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DNA Damage and Repair in different Tissues of Fresh Water Fish, *Channa punctata* after Acute and Subchronic Exposure to bisphenol A

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Abstract

The present study was conducted to investigate the genotoxic effect of Bis-Phenol A (BPA) after acute and subchronic exposure in different tissues of *Channa punctata*. The recovery in DNA damage was also ascertained after 30 days of cessation of exposure. Fish were exposed to different sublethal concentrations of BPA along with two controls i.e., with positive (acetone) and negative (water) controls for 96h (acute exposure) and 60 days (subchronic exposure) and after that fish were allowed to recover for 30 days in freshwater. The blood, liver, and gill tissue samples were collected at 24, 48, 72 and 96h for acute exposure and after 20, 40, and 60 days post-exposure for subchronic exposure. Exposed groups showed significantly higher DNA damage in both acute and subchronic exposure as compared to control groups. In the case of acute exposure, the highest damage was observed at 24 h of exposure followed by a decline in the value of all the parameters, while in the later hours of exposure these values further increased. On the other hand, in the case of sub-chronic exposure, the highest damage was observed after 60 days of exposure. Recovery experiment showed a decrease in the values of all the parameters studied. The result of the study clearly showed that BPA caused DNA damage in *Channa punctata* after acute as well as subchronic exposure.

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1. Introduction

Industrial effluents from different industries such as textile mills, chemical manufacturing, pharmaceuticals, plastic industry, paper industry, etc. usually add several contaminants like pesticides/insecticides, heavy metals, flame retardants, plasticizers, etc to the water bodies. Among different chemicals, attention is increasing worldwide towards Endocrine-Disrupting Chemicals (EDCs). Some of the major popular classes of endocrine disruptors are polychlorinated biphenyls, pesticides, synthetic and natural hormones, and bisphenols[1,2]. The human population gets exposed to these chemicals in daily life from various sources like the use of pesticides/herbicides, plastics, flame retardants, industrial and household products, detergents, and ingredients of personal care products[3]. These endocrine disruptors tend to bioaccumulate and thus alter the endocrine system of both humans as well as wild animals and can be hazardous even at very low concentrations[4]. Endocrine-disrupting chemicals being ubiquitous substances are known to interfere with hormone signaling, action, and metabolism, thus interrupting hormone homeostasis and A potent factor in increasing the incidence rate of breast cancer[5,6]. EDCs are potential mimics of some naturally occurring hormones like androgens and estrogens. They exert their toxicity by interfering with hormonal homeostatic mechanisms that are involved in the growth and development of various tissues[7].

Bis-Phenol A (BPA) is an endocrine-disrupting chemical found in polycarbonate plastic products, linings inside food cans, epoxy resins, thermal receipts, and micro/nanoplastics[8-12]. Not only this, but it has also found its importance in the packaging of food items such as baking powder, cereals, and yeast[13]. BPA has been used as an additive in polycarbonate plastics and epoxy resins to harden the products for their usage as water bottles and food storage containers, due to its cross-linking properties[14]. Mileva *et al.*[15] reported that the production of these products can reach up to three million tons annually, on a global scale. According to recent reports by the Environment Protection Agency (EPA) more than one billion pounds of BPA leaks into the environment every year, thus illustrating the extent of potential exposure problems that can be faced[16]. Porreca *et al.*[17] reported that BPA exposure can be associated with an increased risk of carcinogenesis and an increase in the sensitivity of certain cell types. Recently, BPA has also been shown to cause genotoxicity either directly or indirectly in various *in vitro* systems[18].

Therefore, the present study has been conducted to investigate the DNA damaging effects of BPA in different tissues of *Channa punctata* after acute and subchronic exposure. Further repair of the damage induced was also deciphered.

2. Materials and Methods

2.1 Experimental Fish Specimens and Chemicals

Freshwater fish *Channa punctata* (weight 25-30g, length 11-13cm) were obtained from the local market and carried in oxygen-filled plastic bags. They were transported to glass aquaria (dimensions: 60×60×60 cm) topped up with tap water (dechlorinated) with filters and aerators. Prophylactic treatment was done to avoid any dermal infection. Floating fish pellets were fed to the fishes. Fishes were acclimatized for 20-22 days. Water was changed on alternate days and any kind of debris was removed daily to remove unutilized feed and to reduce ammonia content. Only healthy and active fishes were used for the experiment after acclimatization. BPA used in the present study was obtained from Sigma Aldrich (CAS 80-05-7 and purity 99%). The stock solution of BPA was prepared in acetone as BPA is less soluble in water. For this 1 mg of BPA was dissolved in 10 µl of acetone.

2.2 *In Vivo* Experiment

After calculating the 96-hour LC_{50} value[19], ¼ LC 50 (1.91mg/l) and ½ LC 50 (3.81mg/l) for acute exposure and 1/10th LC 50 (0.76 mg/l) for subchronic exposure was used for the *in vivo* experiment. Experiment was divided into 3 groups: two control groups (positive and negative) and one exposed group. Positive control was treated with acetone and negative control contained only water. For acute exposure, sampling was done after 24, 48, 72, and 96h and for subchronic exposure; sampling was done after 20, 40, and 60 days of exposure. The experiment was performed in triplicates. To study the repair potential of fish, the exposed fish were kept in fresh water for 30 days and recovery was ascertained using the same parameters.

2.3 Single-Cell Gel Electrophoresis (COMET Assay)

The alkaline Single-Cell Gel Electrophoresis (SCGE) was carried out using the method of Ahuja and Saran[20] with some modifications. 10µl of blood (from the heart) was immediately diluted in 1 ml of Phosphate Buffer Saline (PBS). Liver and gill tissues were homogenized in PBS followed by centrifugation at 3000 rpm for 10 minutes. Microscope slides were coated with 1% of Normal Melting Point Agarose (NMPA), and then the slides were incubated at 37˚C, overnight. 0.5% of Low Melting Point Agarose (LMPA) mixed with 20µl of blood was added to the coated slides. The slides were then kept in the refrigerator at 4˚C for 10-15 minutes. Third layer of 0.5% LMPA was poured on the slides and returned to the refrigerator at 4˚C for another 10 minutes. Slides were then placed in lysis buffer for about 3h in the refrigerator. After the lysing step the slides were incubated in electrophoretic buffer followed by electrophoresis for 20 minutes at 300 mA and 24 V. Neutralization buffer was used to neutralize the slides for 15 minutes. Slides were kept for drying. Ethidium bromide was used to stain the slides. Blotting paper was used to remove an excess of stain, Fluorescent microscope with 40X magnification, excitation filter of 515-560 nm, 590 nm barrier filters were used for scoring of slides. CASP LAB image analysis software was used for examining the comets.

2.4 Statistical Analysis

Statistical analysis was performed using a software program (SPSS 16.0 for windows). All data were presented as mean ± SE. One-way ANOVA followed by Posthoc Tukey's test was used to assess the significance of the difference between control and treatment groups. Student t-test was applied to ascertain the significance of the difference between 60 days exposure and 30-day recovery groups. P values less or equal to 0.05 were considered statistically significant.

3. Results

The DNA damaging effect of different sub lethal concentrations of BPA after acute and subchronic exposure in different tissues of *C. punctata* are given in Figures 1-6. Also, the recovery of DNA damage induced after subchronic exposure of 60 days was assessed (Figure 7). The DNA damaging effect of different concentrations of BPA (1.91 mg/l and 3.81 mg/l) at different hours of

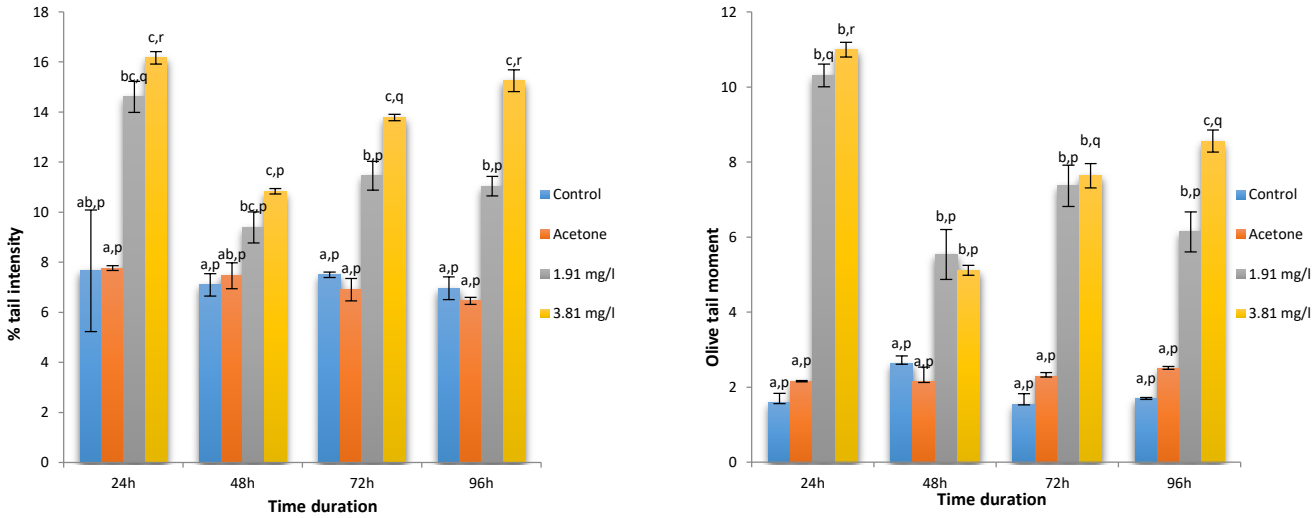


Figure 1. Effect of BPA on: **(a)** tail intensity, and **(b)** Olive Tail Moment (OTM) in blood under different treatment at different durations of exposure. Error bars represent Standard Errors (SE). Different letters a,b,c, signify ($p<0.05$) the effect of treatment at the same time interval and p,q,r signify the effect of duration of exposure.

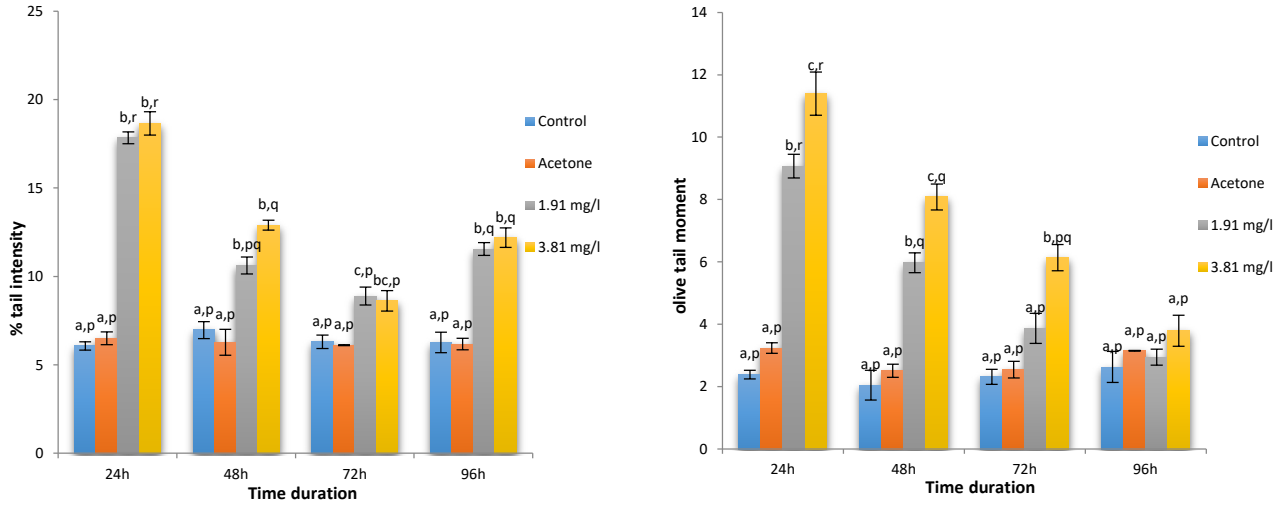


Figure 2. Effect of BPA on: **(a)** tail intensity, and **(b)** Olive Tail Moment (OTM) in liver under different treatment at different durations of exposure. Error bars represent Standard Errors (SE). Different letters a,b,c, signify ($p<0.05$) the effect of treatment at the same time interval and p,q,r signify the effect of duration of exposure.

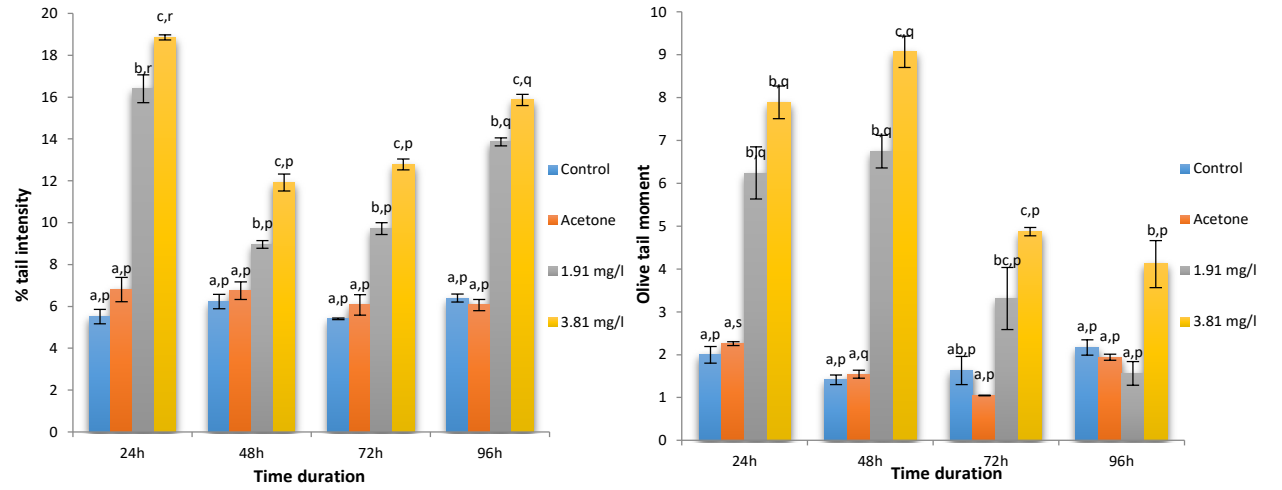


Figure 3. Effect of BPA on: **(a)** tail intensity, and **(b)** Olive Tail Moment (OTM) in gillunder different treatment at different durations of exposure. Error bars represent Standard Errors (SE). Different letters a,b,c, signify ($p<0.05$) the effect of treatment at the same time interval and p,q,r signify the effect of duration of exposure.

exposure in the blood, liver and gill cells of *C. punctata* are shown in Figures 1-3. In case of blood cells the maximum damage was observed at 24 h of exposure with the highest concentration of BPA (3.81 mg/l) where % tail intensity increases 2.11 times and Olive Tail Moment (OTM) increases 6.99times as compared to control followed by a decrease at 48 h (Figure 1). Further at 72 and 96 h of exposure increase in the values was observed. The effect of duration of exposure was also found to be significant (One way ANOVA, $p<0.05$). Similar results

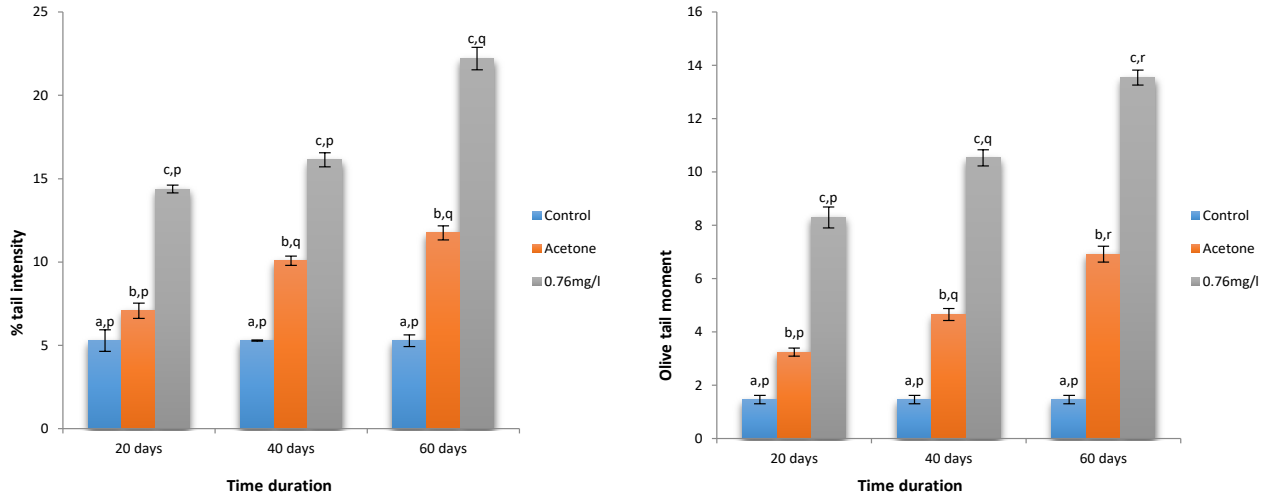


Figure 4. Effect of BPA on: **(a)** tail intensity, and **(b)** Olive Tail Moment (OTM) in blood under different treatment at different durations of exposure. Error bars represent Standard Errors (SE). Different letters a,b,c, signify ($p<0.05$) the effect of treatment at the same time interval and p,q,r signify the effect of duration of exposure.

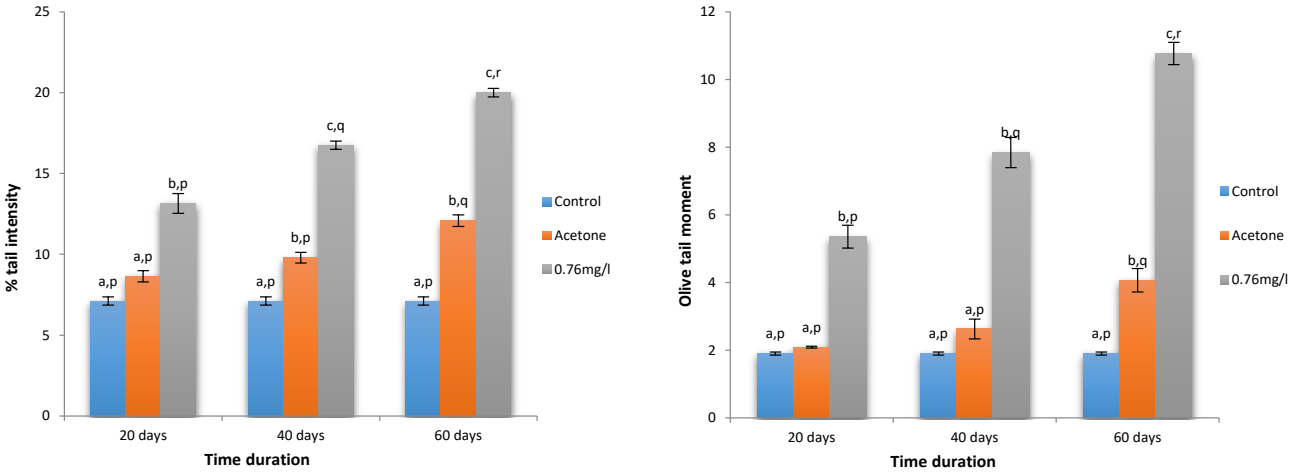


Figure 5. Effect of BPA on: **(a)** tail intensity, and **(b)** Olive Tail Moment (OTM) in liver under different treatment at different durations of exposure. Error bars represent Standard Errors (SE). Different letters a,b,c, signify ($p<0.05$) the effect of treatment at the same time interval and p,q,r signify the effect of duration of exposure

were observed in liver also (Figure 2). Maximum values of both the parameters were observed after 24 h of exposure which further decreased up to 72 h and then slightly increased after 96 h of exposure except for OTM which showed further decrease at 96 h. Overall the effect of duration of exposure was also found to be significant for both treatment groups ($p<0.05$). In case of gill cells, for %tail intensity the maximum effect was observed at 24 h of exposure followed by a decrease at 48 h while for the olive tail moment the maximum effect was found at 48 h of exposure which further decreased at 72 and 96 h (Tukey's test) (Figure 3). The effect of duration of exposure

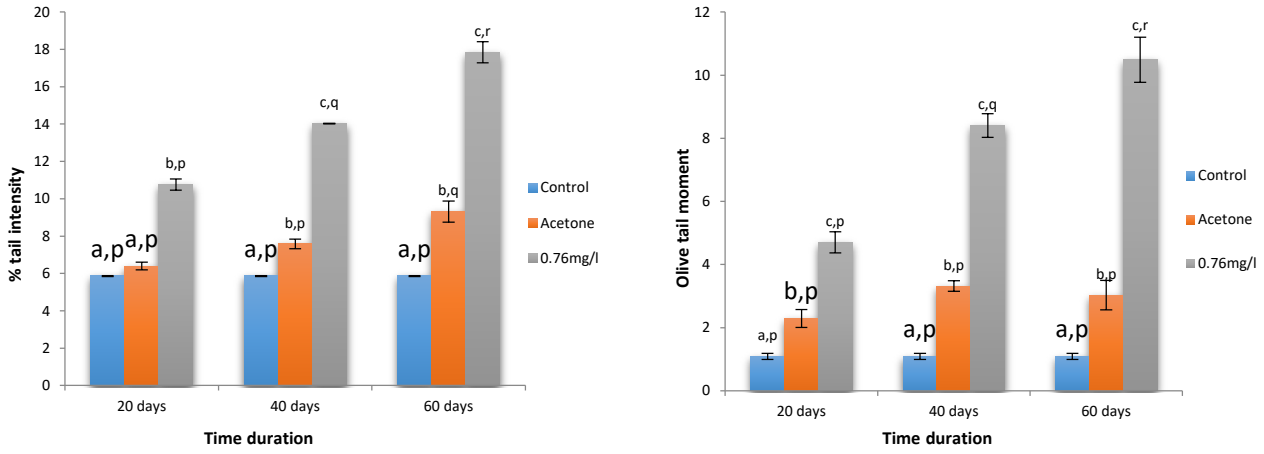


Figure 6. Effect of BPA on: **(a)** tail intensity **(b)** Olive Tail Moment (OTM) in gillunder different treatment at different durations of exposure. Error bars represent Standard Errors (SE). Different letters a,b,c, signify ($p<0.05$) the effect of treatment at the same time interval and p,q,r signify the effect of duration of exposure.

is found to be significant and reveals the significant changes in DNA damage with increase in the duration of exposure (ANOVA).

Perusal of Figures 4-6 revealed the effect of 0.76 mg/l concentration of BPA on different parameters of comet assay in blood, liver and gill cells of *C. punctata*. The values of both the parameters (% TI and OTM) increased significantly after treatment with BPA as compared to both control groups after different durations of exposure. In blood cells, % tail intensity increased from 5.28±0.35 (negative control) to 14.38±0.23 and olive tail moment 1.46±0.16 (negative control) to 8.29±0.39 after 20 days of exposure. Further increase in the values of all the parameters was observed up to 60 days (Figure 4). In case

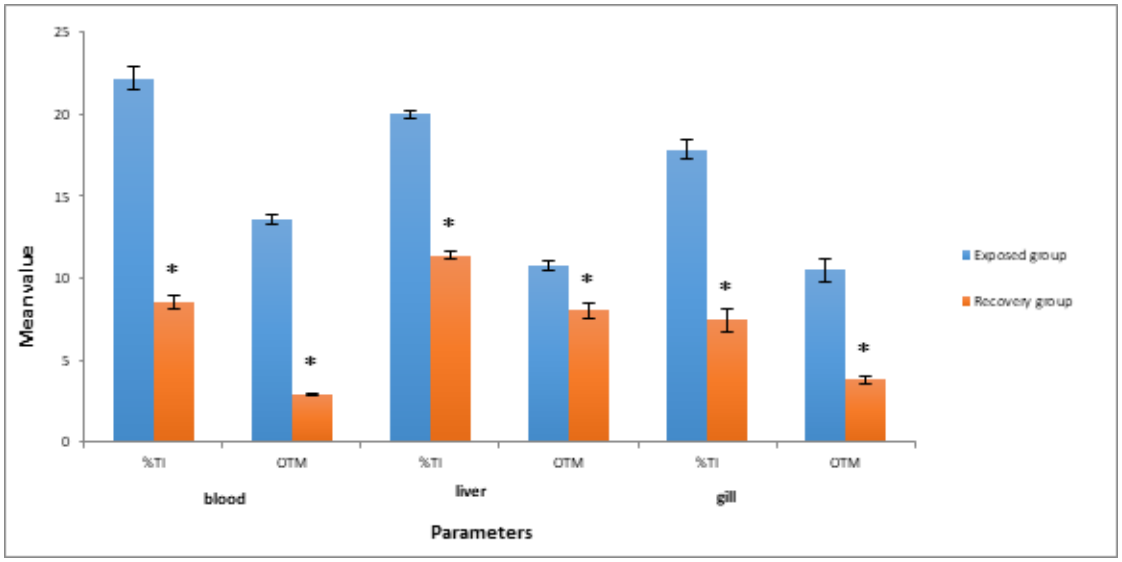


Figure 7. Recovery in different comet parameters after 30 days in blood, liver and gill cells. The values are represented as mean ± SE. * significantly different ($p<0.05$) as compared to exposed group (student t-test).

of liver cells, % tail intensity increased from 5.25±0.08 (negative control) to 17.85±0.58, and olive tail moment from 1.04±0.01 (negative control) to 10.49±0.71 (Figure 5). Whereas in gill cells tail intensity increased 1.85 folds and olive tail moment increased 2.83 folds after 60 days of exposure (Figure 6). Analysis of variance showed significant increase in all the parameters with the time of exposure. The maximum effect was observed after 60 days of exposure.

The results of the recovery test showed the values of both the parameters return to normal level after the recovery period, thus *C. punctata* has great potential to overcome stress conditions. After 30 days of recovery, a significant reduction in the level of different comet parameters in blood, liver, and gill tissue was observed as compared to 60 days exposure group (Figure 7). In blood cells values of % TI and OTM decreased from 22.21±0.68 and 13.54±0.28 to 8.49±0.42 and 2.86±0.07 respectively. Liver cells showed 41.62% and 36.03% reduction in values of % TI and OTM respectively while in gill cells the values of % TI and OTM showed 56.77% and 46.56% reduction respectively after 30 days of the recovery period as compared to 60 days exposure group.

4. Discussion

The comet assay or the Single-Cell Gel Electrophoresis (SCGE) technique is a method for measuring DNA strand breaks in individual cells and is used to evaluate genotoxicity, DNA repair, and genome instability. The SCGE is a well-established assay for the assessment and estimation of DNA damage both *in vitro* and *in vivo*, at the individual cell level due to its capability of detecting very low-level oxidative damage induced in DNA[21,22]. Percent tail intensity (% TI) and olive Tail Moment (TM) were considered as parameters for assessing the genetic damage. Significant increase in the values of both the parameters was seen in the case of blood, liver and gill cells of treated groups as compared to both control groups (Tukey's test).

Different researchers observed an increase in DNA damage in different fish species after exposure to various organic compounds[23-26] but the DNA damaging potential of BPA has been mainly investigated *in vitro* studies. Ramos *et al.*[27] studied the effect of Bis-Phenol A (BPA) on Hep-2 and MRC-5 cell lines and found that BPA induced an increase in DNA damage in the Hep-2 cell line and oxidative damage in the MRC-5 cell line. Xin *et al.*[28] reported that BPA caused a significant increase in DNA strand breaks in INS-1 cells. Kose *et al.*[29] observed that RWPE-1 cells exposed to BPA, BPS, and Bis-Phenol F (BPF) showed significantly higher levels of DNA damage as compared to control. Eid *et al.*[30] found a significant increase in DNA damage in the hepatic tissue of female rat offspring exposed to BPA. Wu *et al.*[31] found a significant increase in comet parameters in pachytene spermatocytes of rats exposed to BPA. Anet *et al.*[32] observed significantly increased tail length and % tail intensity in *Drosophila melanogaster* after treatment with BPA.

In the present study, the highest effect was seen at 24h of exposure followed by a drop in the value but at later hours of exposure value again increases. The decrease may be due to the repair of damaged DNA or replacement of highly damaged cells or both[33,34]. Another reason may be gene activation like cytochrome p450 which activates the metabolizing enzymes and provides a defensive mechanism against genotoxicants[35]. It may be due to the removal of apoptotic cells with time, which contribute to the comet in the early stages. Similar results were found by Cavalcante *et al.*[36] who tested blood and gill cells of fish and found high damage at 6h of exposure, but at 24h blood cells showed decreased damage and in contrast to this gill cells showed high damage at 24h. Further Gulsoy *et al.*[37] reported the highest DNA damage at 24 h of exposure in zebra fish (*D. rerio*) treated with borax, and a decrease in values was observed at 48h and 72h and 96 h again the values increased. The effect of concentration was also found to be significant. The highest DNA damaging effect was observed with the highest concentration. Concentration-dependent DNA damage was found in human SK-N-MC cells treated with BDE-47 and BDE-209[38]. In a study, Wang *et al.*[39] Cd_{2+}, Hg_{2+} exposed THP-1 cell lines to BDE-47, HBCD, and TBBPA and observed a significant increase in % tail DNA with increasing exposure concentration. Park and Choi[40] studied the effect of nonylphenol and bisphenol A on *Daphnia magna* and *Chironomus riparius* and found a concentration-dependent increase in OTM in both nonylphenol and BPA exposed group. Chen *et al.*[41] found that the liver of Chinese rare minnows exposed to Tris (1,3-dichloro-2-propyl) phosphate resulted in a

significant increase in DNA damage in a concentration-dependent manner.

In present study, DNA damage might be due to the overproduction of free radicals related to oxidative stress. The underlying mechanism of BPA-induced genotoxicity is not very clear but it has been mainly associated with oxidative stress via Reactive Oxygen Species (ROS) and lipid peroxidation[28,42] which may cause direct DNA abnormalities[43]. DNA damage in different tissues of organisms induced by the generation of free radicals and oxidative stress has been investigated by different researchers[26,44-48].

The results of the recovery test showed the values of all the parameters return to normal level after the recovery period, thus *C. punctata* has great potential to overcome stress conditions. After 30 days of recovery, a significant reduction in the level of different comet parameters in blood, liver and gill tissue was observed as compared to 60 days exposure group (Figure 7). The decrease in the value of all the parameters was found to be significant ($p<0.05$) after 30 days recovery period (Student's t-test). Similar results were observed by Sharma and Chadha[49] in fish *C. punctata* exposed to 4-nonylphenol and a reduction in DNA damage in the liver, kidney, and gill tissue was reported after 30 days of the recovery period. This may be due to the DNA repair system which may lead to the elimination of DNA lesions. Guilherme *et al.*[50] reported that blood cells of *Anguilla anguilla* showed evident recovery at 14 days after exposure to herbicide based pesticide Roundup. The results of the recovery test showed that values of both the parameters return to normal level after the recovery period, thus *C. punctata* has great potential to overcome stress conditions. Recovery is a multifactorial process and it can be determined by the toxicokinetics of genotoxicants[51]. The fish body may metabolize the genotoxicant and release the intermediate products via the kidney and gills. But above all, cessation of exposure leads to suppression of direct genotoxic pressure.

5. Conclusion

The present study thus revealed the ability of bisphenol A to induce DNA damage in different tissues of *Channa punctata* after acute as well as subchronic exposure. It focuses on the use of comet assay in the evaluation of DNA damage and DNA repair. BPA may causeDNA damage due to adduct formation, ROS formation, microtubular disruption activity, and also by impairment of the DNA repair system. A significant recovery in DNA damage after 30 days suggests a great capacity of *C. punctata* to retain its DNA integrity.

6. Conflicts of Interest

The authors have no conflicts of interest.

7. Acknowledgments

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