Original Article

Toxicology International, Vol 25(1), 48-58, January-March 2018, DOI: 10.18311/ti/2018/v25i1/21411

ISSN (Print): 0971-6580
ISSN (Online): 0976-5131

Aldrin Induce Acute Toxicity at Enzymatic and m-RNA Expression Levels in Zebrafish

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Abstract

Aldrin is a systematic toxicant, belongs to cyclodiene class of organochlorine pesticide and banned due to its toxic, bioaccumalative and persistent nature but still detected from environmental components. Present study includes the assessment of aldrin toxicity at two sublethal concentrations in zebrafish. A total 81 zebrafish were divided into three groups viz. group 1: Control, group 2 and 3: Exposed groups which were given 3 mg/mL and 6 mg/mL of aldrin, respectively for 24 hours. The markers of oxidative stress viz. antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) were examined in terms of biochemical activities and gene expression. Acute exposure of aldrin (both concentrations) caused significant alteration in antioxidant enzymes activities and gene expression in liver, kidney and brain tissues, which were more prominent in brain in concentration dependent manner. Study provides a baseline data to understand alterations in enzymatic activity and expression leading to toxic manifestation of aldrin in aquatic animals.

Keywords: Aldrin, Gene Expression, Organochlorine, Oxidative Stress, Zebrafish

1. Introduction

Aldrin is an organochlorine pesticide, widely distributed in environment due to its anthropogenic uses. Aldrin gets converted via epoxidation to dieldrin in air, water, soil, biological tissue and exists majorly in dieldrin form[1]. Aldrin and dieldrin are banned for usage since three decades but still higher levels than acceptable limits are detected in ecosystem which poses threat to human health. Due to persistency and bio-accumulative nature, it is classified as Persistent Organic Pollutant (POP) and biomagnified through food chain[2].

The intensity of aldrin toxicity is well known as it can be easily absorbed due to its lipophilicity. Exposure to aldrin leads to systemic toxicity mainly affecting the central nervous system. It causes neuro-chemical changes at $GABA_A$ receptor. The inhibition of neurotransmitter is caused by blockade at the chloride channels which leads to hyper- excitability, irritability and convulsions[3]. It also causes hepatic necrosis, renal damage and reproduction system disruption. Although, there is evidence that aldrin exposure have toxic effects on nervous system, with GABAergic system as major target site, various studies in different animal models viz. mice, rat, dog, frog, rabbit, chicken, cat, catfish have shown that aldrin causes hepatotoxicity and biochemical alterations[4–7].

Aldrin and dieldrin induces generation of reactive oxygen/free radical species. Free radical species cause loss of membrane stability and lead to oxidative stress. Oxidative stress is believed to occur when there is an imbalance in the biological oxidant-to-antioxidant ratio[8]. As a result of this oxidative stress, fish, like many other vertebrates, try to reduce the damage using the antioxidant defense system. For example, O_2, the parental form of intracellular reactive oxygen species, is a very reactive molecule. Although superoxide dismutase the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion radical to hydrogen peroxide[9]. Further, catalase and glutathione peroxidase trigger the decomposition of endogenously produced hydrogen peroxide to water and molecular oxygen[10]. Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione

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Peroxidase (GPx) are the endogenous enzymes. They show scavenging activity against free radicals as repair mechanism. Thus, the evaluation of altered expression of these enzymes serves as biomarker in toxicological assessment. Studies demonstrated that aldrin can induce alterations in the antioxidant enzymes at cellular level due to reactive oxygen species generation; however, the mechanism involved in it is still unclear[1].

The antioxidant enzymes selected for biochemical and genetic parameters are considerably used as biomarkers for oxidative stress and their functions associating to each other. The biochemical assay is carried out to determine the activity of enzymes. The differential expression of the genes encoding these enzymes has been used to detect biological toxicity to monitor the impact of chemical pollutants such as aldrin. On toxicity at biochemical level, the gene expression data supports the enzymatic activity alterations by up-regulation or down-regulation of expression. RT-qPCR is a popular method that often involves the use of endogenous reference (housekeeping) gene for normalization of data obtained through examined gene. β-actin is commonly selected as a housekeeping gene due to its general expression across all eukaryotic cell types. The expression levels of β-actin do not vary drastically due to cellular treatment.

In view, aldrin is an important pollutant and induces impairment in several biological functions on biota of aquatic ecosystem. Therefore, the acute effects of exposure of aldrin at two sub-lethal concentrations in the liver, kidney and brain of zebrafish on antioxidant enzymes by biochemical assays and further alterations at gene level by qPCR system were evaluated. Literature regarding the effects of aldrin on zebrafish is scant, thus the present study was planned to investigate effects of aldrin at sub-lethal concentrations in liver, kidney and brain tissues. As a model organism, zebrafish is used for toxicity assessment of heavy metals[9], pesticides[2,11], chemicals[12] and industrial effluent discharge[13]. Zebrafish is a small, robust animal, have a transparent embryos and genetic structure similar to humans. These features assist researchers to extrapolate the data to humans.

2. Materials and Methods

2.1 Chemicals and Reagents

Aldrin neat (CAS: 309-00-2), analytical standard grade was procured from Sigma-Aldrich Co. (St. Louis, MO, USA). All chemicals and reagents were of molecular biology grade and purity >99.99%. Ribozol was obtained from Ameresco, Solon, Ohio USA. qPCR Green Master with UNG- blue dyed for quantitative PCR and Verso cDNA synthesis kit were procured from Thermo Fischer Scientific, India. Primers of specific sequence *viz.* superoxide dismutase, catalase, glutathione peroxidase and β-Actin for zebrafish reported by Brammell and Wigginton[14] were procured from Eurofins Genomics India Pvt. Ltd for PCR amplification (Table 1).

2.2 Animals

Certified wild species of adult zebrafish (4–5 months; length 2.8 cm ± 0.5 cm; weight 0.295 – 0.395 g) were procured from a pet shop and handled in accordance with good animal practice as defined by animal welfare bodies. The study was approved by the University Research Committee (No. PhD/FS/RA/03). Zebrafish were acclimatized for 15 days to laboratory environment by keeping them in glass aquaria containing 50 L of de-chlorinated tap water maintained under controlled conditions (temperature = 25°C ± 2; pH = 7.0 ± 0.2; Dissolved Oxygen = 7.2 ± 0.3 mg/L; Hardness = 110.0 mg/L; Conductivity = 0.2 μohm; Alkalinity = 0.25 g/L) with constant aeration and 12 hours alternate light and dark cycle. Water was changed every day. Zebrafish were fed daily on commercial pellets and 100% freeze dried blood worms of Taiyomax.

Table 1. Primer sequences of genes used in study

Gene	Forward Primer	Reverse Primer
Superoxide dismutase 2 (100bp)	**5'-CGCATGTTCCCAGACATCTA**	**5'- GAGCGGAAGATTGAGGATTG**
Catalase (115bp)	**5'- AGTGCTCCTGACGTCCAGCCA**	**5'-TGAAGAACGTGCGCACCTGGG**
Beta actin 1 (138 bp)	**5'- CAACAGAGAGAAGATGACACAGATCA**	**5'-GTCACACCATCACCAGAGTCCATCAC**
Glutathione peroxidase 4b (148bp)	**5'-GGACGATCCAAGCGTGGTGGA**	**5'-CAGCCGTCACACGTCTGGGC**

2.3 Experimental Protocols

2.3.1 Preparation of Stock and Working Solutions

A stock solution of 10 mg/mL (10000 ppm) aldrin in 100 µL groundnut oil followed by adding 900 µL distilled water was prepared. Test solutions of 3 mg/L and 6 mg/L were prepared by diluting the stock solution with de-ionized water i.e. 300 µl and 600 µl of stock solution in 1 L of water for 3 mg/L and 6 mg/L concentration, respectively.

2.3.2 Animal Exposure

The experimental design was in accordance with the guidelines of Organization for Economic Co-operation and Development[15]. During experimentation animals were not provided any food. To observe the effects of aldrin induced oxidative stress, a total 108 fishes were divided into three groups (36 animals in each group). Group 1: Animals were kept in de-chlorinated water and which served as control (CTRL) while animals of Group 2 and 3 were given 3 mg/L (3 ppm) and 6 mg/L (6 ppm) of aldrin (ALD), respectively. The concentrations were selected through preliminary experiments as mentioned in our published work[2].

2.3.3 Collection of Tissues and Homogenate Preparation

Fish were sacrificed after 24 h of exposure. Total 18 fish were used for RNA isolation. For RNA isolation, the liver, kidney and brain (pool of 6 zebra fish) were removed quickly and stored at –20°C in 0.2 mL of ice cold ribozol for 48 h. Remaining 18 fish (pool of 6 zebra fish) were used for biochemical studies, the liver, kidney and brain were collected, minced and homogenized (2.5% w/v) with ice-cold 0.15% KCl–0.1 M phosphate buffer (pH 7.4) using Potter–Elvehjem homogenizer in ice-cold condition and stored at –20°C till analysis.

2.4 Biochemical Assays

All biochemical parameters were analyzed on Multiskan EX Plate Reader (Thermo Fisher Scientific, Inc., USA). Superoxide Dismutase (SOD) activity was measured by the modified method of NADH-phenazine-methosulfate-nitroblue-tetrazoliumformazan inhibition reaction at 550 nm[16]. Catalase (CAT) activity was measured at 570 nm[17]. Glutathione peroxidase (GPx) activity was measured using glutathione as substrate and DTNB as standard at 420 nm[18]. Protein level was assayed using Bovine Serum Albumin (BSA) as the standard at 690 nm[19].

2.5 Total RNA Extraction and Reverse Transcription

The total RNA isolation was performed using ribozol reagent by method of Shukla et al.,[20]. The quantitative and qualitative analyses of extracted total RNA were carried out using NANOGENIUS photometer MAPADA Instruments. The OD_{260}/OD_{280} ratio of all RNA samples used was from 1.8 to 2.1. Total RNA from zebrafish tissue *viz*. liver, kidney and brain tissue were reverse transcribed to complementary DNA (cDNA) with Verso cDNA synthesis kit according to the manufacturer's protocol using Veriti 96 well Thermal Cycler, Applied Biosystems by Thermo Fischer Scientific (U.S.). Briefly, 5 µL of extracted total RNA (01 µg) was gently mixed with 11 µL DEPC-treated water and incubated at 70°C for 5 minutes, followed by placing the tubes on ice for cooling. Afterwards, 4 µL of cDNA synthesis buffer (5x), 2 µL of dNTP mix, 1 µL of random hexamer, 1 µL of RT enhancer and 1 µL of verso enzyme mix were added and mixed gently. cDNA was synthesized by incubation at 42°C for 60 m followed by heating at 92°C for 2 m to stop the reaction.

2.6 Real-time RT-PCR

PCR amplification and quantitation of gene were performed for CAT, GPx, SOD and β-actin as housekeeping gene. Real-time RT-PCR by qPCR Green Master with UNG-blue dyed detection kit was performed using Applied Biosystem ABI 7500 qPCR System, USA. The expected lengths of the PCR products of zebra fish superoxide dismutase, catalase and glutathione peroxidase and β-actin genes were 100–150 base pairs (bp). All the reactions were carried out in a 10 µL reaction mixture in triplicate following the manufacturer's protocol.

2.7 Data Analysis

The data obtained by real time RT-PCR are presented as threshold cycle (Ct). For fold changes in target gene, relative quantification by the 'delta-delta Ct Method' was used for calculation to compare relative expression levels between treatments in real-time RT-PCR[21]. The method is based on the relative expression of a target gene versus that of a reference gene.

2.8 Statistical Evaluation

The data from individual groups were presented as the mean ± standard error of the mean (n = 6). Statistical significance of the mean value of different parameters (superoxide dismutase, catalase and glutathione peroxidase) in liver, kidney, brain tissues for different concentrations (control, aldrin 3 mg/L and 6 mg/L) were determined by one-way analysis of variance (ANOVA). The analysis was repeated for each parameter and for each tissue separately. A probability of less than or equal to 0.05 was considered to be significant.

3. Results

Present study demonstrates significant alterations in expression of antioxidant enzymes in zebra fish due to exposure of sub-lethal concentrations of aldrin. The effects of aldrin at two concentrations of 3 mg/L and 6 mg/L on superoxide dismutase, catalase and glutathione peroxidase activities were observed in liver, kidney and brain tissues of zebrafish at 24 hours exposure.

3.1 Superoxide Dismutase (SOD)

Superoxide Dismutase (SOD) activity due to aldrin exposure is shown in Figure 1a (expressed as Unit inhibition per minute per milligrams of protein), Figure 1b (Ct values) and Figure 1c (expressed as Fold change relative to β-actin {housekeeping/reference} gene). Exposure to aldrin at 3 mg/L showed significant elevation ($p<0.05$) in superoxide dismutase activity in kidney and brain while at 6 mg/L, the activity was depleted as compared to control group. In liver, significant depletion ($p<0.01$) in superoxide dismutase activity was observed at 3 mg/L and 6 mg/L concentrations of aldrin exposure when compared to control (Figure 1a). Likewise, significant down-regulation in expression of superoxide dismutase gene in liver was observed at both concentrations. The alterations were more prominent at high concentration of aldrin (6 mg/L). In kidney and brain tissues, the expression of superoxide dismutase gene was up-regulated at 3 mg/L while it shows down-regulation at 6 mg/L in compare to control (Figure 1c).

3.2 Catalase (CAT)

Catalase (CAT) activity on exposure of aldrin at concentration of 3 mg/L and 6 mg/L in liver, kidney and brain tissue is shown in Figure 2a (expressed as micromoles CAT per minute per milligrams of protein), Figure 2b (Ct values) and Figure 2c (expressed as fold change relative to β-actin {housekeeping/reference} gene). Exposure to aldrin at 3 mg/L concentration showed depletion in catalase activity while at 6 mg/L, significant elevation ($p<0.005$) was observed in liver, kidney and brain (Figure 2a). The expression of catalase gene was observed to be down-regulated in liver at both concentrations of aldrin. The mRNA level in kidney was up-regulated at 3 mg/L concentration but down-regulated at concentration of 6 mg/L. In brain, up-regulation catalase expression was observed at both concentrations (Figure 2c).

3.3 Glutathione Peroxidase (GPx)

Glutathione Peroxidase (GPx) activity of aldrin exposure at 3 mg/L and 6 mg/L in liver, kidney and brain tissue is shown in Figure 3a (expressed as micromoles GSH consumed per minute per milligrams of protein), Figure 3b (Ct values) and Figure 3c (expressed as fold change relative to β-actin {housekeeping/reference} gene). Zebra fish exposed to aldrin at 3 mg/L concentration, does not show significant alteration in glutathione peroxidase activity in liver while at 6 mg/L, the activity was significantly depleted ($p < 0.05$) as compared to control group. The activity of glutathione peroxidase in kidney showed significant depletion ($p < 0.01$) at 3 mg/L while significant elevation ($p < 0.05$) was observed at 6 mg/L. In brain, the activity was elevated at both concentrations, which were more prominent ($p < 0.005$) at 6 mg/L (Figure 3a). Transcription of glutathione peroxidase gene was up-regulated in all examined tissue at both concentrations. Though, it was significantly promoted in kidney and brain of zebra fish exposed at 3 mg/L concentration (Figure 3c) when compared to 6 mg/L exposed group.

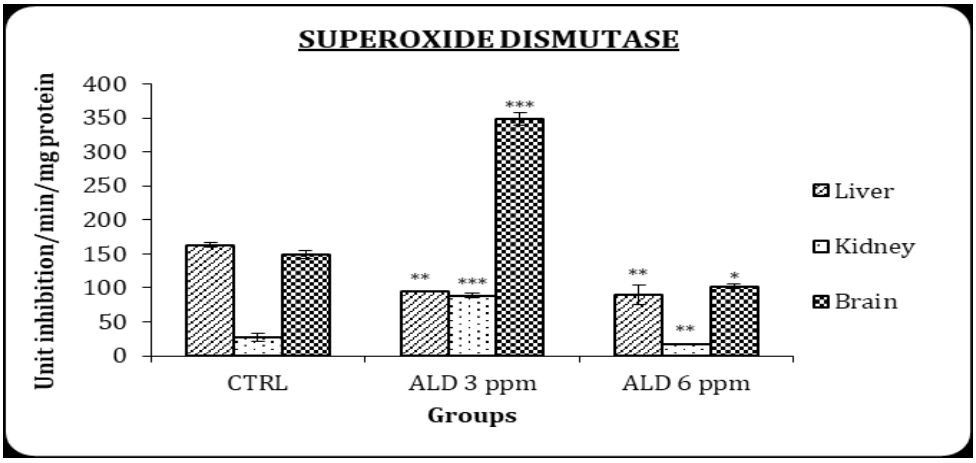


Figure 1a.

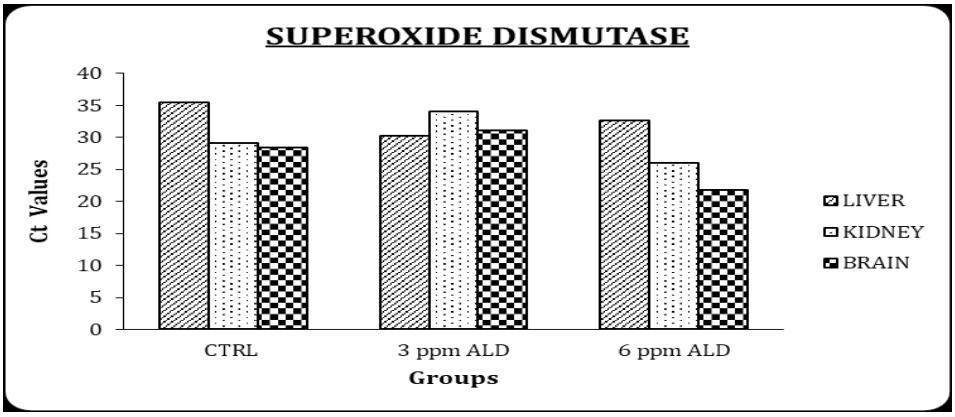


Figure 1b.

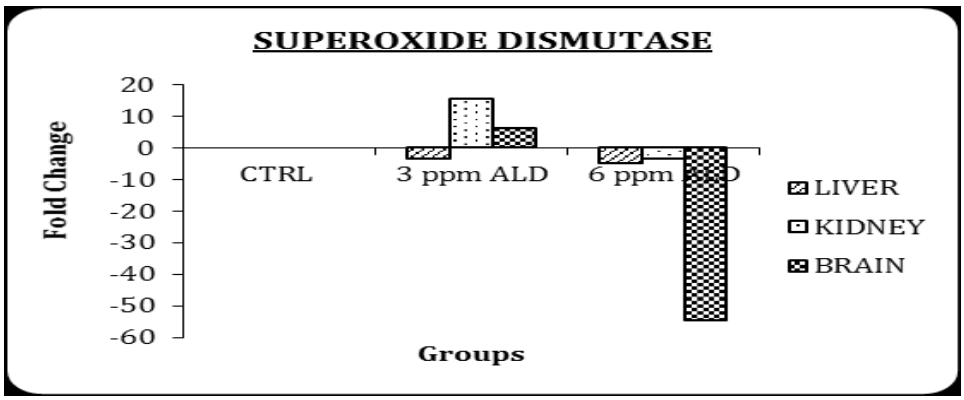


Figure 1c.

Figure 1: Effect of different concentrations of Aldrin (ALD) 3 mg/L (3 ppm) and 6 mg/L (6 ppm) groups after 24 hours exposure on Superoxide Dismutase (SOD): Figures 1a, 1b and 1c; in liver, kidney and brain tissue of zebrafish in compare to Control (CTRL); where a: Biochemical Alterations b: Ct Values C: Gene Expression.

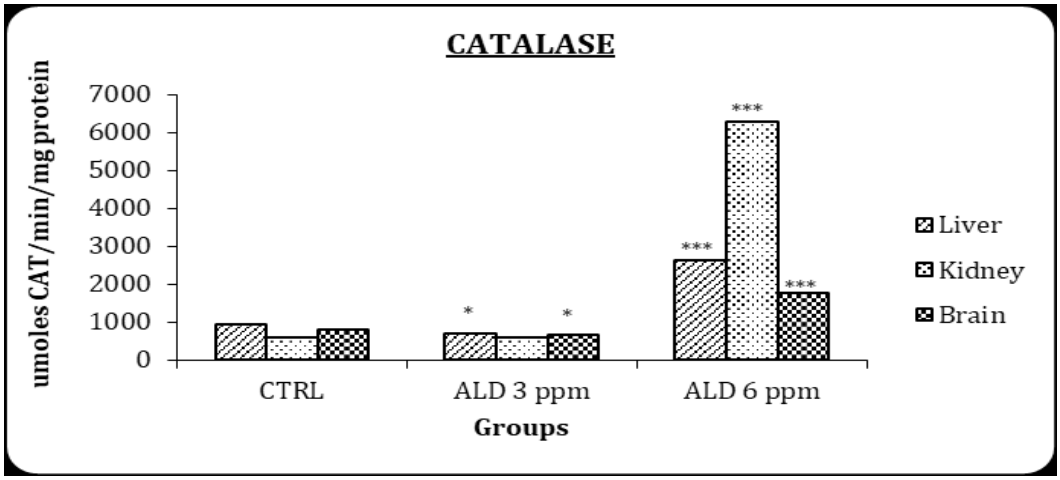


Figure 2a.

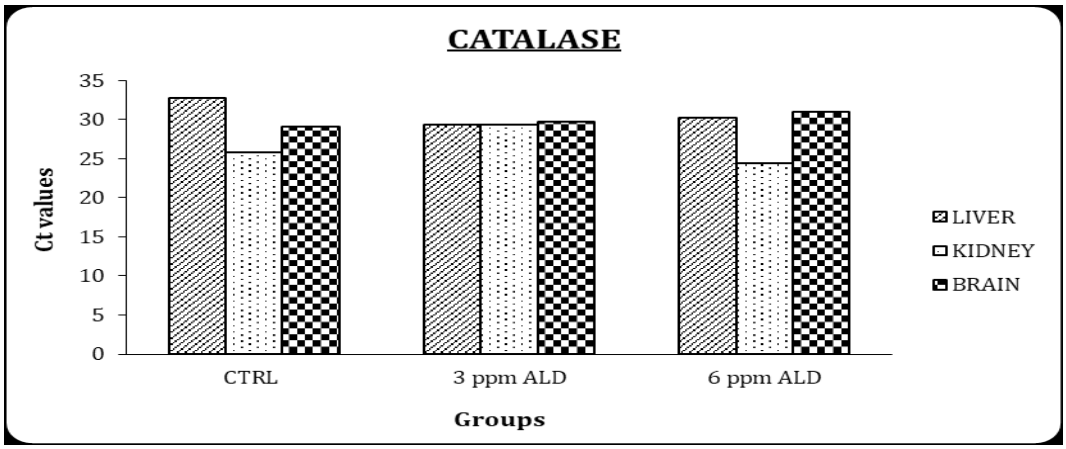


Figure 2b.

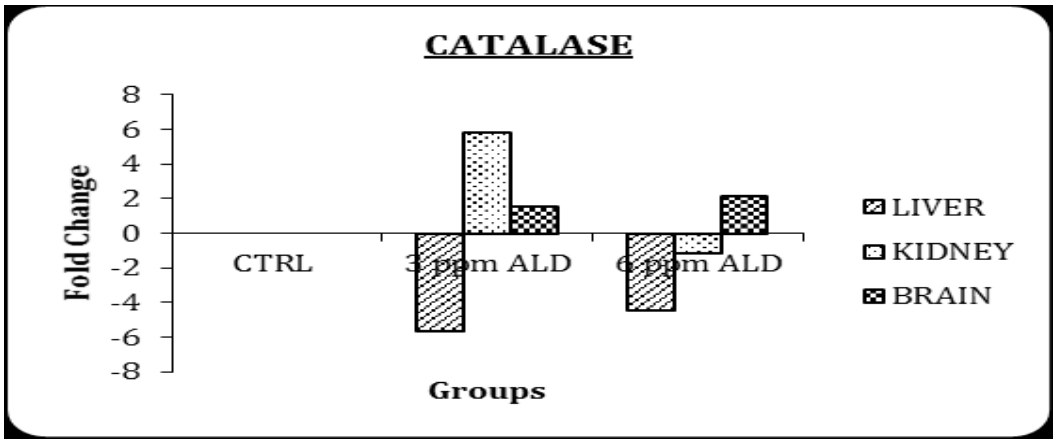


Figure 2c.

Figure 2: Effect of different concentrations of Aldrin (ALD) 3 mg/L (3 ppm) and 6 mg/L (6 ppm) groups after 24 hours exposure on Catalase (CAT): Figures 2a, 2b and 2c; In liver, kidney and brain tissue of zebrafish in compare to Control (CTRL); where a: Biochemical Alterations b: Ct Values C: Gene Expression.

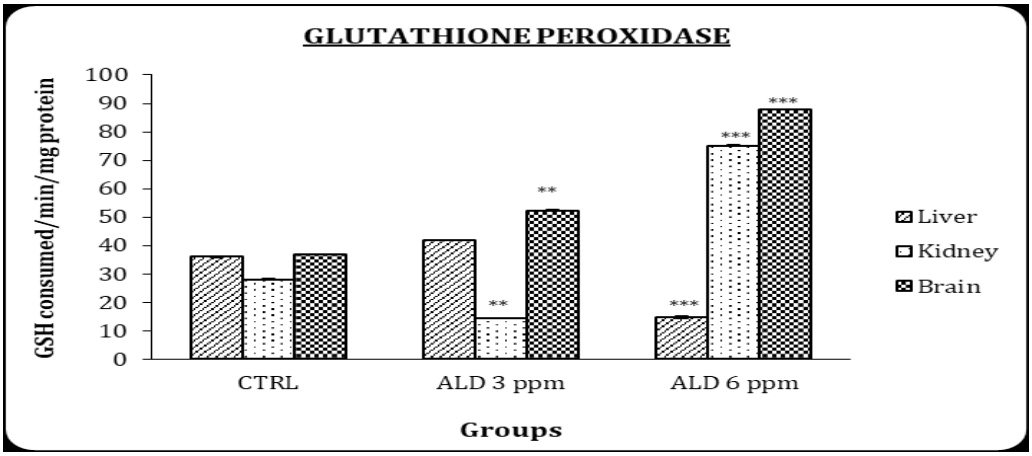


Figure 3a.

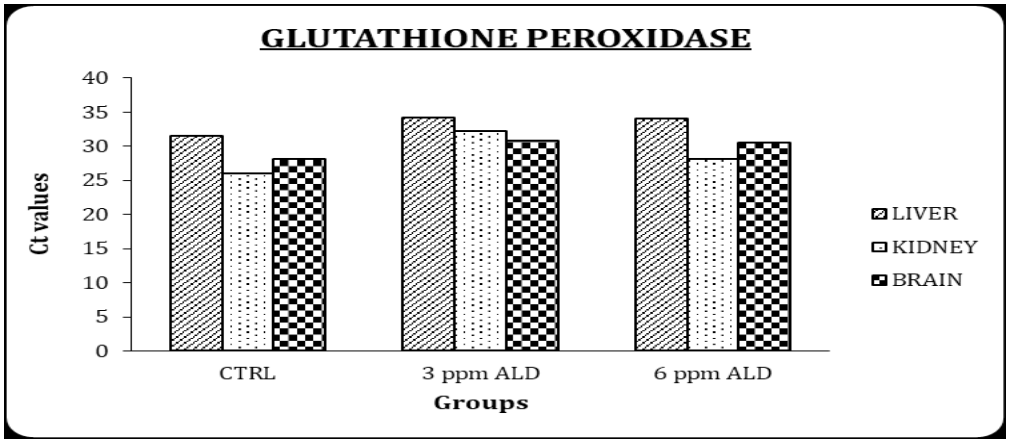


Figure 3b.

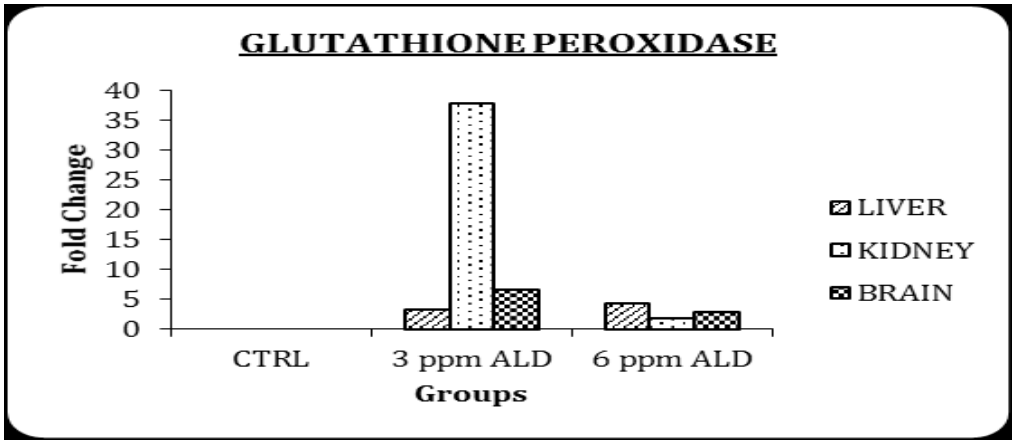


Figure 3c.

Figure 3: Effect of different concentrations of Aldrin (ALD) 3 mg/L (3 ppm) and 6 mg/L (6 ppm) groups after 24 hours exposure on Glutathione Peroxidase (GPx): Figures 3a, 3b and 3c in liver, kidney and brain tissue of zebrafish in compare to Control (CTRL); where a: Biochemical Alterations b: Ct Values C: Gene Expression.

4. Discussion

Antioxidant enzymes activities are altered by pesticides exposure; it may be either stimulated or inhibited depending upon concentration, intensity ~~of~~ or duration of exposure and susceptibility of animal model[22]. It is well known that environmental pollutants can induce reactive oxygen species in organisms[11] and aldrin, an organochlorine pesticide causes toxicity in same manner[1,2]. An increase in level of reactive oxygen species viz. superoxide radicals and hydroxyl radicals can cause oxidative damage to lipids, proteins, carbohydrates and nucleic acids by disrupting the cell membrane and altering the membrane bound enzymes.

Due to the inhibitory effects on oxyradical formation, the SOD-CAT system provides the first line defense against prenatal oxygen toxicity and usually used as a biomarker indicating reactive oxygen species production. Increase in these enzyme activities are probably a response towards increased reactive oxygen species generation in pesticide toxicity[23]. This is also documented by Shao et al., who reported simultaneous response in superoxide dismutase and catalase activities in zebra fish exposed to endosulphan[23]. Therefore, examining the alterations in activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase is considered as an effective method of denoting oxidative stress. The differential expression of the genes encoding these enzymes has been used to detect biological toxicity and/or to monitor the impact of chemical pollutants[12,24]. The alteration of superoxide dismutase, catalase and glutathione peroxidase mRNA gene expression, at two sub-lethal concentrations of aldrin could be explained by enhanced oxidative injury occurred in the zebra fish tissue as shown by altered biochemical parameters. Laboratory studies have also demonstrated that aldrin can induce oxidative stress in animals[1,8].

Dieldrin induces superoxide radicals serve as marker to initiate apoptosis[25]. In present study, superoxide dismutase activity was induced in kidney and brain at low concentration. The increased superoxide dismutase activity indicates an elevated antioxidant status that attempting to neutralize the reactive oxygen species. These results are in agreement with Banerjee et al., that the exposure to pesticides can elicit pro-oxidant conditions that trigger adaptive responses such as increases in the activity of the antioxidant enzymes[22]. Similar to the findings of Slotkin and Seidler our findings show that the elevation of SOD activity at lower concentration may possibly a shift towards detoxification process in aldrin exposed condition[12].

In 6 mg/L, contradictory to aldrin exposure at 3 mg/L concentration, the activity and expression of superoxide dismutase decreased significantly which may represents the severity of aldrin toxicity in a concentration dependent manner as Shao et al.,[23] have also reported endosulphan induces reactive oxygen species and inhibited superoxide dismutase activity in zebrafish in a similar fashion. This phenomenon can be explained that aldrin induced surplus reactive oxygen species exhausted cellular antioxidant defense system, hence prevent repairing/detoxification mechanism. Kim et al., has reported that superoxide dismutase enzyme inhibition might be due to oxidation of cysteine by hydrogen peroxide radicals triggered by increased transformation of superoxide radicals to hydrogen peroxide[26]. Interestingly, higher concentration of aldrin (6 mg/L) showed the down-regulation of superoxide dismutase gene in kidney and brain tissue. It indicates the reverse action that aldrin cause tolerance at optimum concentration[27,28]. The positive correlation of elevated SOD and up regulation of gene get supported by Hansen et al., as it is reported that in case of environmental toxicant there is positive correlation between enzyme activity and gene expression pattern.

Catalase is a hemoprotein with four atoms of iron per molecule attached to the protein. Catalase is an enzyme responsible for hydrogen peroxide detoxification. The induction of catalase activity at 6 mg/L might be associated with increase in production of hydrogen peroxide due to depletion of activity at 3 mg/L. The results of catalase were in accordance to reported study showing effect of organochlorines on fish species in artificial reservoirs[23]. The suppressed activity indicates inefficient scavenging of hydrogen peroxide due to deactivation of enzyme.

Induction of catalase mRNA expression by organic pollutants has been widely reported[23,24]. However, suppression of catalase gene due to oxidative stress is also known[29]. Similarly, catalase transcription was inhibited by aldrin in liver at both concentrations (Figure 2c). The resemblance at biochemical level and expression of gene was observed in liver at 3 mg/L and brain at 6 mg/L exposure. However, catalase transcription was down-regulated while its activity was elevated in liver and kidney tissue at 6 mg/L. Opposite results are seen with kidney SOD activity and mRNA expression. Pollutant induced catalase activity and changes in catalase gene

expression have been investigated in the brown trout[30] and no correlation between the two indices was found. Such inconsistent trends in transcript expression of a gene and catalytic activity were also observed by Karacca et al.,[31]. Down-regulated expression of catalase gene might be due to transcriptional inhibitors in the system or by inconsistent correlation between gene expression and enzyme activity[2]. Results indicate a negative correlation of biochemical assay and gene expression between catalase and superoxide dismutase at both concentrations in kidney and brain. In liver, at 6 mg/L the same correlation was observed which has not been observed at 3 mg/L (Figures 1a, 2a).

Glutathione peroxidase acts as a defense system against hydrogen peroxide. The activity of glutathione peroxidase is expressed in terms of GSH consumed, which utilize reduced GSH as substrate for scavenging the hydrogen peroxide molecule and to convert into water molecule. Glutathione peroxidase activity shows elevation in kidney and brain at higher concentration. Increase in glutathione peroxidase enzyme activity as a result of environmental pollutants exposure have been investigated in different tissues of several fish[22–24], revealing that glutathione peroxidase activity varies considerably between fish species and tissues. The suppression indicated deficiency of glutathione substrate or impairment of activity due to increase production of reactive oxygen species as same has also been reported by Sakuragui et al.,[24]. Findings of glutathione peroxidase were in accordance with Bachowski et al.,[8]. Hansen et al., investigated changes in glutathione peroxidase activity and glutathione peroxidase gene expression in brown trout liver and found a correlation between the two indices[30]. The gene expression findings showed similarity with biochemical parameter in liver at 3 mg/L, in kidney at 6 mg/L and in brain tissue at both concentrations.

In our study, glutathione peroxidase expression was induced by aldrin in all the three tissues, but enzyme activity was inhibited in kidney at 3 mg/L and liver at 6 mg/L. Ahmad et al., reported a low activity of glutathione peroxidase in different fish tissues after exposure to paper mill effluent, indicating an inefficiency of these organs to neutralize the peroxide impacts[32]. A similar suppression in glutathione peroxidase activity in rat liver was reported after 90 days of exposure to lindane, an organochlorine pesticide[33]. A reduced glutathione peroxidase activity could indicate that its antioxidant capacity was surpassed by the amount of hydroperoxide products generated due to lipid peroxidation[22]. Up-regulation of GPx gene and decreased enzyme activity in kidney tissues at 3 ppm concentration shows that the mRNA induction patterns were not in accordance with those of antioxidant enzyme changes. This also get support by Jin et al., who reported that same in case of environmental toxicant i.e. atrazine[34].

The biochemical results showed elevation of catalase and glutathione peroxidase indicating positive correlation in all tissue exposed at 6 mg/L except the glutathione peroxidase in liver tissue (Figures 1a; 2a; 3a). Results empowered the positive correlation of up-regulation among all three enzymes expressed in kidney and brain tissue at 3 mg/L concentration. However, the expression of glutathione peroxidase showed negative correlation to superoxide dismutase and catalase in liver tissue at both concentrations and brain at 6 mg/L concentration (Figures 1c; 2c; 3c).

In all, although aldrin is a known neurotoxic compound[3], however present study results indicate significant alterations in liver and kidney along with brain, as Liu et al., reported that acute kidney injury is related to anatomic lesion and functional disturbances of brain[35]. Ikeda et al., have reported that in damage pathological brain condition, the histological alterations also occurred in myocardium and liver along-with renal tubular injury and all degrees of asphyxial damage[36]. Therefore, alteration of these examined parameters indicated the presence of an elevated level of reactive oxygen species, suggesting that resulted for the onset of oxidative stress related processes were involved in the due to aldrin induced in vivo toxicity. Thus, from the findings it can be concluded that aldrin altered activity and expression of superoxide dismutase, catalase and glutathione peroxidase depend upon the concentration at which animal is exposed, which may finally cause physiological alterations leading to disease or ill health condition.

5. Acknowledgments

The authors are grateful to Director General, Gujarat Forensic Sciences University for overall support. Ms. Reena C. Jhamtani is grateful to Department of Science and Technology, New Delhi, India for providing DST-INSPIRE Junior and Senior Research fellowships (IF140059).

6. Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not–for-profit sectors.

7. Declaration of Conflict of Interest

The authors declare there is no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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