni post- tests. Values of $P < 0.05$ were considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3. Results

No mortality was observed during the acclimatization and exposure periods, and there was no significant difference between the blank (water) and solvent control (DMSO) for any of the biomarkers during the exposure. Hence, the water control group was maintained as the reference group.

3.1. Effects of BUV-328 on antioxidant enzymatic activities and lipid peroxidation

The activities of SOD, CAT and GPx in the liver of adult zebrafish exposed to BUV-328 are depicted in Fig. 1a, b and c, respectively. It is evident that when compared with the control, the above enzyme activities were significantly increased when exposed to higher concentrations (0.1 and 1 mg/L) of BUV-328 for 14 and 28 days. The only exception was the lower concentration (0.01 mg/L) treated group on day 14. As the exposure time extended to 42 days, the activities of these enzymes decreased gradually in all the treated groups, except the lower dose (0.01 mg/L) treated group. It is clear from Fig. 1d that the GST activity of the BUV-328 treated samples were enhanced gradually (relative to their activities in the control) in a concentration and time-dependent manner. MDA level was also more elevated in all the dose groups on days 14, 28 and 42 than in the control groups (Fig. 1e).

3.2. mRNA expression levels of genes involved in stress response

The effects of BUV-328 exposure on the expression of genes involved in stress response were determined by qRT-PCR (Fig. 2). The analysis revealed that *cyp1a* (Fig. 2a) was up-regulated in response to 0.01, 0.1 and 1 mg/L BUV-328 on days 14, 28 and 42 with respect to the control group. Gradual up-regulation of *hsp70* (Fig. 2b) expression was also observed in groups exposed to 0.01, 0.1 and 1 mg/L BUV-328 when compared with the control. In addition, the transcription levels of the genes encoding antioxidant enzymes (*sod*, *cat*, *gpx* and gst: Fig. 2c-f) were raised significantly on day 14. Interestingly, as the exposure period

extended to 28 and 42 days, the transcriptional levels of these genes (except *sod* on 28 day) were significantly inhibited by BUV-328.

3.3. Histopathological changes

To understand the toxicity of BUV-328 in a better way, the histological photomicrographs of liver sections from adult zebrafish specimens have been shown in Fig. 3. The sample in the control group appeared to be normal. The hepatocytes and nuclei were uniform in size and shape (Fig. 3. Ph.m. A). Fish exposed to 0.01 and 0.1 mg/L BUV-328 for 14 days showed less histological lesions such as liver sinusoids, with a little degree of hepatocyte vacuolation and nuclear enlargement (Fig. 3. Ph.m. B & C). These changes were observed throughout the liver tissue. In addition, fish exposed to 1 mg/L of BUV-328 displayed various injuries namely eosinophilic granules, pyknotic nuclei, cytoplasmic vacuolation and degeneration, dilated sinusoids, nuclear degeneration and hypertrophy (Fig. 3. Ph.m. D). At the end of 28th day, similar lesions were more prevalent, accompanied by lipid vacuolization in all the three treatments (Fig. 3. Ph.m. E-G). In addition, cloudy swelling of hepatocytes and severe liver necrosis were evident in many places in fish exposed to 0.1 and 1 mg/L of BUV-328. As the exposure time extended to 42 days, the histological changes observed in the fish become more severe with increasing concentration (Fig. 3. Ph.m. H-J). Hemorrhage in the veins, pyknosis on the nuclei, necrosis with sinusuoidal lesions and complete degeneration of hepatocytes were the maximum alterations observed. Furthermore, blood sinusoid and melanomacrophage aggregates were noticed in fish exposed to 1 mg/L of BUV-328 at 42nd day interval.

4. Discussion

In this work, we report the potential of BUV-328 to alter the activity of antioxidant enzymes and the expression of genes related to oxidative stress as well as hepatic histology in adult zebrafish. Our findings will help to understand the molecular mechanisms involved in the toxicological effects of BUV-328 in aquatic organisms.

The hepatic antioxidant system is the chief defense mechanism against environmental stress since it can degrade the ROS that can damage many of the intracellular macromolecules (Morales et al., 2004; Huang et al., 2007). Antioxidant enzymes such as SOD, CAT, GST and GPx offer effective protection from ROS (Yu, 1994; Adeyemi, 2014). SOD safeguards the cells against free radical induced oxidative damage by catalyzing the conversion of superoxide anion (O^{2-}) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), while CAT is responsible for removing the H_2O_2 from the system by converting it to water (H_2O) and oxygen (O_2) (Zhu et al., 2008). The findings of the present study indicate that exposure to higher concentrations (0.1 and 1 mg/L) of BUV-328 for 14 days have promoted the liver's SOD and CAT to eliminate the overproduced ROS as a protection mechanism against oxidative stress. However, no statistical difference in SOD and CAT activity between the control and BUV-328 exposed fish was observed in the lower dose (0.01 mg/L) group, which may be due to the simultaneous elevation of antioxidant enzymes and the oxidative modification of SOD and CAT caused by excess ROS (Ni et al., 2019). In all higher concentration treated groups, at the end of 28 and 42 days of exposure, the enzyme activities were decreased to the levels of the control fish, which can be interpreted as the inability of the antioxidant enzymes to overcome extremely high oxidative stress (Bagnyukova et al., 2006). Similar findings have also been reported by Liu et al. (2015) and Liang et al. (2019), who found UV filter and benzotriazole ultraviolet stabilizer-induced overproduction of H_2O_2 in *Carassius auratus* and zebrafish embryos, respectively.

The main function of GPx is to eliminate the radical species and maintain the membrane protein thiols (Kavitha et al., 2011). Parallel to the SOD and CAT enzyme activities, the GPx activity was also increased initially but decreased in the later stages. The enhanced GPx activity during the study period (14 and 28 days) indicates increased production of H_2O_2, which also coincides with the high lipid hydroperoxide level responsible for toxicant induced oxidative damage. The unaltered GPx activity may be due to the stimulative and suppressive effects counteracting each other at a lower concentration of BUV-328. Accentuated GPx activity might be due to the surplus amount of oxidized glutathione (GSSG) (Narra, 2014). Furthermore, reduced CAT activity can be considered as a factor in the decreased GPx activity (Lumaret et al., 2012). Similar results have also been reported for the freshwater fish *Labeo rohita* (Hemalatha et al., 2019) and zebrafish embryos (Nataraj et al., 2020) after exposure to triclosan and octyl methoxycinnamate and its photoproducts, respectively.

GST, a phase II biotransformation enzyme, plays an important role in the detoxification processes (Thom et al., 2001) as it catalyzes the conjugation of several xenobiotics with glutathione (GSH). In the current study, we found that BUV-328 exposure significantly elevated GST activity, implying the involvement of GST in the biotransformation of toxic metabolites and the metabolization of lipid peroxides formed by Fenton reaction (Modesto et al., 2010). Similar results have also been noted by Liu et al. (2015) in *Carassius auratus* exposed to benzophenone UV filters and in zebrafish embryos exposed to 4-methylbenzylidene camphor (Quintaneiro et al., 2019). Lipid peroxidation can produce malondialdehyde (MDA) (Liu et al., 2016), which may severely damage the cell membranes (Liu et al., 2007; Modesto et al., 2010). Measurement of MDA content can indirectly reflect the degree of LPO, besides revealing the oxidative stress (Chen et al., 2017; Zhang et al., 2017). In the present investigation, MDA content was found to be higher in the

liver of the treated groups than in the controls. This can be interpreted as the induction of ROS, which increases the oxidation of polyunsaturated fatty acids and leads to lipid peroxidation. This finding is consistent with previous studies by He et al. (2019) and Giraudo et al. (2017) in which similar responses were observed in *Chlamys nobilis* exposed to benzotriazole and in *Chlamydomonas reinhardtii* exposed to BUV-328, respectively.

Oxidative stress and antioxidant-related gene expression have been used effectively to study the impact of xenobiotics on fish (Twaroski et al., 2001; Na et al., 2009). Transcriptional responses are sensitive indicators of stress and thus can serve as early warning signs of a specific material (Causton et al., 2001; Zheng et al., 2018). However, only a limited number of studies have reported the effect of BUV-328 induced toxicity on antioxidant responses and related gene expression in zebrafish. *Cyp1a* is one of the most studied xenobiotic-metabolizing isoforms in fish (Havelkova et al., 2007). *Cyp1a* mRNA expression was gradually up-regulated in all the treatment groups. Previous research (Swanson et al., 2002) has documented that the expression of *cyp1a* is transcriptionally up-regulated through the ligand-activated aryl hydrocarbon receptor (AHR) that can form a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). Therefore, the up-regulation of *cyp1a1* mRNA in this study might have been influenced by the pregnane x receptor (PXR) and constitutive androstane receptor (CAR) proteins. Upon activation, they move to the nucleus and bind to the DNA response elements in the promoters to induce the expression of many phase I and phase II metabolizing enzymes (Staudinger et al., 2011). Up-regulation of *cyp1a1* mRNA in zebrafish embryos due to the activation of AHR pathway has also been observed after BUVSs exposure (Fent et al., 2014).

Hsp70 plays a major role in the cellular stress response (Hartman and Gething, 1996). The protein assists in the folding of new polypeptide chains that act as molecular chaperones and participates in the degradation and repair of altered or denatured proteins (Basu et al.,

2002). In the present study, the exposure of adult zebrafish to different doses of BUV-328 substantially up-regulated the mRNA levels of *hsp70* in liver, signifying the damage in this tissue caused by chronic exposure. Similarly, Ozáez et al. (2016) and Zheng et al. (2017) have demonstrated a dose-dependent up-regulation of *hsp70* gene in the embryos and larvae of *Chironomus riparius* treated with UV filter 4-methylbenzylidene camphor and cadmium, respectively.

The transcriptional levels of genes such as *sod*, *cat*, *gpx* and *gst* in the zebrafish were significantly increased in the BUV-328 exposure groups (on 14th day), suggesting the mobilization of the antioxidant defense system in the liver to resist the oxidative damage. On days 28 and 42, these genes (except *sod* on the 28th day) were significantly inhibited by BUV-328, indicating that the normal transcriptions were strongly inhibited as the exposure period extended. Our results are in line with the findings of Ni et al. (2019) who observed similar changes in zebrafish treated with maduramicin. Contrary to our current finding, other studies have shown unchanged or opposing transcriptional levels of these genes in *Daphnia magna* and *Chlamydomonas reinhardtii* after BUV-328 exposure (Giraudo et al., 2017). This difference might be attributed to the varying BUV-328 concentration, different treatment durations and alterations between different species. Based on the above results, we speculated that BUV-328 induced oxidative stress and repressed the function of antioxidant enzyme systems in the liver, which were agreement with the previous study in zebrafish by Li et al. (2018).

In the present study, the structure of liver in the control group was normal (Fig. 3A). However, dose and time-dependent histological changes were noticed in fish exposed to different concentrations of BUV-328 (Fig. 3. Ph.m. B-J). The most noticeable structural changes were hypertrophy, cellular and nuclear enlargement, cytoplasmic and nuclear degeneration, necrosis with pyknotic nuclei, lipid and cytoplasmic vacuolization and nuclear

displacement to the periphery, blood sinusoid dilation and aggregation of melanomacrophages close to a vessel. The observed hepatocyte vacuolization after 14 days of BUV-328 exposure suggests the interference in the lipid metabolism, inhibition of protein synthesis and energy depletion of fish (Macêdo et al., 2020). Hypertrophy might have occurred as a cellular response to the increased inflow of toxicants to the liver cells (Fontagne et al., 1998). Furthermore, the occurrence of necrotic areas in the liver was severe in case of fish treated with higher concentration group (0.1 and 1 mg/L) on 28^{th} and 42^{nd} days, which reflects the failure of cellular protective mechanisms in the presence of chemical stress (Olufavo and Alade, 2012). In the present study, melanomacrophage aggregation were observed in 1 mg/L treatment group of 42^{nd} day indicate that this organ has undergone structural and metabolic damage due to the chemical exposure. Similar results have also been noted in zebrafish exposed to 17α-ethynylestradiol and dibutyl phthalate for 21 days (Xu et al., 2014). Likewise, Liu et al. (2015) have investigated the effects of benzophenone UV filters on *Carassius auratus* and found similar impacts on the liver. Taken together, our observations reveal that the accumulation of BUV-328 in the liver might have caused damage to this organ and altered the normal physiological function of zebrafish.

5. Conclusion

In summary, it can be concluded that chronic exposure of BUV-328 induced oxidative stress in the liver of adult zebrafish, significantly affected the antioxidant enzyme (SOD, CAT, GPx and GST) activities and MDA contents, as well as the transcriptional levels of genes (*cyp1a, hsp70, sod, cat, gpx* and *gst*) related to oxidative stress responses. In addition, BUV-328 exposure caused severe tissue damage after 42 days of exposure. Collectively, our results demonstrate that the selected BUV-328 concentrations exerted detrimental effects on adult zebrafish over a long period of exposure. Both time and concentration had significant effects on the changes in the tested biomarkers. Moreover, these results also provide evidence

for the oxidative stress and tissue damage resulting from BUV-328 exposure. Thus, these findings will contribute profoundly to our understanding of the chronic toxic effects of BUV-328 in fish. Specific toxic effects, such as developmental toxicity and endocrine disruptive effects are our next research goals.

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Figure captions

Fig. 1. The activities of antioxidant enzymes [SOD (a), CAT (b), GPx (c), GST (d)] and lipid peroxidation [LPO (e)] level in the liver of zebrafish exposed to BUV-328. The data are presented as mean ± SD. The asterisks represent significant difference between treatment and control group at the same timepoint ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***).

Fig. 2. Effects of BUV-328 on the mRNA expression levels of *cyp1a* (a), *hsp70* (b), *sod* (c), *cat* (d), *gpx* (e) and *gst* (f) in the liver of adult zebrafish. The data are presented as mean ± SD. The asterisks represent significant difference between treatment and control group at the same timepoint ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***).

Fig. 3. Light micrographs of sections through liver of zebrafish showing histological structure of the control group **(Ph.m. A)** and animals treated with 0.01 **(Ph.m. B, E, H),** 0.1 **(Ph.m. C, F, I)** and 1 mg/L **(Ph.m. D, G, J)** of BUV-328 for 14, 28, 42 days. Samples were stained with hematoxylin and eosin and photomicrographs were taken using 400X magnification. H-Hypertrophy, S- sinusoids, CV- Cytoplasmic vacuolation, DS-Dilated sinusoids, NE-Nuclear enlargement, CD-Cytoplasmic degeneration, LV-Lipid vacuolization, ND-Nuclear degeneration, SD-Sinusoidal dilation, PN-Pyknotic nuclei, EG-Eosinophilic granules, CS-Cloudy swelling, N-Necrosis, CND-Cytoplasmic and nuclear degeneration, CS-Cloudy swelling, CN-Condensation of the nuclei, BS-Blood sinusoid, melanomacrophage aggregates (star symbol).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Credit Author contribution statement

Devan Hemalatha	: Conceptualization, Validation, Investigation, Methodology, Data curation; Writing - original draft
Basuvannan Rangasamy	: Investigation, Methodology, Data curation; Review & Editing
Bojan Nataraj	: Investigation, Methodology, Data curation; Writing- review & editing
Kannan Maharajan	: Investigation, Methodology, Writing- review & editing
Narayanaswamy Arul	: Writing- review & editing
Mathan Ramesh	: Supervision, Funding acquisition, Resources, Data curation, Writing- review & editing

Graphical Abstract

Highlights

- The adverse effect of BUV-328 was investigated in liver of zebrafish
- The effects were based on antioxidant, transcriptional and histological responses
- BUV-328 exposure alters the liver antioxidant enzymes in all the concentrations
- BUV-328 induced changes was observed in stress responsive gene expression
- The severity of liver damage was time- and concentration-dependent

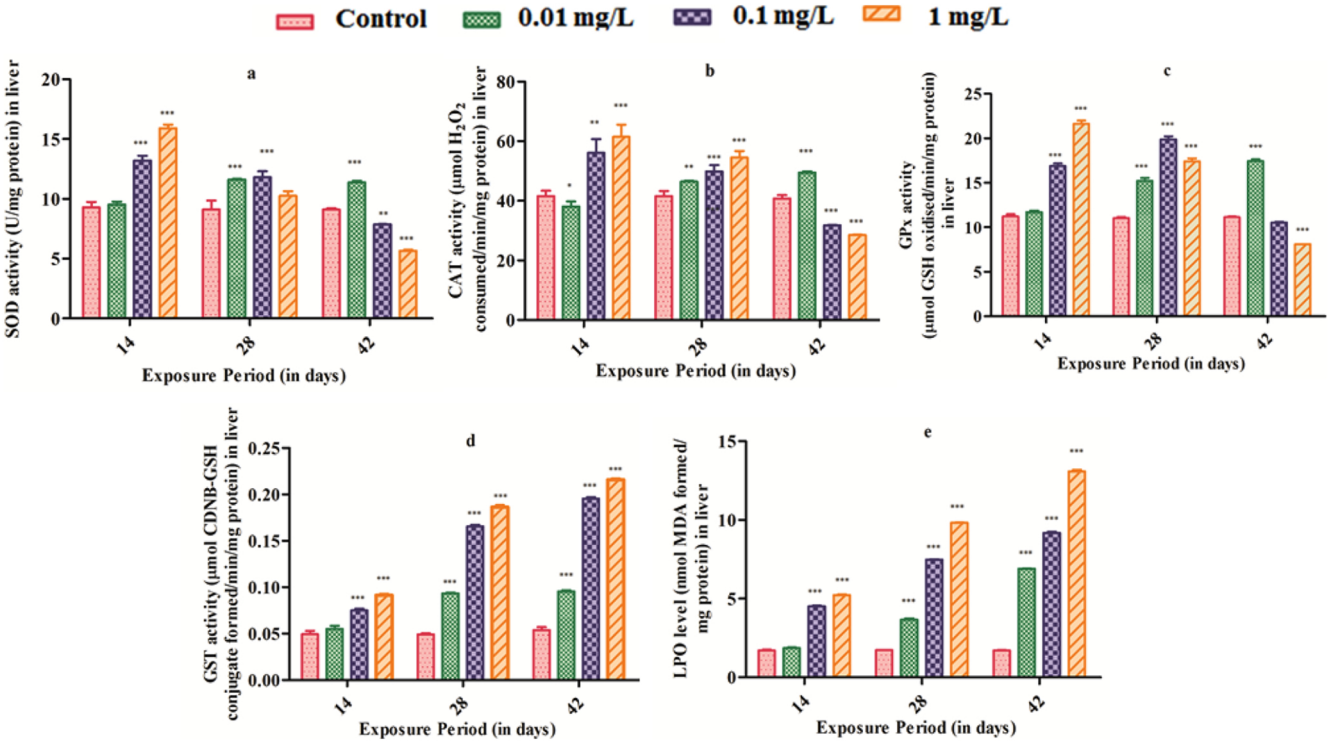

Control
0.01 mg/L
0.1 mg/L
1 mg/L
a
SOD activity (U/mg protein) in liver
Exposure Period (in days)
b
CAT activity (µmol H_2O_2 consumed/min/mg protein) in liver
Exposure Period (in days)
c
GPx activity (µmol GSH oxidised/min/mg protein) in liver
Exposure Period (in days)
d
GST activity (µmol CDNB-GSH conjugate formed/min/mg protein) in liver
Exposure Period (in days)
e
LPO level (nmol MDA formed/ mg protein) in liver
Exposure Period (in days)

Figure 1

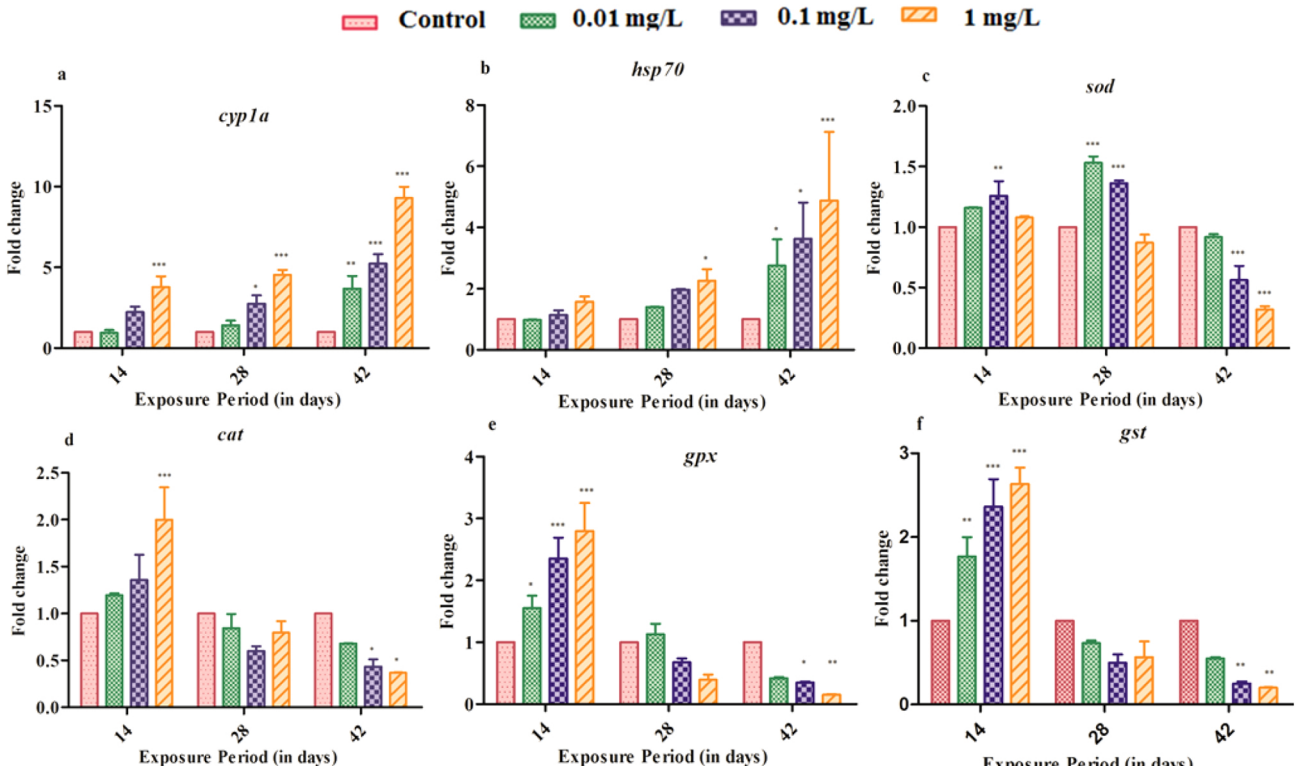
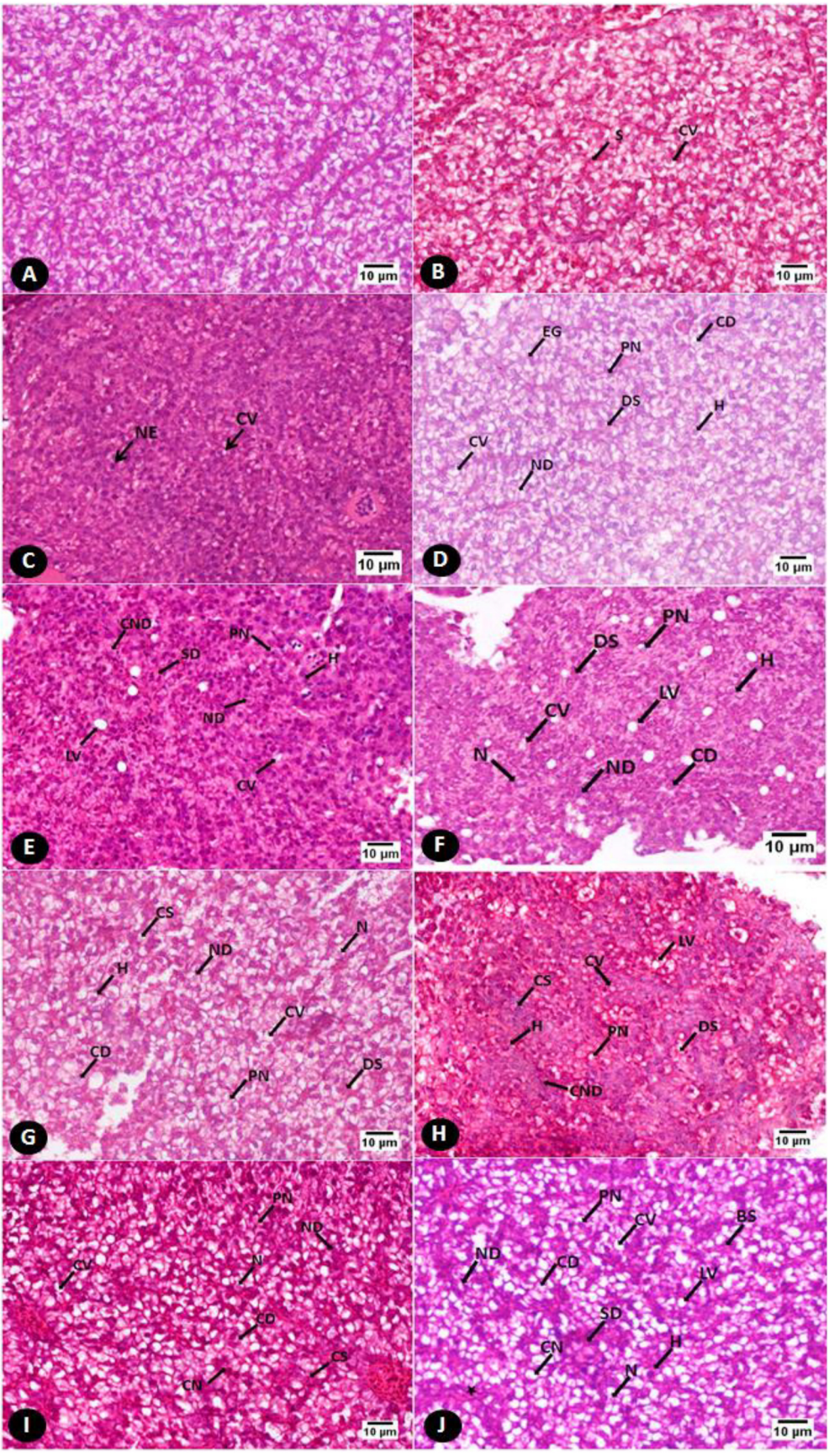

Control
0.01 mg/L
0.1 mg/L
1 mg/L
a
cyp1a
b
hsp70
c
sod
d
cat
e
gpx
f
gst
Fold change
Exposure Period (in days)
14
28
42

Figure 2


A
10 µm
B
S
CV
10 µm
C
NE
CV
10 µm
D
EG
PN
CD
DS
H
CV
ND
10 µm
E
CND
PN
SD
H
ND
LV
CV
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DS
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CV
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LV
SD
CN
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N
10 µm

Figure 3