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Bisphenol A induced decline in male fertility: Oxidative stress play important role in sperm DNA damage.

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ABSTRACT
Bisphenol A has adverse effect in male reproductive health, resulting into decrease in sperm count, viability, abnormal sperm motility, morphology and reduction in reproductive organ weight. Studies have affirmatively revealed that BPA causes overall reduction in male fertility rate. Present study focussed on the, role of oxidative stress in the newly formed sperm DNA damage was assessed and compared with level of 8-OHdG in testicular tissues. Healthy male rats of normal fertility were randomly distributed in to four groups consisting of 10 animals each. Group I: Sham control, Group II-IV: Daily oral administration of 10, 50, and 100 mg BPA/kg, respectively for 45 days. Body and organ weight were estimated to confirm gross toxicity. Fertility record was maintained throughout the investigated period. Comet assay was performed on testicular sperms to investigate DNA damage along with evaluation of oxidative DNA damages through 8-OHdG marker. Results of the study indicated dose dependent reduction body and testicular weight. A significant decline in fertility rate of BPA exposed rat was evident, following 45 days of daily administration where Group III and IV showed 30% reduction in fertility. Level of 8-OHdG significantly increased in BPA exposed rats comparing to control. Significant increase in oxidative DNA damage was evident between doses of BPA. No significant sperm DNA damage was evident in rats exposed to 10 mg and 50 mg BPA/kg body weight, whereas, nearly 5-folds increase in sperm DNA damage was apparent in rats exposed 100 mg BPA/kg comparing to control. It was concluded that high daily doses of BPA can induce DNA damage in newly formed sperms. Besides common adversities, reduction in fertility of exposed male rats may also be contributed by DNA damages in sperm.
Key Words: Bisphenol A, Comet assay, Male fertility, 8-OHdG marker.

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INTRODUCTION

There are concrete evidences that bisphenol A (BPA) has negative impact on human fertility [1,2,3]. One of the most acceptable reasons of its antifertility activity is its endocrine-disrupting properties [4]. BPA uniquely and deliberately act on male fertility. It particularly binds with androgen receptor and act as an antagonist [5]. Previous data suggested that doses which were considered safe earlier, were also compromised spermatozoa, and disrupted hypothalamic-pituitary-gonadal axis [1]. Testicular dysgenesis syndrome and cryptorchidism have been linked to BPA exposure [5]. In addition, BPA has been associated with Leydig cell proliferative activity and decrease in androgen secretion [6]. The European Chemical Agency has enrolled BPA as substance of very high concern, specifically for its endocrine disruption ability [7].

Deleterious effects induced by BPA toxicity have intrinsic relation with oxidative stress. Oxidative stress here, is not only restricted to exclusive radicals generated through BPA exposure but the pre-existing homeostasis between antioxidants and normal oxidative by-products also play important role. There is ample information available that suggested production of excessive oxidative stress and significant decrease in antioxidant defence contribute to BPA led cellular toxicity [7,8,9]. Oxidative stress alone plays important role in testicular functions. Testicular cells go through high rate of cell divisions and therefore, consequential increase in consumption of oxygen in mitochondria is inevitable [10].Moreover, supply of

oxygen through testicular arteries is demanding, hence, competition for oxygen between cells is extremely high [11]. Increased production of peroxides under extreme competition has been related to sperm functions [12]. Previous studies have profoundly mentioned that free radicals modulate sperm count, motility, and morphology [13].Oxidative stress can also significantly increase DNA damage in sperm which is protected by protamines inside the sperm head [14].

BPA has been correlated with impairment of spermatogenesis[15,16,17] whether it damages DNA inside the sperm head during spermatogenesis is still elusive. A study by [18] reported that BPA concentration between range of 0.8-2.5 ng/ml in urine was associated with higher DNA damage in sperm. In the present study, an attempt was made to investigate associations between fertility status, BPA induced oxidative stress and sperm DNA damage in testicular tissues of male albino rats.

MATERIAL AND METHODS

Test material

Bisphenol A (2,2-bis (4-hydroxyphenyl) propane or $C_{15}H_{16}O_2$) of ≥99% purity was purchased from Sigma Aldrich (CAS Number 80-05-7) (MO, USA).

Test animals

Male Wistar albino rats (*Rattusnorvegicus*) of age 3 months, weighed in range of 150-200 g were used in the present study. Rats were maintained in veterinary supervised departmental animal's house. Animal house was ensured with 12 h light and 12 h dark schedule. Based on groups test animals were kept in polypropylene cages of size 43×27×15 cm, throughout period of investigation. Experiments performed in this study was strictly followed guidelines of Committee for the Purpose of Control and Supervision of Experiments and Animals [19]. In addition, protocol used in the study was approved by the Institutional Animal Ethics Committee (IAEC). All experiments were performed under guidelines of Indian National Science Academy (INSA), New Delhi for Care and Use of Animals.

Experimental design

Male rats of proven fertility were randomly divided into four groups consisting of 10 animals each. Group I designated as placebo control, administered with olive oil used as a vehicle to dissolve BPA. Group II administered with 10 mg BPA/kg dissolved in olive oil, Group III was administered with 50 mg BPA/kg and Group IV was given 100 mg BPA/kg body weight. BPA was dissolved in olive oil in 1:1 ratio and administered daily through oral gavage technique. All groups were investigated in parallel with each other for 6 consecutive weeks.

Body and reproductive organ weight

Initial and final body weight of all animals were measured at commencement and termination of experiment. The weight of testis was obtained at the time of euthanization schedule of each group.

Fertility test

Periodical fertility tests were conducted for all groups by cohabiting the male with female rats in 1:2 ratios. Mating was confirmed by visual appearances and presence of spermatozoa in the vaginal smear. Following mating pregnant animals were allowed to complete the term and pregnancy record was maintained.

Single cell gel electrophoresis analysis of sperms

Comet assay was carried out according to the methods of [20], with few modifications. In brief, A 80 µl of 0.7% agarose was placed on to the frosted glass slides, a coverslip was placed on top of the agarose and allowed to settle. Once settled, coverslip was removed gently, and second layer of 80 µl of agarose containing 10 µl of sample was placed on top of the first layer. Sperm samples were pre-treated with freshly made lysis buffer (DDT, 10 mM-30 min at 4 ºC; LIS, 4 mM- 90 min at 20 ºC). A coverslip was then placed on top of the second layer and allowed to settle. Once settled, coverslip was gently removed and a third layer of 0.7% agarose was then placed on top of the second layer and coverslip was placed for even distribution. Coverslip was gently removed once the gel was settled. Sandwiched sample was washed into pre-chilled distilled water, and placed into electrophoresis chamber. Electrophoresis buffer was then poured into the chamber until slides were completely submerged. Electrophoresis was carried out at 24 V (0.7 V/cm) and 330 mA for 30 minutes. Following electrophoresis sample containing slides were neutralized in neutralizing buffer (0.4 M Tris-HCL-pH 7.4). Slides were later precipitated by ethanol and air dried for further analysis. Comets were examined by staining with ethidium bromide and observed under confocal fluorescent microscope with dual filter excitation wavelength. Analysis of comets were carried out by freeware software Comet Score (TriTek Software v1.5, USA). A total of 25 comets were analyzed per rat. Olive moment, tail length and percentage DNA were evaluated for each comet.

Detection and localization of oxidative stress marker

Measurement of oxidative stress marker, 8-hydroxy-2'deoxyguanosine (8-OHdG), in testicular tissues was conducted through PCR/ELISA as instructed by kit manufacturer (abcam, Cambridge, UK). Briefly,

reagent and standard were prepared according to instruction given in the kit. Samples were homogenized and DNA was extracted by combination of bead-beating and Proteinase K (Thermo Fisher, MA, USA) digestion technique. Extracted DNA was digested by nuclease P1 and added with alkaline phosphatase than incubated at 37 ºC for 30 min. This solution was boiled for 10 min and thereafter placed into ice. Assay was performed by adding 50 µl of 8-OHdG standard, blank, and samples. To this 50 µl of 8-OHdG antibody to each well. Plate was covered and incubated for 1 hr at room temperature. Each well was washed thrice with wash buffer. TMB solution was added to each well and allowed for colour to develop for 30 min at room temperature and absorbance was measured at 450 nm.

Statistical analysis

Results of the study were presented in standard error of mean (SEM). Analysis of variance was estimated by One-way ANOVA in conjunction with Tukey's multiple comparison test (MINITAB, Pennsylvania, US). Significance level was assessed at 0.05, 0.01, and 0.001 and considered as significant, highly significant and extremely significant, respectively. Coefficient of determination (R^2) was estimated to examine dependence in variables of % DNA damage and regression in testicular weight by increase in level of 8-OHdG in testicular tissues.

RESULTS

Body and organ weight

In comparison to Group I, body weight of groups II and III indicated significantly decline over the time of investigation. Initial body weight in Group I was measured as 251±7.95 g, whereas, final weight was measured as 280±3.94 g. Despite similar initial body weight, groups II and III showed substantial reduction in final weight (Figure 1). Mean of average of left and right testes indicated significant decline in all test groups when compared with control (Figure 2). Average of both testes in Group I was measured as 1.58±0.04 g, whereas, 1.44±0.04, 1.29±0.02, and 1.21±0.02 g was measured for groups II-IV, respectively.

Role of BPA on fertility of male rats

Periodical fertility was estimated on day of commencement, 15th day, 30th day, and 45th day. Control male rats showed cent percent fertility on all days of investigation. Group II animals also showed cent percent fertility on 0th, 15th, and 30th day. However, on 45th day only 90% fertility was observed. Administration of BPA in Group III indicated rapid decline in fertility rate which was observed from 15 days onward. On 15th day 90% fertility was observed, followed by 80% and 70%, on 30th day and 45th day of administration, respectively. Despite, a 2 folds increase in dose Group IV animals did not show significant decline in fertility rate comparing to Group III. Following 45 days consecutive administration fertility was recorded to be 70% in Group IV. Similarly, 70% fertility was also observed on 30th day of investigation which remained unaltered until 45th day of investigation (Table 1).

Single cell gel electrophoresis assay

Single cell DNA damage quantification of testicular sperms were carried out by comet assay. Images of comets were used for measurement of olive moment (length between centre of the cell to end of the tail) and tail length (length between edge of the cell to edge of tail). Percentage DNA was estimated automatically through Comet Score (TriTek Software v1.5, USA). Noise reduction (65%) was applied to reduce unwanted pixels (px) from original images (Figure 3). Intensity of pixels were measured automatically by the software and respective olive moment, tail length, and percentage DNA in tail was displayed. Mean of olive moment, and tail length were estimated in test groups and compared with control. Olive moment and tail length in Group I animals were measured as 3.64±0.18 and 12.70±0.92 px, respectively. Extremely significant increase in olive moment and tail length was recovered in Group IV which was noted as 13.96±0.37 and 50.36±3.84 px, respectively. Nonetheless, no alteration in both parameters were observed in groups II and III (Figure 4). Likewise, mean of percentage DNA damage was measured in Group I as 6.21±0.29%, whereas, 5.45±0.65%, and 5.93±0.57% was estimated in groups II and III, respectively. No alteration in percentage DNA damage was observed in groups II and III, with respect to control. However, extremely high percentage of DNA damage was evident in Group IV testicular sperms. The percentage DNA damage in Group IV was noted as 28.88±1.60% (Figure 5).

Evaluation of oxidative stress by 8-OHdG marker

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) is an oxidized derivative of deoxyguanosine formed following major DNA oxidation. Steady level of DNA damage is a balance between normal damage and repair. Level of 8-OHdG in testicular tissues of Group I was noted as 0.71±0.16 ng/ml, whereas, 1.50±0.25, 2.94±0.33, and 3.45±0.32 ng/ml. A gradual increase in oxidative damages was observed according to the increase in doses of BPA (Figure 6). Each test group showed significant increase in level of 8-OHdG with respect to doses i.e. Group II-III ($p=0.001$), Group III-IV ($p=0.019$), and Group II-IV ($p=0.0002$).

Coefficient of determination between data through regression prediction

Analysis of relatedness between three separate variables such as; level of 8-OHdG, % DNA damage in testicular sperms, and testicular weight was carried out. Since dose dependency in the level of 8-OHdG was clearly evident, it was used to predict goodness of fit between other two variables i.e. % DNA damage, and testicular weight. Results showed strong relatedness between variables of testicular weight and level of 8-OHdG in testicular tissues (R^2=0.988). However, comparatively weak likeness (R^2=0.458) between level of 8-OHdG and %DNA damages was apparent (Figure 7).

DISCUSSION

DNA damage due to oxidative stress is a consequence of cellular metabolism subsequent to increased level of toxicity. Among the reactive oxygen species, hydroxyl radical reacts with DNA by forming double bonds and releasing hydrogen from methyl group of thymine and 2'-deoxyribose [21]. BPA can impair the antioxidant-oxidative radical homeostasis through direct and/or indirect mechanism [22,23]. Phenoxyl radicals are generated by BPA, it can react with reduced nicotinamide adenine dinucleotide phosphate (NADPH) [24]. The oxidative radical induced NADPH oxidases (NOXs) have been recognized as main source of reactive oxygen species. A study by [25], revealed that NOX4 plays important role in oncogenic Ras induced DNA damage. Earlier BPA has been associated with DNA damage, as revealed by a study that upon administration of 50-100 μM of BPA, pancreatic cells undergo higher DNA damage [26]. Despite, various studies on BPA toxicity in male reproductive organs[27] and spermatogenesis[28], its exclusive effect on sperm DNA integrity is still elusive. Present study examined DNA damage in the newly produced sperms in testis of rats administered with various doses of BPA.

Body weight in all BPA administered groups declined significantly comparing to initial body weight. Sudden reduction in body weight is a sign of gross toxicity. In 6 weeks of investing period, 10-25% decline in body weight of BPA administered animals was found. Whereas, at least 10% increase in body weight of control animals was highly significant. Although BPA has been associated with increased risk of metabolic disorder and obesity due to its potential effect on the endocrine-metabolic pathways[29,30], higher doses of BPA may lead to significant reduction in body weight [31]. Similar results were observed in female rats, where administration of 4-5 mg BPA/day for 15 days has been reported with reduction in body weight gain [32]. In the present study, daily administer of 10, 50 and 100 mg BPA/kg was expected to induced significant decline in body weight. Since significant decline in body weight of BPA administered rats was evident, decrease in organ weight was anticipated. A study by [33]reported that a 9% decline in body weight can reflect in reduction of vital organs by almost 6%. This study witnessed significant testicular atrophy following 6 weeks administration of BPA. Previous studies have also reported testicular atrophy in BPA treated males [27].

Fertility status of males administered with BPA indicated a dose dependant response. However, dose dependency was only visible until 30 days of daily administration. Following 45 days of administration similar response was observed in both 50 mg and 100 mg BPA administered animals. It was also interesting to note that with 10 mg BPA no decline in fertility was observed until 30 days of administration. Present study indicated that despite high daily doses of BPA, decline in fertility rate was slow and limited. Animals administered with 100 mg BPA/kg showed 70% fertility following 30 days of administration, whereas, following 45 days of daily doses the rate remained same. It shows stabilizing effect of BPA on decline in rate of fertility. To this day there are no reports which claim cent percent sterility due to BPA exposure. Although there are numbers of studies associated BPA with reduced sperm count, motility, sperm functions, steroidogenesis, normal morphology etc [4]. Therefore, it can be speculated that although BPA does affect fertility its role is largely limited to testicular functions.

Comet assay of testicular sperms indicated similar results in animals administered with 10 and 50 mg BPA/kg which comparable to control. It reflects no interference of BPA at these dose levels in post-meiotic or spermiogenesis phase. During post-meiotic phase sperm stem cells contain histone as condensation protein for genetic material which is replaced by transition protein in round spermatids and further replaced by protamines in elongating spermatids [34]. Interestingly, percentage sperm DNA damage in 100 mg BPA/kg administered group showed extensive increase. Spermatogenesis is a complex process generate nearly 1000 sperm per second [35]. High numbers of cellular division during spermatogenesis require higher oxygen consumption leading to competition for vital elements within testicular compartments. At such scenario chemically induced oxidative stress can cause severe impairment. Previous studies have noted BPA induced disruption in meiotic progression such as; chromosome segregation, end to end chromosome association and also delay in meiotic progression [36,37,38]. It is assumed that 10 and 50 mg BPA/kg could not generate sufficiently enough oxidative stress that may penetrate pre-existing anti-oxidative mechanism. Sperm genetic materials are unique in many sense from any other eukaryotic cells [39]. Protamines, a sperm-specific nuclear protein, plays important role in packaging and structural stability of genetic materials [40].Decrease in protamine

content has been associated in DNA damage in sperm [41]. DNA damages in sperm indicate dysfunctional packaging system. Thus, it is highly likely that 100 mg BPA/kg causes damages to genetic materials while sperm undergo packaging of genetic materials, specifically, during transition of histone protein to transitional protein and to the protamines.

There is a direct correlation between oxidative stress and testicular weight [42].Likewise, level of 8-OHdG in testicular tissues also indicate extent of imbalance between antioxidants and oxidative radicals. Moreover, it relates oxidative insult with oxidative DNA damage. In normal somatic cell DNA damage corresponds to the inability of DNA repair mechanisms in minimizing number of single-strand breaks. Single strand breaks are more common in histone bound chromatin comparing to protamine bound [43]. Results of single cell gel electrophoresis indicated extensive DNA damage in testicular sperm is indicative of major interference by BPA induced oxidative stress in the post-meiotic spermatogenic phase. Through coefficient of determination, power of respective related ness between 8-OHdG, testicular weight, and percentage DNA damage in sperm can be elucidated. Present study showed that there is strong dependence between level of 8-OHdG and testicular weight, which interpreted as exclusive role of oxidative stress in inducing higher rate of DNA damage and testicular atrophy. However, weak relatedness between level of 8-OHdG and percentage DNA damage in testicular sperm was evident. Level of 8-OHdG does not completely corresponds to percentage DNA damage in sperm, as the DNA repair mechanism constantly repairs the single strand breaks. Presence of higher DNA damage in sperms is most likely due to exhaustion of repair mechanism or apoptosis. Though apoptosis is uncommon in spermatozoa, previous studies suggest that exposure of phosphatidylserine on the plasma membrane leads to sperm apoptosis [44,45].The present study indicated that reduction in fertility of rats exposed to 100 mg BPA/kg may have been contributed by DNA damage in testicular sperms.

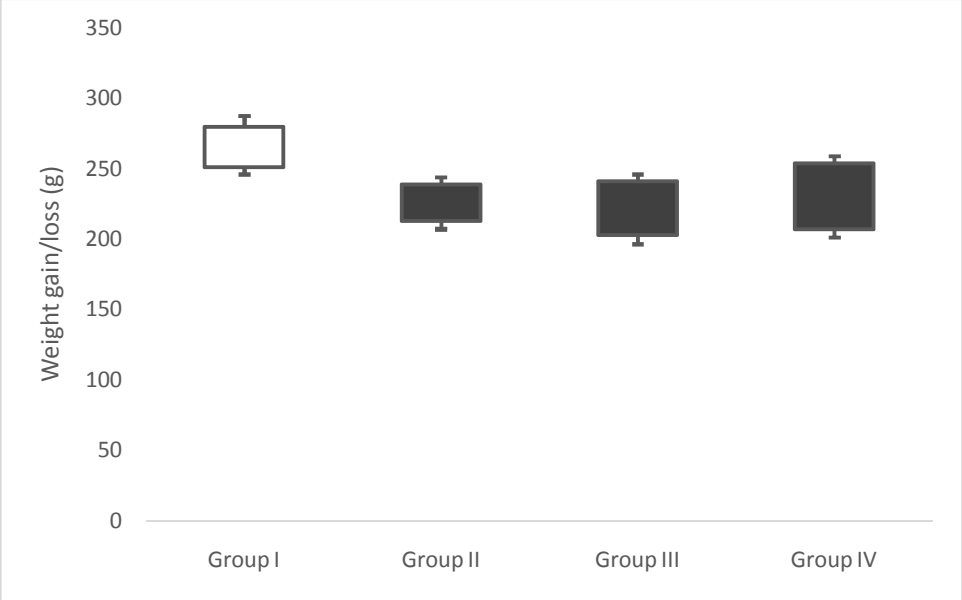

Figure 1: Weight gain/loss in groups administered with BPA, along with parallel sham control.

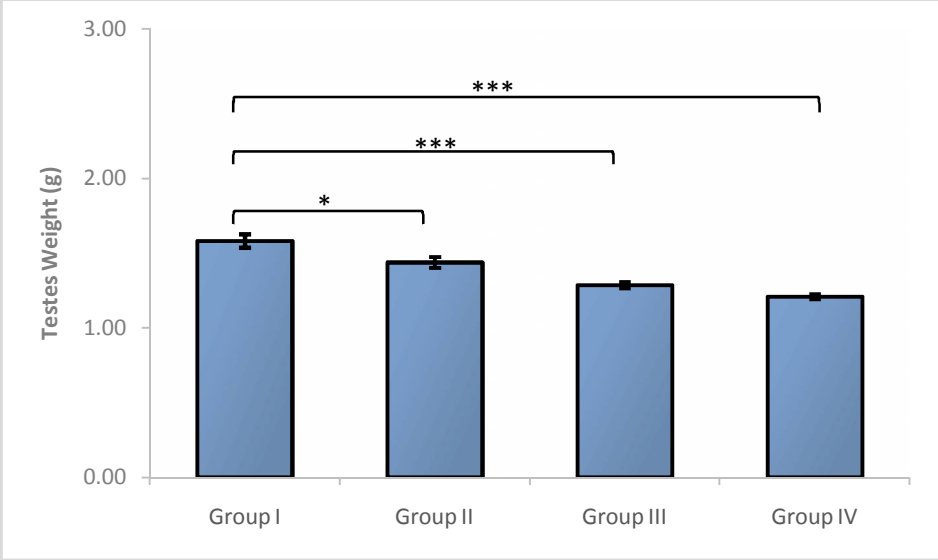

Figure 2: Mean of average of both testes (left and right) of test groups and respective control group.

Table 1: Fertility rate in animals treated with various doses of BPA against control. Values are represented in percentage (%).

	Group I	Group II	Group III	Group IV
0 days	100	100	100	100
15 days	100	100	90	80
30 days	100	100	80	70
45 days	100	90	70	70

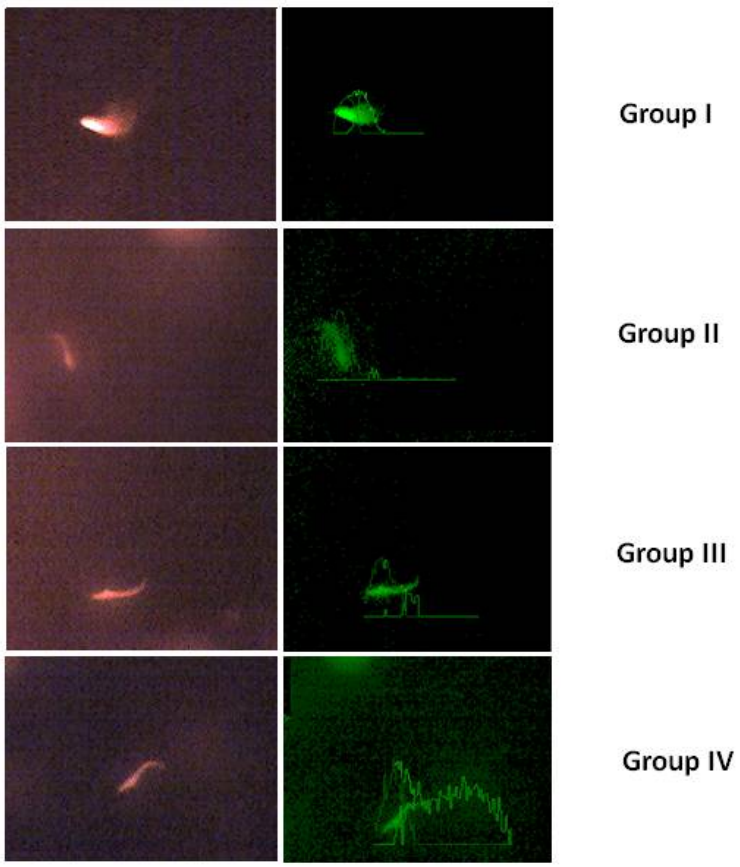

Figure 3: Representative group-wise images of comets observed in testicular sperm. A. Original images of sperm following EtBr staining, B. Images followed by 65% noise reduction and software acquisition.

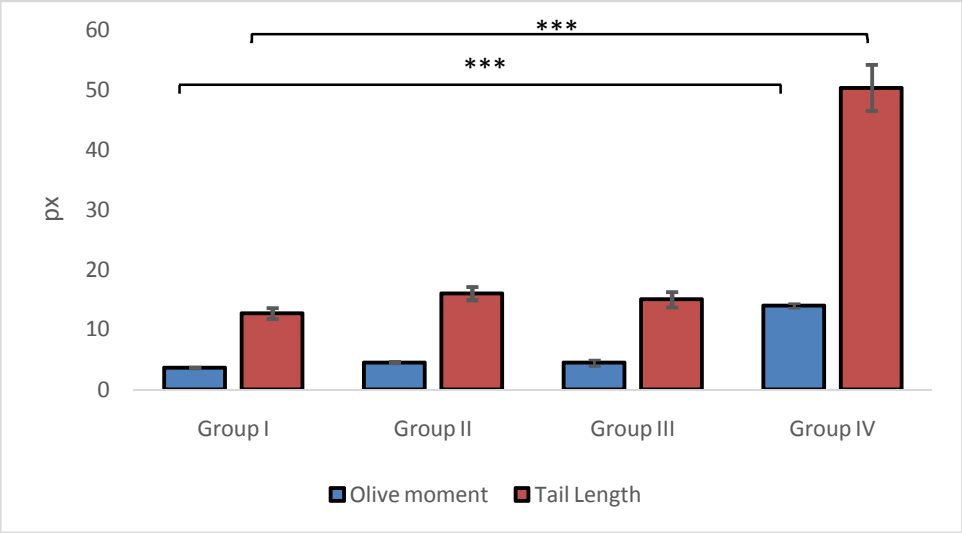

Figure 4: Olive moment and tail length in testicular sperms of groups administered with BPA along with the parallel control. Values are in pixels (px) which was used by Comet Score (TriTek Software v1.5, USA) as measuring unit.

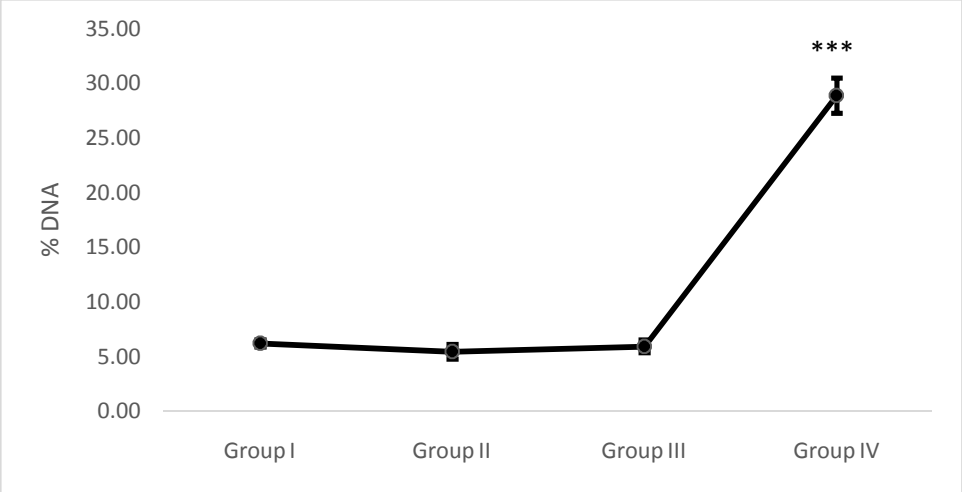

Figure 5: Percentage DNA in tail of comets analyzed in test groups and control percentage DNA in tail is considered as percentage of DNA damaged in single sperm.

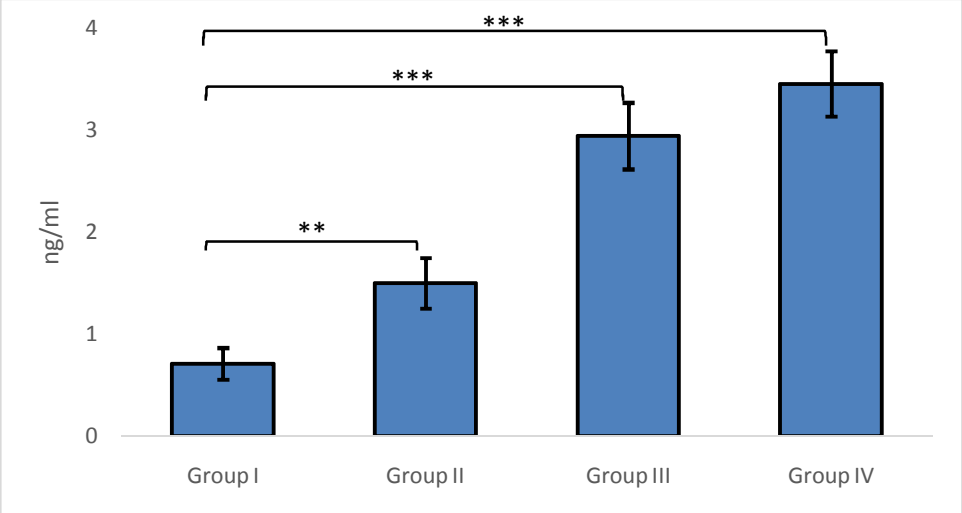

Figure 6: Level of 8-OHdG in testicular tissues following administration various doses of BPA against control.

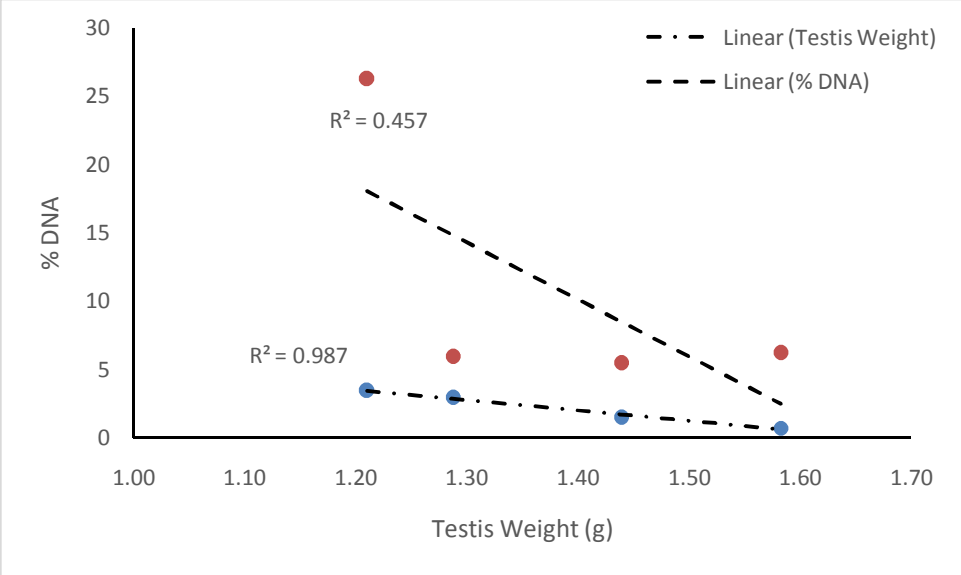

Figure 7: Coefficient of determination between three separate variables were analyzed. Variables of level of 8-OHdG was examined against testicular weight and %DNA damage in testicular sperm.

CONCLUSION

The present study concludes that BPA induced oxidative toxicity not only interferes with spermatogenesis and reduce male fertility but also induce oxidative damages in genetic materials those are about to package in the newly formed sperm. Daily doses of 10 and 50 mg BPA/kg has no impact on sperm DNA irrespective of significantly higher level of 8-OHdG in testicular tissue comparing to control. Which indicated toleration against oxidative damages in sperm DNA preserving genetic integrity. Nevertheless, extensive DNA damage in testicular sperm of rats administered with 100 mg BPA/kg, revealed insufficiency of DNA repair mechanism and interference in post-meiotic phase of spermatogenesis. Therefore, decline in male fertility due to BPA induced oxidative insult is likely to be complimented with subsequent DNA damage in newly formed sperms.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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