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Bisphenol-A Induced Genotoxicity in *Channa punctatus* (Bloch)

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ABSTRACT:
Bisphenol A is a high volume, synthetic monomer that has been reported in all environmental matrices. Its structural analogy to Diethylstilbestrol, a high risk estrogen mimic, raises concern about its genotoxic potential. This study investigated the genotoxic potential of Bisphenol-A in the snake headed murrel, Channa punctatus. The effect of three sublethal concentrations of Bisphenol A on C. punctatus was studied for two different durations i.e., 15 days and 30 days. Micronucleus assay of peripheral erythrocytes and comet assay of liver cells were used to estimate the genotoxic potential of Bisphenol-A. An increase in micronucleus frequency was observed in all Bisphenol A exposed groups after both durations. Tail DNA% was significantly higher in liver cells following Bisphenol-A exposure. These results suggest that Bisphenol-A exposure probably causes DNA damage due to double strand breaks. This study highlights the genotoxic potential of Bisphenol A. These results also suggest that micronucleus test and comet assay of C. punctatus tissue can be used as useful tool to estimate the exposure of aquatic fauna to ambient genotoxins.

Keywords: *Genotoxicity, Bisphenol-A, BPA, Micronucleus, Comet, SCGE*

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INTRODUCTION

Bisphenol-A (BPA) is ubiquitous synthetic monomer. It is used in the production of polycarbonate plastics, epoxy resins, dental sealants and thermal printer paper (Seachrist et al., 2016; Yadav, 2021). More than 1 million pounds of BPA are released into the environment every year (Song et al, 2020). Several studies have reported the presence of BPA in air, water, soil and biological fluids like serum, urine, breast milk etc. (Chen et al, 2016). BPA has been linked to various health issues such as diabetes, cancer, obesity, cardiovascular problems, lowered immunity etc. (Canesi and Fabbri, 2015; Jenkins, Wang, Eltoum, Desmond and Lamartiniere, 2011).

BPA is an important endocrine disrupting chemical. BPA lacks the characteristic steroid rings of 17β Estradiol but the span of the outer hydroxyl groups of BPA bestow it with estrogen like properties, allowing it to fit into estrogen receptor (ER) pocket (Seachrist et al., 2016; Kuiper et al, 1998). In addition to this BPA can act as a thyroid hormone antagonist and an antiandrogenic agent (Vandenberg, Maffini, Sonnenschein, Rubin and Soto, 2009).

Structurally, BPA is analogous to Diethylstilbestrol (DES), another estrogen mimic that poses serious health risks. Due to its proven teratogenicity, carcinogenicity and

genotoxicity, the use of DES has been banned worldwide (Naik and Vijayalaxmi 2009). This raises serious concern about the adverse effects of BPA. Though several studies have investigated BPA's genotoxic potential *in vitro* and *in vivo*, many of these have contradictory results (Audebert, Dolo, Perdu, Cravedi and Zalko, 2011; Xin et al., 2015). While some studies have not reported any mutagenic potential of BPA (Chen, Ike and Fujita, 2002; Fic, Sollner Dolenc, Filipič and Peterlin Mašić, 2013) others have reported a significant increase in micronucleus (MN) frequency, chromosomal aberrations and DNA damage following BPA exposure (Xin et al., 2015; Tiwari et al., 2012).

Fish are useful models for studying genotoxicity (Al-Sabti and Metcalfe, 1995). *Channa punctatus* is widely used in controlled laboratory settings to evaluate the genotoxic potential of xenobiotics. It has been employed to assess the genotoxic potential of pesticides (Ali et al., 2009), heavy metals (Yadav and Trivedi, 2009) and endocrine disrupting chemicals (Sharma and Chadha, 2017).

Widespread occurrence of BPA, its structural similarity to DES, a potent genotoxic agent and the limited availability of literature pertaining to the genotoxicity of BPA *in vivo* led us to evaluate the genotoxic potential of this xenoestrogen in *Channa punctatus* by employing two different assays- Micronucleus assay and Comet Assay.

METHODS

Fish Procurement and Acclimitization: Healthy specimens of the snake headed murrel, *Channa punctatus* (14 ± 2 cm in length and 25 ± 3 g in weight) were procured from Sumera reservoir, district Aligarh, India and transported to the Toxicology laboratory, D.S. College, Aligarh with utmost care (Jalal, Surendranath, Pathak, Yu and Chung, 2018). The fish were dipped in 0.05% potassium permanganate solution for two minutes to treat dermal infections (Yadav, 2021) and allowed to acclimatize to laboratory conditions for two weeks. Physiochemical parameters of water (Temperature 23-25°C, pH 7.6-7.8, Dissolved oxygen 7.2-7.6mg/L, Conductivity 270-300 µS/cm, Total hardness 175-185 mg/L) were maintained throughout the study in accordance with APHA (1998) guidelines. The fish were fed commercial fish diet at twelve hours interval and natural photoperiod was maintained.

Preparation of BPA stock solution: Bisphenol A (purity > 97%) was purchased from HiMedia Laboratories, Pvt. Ltd. Mumbai, India. A 20% stock solution of BPA was prepared in 100% analytical grade ethyl alcohol (Nangia, 2020a; Nangia, 2020b). Suitable dilutions of the stock solution were used for treatment.

Sublethal Concentrations of BPA: In a previous study from our laboratory the 96 hours LC 50 of BPA to *Channa punctatus* was estimated to be 13.075 mg/L (Nangia, 2020a; Nangia, 2020b). Based on this, three sublethal concentrations of BPA were chosen for the study, namely, 10, 20 and 30 % of 96 hours LC50. These concentrations were 1.31 mg/l, 2.62 mg/L and 3.92 mg/L, respectively.

***In vivo* exposure experiment:** Acclimatized fish were randomly assigned for exposure to the aforementioned sublethal concentrations of BPA for two durations i.e., 15 days and 30 days using a static renewal system. Besides the BPA exposure groups a negative control group (no BPA, no ethyl alcohol) and a solvent control group (ethyl alcohol solvent without BPA) were also maintained for the aforementioned durations. After the desired time period fish were taken out and anaesthetized by immersing in 50 mg/L benzocaine solution (Laird and Oswald, 1975; Yadav and Trivedi, 2009).

Micronucleus Assay: The micronucleus assay was performed on peripheral erythrocytes by the method of Palhares and Grisolia (2002) as described by Ali et al (2009). Briefly, a drop of blood was applied to a clean slide and a smear was prepared. The blood smear was air dried and fixed in methanol for 10 minutes. Thereafter, the slides were left overnight in a dust free chamber. Slides were stained with 6% Giemsa stain for 20 minutes. The slides were viewed using Olympus Magnus MLX microscope and images were captured using Capture Pro 4.6 software. Fenech's (2000) criteria for scoring micronuclei were adopted. Only structures that were clearly demarcated from the nucleus, were of the same colour and intensity and were less than one-third the size of the main nucleus were considered as

micronuclei. The number of fish and cells counted in each treatment group is summarized in Table 1. Micronucleus frequency (MN%) was calculated as:

$$MN\ \% = \frac{Number\ of\ cells\ with\ micronuclei}{Total\ number\ of\ cells\ counted} X\ 100$$

Single Cell Gel Electrophoresis (SCGE) or Comet Assay: Single cell gel electrophoresis (SCGE) was performed on liver cells using the method of Olive (2002) with slight modifications as described by Ahmad and Ahmad (2016). Briefly, the liver was chopped into small pieces. A single cell suspension was obtained and was centrifuged at 3000g at 4°C for 5 minutes. The cell pellet was collected and used for cell viability assay and comet assay. Trypan blue dye exclusion test was used to determine cell viability. Slides were treated with ice cold 0.4 M Tris (pH 7.5), and stainedwith 80 µL ethidium bromide (20 µL./mL). For positive control, cells from control fish were treated with 100 µM H_2O_2 for 10 minutes prior to comet assay (Pandey, Nagpure, Trivedi, Kumar, and Kushwaha, 2011). Image scoring and analysis was conducted using Komet 5.5 image analysis system (Kinetic Imaging, Liverpool UK). An Olympus Fluorescent Microscope (CX41) with integrated CC camera COHU 4910 was used for this purpose. Tail DNA percentage was used as the parameter of choice to estimate DNA damage. Statistical package SPSS (version 16.0, SPSS Inc. Chicago, IL, USA) was used for statistical analysis.

RESULTS

Micronucleus Assay- A dose dependent increase in micronucleus percentage (MN%) was observed after exposure to BPA. This increase was significant at the two higher doses of exposure after both 15 days and 30 days duration. These results have been summarized in table 1 and in figures 1 and 2. Comet Assay-All three BPA exposure groups showed significant increase in tail DNA% of liver cells vis-à-vis control group after both 15 days and 30 days of exposure. These results have been summarized in table no. 2 and in figures 3, 4 and 5.

Table 1: MN% in peripheral blood erythrocytes of *Channa punctatus* after exposure to BPA

Exposure Duration	Treatment Groups	No. of Fish	No. of Cells Observed	MN (%) ± SEM
15 Days	Group I (Control)	5	10109	0.059 ± 0.019^{a}
	Group II (Solvent Control)	5	10099	0.069 ± 0.012^{a}
	Group III (BPA 1.31 mg/L)	5	10089	0.159 ± 0.024^{ab}
	Group IV (BPA 2.62 mg/L)	5	10098	0.287 ± 0.033^{bc}
	Group V (BPA 3.92 mg/L)	5	10091	0.347 ± 0.035^{c}
30 Days	Group I (Control)	5	10101	0.079 ± 0.019^{a}
	Group II (Solvent Control)	5	10071	0.070 ± 0.020^{a}
	Group III (BPA 1.31 mg/L)	5	10107	0.168 ± 0.025^{ab}
	Group IV (BPA 2.62 mg/L)	5	10105	0.248 ± 0.035^{b}
	Group V (BPA 3.92 mg/L)	5	10109	0.297 ± 0.035^{b}

Note: Values are means ± SEM (n = 5). Values with different alphabet (a, b, c) superscripts differ significantly ($p \leq 0.01$) between concentrations and within duration as analysed by one-way ANOVA followed by TUKEY's multiple comparison test. There was no significant difference between 15 days and 30 days data.

Table 2: Tail DNA% in liver cells after exposure to different concentrations of BPA

Exposure Duration	Tail DNA%					
	Group I Control	**Group II** Solvent Control	**Group III** BPA 1.31 mg/L	**Group IV** BPA 2.62 mg/L	**Group V** BPA 3.92 mg/L	**Group VI** Positive Control
15 Days	4.874 ± 0.33^{ap}	5.038 ± 0.39^{ap}	10.438 ± 0.48^{bp}	13.789 ± 0.61^{cp}	14.246 ± 0.72^{cp}	8.837 ± 0.55^{bp}
30 Days	5.144 ± 0.33^{ap}	5.521± 0.29^{ap}	12.993 ± 0.54^{bq}	16.327 ± 0.79^{cq}	17.422 ± 0.87^{cq}	8.613 ± 0.28^{dp}

Note: Values are means ± SEM (n = 5). Values with different letter superscripts (p, q) differ significantly ($p \leq 0.05$) between durations within concentration as analysed by t-test. Values with different letter superscripts (a, b, c, d) differ significantly ($p \leq 0.05$) between concentrations within duration as analysed by one-way ANOVA followed by TUKEY's multiple comparison test.

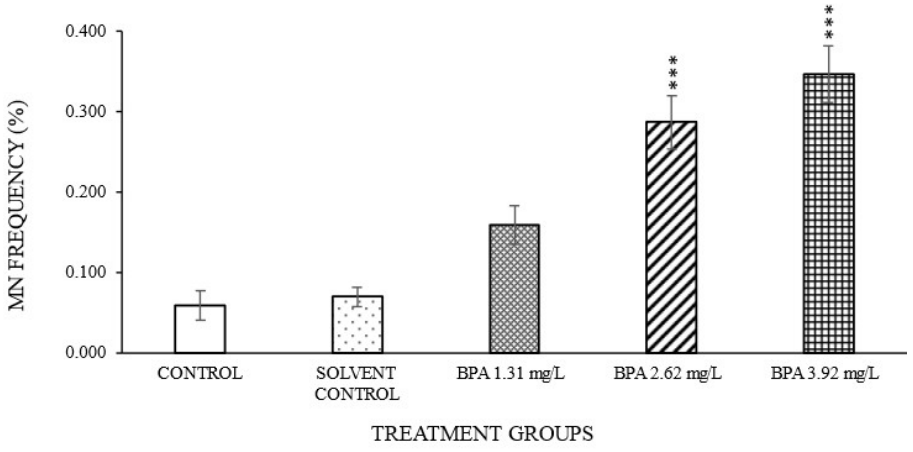

Figure 1: Micronucleus (MN) % after exposure of *C. punctatus* to BPA for 15 days
* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 represent significant difference between control and treatment.

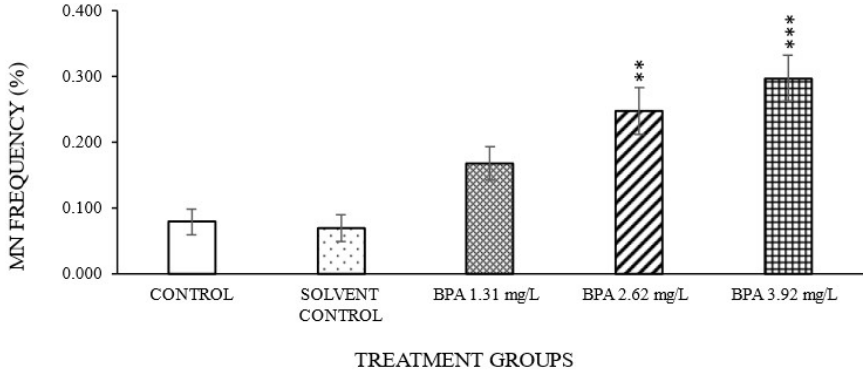

Figure 2: MN frequency after exposure of *C. punctatus* to BPA for 30 days
* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 represent significant difference between control and treatment

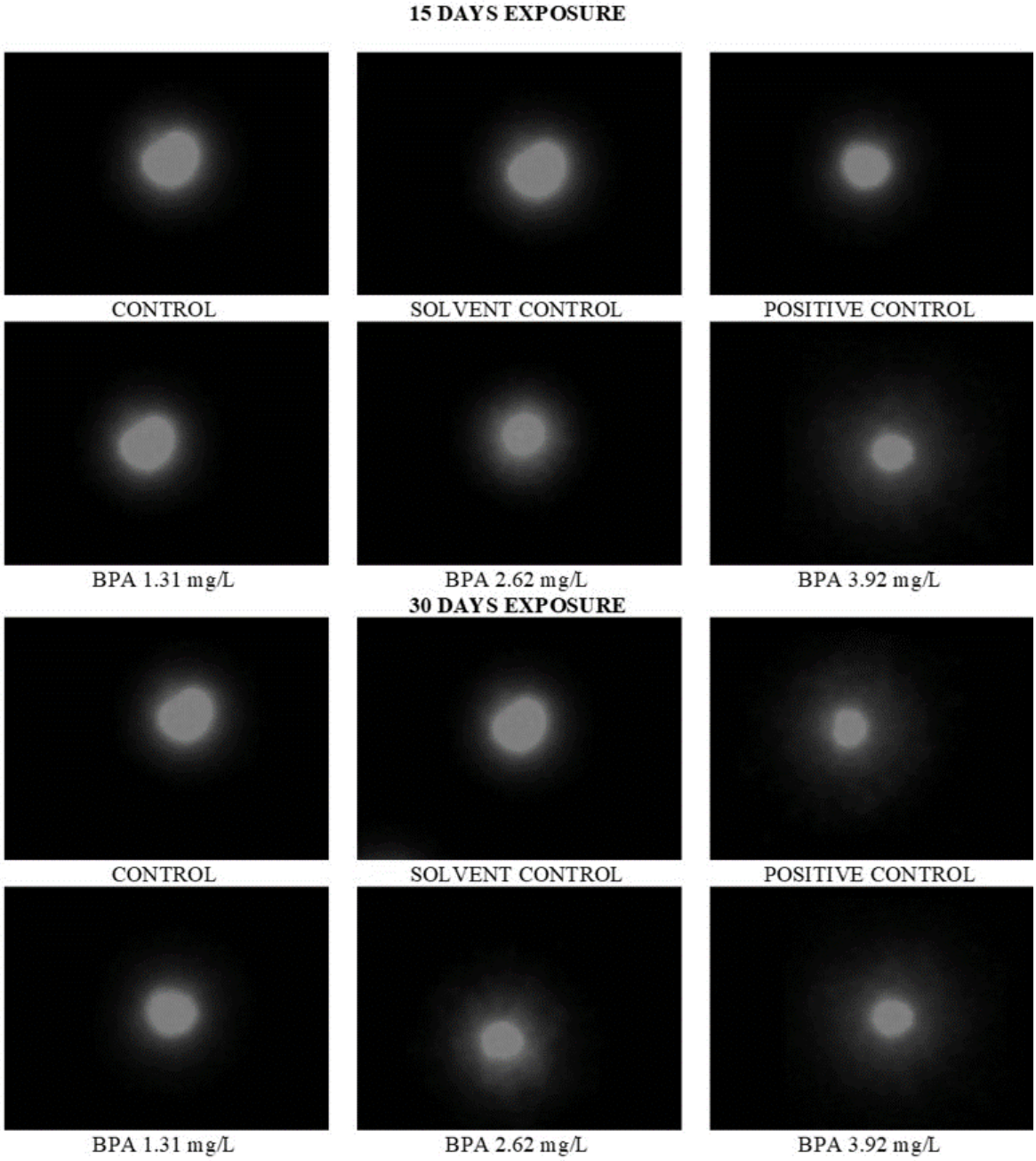

Figure 3: Representative images of Comet Assay of liver cells after BPA exposure

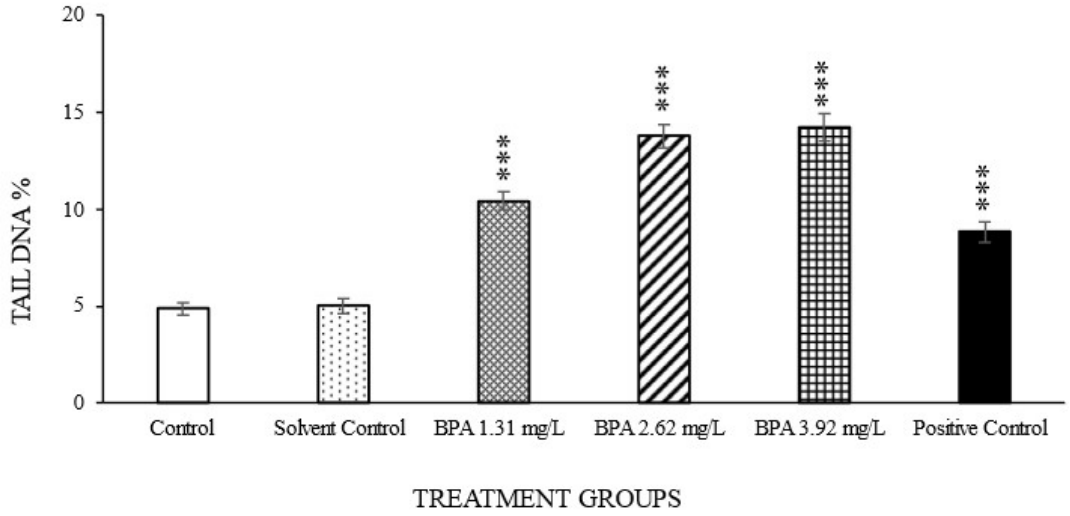

Figure 4: Tail DNA% in liver cells of *C. punctatus* after BPA exposure for 15 days
* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 represent significant difference between control and treatment

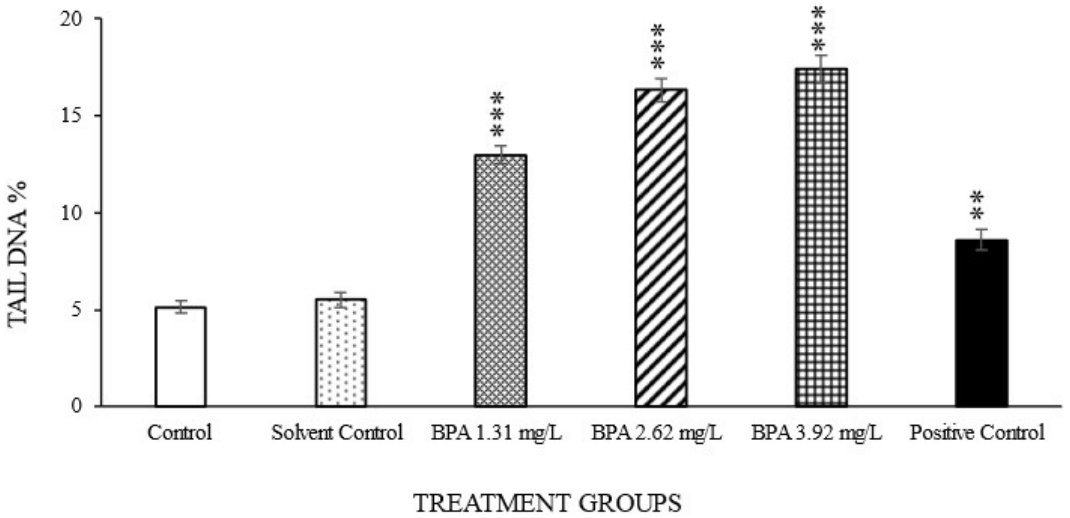

Figure 5: Tail DNA% in liver cells of *C. punctatus* after BPA exposure for 30 days
* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 represent significant difference between control and treatment.

DISCUSSION

There are a few reports that BPA exposure results in increased mutations in human RSa cells (Takahashi et al., 2001), in *S. typhimurium* revertant test using TA102 strain (Tiwari et al., 2012) and in rat spermatids and spermatocytes (Tiwari and Vanage, 2013). Among fish species, the genotoxic potential of BPA has been assessed on Atlantic cod and turbot (Barsiene, Dedonyte, Rybakovas, Andreikenaite and Andersen, 2005), zebrafish embryo (Wang et al., 2013), sperm DNA of *Acipenser* (Hulak, Gazo, Shaliutina and Linhartova, 2013), juvenile rock bream, *Oplegnathus fasciatus* (Choi et al., 2016) etc. These studies suggest that BPA is a weak genotoxic agent that BPA exposure may lead to DNA strand breaks and increase in micronuclei frequency.

In this study, 15 days exposure of *C. punctatus* to sublethal doses of BPA resulted in a dose dependent increase in MN%. However, there was no significant difference between the MN% after 15 days and 30 days exposure to BPA. These results are supported by the work of Negintaj, Archangi, Movahedinia, Safahieh and Eskandari (2015) in yellowfin seabream, *Acanthopagrus latus*. They reported a dose dependent increase in MN frequency after intraperitoneal injections of BPA for 2 weeks.

The increase in MN frequency after BPA exposure could be the result of DNA strand breaks or errors in the mitotic machinery. Comet assay was conducted to determine if the increased MN frequency after BPA exposure was due to DNA strand breaks. A significant, dose dependent increase in tail DNA% was observed in liver cells after exposure to BPA for both 15 and 30 days. These results are supported by the work of Tiwari et al. (2012) in rats, Choi et al. (2016) in juvenile rock bream, *Oplegnathus fasciatus*, Wang et al. (2013) in zebrafish embryos and Negintaj et al. (2015) in yellow fin sunbream (*Acanthopagrus latus)*.

Choi et al (2016) hypothesized that the increase in tail DNA % was probably due to increased oxidative stress after BPA exposure. RAD54 is involved in DNA double strand break (DSB) repair (Dronkert et al., 2000). Studies by Lee, Liu, Takeda and Choi (2013)on $RAD54^{-/-}$ DT40 cell line suggest that BPA exerts a genotoxic effect by increasing DSBs in DNA. Endocrine disrupting chemicals (EDCs) like nonylphenol, octylphenol, etc. are reported to cause genotoxicity (Sharma and Chadha, 2017; Sreedevi and Chitra, 2014). The results presented in this study suggest that BPA exposure leads to increased DNA damage in *C. punctatus* probably due to increased double strand breaks in DNA.

CONCLUSION

C. punctatus is known to inhabit the Delhi section of the river Yamuna which has the highest reported concentration of BPA in any river in the world (Lalwani et al., 2020). The results presented in this study suggest that comet assay may be employed as a sensitive tool to assess the genotoxic potential of BPA and that *C. punctatus* may be a suitable model organism to assess the genotoxic potential of EDCs like BPA in in aquatic ecosystems. Besides their role as 'sentinel' organisms used for environmental biomonitoring, fish are also an important part of human diet and therefore a potential source for the vertical transfer of toxicants from polluted water bodies to human beings. This study raises concern over the indiscriminate release of BPA containing sewage and industrial effluents and the potential hazards BPA and other EDCs pose to living organisms.

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Conflict of Interest: None

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