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ASSESSMENT OF DELETERIOUS EFFECTS OF BISPHENOL A (BPA) ON STEROIDOGENESIS, SPERM COUNT, AND SPERMATOGENESIS IN A MAMMALIAN MODEL

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ABSTRACT

The current study was designed to demonstrate spermatotoxicity of Bisphenol A (BPA) in adult albino male mice, 40 adult albino mice (8 weeks old; weighing 25±5 gm) were randomly categorized into four groups following Completely Randomized Design i.e., G-I (Untreated) G-IV (Vehicle control) G-II (600mg/Kg of B.W) and G-III (300mg/Kg of B.W) respectively. Experimental groups (G-II & G-III) were administered with desired BPA doses (600mg/Kg & 300mg/Kg of B.W) for four weeks routinely through oral gavage, meanwhile, G-I was not given any treatment and G-IV was given soya bean oil. Mice were dissected the next day of the last treatment under deep anesthesia for the collection of blood and testis tissues for further analyses. Microscopic and micrometric examinations showed that BPA had reduced the body as well as testes weight as compared to control. It was also revealed by the biochemical analysis that testosterone hormone level was lowered significantly in BPA exposed groups, which resulted in decreased sperm cell count against control. It severely damaged the seminiferous tubules and interstitial cells. Histological observations showed deformations like Leydig cell apoptosis, oligospermia, tubular destructions in BPA exposed HE stained testis sections. The parameters i.e., body and testes weights, testosterone level, sperm count cells, and Johnson scoring had been found significantly ($P \leq 0.05$) altered after BPA intoxication. Numerical data were analyzed statistically via analysis of variance (ANOVA) by using software SPSS (Statistical Program for Social Sciences, version 20) followed by Tukey test to see differences among groups. These results revealed that BPA is a reproductive toxicant in mice.

Keywords: Bisphenol A, Spermatotoxicity, Seminiferous tubules, Histology, Sperm count.
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INTRODUCTION

Urbanization as well as modernization tends to a drastic change as well as demands for a suitable and compensative alternate of metals for the industries. Plastic was introduced appropriate and reliable more importantly cheaper option, to meet the metal shortage in industries. Bisphenol A (BPA) is the major constituent in plastic compositions (Galloway, 2015). Studies reported BPA as well as phthalates are toxins, which leak out from the packing into the food and water contents (Rudel *et al*., 2011). BPA is being consumed in every field all over the world ranging from medical appliances, food items packing electronic, printing industries and currencies (EFSA Panel on Food Contact Materials and Aids, 2015; Schecter *et al*., 2010). In addition to the above direct exposure, there are several indirect sources like leaching through polycarbonate products, thermal paper recycling causes the incorporation of BPA to the living beings (Ragavan *et al*., 2013).

It was estimated that BPA average production would reach near 9.6 million metric tons in 2020 (Analysts, 2016). The safe tolerable daily intake (TDI) of BPA reported by the European Food Safety Authority was 4 µg/kg of BW/day (EFSA Panel on Food Contact Materials and Aids, 2011).

The easiest way of BPA incorporation in human society is via food packing. BPA leaks out from artificial synthetic Crockery and becomes a part of the food (Faheem *et al*., 2016; Omari *et al*., 2018). BPA is also used in epoxy resin, material consumed for internal lining in metallic cans to prevent mixing of eatable with manufactured material (vom Saal and Welshons, 2014), So recurrent exposure to BPA is unavoidable. The neonates were found to be habitual to use feeding bottles by their mothers. Sometimes people compromise the quality of plastic used in the manufacturing of the feeding bottles. BPA-used plastic of low quality becomes drastic for the new incumbent even at a small quantity at this sensitive age (Russo *et al*., 2018; Speidel *et al*., 2018). In Pakistan too, poor quality of plastic was being used in industries and reported lethal for human society (Taskeen, 2012; Fawzy *et al*., 2018).

The prevalence of BPA toxicity is found proportional to the repetitive exposure to the living ones along with intensity, quantity, and duration. Another negative effect of BPA as it masks the androgenic role during endogeneity activity. BPA have destructive impacts on organ especially kidney, liver, heart, and testis

(Karnam *et al*., 2014; Tiwari and Vanage, 2017). This was not only a causative agent for deleterious effects in tissues but also alters the basic mechanism of various body functions. BPA is involved in neural, gene toxicity, estrogenic, behavioral alternations, carcinogenesis, and developmental toxicity in addition to the cardiac and hepatic damages. Sometimes BPA along with other pollutants, causes extensive damage to disrupt the normal mechanism by interacting with estrogen receptors α and β, lead to change in cellular proliferation, migrations as well as apoptosis (Paulose *et al*., 2015; Watanabe *et al*., 2016).

A lot of researches are going on the carcinogenicity of BPA. Several types of research have investigated reproductive toxicity of BPA (Yousaf *et al*., 2016; Ijaz *et al*., 2019), and extensive reviews were published to address the strength of the evidence regarding BPA-induced organ toxicity (Peretz *et al.,* 2014). The panel of experts focused on both *in vitro* and *in vivo* studies found that from the standpoint of reproductive toxicity data is not yet conclusive due to various exposed concentrations in various models. So the study was designed to specify testicular damages in mammalian tissues on various BPA concentrations to explore the minimum tolerable limit.

MATERIALS AND METHODS

Animal Husbandry and Chemicals: Bisphenol A ((BPA) 99.9% pure) obtained from "Duskan" USA chemicals. Pure and fresh soya bean Oil obtained from the local market, Akbari Mandi Lahore Pakistan. Forty adult albino mice (*Mus musculus*) of Webster strain were utilized in research work as experimental animals having body weights between 25±2 gm. These mature male mice were purchased from the University of Veterinary and Animals Sciences, (UVAS), Lahore Pakistan. Mice were shifted and kept for a week undisturbed under standard conditions at Animal House of the Department of Zoology, University of the Punjab, Quaid e Azam Campus, Lahore, Pakistan, for acclimatization as reported previously (Khawar *et al*., 2020). To cut down the risk of BPA exposure, stainless steel cages were used instead of plastic cages. Glass bottles and metallic bowls were used to provide water and feed. Chick feed (National No. 14) and water were supplied *ad libitum* to all animals.

Dose preparation and administration: Two Sub-lethal doses, 12.5 and 25% of LD_{50} (2400 mg/Kg/BW) were prepared (Hussein and Eid, 2013). BPA pellets were subjected to fine powered and dissolved with soya bean oil. Forty mice (8 weeks old; weighing 25±5 gm) were divided equally following Completely Randomized Design (CRD) in four groups (n=10). Group G-I was untreated. Mice of the groups G-II and G-III were exposed to a high dose (600 mg/kg/body weight and low dose (300 mg/kg/body weight) respectively. Group G-IV was termed as vehicle control, was given soya bean oil 0.1 ml per animal/day.

Dissection and samples recovery: There was oral administration of BPA and soya bean oil via gavage for consecutive four weeks. After 24h of the last treatment mice we euthanized by anesthetic inhalation (5% isoflurane) blood was taken out through cardiac puncture and testes were dissected out for further studies. Mice husbandry, necropsy, and other analyses were carried out in Developmental Biology Laboratory, Zoology Department, University of the Punjab Lahore, following NIH Publication "Guide for the Care and Use of Laboratory Animals" (NRC 2004) and guidelines by the local bioethical committee of the University on animal experimentation.

Histopathology: To observe the testis's histology several laboratory phases needed for slides preparation; testes specimens were fixed in 10% formalin. Samples were dehydrated by series of ethanol grade, clear with xylene, embedded in paraffin wax, sliced 5 µm sections by a rotatory microtome. H & E (Hematoxylin and Eosin) staining technique was followed by protocols (Bancroft and Gamble, 2008). Microphotographs of histological sections were taken at the following magnification i.e., 40X, 100X, 400X using microscope model no M4000-D Swift, Japan supported by portable camera "Ease-i-Imageur universal".

Jonson's scoring analysis: Besides, to observe histopathological defects, seminiferous tubules were also analyzed according to the Modified Johnson scoring system. In this process, certain numerical values were given a score from 1 to 10 based on the physical examination of seminiferous tubules (Johnsen, 1970). To avoid confusion and mishandling to observe seminiferous tubules slide was rotated in a zigzag manner at the microscope stage. Johnsen scoring was observed in all groups.

Epididymal sperm count: Epididymides were carefully excised from each group separately and macerated in trisodium citrate solution then a drop of this solution was shifted to the Neubauer hemocytometer. The number of sperms was counted in 5 squares (4 corners and the center) in the center grid of both sides and were calculated following the method by (Val and Robledano, 2013).

Hormonal assay: Collected blood was centrifuged at 3000 rpm for 15 min. Obtained serum was stored at -20°C until assayed. The serum testosterone level was quantified through ELISA using a commercially available testosterone reagent kit, following kit instructions (Beijing Sino-UK Institute of Biological Technology).

Statistical Analysis: Numerical data were analyzed statistically via analysis of variance (ANOVA) by using software SPSS (Statistical Program for Social Sciences, version 20) followed by Tukey test to see differences among groups and ($P \leq 0.05$) was considered significant.

RESULTS

All experimental animals were remained normal and not any moribund mice were found during experimentation. Both BPA treated groups (G-II and G-III) showed a clear and remarkable reduction in the body as well as testes weight compared to control (Table 1). Average testes size and diameter of seminiferous tubules were reduced in the dose-dependent fashion in treated groups against control.

Histological sections from control and vehicle control groups showed a regular process of spermatogenesis. Lumens of seminiferous tubules were filled with sperm tails and well-formed interstitial cells were seen in between the seminiferous tubules (Fig.1 A to F). Histopathological observations showed destructive changes in seminiferous tubules along with a decrease in Leydig cells. Debris and slough off cells were observed in between and within the seminiferous tubules of BPA exposed groups. BPA disrupts the growth of spermatocytes causing no or a smaller number of sperms in the lumen. Mostly lumina were vacant unlike the control; there was a rare prediction of all phases of germ cells. In other words, treated groups showed clear signs of necrosis in whole testicular sections in BPA-treated groups (Fig. 1 G-L).

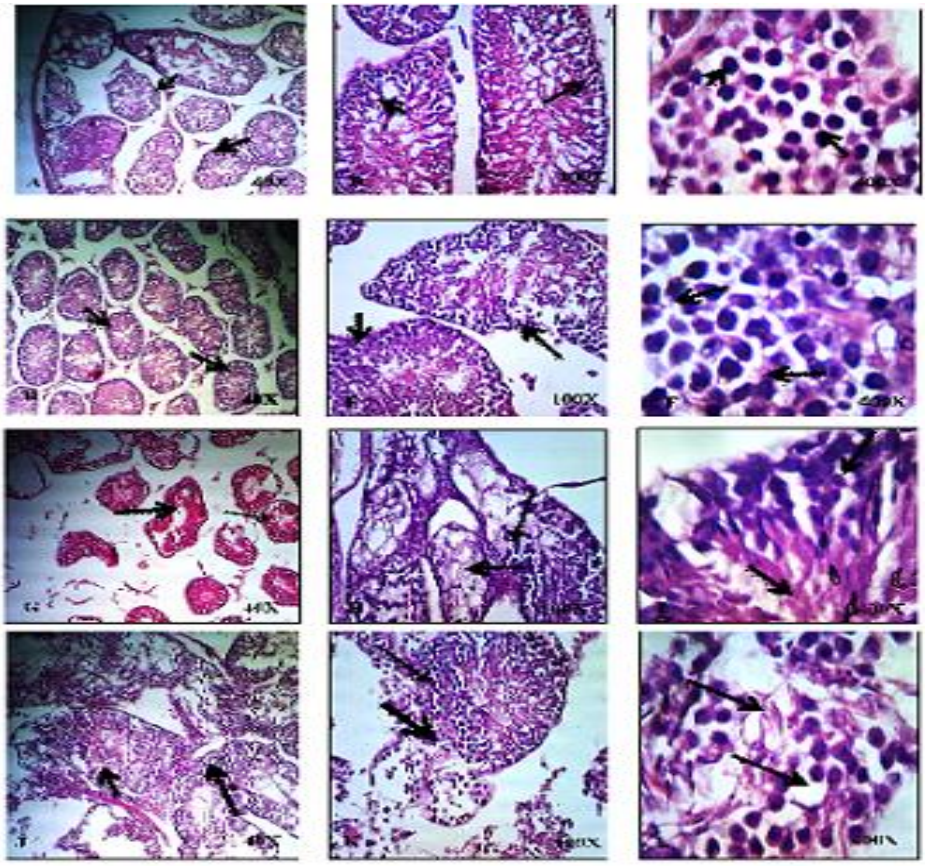

Figure 1: Histological images from control (A-C), vehicle control (D-F). and BPA treated (G-I, high dose); (J-L, low dose) groups respectively at different magnifications. It was shown by the slides microphotographs basement membrane were intact firmly confined to the seminiferous tubules (A-C). Almost all the seminiferous tubules were seen loaded with spermatozoa, primary spermatocytes as well as spermatogonia. The shape and morphology of sperm cells are normal, easily visualize in figures (C, F). Contrary to this, figures (G-L) are consequences of BPA toxicity there was swear destruction indicated by the BPA the Thin & thick arrows. In the above-shown microphotographs, most of the seminiferous were deprived of sperm cells, and lumens are empty, lacking the germ cell in them. In higher magnification, it is evident that from the figure (I, L) that mostly seminiferous tubule cells are lying vacant, Leydig cells

undergo apoptosis. Most of the sperms cell morphology was abnormal, remaining have the size of the caps in sperm cells were slightly larger than the normal size. Even in some regions in the testis' basement membrane was also a rip-off. Stains colorations indicate slough off cells, as well as blood vessels, are ruptured via BPA toxicity. Unlike the (A-F) Lumen formed are mostly empty. (Photographs 40X, 100X, 400X and H &E stained)

After Morphological and anatomic deviations, the biochemical analysis also showed a significant decrease ($P \leq 0.001$) in testosterone levels in BPA treated groups as compared to control, consequently lowering the sperm cell count and inter-related parameters. Sperm count evaluation reported a remarkable decline in the production of sperms in seminiferous tubules. (Table 1).

Table 1. Showing the effect of BPA on morphometric, physical parameters, testosterone level, and sperm count among the various group.

Parameters		Group –I (Control)	Group –II (High dose) (600 mg/Kg Body Weight)	Group III (Low dose) (300 mg/Kg Body Weight)	Group IV (V. Control) (0.1ml /Animal/Day)
Mean body weight initial (gm)		$28.10^{a} \pm 02$	$29.86^{a} \pm 02$	$28.00^{a} \pm 02$	$27.90^{a} \pm 02$
Mean body Weight final (gm)		$29.58^{a} \pm 02$	$26.79^{b} \pm 03$	$27.64^{b} \pm 01$	$28.50^{a} \pm 02$
Mean weight of Testes (gm)		$0.78^{a} \pm 0.2$	$0.59^{c} \pm 0.1$	$0.61^{b} \pm 0.2$	$0.76^{a} \pm 0.2$
Mean testes length (mm)		$09.78^{a} \pm 0.2.$	$07.18^{b} \pm 0.2$	$07.50^{c} \pm 0.7$	$09.15^{a} \pm 0.1$
Mean testes width (mm)		$05.68^{a} \pm 0.1$	$4.12^{b} \pm 0.1$	$04.45^{c} \pm 0.1$	$05.15^{a} \pm 0.1$
Mean diameters of seminiferous tubules (µm)		$241^{a}.30$	$229^{c}.10$	$237^{b}.89$	$239^{a}.10$
Testosterone (ng/ml)		$1.389^{a} \pm 0.67$	$0.348^{c} \pm 0.133$	$0.653^{c} \pm 0.29$	$1.308^{b} \pm 0.054$
SPERM COUNT					
Microscopic examination	**Total Sperm count** million/ml	$45^{a} \pm .80$	$28^{c} \pm .77$	$32^{b} \pm 0.51$	$43.5^{a} \pm 0.56$
Morphology analysis	**Abnormal%**	09	80	73	09
	Normal%	91	20	27	91

Johnson scoring value was observed less in treated (G-II & G-III) as compared to the control (G-I) and vehicle control (G-IV). This showed the frequency of histopathological defects and quantified deterioration of germ as well as supporting cells within the seminiferous tubules (Fig. 2).

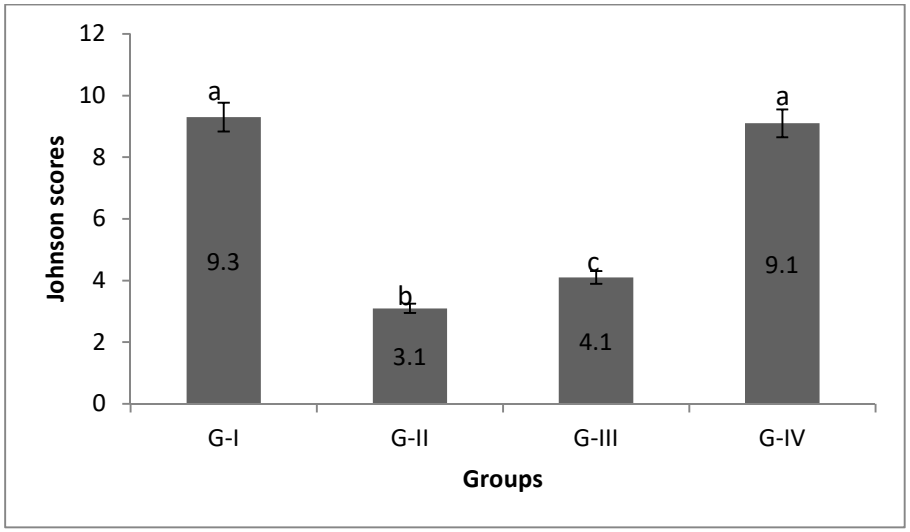

Figure 2: Histogram indicating Johnson scores variations among BPA intoxicated and control groups' alphabets specifying the bar showed whether these groups are significantly or non-significantly different from each group. A significant decrease was found in G-II and G-III against control, while a non-significant difference in G-I and G-IV.

DISCUSSION

The present study reported BPA as a toxicant, causing serious reproductive hazards in mice. Body and testes weight of BPA treated mice showed a significant reduction in the final body weights, however, the intensity of reduction was greatly related to BPA concentration. The resultant decrease in body weight due to BPA was comparable with the results of Munir *et al.,* (2017). Such a peculiar change in the body weight of mice might be due to the oxidative stress caused by BPA. BPA influences many homeostatic pathways (Elgawish *et al*., 2020), this may be associated with the decrease in body weight.

Our results showed that average testes weight and size decreased in BPA intoxicated groups contrary to control and vehicle control. Munir *et al*., (2017) reported the disintegration of testes and reduction in the testis's weight similar to our findings. Moreover, upon BPA treatment a reduction in the size of seminiferous tubules as well as spermatogenic cells (Munir *et al*., 2017) was also observed in the present investigation. Zahak and Saraswat, (2020) also reported that BPA caused a reduction in the germ cells in the seminiferous tubules.

Testosterone level was significantly altered in a dose-dependent way in BPA groups as compared to control. According to Wisesa *et al*., (2020), exposure to BPA caused the depletion in serum testosterone levels, which is mainly attributed to the dysfunction of the reproductive system of animals. These alterations are caused by mitochondrial dysfunction (Hassanin *et al*., 2019; Zahak and Saraswat., 2020).

Results demonstrate that mice exposed to BPA suffered serious disruption in testes which reduced spermatogenesis (Kaur *et al*., 2020), these results also support our present findings. They reported testicular lethality after BPA exposure. Furthermore, it was found that Johnson scores dropped in treated groups, however, such a trend was not observed in the control group (Behmanesh *et al*., 2018). The control group has a high Johnson score indicating the normal process of spermatogenesis. However, the low Johnson scores represent degeneration in the production of spermatid or spermatozoa. This correlates with the results of Sobhani *et al*., (2019) who described low Johnson Score as representing vacant seminiferous tubules in BPA treated groups.

According to our study, BPA significantly disrupted sperm count. The number of mature sperms remarkably reduced in intoxicated groups contrary to controls. Preliminary scientific results by Ullah *et al*., (2019) and Moazzam *et al*., (2015) proved after semen analysis that BPA disturbed various parameters along with sperm number.

Microscopic studies of testes from the control group showed no alternation in their basic architecture, morphology, or their accessory structure. However, histopathological findings from treated groups demonstrated destructions in the regular structure of the testes. We observed histopathological defects like the defective orientation of germ cells, hyalinization, tubular degenerations, in testes indicating variable damage after BPA administration in the respective BPA doses. It was noted in the work of Hassanin *et al* (2019) that there was a serious distortion in testes of experimental animals treated with BPA. Another study also showed detached and degenerated primary spermatogonia in seminiferous tubules of BPA intoxicated rats. There was a decrease in the diameter of seminiferous tubules along with a loosely bounded basement membrane, paired with a reduction in sperm cells (Kamel *et al*., 2018).

By this study, it is deduced that repeated intake of BPA even at sub-lethal levels may result in varying degrees of biochemical and histopathological alterations in mice testis thus proving as a reproductive toxicant. This is a multiparametric study showing the dose-dependent response of animals and each parameter affirmed the other. But still, further studies on other animal models and in different environments are required to awake authorities to monitor and regulate its acceptable limits that are not defined yet.

Conflict of interest: There is no conflict of interest of any kind.

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