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Bisphenol A induced oxidative stress mediated genotoxicity in *Drosophila melanogaster*

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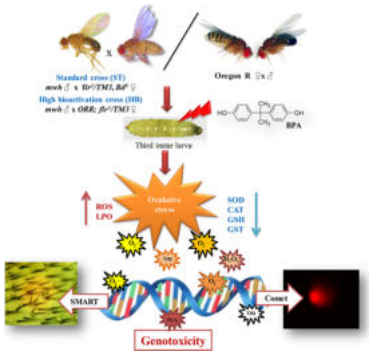
GRAPHICAL ABSTRACT



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ABSTRACT

This study investigates Bisphenol A (BPA) induced oxidative stress that mediates the genotoxicity in *in vivo* model *Drosophila melanogaster*. The calculated LC_{50} for BPA was 12.35 µg/mL. The strains of *D. melanogaster* were reared in 0.1, 1.0, 2.5 and 5.0 µg/mL BPA treated food media from the embryonic stage (egg); oxidative stress and genotoxicity parameters were analyzed. Food intake analysis confirmed that BPA is not an anti feedant for *Drosophila* larvae and it consumed BPA containing food. Increased reactive oxygen species (ROS) and lipid peroxidation (LPO) and depletion of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione-s-transferase (GST) antioxidant activities were observed in BPA treated groups compared to control. Positive single spots/wing frequencies were observed in standard (ST) and high bioactivation (HB) crosses of marker heterozygous (MH; *mwh/flr3*) and balancer heterozygous (BH; *mwh/TM3*) genotype flies indicating BPA is mutagenic and not recombinogenic. A significant increase in tail length and % tail DNA in Comet assay after BPA treatment reveals that BPA has a potential to induce the genotoxicity. Present study suggests that BPA exposure induces oxidative stress, which could be one of the possible mechanisms for induction of genotoxicity.

1. Introduction

Bisphenol A (BPA), 2,2-bis (4-hydroxyphenyl) propane, is a xenoestrogen and one of the most used industrial monomer in the production of polycarbonate plastics and epoxy resins in the manufacture of food and beverage packaging material or containers, can

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linings, baby feeding bottles, toys, sealants, eyeglass lenses, several paper consumer products, and so on [1]. The worldwide production of BPA is about 8 million tons per year and approximately 100 tons may be released into the atmosphere in a year [2]. It is a pollutant ubiquitously present in global environment as a result of high production, consumption and its frequent environmental introduction [3]. Human beings are getting exposed to BPA as its residual monomer leaches from the inner lining of tin, cans and microwave containers during heating or exposure to UV light into the food materials, from dental sealant into saliva and into beverages from polycarbonate bottles due to the repeated usage (ageing) or contact with any acidic or basic compounds [4,5]. BPA is detected in food samples (0.2–106 ng/g) [6]; in environment: water bodies (8–21 ng/mL), air (2–208 ng/m^3), thermal paper (54–79 μg/cm^2), cans (2–82 ppb) and dental materials (0.013–30 mg) [7]; and in human blood and serum (0.5–10 μg/L) [2], placenta (0.14–4.76 μg/L) [8], urine (0.12–59.72 μg/L) [9] and breast milk (0.22–10.8 ng/mL) [10]. Although, the number of epidemiological studies in humans is trivial, presence of BPA in humans has been correlated with increased reproductive (recurrent miscarriages) and genetic abnormalities, (abnormal embryonic karyotype) and onset of various diseases such as diabetes, obesity, cardiovascular disease, and cancer [7–11]. Presence of BPA in biological samples has raised a great concern regarding human health, since BPA shares similarities in structure, metabolism and action with diethylstilbestrol (DES), an estrogenic drug, which has been banned worldwide due to its potential health risks [12]. Both DES and BPA have been shown to interfere with the assembly of microtubules [13], a process by which DES is thought to promote induction of aneuploidy and chromosomal aberrations.

The possible genotoxicity of BPA has been studied extensively in many systems, but the results remain controversial due to inconsistent data. Studies showed that BPA can induce chromosome aberrations and DNA adducts formation in Syrian hamster embryonic (SHE) cells [14] or micronuclei formation [15] as well as aneuploidy and DNA adduct formation [16]. BPA also induced structural changes (achromatic gaps) in bone marrow cells of mice [17]. In contrast to this, a study clearly demonstrated that BPA is not mutagenic but exhibit significant genotoxic and cytogenetic effects in Chinese hamster ovary cells [18]. However, studies are showing that BPA exposure fail to induce chromosomal aberrations [19], micronucleus formation [17] or gene mutations at the HGPRT locus [20]. Taken together, all these reports remain unclear as to whether BPA exposure promotes genotoxicity *in vitro* and *in vivo*. Despite these inconsistencies, BPA exposure has been shown to cause genotoxic effects linked to the generation of reactive oxygen species (ROS) and induction of oxidative stress [21]. Even though the ubiquity of BPA exposure can increase the genomic DNA damage and their implications for onset of human disease and progression, the mechanism involved in BPA induced oxidative stress mediated genotoxicity is unclear. These discrepancies warrant the need to study the genetic toxicity of BPA, as it is present in environment and/or in foodstuffs that humans can be exposed to it and therefore, the potential genotoxicity of this compound has to be better understood.

Thus, the objective of this study was to examine the BPA induced oxidative stress that mediates the genotoxicity in *in vivo* model *Drosophila melanogaster*. BPA induced oxidative stress was analyzed by measuring the generation of ROS, lipid peroxidation (LPO) as a oxidative stress markers and level of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione-S-transferase (GST) antioxidants. In order to evaluate the involvement of oxidative stress induced genotoxicity of BPA Somatic Mutation and Recombination Test (SMART) commonly known as wing spot test and Comet assay were employed. Wing spot test revealed the mutagenic and recombinogenic potential of the chemical. Oxidative stress induced genotoxic potential of BPA was assessed by alkaline Comet assay.

2. Materials and methods

2.1. Chemicals

Chemicals used for the experiments are followed by its CAS Registry number and purity in bracket. Bisphenol A (BPA) 80-05-7 (97%), Acetone 67-64-1 (99.8%), Quercetin 6151-25-3 (99%), Glutathione Reduced (GSH) 70-18-8 (99.5%), Phenylthiourea (PTU) 103-85-5 (97%), Sodium chloride (NaCl) 7647-14-5 (99.9%), Potassium chloride (KCl) 7447-40-7 (99.5%), Sodium carbonate ($NaHCO_3$) 497-19-8, (99.9%), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) 7365-45-9 (99.5%) were purchased from Sisco Research Laboratories, Mumbai, India. Sodium lauryl sulphate (SDS) 151-21-3 (99.0%), Ethyl methanesulphonate (EMS) 62-50-0 (98.0%), 5-5′-Dithiobis 2-nitrobenzoic acid (DTNB) 69-78-3 (98%), 1-chloro-2, 4-dinitrobenzene (CDNB) 97-00-7 (99%), Hydrogen peroxide (H_2O_2) 7722-84-1(98.0%), 1,1,3,3- tetramethoxy propane 102-52-3 (99%) were purchased from Sigma Chemicals Co. St Louis, USA. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) 4091-99-0 (98%) was purchased from Thermo Fisher Scientific, USA. *N,N,N,*N tetramethyl ethylene diamine (TEMED) 110-18-9 (99%), Ethylene diaminetetraacetic acid (EDTA) 6381-92-6 (99%), Thiobarbituric acid (TBA) 504-17-16 (98%), Trichloroacetic acid (TCA)76-03-9 (98%), Acetic acid 64-19-7 (99.5%), Ethidium bromide 1239-45-8 (95%), Triton X-100 9002-93-1 (99%), Tris base 77-86-1 (98%), Dimethyl formamide 68-12-2 (99%), normal melting point agarose (NMPA) and low melting point agarose (LMPA) 9012-36-6 (98%) were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India.

2.2. Drosophila strains

Wild type Oregon-R *Drosophila melanogaster* strain was used for the toxicological, biochemical and Comet assay studies. The following parental *D. melanogaster* strains and markers were used for the crosses to perform wing somatic mutation and recombination test: (i) *mwh/mwh* - the multiple wing hairs (*mwh*, 3–0.3), (ii) $flr^3/In(3LR)TM3, ri\ p^{p`}sep\ l(3)89Aa\ bx^{34e}\ e\ Bd^s$ (flr^3, 3–38.8) is a recessive mutation that affects the shape of wing hairs, producing malformed wing hairs that have a shape of flare and (iii) $ORR/ORR, flr^3/In(3LR)TM3, ri\ p^p sep\ l(3)89Aa\ bx^{34e}\ e\ Bd^s$ - strain (ORR), which constitutively overexpresses *Cyp* genes with increased constitutive levels of cytochrome P450 (CYP450) dependent xenobiotic metabolism which facilitates the detection of promutagens. More detailed information of these genetic markers and phenotypic descriptions of the strains are given in Lindsley and Zimm [22].

2.3. Drosophila culture

The isogenic line of all strains were maintained and cultured in bottles (50 mL) at the *Drosophila* laboratory, Department of Animal Science, Central University of Kerala, Kasaragod, India under the standard conditions 24 ± 1 °C and 60–70 % relative humidity conditions for 10 days, 12 h/12 h light and dark cycle and on a standard wheat cream -agar diet with yeast granules as the protein source [23].

2.4. Crossing experiment for toxicological, biochemical and comet assay studies

Isogenic line of *Oregon R* flies was reared in regular standard *Drosophila* food medium. The 7 days old virgin flies were allowed to mate and lay eggs on grape juice medium (containing 3% agar-agar, 1.2% sucrose, 2% ethanol, 1% acetic acid, and 27.2% grape juice without any preservative) for 2 h. Collected eggs from isogenic fly lines were used for toxicological, biochemical and Comet assays.

2.5. Crossing experiment for wing spot test

Two different strains, a standard (ST) and a high-bioactivation (HB) cross, were used to investigate the possible role of metabolism on the genotoxic effects of the BPA. The use of HB strains of *D. melanogaster,* which are characterized by increased cytochrome P-450-dependent bioactivation capacity, facilitates the detection of promutagens. The standard (ST) and high bioactivation (HB) crosses were made with virgin females of the *flare*^{3} and *Oregon-flare*^{3} strains respectively, mated to *mwh/mwh* males. Both crosses originated eggs (offspring) with two different genotypes, namely marker-heterozygous (MH) (*mwh* +/+ *flr*^{3}) and balancer-heterozygous (BH) (*mwh* +/ *TM3, Bd*^{s}). Eggs from the respective crosses were collected separately on a grape juice medium for 2 h. Eggs derived from the ST or HB crosses were transferred to food bottles and were maintained at 24 °C and 60–70% relative humidity. Two days later, the second instar larvae (48 ± 2 h) were washed out of the bottles with tap water (24 °C) through a fine nylon mesh. Two-day-old larvae derived from both crosses were used as experimental larval populations for wing spot test.

2.6. Preparation of BPA treated Drosophila food media

BPA solution was prepared from solid compound. A known amount of BPA was dissolved in 0.1% acetone and it was mixed with liquid food medium to obtain a final concentration of BPA treated food medium. The preliminary toxicity experiment conducted in our laboratory showed that 0.1% acetone in the food medium (solvent control) had no significant toxic effect on the larvae of *Drosophila* compared to the control (without the 0.1% acetone) food medium. Hence the solvent (0.1% acetone) in the food medium was considered as control in all other experiments. BPA or Ethyl methanesulphonate (EMS) was added in desired concentration to freshly prepared 50 mL of *Drosophila* food, thoroughly mixed, poured into experimental bottles and allowed to solidify. For each experiment, the negative control (without BPA) and EMS (0.1 mM) a well-known mutagen, was used as a positive control (for wing spot and Comet assay) and the BPA treated food were prepared uniformly in the same batch.

2.7. Toxicity analysis and selection of BPA concentrations for assays

Toxicity assay was conducted to assess the BPA toxicity in *Drosophila* following the method established in our laboratory [24]. The calculated LC_{50} for BPA was 12.35 µg/mL and the lowest observed effect concentration (LOEC) was 0.1 µg/mL (based on the viability assay). Hence, less than half of the LC_{50} concentrations of BPA 0.1, 1.0, 2.5 and 5.0 µg/mL were selected for analyzing the BPA induced biochemical and genetic effects in a concentration dependent fashion.

2.8. Food intake analysis

To confirm intake of BPA containing food, we performed feeding assay with modifications of Lee et al. [25]. Third instar larvae (72 ± 2 h) were allowed to feed for 4 h in a food media treated with 0.1, 1.0, 2.5 and 5.0 µg/mL BPA and control (without BPA). Fifty larvae from each group were transferred into vials containing different concentrations of BPA diet with orange-red synthetic food dye (Tiger, Manju Chemicals Pvt. Ltd, India). Feeding was continued for 4 h and the fed larvae were collected. Larvae were washed with phosphate buffered saline (PBS) (pH 7.2). Larvae were homogenized in 1 ml of distilled water, centrifuged at, 15000 g for 10 min. The diluted (100 times) supernatant was used to measure the absorbance at 595 nm using spectrophotometer. Food intake by larvae was also confirmed by image analysis. Third instar larvae (72 ± 2 h) were allowed to feed for 4 h in food media treated with 0.1, 1.0, 2.5 and 5.0 µg/mL BPA and control (without BPA). Larval images were captured using the stereo microscopic camera (Carl Zeiss, Germany) and images were converted to gray scale and a threshold value was set that corresponded to the darkest pixels where food was present in the gut. Selections of specific areas of the gut tube were made using the Image J wand tool and mean gray values for the selections were determined. Mean gray values for each control and BPA treatment groups were averaged and compared.

2.9. Biochemical assays

Biochemical assays were performed using third instar larvae (72 ± 2 h) of *Drosophila* reared in control or in BPA treated medium (0.1, 1.0, 2.5 and 5.0 µg/mL BPA) from embryonic stage (usually eggs of *Drosophila* is considered as embryo).

2.10. Sample preparation for oxidative stress assays

Samples were prepared by homogenizing 30 larvae (3rd instar; 72 ± 2 h) in 200 µL of ice cold sodium phosphate buffer (0.1 M; pH 7.4) containing protease inhibitor cocktail, followed by centrifugation at 2500 *g* for 10 min at 4 °C. Filtered supernatant was collected for biochemical analysis. All biochemical assays were performed in triplicate and the experiments were repeated thrice.

2.10.1. Reactive oxygen species (ROS)

The level of ROS generation was measured by the method of Driver et al. [26]. An aliquot of homogenized larval sample (50 µL) was dispensed into tubes containing Locke's buffer solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 2 mM $CaCl_2$ and 10 mM glucose pH 7.4) to which 10 µL of DCFH-DA (5 µM) was added and incubated for 30 min at room temperature. The fluorescence was measured with excitation and emission wavelengths at 480 and 530 nm. Values were expressed as picomole of dichloro fluorescein formed/mg protein/min.

2.10.2. Lipid peroxidation (LPO)

The extent of LPO was measured by the method of Ohakawa et al. [27] by measuring thiobarbituric acid reactive substances (TBARS) in larval homogenate. Reaction mixture contained 500 µL larval homogenate, 1.5 mL acetic acid (pH 3.5, 20% v/v), 1.5 mL of TBA (0.8% w/v), 200 µL SDS (8% w/v). The mixture was heated in a boiling water bath for 45 min and adducts formed were extracted into 3 mL of 1-butanol and the color intensity was measured at 532 nm and quantified as malondialdehyde (MDA) equivalents using 1,1,3,3- tetramethoxy propane as standard. The values were expressed as µmole MDA/mg protein.

2.10.3. Superoxide dismutase (SOD)

SOD activity was determined following the method of Kostyuk and Potapovich [28] by monitoring the inhibition of quercetin auto oxidation. To 1 mL reaction mixture containing 3–5 µg protein; 0.016 M sodium phosphate buffer (pH 7.8), 8 mM TEMED and 0.08 mM EDTA was added. Adding 0.15% quercetin dissolved in dimethyl formamide started the reaction. The rate of quercetin auto oxidation was monitored for 3 min at 406 nm. Following the addition of sample, the decrease in absorbance was monitored. The amount of protein that inhibits quercetin oxidation by 50% was defined as one unit. Values were expressed as unit/mg protein.

2.10.4. Catalase (CAT)

The CAT activity [29] was determined as a measure of H_2O_2 (final concentration 8.8 mM) decomposition by the enzyme. It was monitored by the addition of an aliquot (20 µL) of the larval homogenate. The decrease in H_2O_2 was monitored for 3 min at 240 nm and resulted values were expressed as µM of H_2O_2 decomposed/min/mg protein.

2.10.5. Glutathione (GSH)

Total GSH content was measured following the method of Mokrasch

and Tschke [30]. 30 larvae (3rd instar; 72 ± 2 h) of *Drosophila* were homogenized in ice-cold 10% TCA and 10 mM EDTA (1:1) and centrifuged at 5000 *g* (15 min at 4 °C). 200 µL of the supernatant was added to the 3 mL reaction mixture containing Tris-buffer (0.2 M; pH 8.0) and 50 µL of DTNB. After 10 min of incubation at room temperature, the absorbance was read at 412 nm and values were expressed as µg GSH/mg protein.

2.10.6. Glutathione-S-transferase (GST)

GST activity was assayed as per the procedure of Habig and Jakoby [31] using CDNB as substrate. The assay reaction mixture was made up of 270 µL of a solution containing (20 mL of 0.25 M potassium phosphate buffer, pH 7.0, with 2.5 mM EDTA, 10.5 µL of distilled water, and 500 µL of 0.1 M GSH at 25 °C), 20 µL of sample (1:5 dilution), and 10 µL of 25 mM CDNB. The increase in the optical density was recorded for 3 min at 340 nm and the activity was expressed as µmol GS-DNB formed/min/mg protein using the molar extinction co-efficient (ε = 9.6 mM/cm).

2.10.7. Protein estimation

Protein concentrations in all the required fractions was estimated using BSA as a standard [32].

2.11. Genotoxicity tests

2.11.1. Wing spot test

2.11.1.1. Treatment procedure, phenotypic characterization and preparation of microscopic slide. To perform wing spot test in *Drosophila,* the second instar larvae (48 ± 2 h) derived from the ST or HB crosses were reared in negative, positive control (0.1 mM EMS) or sublethal concentrations of 0.1, 1.0, 2.5 and 5.0 µg/mL BPA treated food media. Advantage of selecting the second instar larvae (48 ± 2 h) for the wing spot test is that large number of cells in the wing discs are exposed simultaneously to the chemical (BPA or EMS) that would develop large, recognizable wing spot helpful for easy interpretation of data. The five-bottles/experimental group (100 larvae/bottle; total 500 larvae/group) was maintained. Larvae were maintained in the respective treated and control food media where they fed for the remainder of their larval life, pupated and emerged as adult flies. After completion of metamorphosis, all surviving flies were scored irrespective of sex and classified according to the presence/absence of the Bd^S phenotype. Adult flies produced from the experimental progeny of ST or HB crosses consisted of marker-heterozygous (MH) flies (*mwh* +/+ flr^3) with phenotypically wild-type wings and balancer-heterozygous (BH) flies (*mwh* +/+ *TM3* Bd^S) with phenotypically serrate wings. These flies were stored in a 70% ethanol solution. All experiments were performed under standard conditions 24 ± 1 °C and 60–70% relative humidity conditions, 12 h/12 h light and dark cycle. Since, positive responses were obtained in MH progeny, BH progeny was also analyzed. Therefore, for observation of mutant spots in the wings of MH and BH flies of two crosses were removed and mounted on slides using Faure's solution (30 g gum arabic, 20 mL glycerol, 50 g chloral hydrate, 50 mL distilled water) [33].

2.11.1.2. Wing spot analysis. Mounted wings (40 flies, i.e. 80 wings/experimental group) were analyzed under an optic microscope at 40X magnification for the occurrence of single and twin spots. Mutant clones were classified into three types during microscopic analysis: (i) small single spots, consisting of 1 or 2 *mwh* or flr^3 cells; (ii) large single spots, consisting of three or more cells; and (iii) twin spots consisting of adjacent *mwh* and flr^3 cells [33]. Induced loss of heterozygosity on MH flies leads to two types of mutant clones: (i) single spots, either *mwh* or flr^3, which can be produced by somatic point mutation, and chromosome aberration such as deletion, and (ii) twin spots, consisting of both *mwh* and flr^3 sub clones, which originated exclusively from mitotic recombination. On the wings of BH flies, only mwh single spots can be recovered. These spots are all due to mutational events because recombinational events are suppressed in inversion-heterozygous cells with the multiple-inverted *TM3* balancer chromosome [34]. So, as a rule, the frequencies of *mwh* clones observed on the wings of BH flies are always lower than those observed on the wings of MH flies [35]. For this reason, wings of BH flies were mounted and analysed whenever a previous positive response was obtained in the MH progeny. A comparison of the results obtained from MH and BH flies was used to quantify the mutagenic and recombinagenic potential of the test sample.

2.11.2. Detection of DNA damage in hemocytes by comet assay

Third instar (72 ± 2 h) larvae were used for the collection of hemolymph to perform comet assay. These larvae were reared in the negative control or positive control (0.1 mM EMS) or sub lethal concentrations of 0.1, 1.0, 2.5 and 5.0 µg/mL BPA treated food media from the embryonic stage. The experiment was conducted in triplicate for each treatment and same experiments were repeated thrice.

2.11.2.1. Collection of hemocytes. Hemocytes from larval hemolymph were collected according to the method of Marcos and Carmona [36]. Briefly, larvae (72 ± 2 h) were removed from food media, washed in distilled water, sterilized in 5% bleach and dried. The cuticle from each (n = 50) larva was then disrupted with two fine forceps under a stereomicroscope. The hemolymph was directly collected into a drop of cold PBS solution containing 0.07% PTU in 1.5 mL microcentrifuge tube. Pooled hemolymph was centrifuged at 300 *g* for 10 min at 4 °C, the supernatant was discarded, and the pellet containing hemocytes was resuspended in 20 µL of cold PBS.

2.11.2.2. Comet assay procedure. Alkaline Comet assay was performed following the method of Dhawan et al. [37] with some modifications. Comet assay slide preparation was performed by mixing 20 µL of hemocyte cell suspension in 75 µL of 0.75% low melting point agarose (LMPA). 20 µL of the mixture was layered on top of the slides that were pre-coated with 1% normal melting point agarose (NMPA). The cell suspension was uniformly smeared with cover slips, and the slides were stored at 4 °C for 5 min until agarose solidification. After agarose solidification coverslips were removed and the slides were immersed for 2 h in freshly prepared, chilled lysing solution (2.5 M of NaCl, 100 mM of EDTA, 0.26 M NaOH, 10 mM of Tris, and 1% Triton X-100, pH 10) at 4 °C in dark chamber and lysis was performed. For the measurement of DNA single strand breaks and alkali labile base damage, the slides after lysis were placed in chilled electrophoresis buffer (0.03 M NaOH, 1 mM Na_2EDTA, pH > 13) for 10 min for DNA unwinding. Subsequently, electrophoresis was conducted in chilled electrophoresis buffer for 15 min at 0.7 V/cm (300 mA/25 V) at 4 °C. The slides were gently washed three times with 0.4 M Tris buffer (pH 7.5) to neutralize excess alkali. ***Staining-*** Before staining, slides were dehydrated with ethanol for 10 min each and air dried overnight. Slides were then stained with ethidium bromide (20 µg/mL: 25 µL per slide) for 10 min in dark. After staining, slides were dipped once in chilled distilled water to remove excess stain and coverslips were placed over the slides. Then the slides were examined using a fluorescence microscope (Olympus, Japan) at 40X magnification. Normal and Comet images were captured. Images were transferred to a computer through a charge coupled device (CCD) camera and analyzed using CASP 1.2.3 software (CASPlab). One hundred and twenty cells from each group (20 cells per slide with two slides/experimental group in triplicates) were examined. Tail intensity (% of DNA in tail) and tail length (TL) (estimated leading edge from the nucleus; µm) were used as parameters of BPA induced DNA damage.

2.12. Data evaluation and statistical analyses

2.12.1. Biochemical assays

All the analysis was made in respective replicate and values are represented as mean ± SEM. A significant difference between the control and BPA treated groups was obtained by one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test using SPSS (version 16.0) (SPSS Inc., Chicago, IL, USA).

2.12.2. Wing spot test

Wing spot data was evaluated with SMART PC-Version 2.1. This computer program analyses the wing spot data by routinely used statistical diagnosis in SMART assay i.e., multiple decision procedure (Null and Alternative hypotheses). This method decides whether a chemical is positive, weakly positive, inconclusive or negative in inducing its mutagenicity [35]. Wing spot data of treated and control series were compared by conditional binomial test [38] with significance levels set at $\alpha = \beta = 0.05$. The clone formation frequency 10^5 cells per cell cycle were calculated [34]. Clone formation frequency per 10^5 cells higher than 2.0 are indicative of genotoxic effect of the chemical [33]. For final statistical analysis of all positive outcomes, the non-parametric Mann-Whitney *U* test with significance levels $p < 0.05$ was used to exclude false positives [34].

2.12.3. Comet assay

Tail length and % tail DNA data were obtained from 120 independent images per treatment group. The distribution of data is shown as box and whisker plots, the box representing the 1st quartile (25th percent) and the 3rd quartile (75th percent), the line in the box representing the median. Medians indicated those that are different from control. Non-parametric Kruskal Wallis test was applied to analyze the data for significance; and also analysis of variance (ANOVA) followed by Tukey HSD post hoc test .

3. Results

3.1. Consumption of BPA treated food

Food intake was analyzed in third instar larvae (72 ± 2 h) reared in control and in different concentrations of BPA treated food media mixed with orange-red synthetic food dye. Representative larval images are presented in the Fig. 1. There was no significant difference in optical density values and mean gray values ($p > 0.05$) in different concentrations of BPA compared to control (without BPA) food mixed with dye (Fig. 1), showing that there was no significant difference in food consumption of the larvae. Food intake analysis confirmed that BPA is not an anti-feedant for *Drosophila* larvae and consumed BPA containing food.

3.2. BPA induced oxidative stress

The elevated ROS generation and increased level of LPO was noticed in third instar larvae reared in different concentrations of BPA than control. Significant increase in ROS generation was noticed in the highest concentration (5.0 µg/mL) of BPA treated group compared to control ($p < 0.05$) (Fig. 2a). Significant increase in the level of LPO was observed in 2.5 ($p < 0.05$) and 5.0 µg/mL BPA ($p < 0.001$) treated groups compared to control (Fig. 2b). The percent increased generation of ROS is 12.03%, 38.21%, 54.44%, and 75.39%; and level of LPO is 9.87%; 41.11%; 57.85% and 88.30% ($p < 0.0001$) higher in BPA treated larvae compared to control.

3.3. BPA induced depletion of antioxidant defense system

A significant reduction of SOD and CAT enzyme activities were recorded in the third instar larvae reared in different concentrations of BPA treated food medium compared to controls. Greater depletion of SOD and CAT enzyme activities were observed in larvae reared in 2.5 and 5.0 µg/mL BPA treated food media (Fig. 3a and b). Similarly, a reduction of intracellular antioxidant GSH content and GST enzyme activity were noticed in third instar larvae reared in BPA treated food media (Fig. 3c and d). Significant level of GSH content was reduced in the larvae reared in 2.5 ($p < 0.05$) and 5.0 µg/mL BPA ($p < 0.001$) treated food media compared to control. The significant reduction of GST was recorded in larvae reared in 1.0, 2.5 and 5.0 µg/mL BPA treated food media.

3.4. BPA induced wing mutant spots

Negative control of ST and HB crosses gave wing mutant spots/wing frequencies less than the BPA treated groups and positive control in this *in vivo* assay. EMS (positive control) showed a clear response, which supports the validity of the negative results found for the BPA tested. Negative control frequencies of total spots per wing range from 0.21 to 0.25 for the *mwh/flr*^{3} genotype and from 0.18 to 0.22 for the *mwh/TM3* genotype. BPA treatments gave inconclusive total spots/wing frequencies in both ST and HB crosses (Tables 1 and 2). In the ST cross of marker heterozygous (MH; *mwh/flr*^{3}) genotype flies all the BPA treated concentrations produced positive results for small single spots and positive large single spots were observed in 2.5 and 5.0 µg/mL BPA treated groups. Positive results were observed in small single spots in the ST cross of balancer heterozygous (BH; *mwh/TM3*) genotype flies (Table 1). Positive small single mutant spots were recorded in the HB cross of both MH and BH genotypes (Table 2). Increased mean *mwh* clone size induction response was observed in BPA treated groups compared to neagtive control in both ST and HB crosses of marker-heterozygous and balancer-heterozygous wings (Tables 1 and 2).

3.5. BPA induced DNA damage

Third instar larvae exposed to 0.1 mM EMS (positive control) showed a significant increase ($p < 0.0001$) in DNA migration compared to negative controls which was evident in tail length and % tail DNA in their hemocytes. Increase in DNA migration was observed in the Comet parameters indicating BPA induced genotoxicity. Significant increase in tail length was observed at 2.5 and 5.0 µg/mL BPA ($p < 0.0001$) treated groups and % tail DNA at a highest concentration of (5.0 µg/mL) BPA treated group compared to those of controls (Fig. 4).

4. Discussion

4.1. BPA induced oxidative stress and depletion of antioxidant defen se system

In this study, BPA caused overproduction of reactive oxygen species (ROS) in third instar larvae reared in BPA treated food media (Fig. 2a). BPA have the potential to cause oxidative stress by disturbing the redox status in cells by excess production of ROS [21]. Oxidation of BPA resulted in the formation of several by-products of which some contained additional hydroxyl-groups and other formed by a ring opening of the original compound [39]. It has been demonstrated that injection of BPA induces overproduction of hydrogen peroxide (H_2O_2) in the mouse organs [40]. Increase in ROS production caused by BPA, induces oxidative stress by the imbalance between ROS production and the cellular antioxidant defense capacity [41]. An elevated intracellular build-up of ROS concentrations can induce oxidative damage in cellular macromolecules such as nucleic acids, proteins, lipids, and carbohydrates [42], resulting in the onset of many diseases, namely, cancer, infertility, and neurodegenerative diseases [43]. Overproduction of ROS causes cell death; DNA mutation, replication errors and genomic instability can occur if the oxidative DNA damage is not repaired prior to DNA

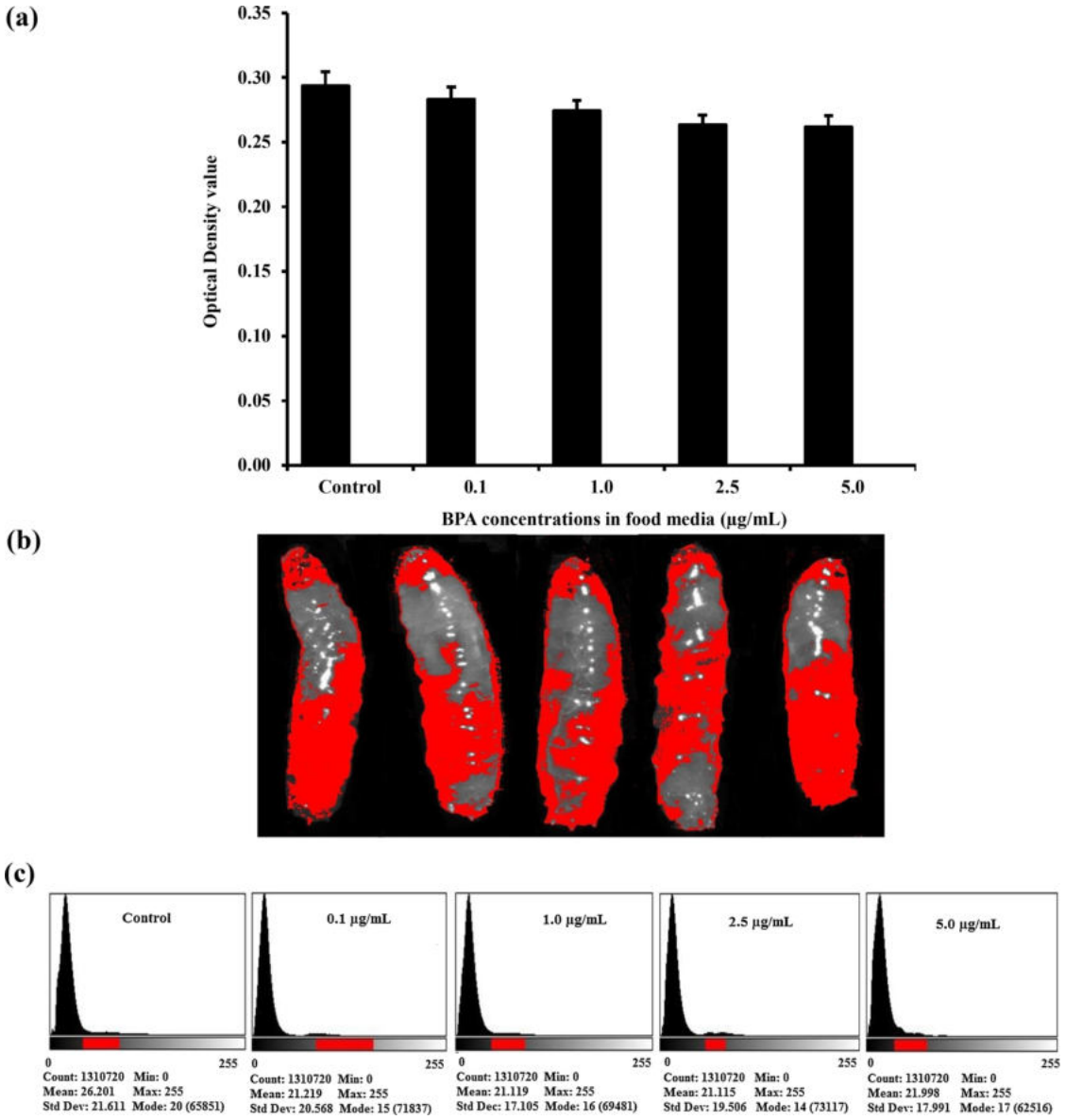

Fig. 1. Food intake was analyzed in third instar larvae (72 ± 2 h) reared in control and different concentrations of BPA treated food media. (1a) Optical density values in the control and BPA treated groups as guesstimate of food consumption. (1b) Control and BPA treatment groups representative of third instar larval images. Larval images were analysed for the determination of food consumption. (1c) The mean gray values for control and BPA treated groups were averaged and compared. Values are mean ± SE; the data analyzed by analysis of variance (ANOVA) followed by multiple comparisons made by Tukey HSD test. Values without the asterisk (*) is not significant compared to control at $p > 0.05$.

replication [44]. Enhanced production of free radicals/ROS leads to membrane lipid bilayer disruption, elicited by oxidation of polyunsaturated fatty acids known as LPO [45]; which leads to the formation of many toxic metabolites, including malondialdehyde (MDA), a widely used biomarker of oxidative stress [46]. An elevated LPO level in the larvae suggests increased cellular oxidative stress as a result of ROS generation and depletion of antioxidant scavenger system (Fig. 2b). The present study further confirms the increased free radical production and/or reduced antioxidant enzymes like SOD, CAT, GSH and GST, which probably make the organism more susceptible to oxidative damage.

Significant depletion of SOD and CAT enzyme levels (Fig. 3a and b) observed in this study is due to BPA-induced inhibition of enzyme activities. The inhibitory effects of BPA on SOD and CAT enzymes would probably result in impaired antioxidant defenses in cells and render cells more vulnerable to oxidative attacks or more functioning in combating the oxidative stress. Superoxide dismutase protects cells from oxidative stress by catalyzing the conversion of superoxide anion radical into the less toxic H_2O_2, a more stable ROS [47]. A decrease in SOD level can result in increased superoxide-free radical as well as escalation of ROS generation and LPO level. Catalase catalyzes the conversion of H_2O_2, a more reactive ROS, into water, providing protection against ROS [48]. The reduction in the activity of CAT may reflect the inability of cells to eliminate H_2O_2 generated in the cells. This may be due to enzyme inactivation caused by excess ROS production and free radical generation in cells.

Reductions of GSH in third instar larvae reared in BPA treated food medium in the present study (Fig. 3c) could be due to impairment of GSH synthesis [48]. In the presence of reactive species, GSH is oxidized rapidly to disulphide (GSSG) forms resulting in a decrease in GSH content. Therefore decreased non-enzymatic antioxidant, GSH is commonly used as an indicator of oxidative stress. This finding, along with increased ROS and LPO in larvae, suggests that BPA-induced formation of reactive byproducts, which, in turn, cause a decrease in GSH content.

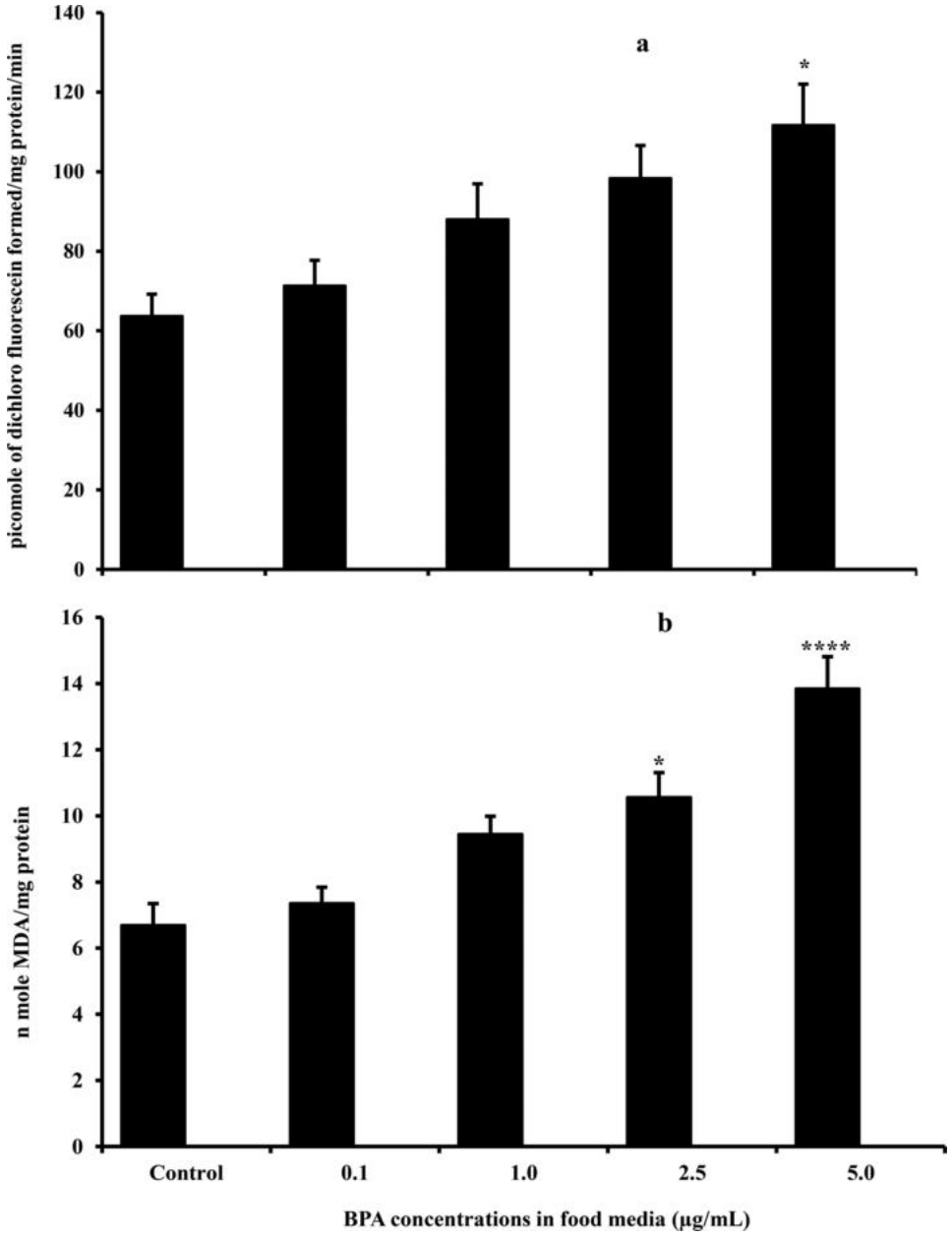

Fig. 2. Reactive oxygen species (ROS) generation (2a) and Lipid peroxidation (LPO) level (2b) in third instar larvae (72 ± 2 h) of *Drosophila* reared in the control or BPA treated groups. Values without the asterisk (*) is not significant compared to control at $p > 0.05$; and significant at $^{*}p < 0.05$; $^{****}p < 0.0001$ level.

Significant reduction in the activity of GST in BPA treated groups (except in 0.1 µg/mL) (Fig. 3d) could be due to increased free radicals/ROS and LPO. In general, xenobiotics can be metabolized by two detoxification pathways, in which insects eliminates or neutralizes toxic substances. Phase I reactions primarily process xenobiotics to undergo phase II reactions. This is accomplished *via* oxidative, hydrolytic and reductive reactions by utilizing the microsomal cytochrome P450 family of enzymes. Phase II detoxification involves the GSTs, which conjugates the products of Phase I detoxification for solubilization and transport. These pathways ensure the xenobiotic metabolism and final excretion in turn preventing reactive free radical build up and cellular damage [49]. In this study, once the phase I detoxification system converts BPA to reactive species; a decrease in the activity of GST may lead to accumulation of free radicals. It is also suggesting that the phase II detoxification system was not activated under this condition. The BPA-induced reduction in total GST activity impairs capacity of the larvae to completely detoxify BPA [50].

4.2. BPA induced wing mutant spots

Wing spot test is used to detect mutagenic and recombinogenic properties of the chemical [51], thus, this test was used to detect the genotoxic potential of BPA. The negative control frequencies of total spots per wing obtained in the *mwh/flr*^{3} and *mwh/TM3* genotype of ST or HB cross larvae (Tables 1 and 2), which is in accordance with the usual range previously reported [52]. An increased frequency of small single spots at all the tested BPA concentrations has demonstrated the mutagenic activity of BPA under *in vivo* conditions in somatic cells of *Drosophila.* EMS was used as a positive control in this assay and gave positive results for their mutagenic and recombinogenic activities.

Lack of twin spots and induction of positive small single spots observed on MH or BH progeny of ST and HB cross reared in all the BPA treated groups (Tables 1 and 2) reflects only somatic mutations (point mutation, chromosomal aberration: deletion, non-disjunction) [53]. In the wings of BH flies (*mwh/TM3*) *mwh* single small spots can be

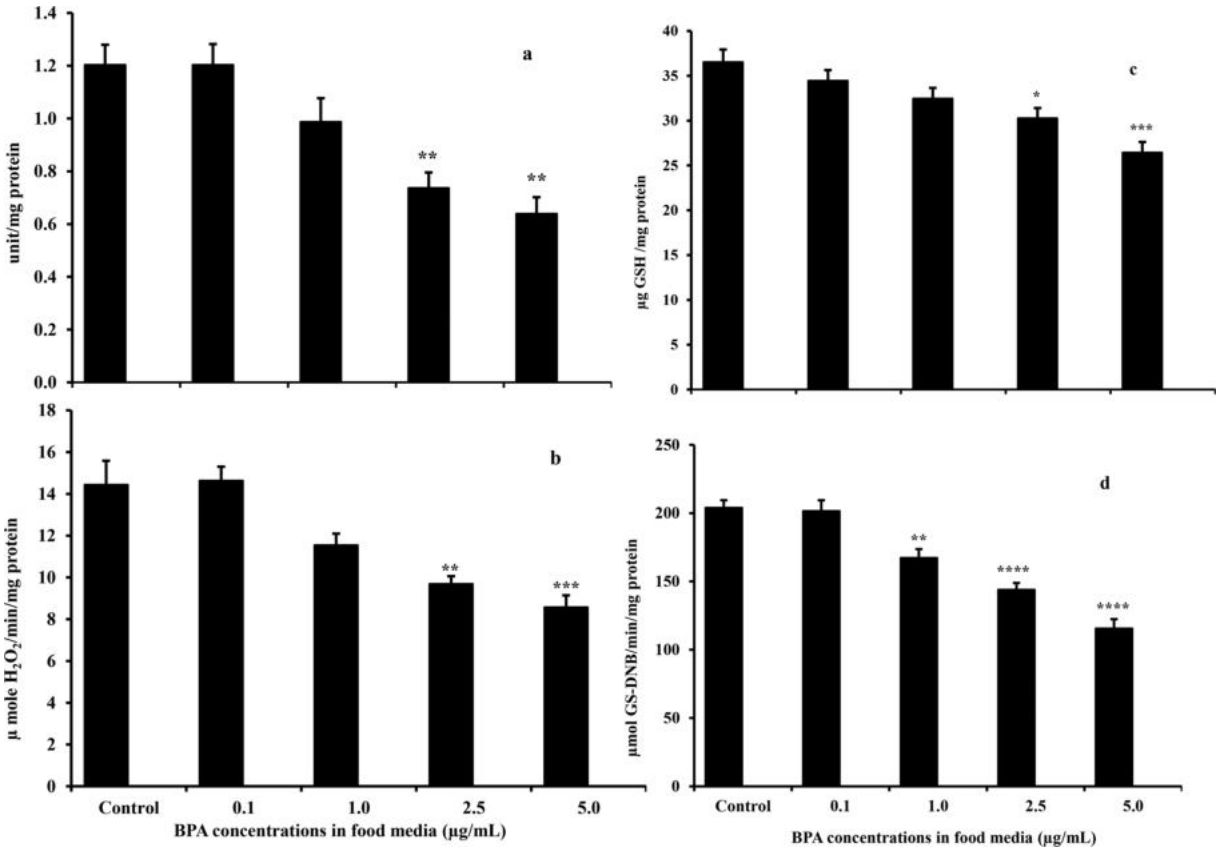

Fig. 3. Superoxide dismutase (SOD) (3a), Catalase (CAT) (3b), Glutathione (GSH) content (3c) and Glutathione-S-transferase (GST) activity (3d) in third instar larvae (72 ± 2 h) of *Drosophila* reared in the control or BPA treated groups. Values without the asterisk (*) is not significant compared to control at $p > 0.05$; and significant at *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ level.

recovered and large single spots are negligible indicating mutational formations [54]. In this study, BPA was incapable of inducing somatic recombination in BH progeny; since the somatic recombination is suppressed in inversion-heterozygous cell with the multiple-inverted *TM3* balancer chromosome and its structurally normal homologue is non-viable in BH progeny [34]. Single spots arise due to mitotic recombination as in twin spots; it can also due to point mutations, deficiencies and non-disjunction events [54]. Lack of twin spots formation in *mwh/flr*^{3} marker heterozygotes in the ST or HB cross in this study exclude the recombinogenic property of BPA.

The frequencies of small and large single spots are less in BH wings than the MH wings in BPA treated groups (Tables 1 and 2). It is a well-known phenomenon that size of the *mwh* clones in BH wings are always smaller than those in MH wings, which may be due to clones with induced segmental aneuploidy that show reduced proliferation capacity [36]. This is best demonstrated in the present study by values of the *mwh* clone size that BPA most probably has mutagenic activity. This conclusion is further supported by the fact that no positive results were obtained for the induction of twin spots (Tables 1 and 2), which are exclusively due to mitotic recombination.

The reduced induction of mutant clone or mean mwh clone size class was recorded in the HB cross of MH and BH genotypes compared to ST cross genotypes (Tables 1 and 2) because its constitutively expressed CYP450 s eliminated the BPA before it could exert any genotoxicity. However, it produced a significant single spots/wing in the ST or HB cross shown to be a genotoxic (Tables 1 and 2). The explanation of the different results in both crosses lies in the CYP450 levels. Activation of any test promutagens is primarily by CYP450-enzyme system that has the capacity of metabolising a wide variety of substances. The HB cross is characterized by a higher sensitivity to progenotoxins, because the *ORR flr*^{3}*/TM3, Bd*^{S} strain carries chromosomes 1 and 2 from a DDT-resistant *Oregon R-(ORR)* line, which has an increased level of CYPs [20]. HB cross of both MH and BH genotypes flies, yielded a positive small single spots/wing frequency in BPA treated groups (Table 2), which could be due to induced genotoxicity at a late stage of development of the wing imaginal discs due to delayed metabolism and also bioactivation (conversion of promutagen to mutagen) process is not required for exerting its mutagenic activity.

Analysis of spots for ST and HB crosses (Tables 1 and 2) showed small single spots/wing frequency is positive. This could also reveal the effect not at the end but at the beginning of treatment, inhibiting more cell division and as a consequence, no large single spots formation. Moreover, BPA does not have recombinogenic activity, because none of the crosses showed significant frequencies for twin spots. Since, both the ST and HB cross showed positive results for small single spots and inconclusive total spots/wing frequency, it seemed to suspect a genotoxic effect of BPA.

During larval growth, imaginal disc-cell groups proliferate mitotically till the point of differentiation of body structures of the adult insect. If genetic alteration occurs in any one of the imaginal disc cells, these changes will be present in all the following cells, subsequently forming a mutant cell clone. This will be seen as a spot of mutant trichome on adult insect wings [54]. BPA is capable of creating variety of free radicals per se and it has been evidenced in the increased generation of ROS and LPO and depletion of antioxidant defence system (Figs. 2 and 3). The production of these free radicals acts directly on the mitotic proliferation thereby generating unfavourable conditions for proliferation. This mechanism appears to be the principal cause for the generation of mutant spots. Previous work carried out by Shilpa et al. [24] and Gaivao et al. [55] has clearly demonstrated the utility of using SMART assay for the evaluation of oxidative stress inducers in generating the wing spot, which supports our result. Since the wing-spot

Table 1
Frequency of wing mutant spots observed in the marker trans-heterozygous descendants (MH; *mwh/flr*^{3}) and balancer heterozygotes (BH; *mwh/TM3*) of *D. melanogaster*, derived from the Standard Cross (ST) treated with different BPA concentrations or positive control (EMS) or negative control.

Genotype	Treatment compound	Con.	Number of wings (N)	Spots per wing (no. of spots) statistical diagnosis[a]				Total *mwh* clones[c] (n)	Mean *mwh* clone size class[c] (i)	Frequency of clone formation per 10^5 cells[d]	
				Small single[b] spots (1–2 cells) (m = 2)	Large single[b] spots (> 2 cells) (m = 5)	Twin spots (m = 5)	Total spots (m = 2)			Without size correction $f_t = (n/NC)*10^5$	With clone size correction $f^1t = (2^{(i-2)})*ft$
MH; mwh/flr^3	NC	0.0	80	0.17(14)	0.03(3)	0.00(0)	0.21(17)	0.18(15)	1.06	0.76	1.46
	PC: EMS (mM)	0.1	80	2.50 (200)+	0.50 (40)+	0.50(40)+	3.5 (280)+	3.41(273)	2.07	13.98	14.72
	BPA (μg/mL)	0.1	80	0.43 (35) +	0.06 (5)i	0.00(0)i	0.50(40)i	0.47 (38)	1.18	1.94	3.43
		1.0	80	0.56 (45)+	0.08 (7)i	0.00(0)i	0.65 (52)i	0.55 (44)	1.28	2.25	3.69
		2.5	80	0.65 (52)+	0.10 (8)+	0.00(0)i	0.75 (60)i	0.67 (54)	1.34	2.76	4.36
		5.0	80	0.95 (76)+	0.11 (9)+	0.00(0)i	1.06 (85)i	1.02 (82)	1.66	4.20	5.29
BH; mwh/TM3	NC	0.0	80	0.16(13)	0.02(2)	[e]	0.18 (15)	0.17 (14)	1.00	0.71	1.43
	PC: EMS (mM)	0.1	80	0.43 (35)+	0.25(20)+		0.68 (55)i	0.58 (47)i	1.66	2.40	3.03
	BPA	0.1	80	0.47 (38)+	0.02(2)i		0.50 (40)i	0.42 (34)	1.10	1.74	3.25
	(μg/mL)	1.0	80	0.52 (42)+	0.03(3)i		0.56 (45)i	0.48 (39)	1.24	1.99	3.38
		2.5	80	0.58 (47)+	0.03(3)i		0.62 (50)i	0.58 (47)	1.30	2.40	3.90
		5.0	80	0.87 (70)+	0.06(5)i		0.93 (75)i	0.88 (71)	1.63	3.63	4.69

NC: Negative control; PC: Positive control; EMS: Ethyl methane sulfonate.

[a] Statistical diagnoses following Frei and Würgler [35]: +, positive; −, negative; i: inconclusive; m: multiplication factor; significance levels $\alpha = \beta = 0.05$.

[b] Including rare *flr*^{3} single spots.

[c] Considering mwh clones from mwh single and twin spots.

[d] Frequency of clone formation per 10^5 cells= (n/NC) X 10^5; n: the number of *mwh* clones, N: number of wings examined for each treatment.C: the number of cells scored in each wing (approx. 24400) Shilpa et al.[24].

[e] Balancer chromosome *TM3* does not carry the *flr*^{3} mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

Table 2
Frequency of wing mutant spots observed in the marker trans-heterozygous descendants (MH; *mwh/flr*^{3}) and balancer heterozygotes (BH; *mwh/TM3*) of *D. melanogaster*, derived from the High Bioactivation cross (HB) treated with different BPA concentrations or positive control(EMS) or negative control.

Genotype	Treatment compound	Con.	Number of wings (N)	Spots per wing (no. of spots) statistical diagnosis[a]						Frequency of clone formation per 10^5 cells[d]	
				Small single[b] spots (1–2 cells) (m = 2)	Large single[b] spots (> 2 cells) (m = 5)	Twin spots (m = 5)	Total spots (m = 2)	Total *mwh* clones[c] (n)	Mean *mwh* clone size class[c](î)	Without size correction $f_h = (n/NC)*10^5$	With clone size correction $f_h^1 = (2^{(i-2)})*f_h$
MH; mwh/flr^3	NC	0.0	80	0.22 (18)	0.02(2)	0.00(0)	0.25 (20)	0.22 (18)	1.05	0.92	1.77
	PC: EMS (mM)	0.1	80	1.5(120)+	0.62(50)+	0.5(40)+	2.62 (210)+	2.31(185)	1.90	9.47	10.13
	BPA (μg/mL)	0.1	80	0.40 (32) +	0.03 (3)i	0.00(0)i	0.43 (35)i	0.41 (33)	1.14	1.69	3.06
		1.0	80	0.48 (39) +	0.03 (3)i	0.00(0)i	0.52 (42)i	0.48 (39)	1.20	1.99	3.47
		2.5	80	0.57 (46)+	0.07 (6)i	0.00(0)i	0.65 (52)i	0.63 (51)	1.28	2.61	4.30
		5.0	80	0.90 (72)+	0.10 (8)i	0.00(0)i	1.00 (80)i	0.96 (77)	1.32	3.94	6.28
BH; mwh/TM3	NC	0.0	80	0.21 (17)	0.01 (1)	[e]	0.22 (18)	0.21(17)	1.00	0.87	1.74
	PC: EMS (mM)	0.1	80	0.37(30)+	0.18(15)+		0.56 (45) i	0.50 (40)	1.87	2.04	2.24
	BPA (μg/mL)	0.1	80	0.38 (31)+	0.01(1)i		0.40 (32) i	0.37 (30)	1.07	1.53	2.91
		1.0	80	0.47(38)+	0.02(2)i		0.50 (40) i	0.43 (35)	1.16	1.79	3.19
		2.5	80	0.58(47)+	0.01(1)i		0.60 (48) i	0.56 (45)	1.25	2.30	3.87
		5.0	80	0.85(68)+	0.02(2)i		0.87 (70) i	0.83 (67)	1.28	3.43	5.65

NC: Negative control; PC: Positive control; EMS: Ethyl methane sulfonate.

[a] Statistical diagnoses following Frei and Würgler [35]: +, positive; −, negative; i: inconclusive; m: multiplication factor; significance levels α = β = 0.05.1

[b] Including rare *flr*^{3} single spots.

[c] Considering *mwh* clones from *mwh* single and twin spots.

[d] Frequency of clone formation per 10^5 cells = (n/NC) X 10^5; n: the number of mwh clones, N: number of wings examined for each treatment. C: the number of cells scored in each wing (approx. 24400) Shilpa et al. [24].

[e] Balancer chromosome *TM3* does not carry the *flr*^{3} mutation and recombination is suppressed, due to the multiple inverted regions in these chromosomes.

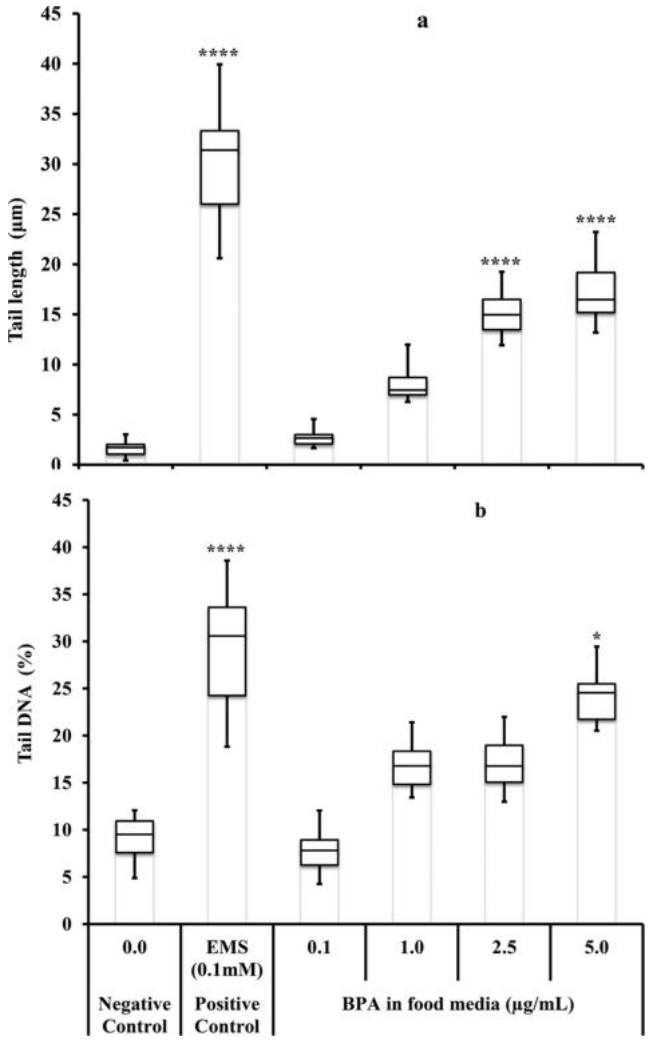

Fig. 4. DNA damage was analysed in third instar larvae (72 ± 2 h) reared in the control or BPA treated groups using alkaline Comet assay. Box plots for two comet assay parameters such as (4a) tail length and (4b) % tail DNA. Each box plot reflects distribution of data indicating 25th and 75th percentile as box, median as a line within the box, maximum and minimum values as whiskers. Box plot without the asterisk (*) are not significant compared to control at $p > 0.05$; and significant at *$p < 0.05$; **** $p < 0.0001$ level.

assay can detect aneuploidy [33] and positive single spots were observed in this study, it can be inferred that significant induction of single spots may be due to non-disjunction. Hence, the genotoxic effects of BPA could be considered as mutagenic in *Drosophila*.

4.3. BPA induced DNA damage in hemocytes

The significant increase in Comet tail length and % tail DNA were observed in hemocytes of *Drosophila* larvae reared in highest concentration of BPA treated group than negative control (Fig. 4a and b). This clearly revealed that BPA caused increased DNA strand-breaks along with DNA migration from nucleus into Comet tail. Present results were also consistent with previous studies conducted, which showed a significant increase in tail length and % tail DNA after BPA treatment [16]. The DNA damage detected by Comet assay in this study reveals that BPA has a potential to induce genotoxicity in *Drosophila*.

The present results provide evidence that DNA damage observed after BPA exposure could be a consequence of free radical attack to DNA. Since elevated expression of ROS and LPO have indicated the generation of oxidative stress by BPA (Fig. 2a and b), one can presume that the DNA damage in this study might be a product of such oxidative stress. Overproduction of ROS is one of the critical factors in the DNA damage induction [18]. An increased level of 8-hydroxydeoxyguanosine and LPO, along with decreased glutathione activity, suggests that oxidative stress could be one of the mechanisms of BPA genotoxicity [16]. Generation of ROS and LPO was shown to cause chemical modification and alteration in DNA including base modification and strand break [56]. The possible mechanism for the occurrence of DNA breaks in the BPA treatments could be due to increase in rate of free radical formation. These strand breaks are directly produced by genotoxic agent or as a consequence of incomplete excision repair sites or alkali-labile apurinic sites [57]. BPA can induce DNA strand breaks detected by the Comet assay in hemocytes of *Drosophila*, and this DNA damage could result in somatic mutation, as evidenced in the wing-spot test (Tables 1 and 2). The observed genotoxic effects in the *Drosophila* model can be explained by oxidative stress induced by BPA treatment. However, the mechanisms of BPA induced oxidative DNA damage need to be further explored.

5. Conclusion

BPA is ubiquitously present in the global environment and its human exposure is unavoidable. Presence of BPA in humans has been correlated with reproductive and genetic abnormalities and onset of several diseases. The strains of *Drosophila* were reared in 0.1, 1.0, 2.5 and 5.0 µg/mL BPA treated food media from the embryonic stage (egg); biochemical and genotoxic effects were analyzed in this study. BPA induces overproduction of ROS and LPO and reduced antioxidant enzyme activities. Positive single spots were observed in wing spot test indicating its mutagenic effect. Increased tail length and % tail DNA in Comet assay reveals BPA induced DNA damage. Induction of oxidative stress mediated genotoxicity of BPA observed in the *Drosophila* could be one of the possible mechanisms for the onset of various diseases in humans upon BPA exposure. Further study is required to decipher the molecular mechanism involved in the BPA induced oxidative free radicals causing DNA damage.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.07.050.

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