LETTER TO THE EDITOR

Hematotoxicity and testicular injury induced by Bisphenol A in *Rattus norvegicus*

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Received April 18, 2020 – Accepted July 10, 2020

*This is a part of Ph.D research work of 1st author under the supervision of last author

To the Editor,

Endocrine disrupters are the chemicals that inhibit or induce change in normal hormonal function of the body and occurrence of these chemicals in the environment is perilous for both the environment and living organism in many respects. Currently, it has been reported that some synthetic chemicals act as endocrine disruptors and affect the reproductive system (1).

Bisphenol A (BPA) is a potential endocrine disruptor that is extensively used in manufacturing polycarbonate plastics and epoxy resins (2). It is a major constituent of several industrial products such as pesticides, plasticizers, paints, dental sealants and thermal stabilizers. Manufacturing, treatment and recycling of plastic and epoxy resins contaminate the ecosystem and food web by releasing its monomers (3). Humans are at high risk of BPA exposure because of leaching of BPA from metal cans to sources of food and water (4). BPA exposure primarily occurs through ingestion, although dermal and inhalation routes may also be a source of exposure, especially in occupational population (5). BPA is metabolized in the liver to form bisphenol A glucuronide and most of the time is excreted with urine (6). BPA inhibits the action of androgen by binding to its receptors. It can also interfere with the synthesis and clearance of hormone (7). It has been reported that reactive oxygen species (ROS) formed by oxidative stress of BPA by attacking DNA and cell membrane caused tissue damage in kidney, liver, brain and other vital organs (8).

Even low dose exposure to BPA may also be of concern as it interferes with metabolic processes and causes cardiovascular problems as well as damage to body tissues. This may alter the efficiency of food, protein, fat and carbohydrate metabolism (9). Currently, this chemical has attained great attention for its association with many health issues. Therefore, the current study evaluated the possible effect of BPA oral administration on hematological and serological parameters along with histopathological alterations in testis of male albino rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (*Rattus norvegicus*) weighing 125-130g were used in the study. The colony of rats was raised in the Department of Zoology, University of the Punjab, Lahore. Rats were kept in well-aerated stainless steel wire cages and were kept in a controlled 12 h light/dark photoperiod at 23±2°C. All rats were fed with commercially available standard rat chow and were given fresh water *ad libitum*. All procedures relating to the use

Key words: bisphenol A; endocrine disruptor; hematology; histopathology; testosterone; toxicosis

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0393-974X (2020)

of experimental animals were performed in accordance with the institutional guidelines for the use and care of animals for research purposes.

Dose preparation and administration

To scrutinize the toxicity of BPA, the animals were randomly divided into five groups (n=8). Different doses of BPA were prepared freshly by dissolving it in corn oil. To induce chronic inflammation, the experimental rats were orally administered specific doses of BPA via oral gavage.

Control Group: The rats were put on commercially available standard rat chow and drinking water.

Group I rats were given 10mg/Kg/body weight/day of BPA for six weeks.

Group II rats were given 25mg/Kg/body weight/day of BPA for six weeks.

Group III rats were given 10mg/Kg/body weight/day of BPA for twelve weeks.

Group IV rats were given 25mg/Kg/ body weight/day of BPA for twelve weeks.

The animals were sacrificed after BPA exposure of 6 and 12 weeks.

Blood sampling and hormonal assay

After 12 hours of receiving the last dose, the animals were weighed and anesthetized with intra-peritoneal injection of ketamine and pyrogen free H_2O. The blood samples were collected by puncturing the heart with the help of sterilized disposable syringes. Blood samples of 3 ml of blood were collected from each rat for serum separation in an anticoagulant vacutainer. Blood samples were incubated for 2-3 h at room temperature and centrifuged for 10 min at 4000 rpm. Then supernatant designated as serum was collected in new eppendorf cups and stored in aliquots at -20^{o}C for hormonal assay. Serum level of testosterone was determined by ELISA kit (MyBiosource, Inc. cat.# MBS775388) according to the manufacturer's instructions.

Hematological assay was performed on SYSME KX-21 Haematology Analyzer of control animals and BPA-treated animals.

Histopathological analysis

Immediately after the blood samples were collected, testes were excised and weighed. A small part was preserved in 10% formalin and then dehydrated in serial dilution of ethanol. Xylene was used to clear tissues. Embedding was carried out in paraffin wax and blocks of tissue were prepared. Tissue sectioning was carried out by microtome. Hematoxylin and eosin stain was used for histological investigation of testes.

Statistical analysis

All collected data were analyzed by Graph Pad Prism 5 software. Statistical significance was calculated by one-way ANOVA test followed by Tukey's post hoc analysis.

RESULTS

Body weight was decreased significantly in the BPA-treated animals of group I (13.97%), II (17.15%), III (28.29%) and IV (29.62%) in comparison to the control group when analyzed by one way ANOVA followed by Tukey's post-hoc test (Fig. 1A). Paired testes weight showed a decreasing trend in the BPA-treated groups I, II, III and IV (20.99%, 25.22%, 35.23% and 37.06%), respectively in comparison to the control group. However, relative tissue weight index of testes had no significant difference in comparison with the control group (Fig. 1 B and C) (Table I).

Testosterone level in serum

Significant decrease in serum testosterone was observed in the BPA-treated groups when compared to the control group (Table II). Testosterone level in serum was significantly decreased in groups II (69.09%), III (58.18%) and IV (80%); group I testosterone level was also reduced when compared to the control group, although this drop was not statistically significant when analyzed by Tukey's post hoc test. A significant decrease was also noticed in groups II, III and IV as compared to group I of low-dose for 6 weeks ($p=0.0003$) when compared by one-way ANOVA (Fig. 1D).

Hematological findings

The red blood cell indices and platelet count of chronically exposed rats is presented in Table III. Significant reduction was observed in Hb level of group III (11.419%) and IV (16.955%) as compared to controls (Fig. 2A). Significant decrease was noted in total RBCs in the BPA-treated groups III (11.42%) and

IV (16.96%) (Fig. 2B).The percentage of RDW-CV of rats was decreased significantly in groups III and IV (7.12% and 9.62%), respectively (Fig. 2C). Hematocrit level was decreased significantly in groups III and IV (13.06% and 17.57%), respectively, compared to the control group, while a nonsignificant decrease was also observed in groups I and II (Fig. 2D). MCV was decreased significantly in groups III (4.546%) and IV (8.64%) in comparison to control animals (Fig. 2E). Significant decrease in MCH percentage value of groups II (5.842%) and IV (9.426%) was noticed while MCHC level was increased insignificantly (Fig. 2F). Platelet count was increased to 39.02% in group IV as compared to controls, whereas MPV decrease was also significant in group IV (19.69%) in comparison to control animals (Fig. 2G).

Effect of chronic exposure of BPA on total and differential leukocyte count

The total and differential leukocyte count of chronically exposed rats is shown in Table III. The WBC count was significantly increase in groups II, III and IV (87.96%, 98.27% and 126.82%), respectively, compared to the control group. Group I also showed an increase in WBC count, however this difference was statistically nonsignificant after analysis of Tukey's post-hoc test (Fig. 2H). Neutrophils exhibited a significant increase in BPA-treated groups III (326.04%) and IV (417.55%) in comparison to control animals (Fig. 2I). A statistically significant decrease in percentage value of lymphocytes was noticed in groups III and IV (9.02% and 11.765%) (Fig. 2J). Percentage value of monocytes declined significantly by 55.57% and 60% in BPA-treated groups III and IV, respectively (Fig. 2K). Eosinophils showed a significant decrease (56.05% and 69.23%) in groups III and IV as compared to controls due to BPA oral administration when analyzed by one-way ANOVA followed by row stats (Fig. 2L).

Histological examination

Testis sections of the control group animals

Table I. *Mean ± S.E.M of body weight (g), paired testes weight (g) and relative tissue weight index (g%) in the control and BPA-treated rats.*

Groups/Parameter	Body weight (g)	Testes weight (g)	RTWI (g %)
Con	340.0±10.00	3.548 ±0.15	1.043±0.04
Group I	292.5±11.50	2.803 ±0.11	0.958±0.04
Group II	281.7±8.29	2.653 ±0.19	0.941±0.07
Group III	243.8 ±6.06	2.298 ±0.12	0.942±0.05
Group IV	239.3 ± 6.56	2.233 ±0.16	0.933±0.07

Table II. *Mean±S.E.M of Serum testosterone level of control and BPA-treated groups.*

Parameter/Groups	Con	Group I	Group II	Group III	Group IV
Testosterone level (ng/ml)	0.275±0.005	0.235±0.005	0.085±0.015	0.115±0.005	0.055±0.025

Table III. *Mean±S.E.M of hematological parameters of control and BPA-treated groups.*

Parameter/Groups	Con	Group I	Group II	Group III	Group IV
Hemoglobin (g/dl)	14.65±0.48	14.00±0.24	13.93±0.37	12.85±0.44	11.25±0.75
RBC (x10^6/uL)	7.22±0.09	7.160±0.19	7.050±0.25	6.400±0.12	6.00±0.20
RDW-CV (%)	13.20±0.27	12.54±0.24	12.40±0.10	12.26±0.15	11.93±0.22
HCT (%)	42.64±0.47	41.23±1.05	40.85±1.85	37.07±0.64	35.15±3.75
MCV (fL)	61.15±0.45	60.53±0.41	58.80 ±0.39	58.37±0.40	55.87±0.79
MCH (pg)	20.37±0.42	20.03±0.22	19.18±0.18	19.60±0.17	18.45±0.55
MCHC (g/dL)	32.70±0.70	33.33±0.35	33.45±0.45	33.82±0.66	34.60±1.30
Platelets (x10^3/uL)	400.3±38.4	456.5±24.2	488.4±19.7	481±8.65	556.5±30.5
MPV (fL)	6.60±0.28	6.075±0.09	5.767±0.32	5.900±0.23	5.300±0.30
WBC (x10^3/uL)	5.533±0.73	9.800±0.50	10.40±0.90	10.97±1.11	12.55±0.45
Neutrophil (%)	3.333±0.33	3.500±0.96	4.500±0.50	14.20±0.74	17.25±1.32
Lymphocytes (%)	89.25±0.63	88.33±1.02	87.80±0.58	81.20±0.49	78.75±2.10
Monocytes (%)	3.00±0.32	2.167±0.17	1.667±0.33	1.333±0.33	1.200±0.20
Eosinophil (%)	6.500±0.50	6.333±0.33	6.00±0.20	2.857±0.59	2.000±0.58

showed normal tissue architecture with normal and active pattern of spermatogenesis. Seminiferous tubules exhibited clear lumen and were lined with germinal epithelium. The intact basement membrane was also observed in the control group. Leydig cells were organized in the interlobular area of seminiferous tubules. Degenerative changes in the germinal layer of BPA-treated rat seminiferous tubules as well as in Leydig cell were observed when compared to the control group. Mild-to-severe atrophy of seminiferous tubules, vacuolization and decrease in Leydig cell was seen as the dose of BPA increased. Seminiferous lumen filled with cellular debris and an irregular ruptured basement membrane and separation within the spermatogenic cells was also found in all BPA-treated groups. The testes of group IV animals showed extensive histopathological variations including atrophy, sloughing, loss of spermatogenesis, reduction in spermatogenic cells and poorly developed Leydig cells. Immature sperms were found in seminiferous tubules lumen. Loose intercellular bridges between the germ cells and Sertoli cells were found in all BPA-treated groups.

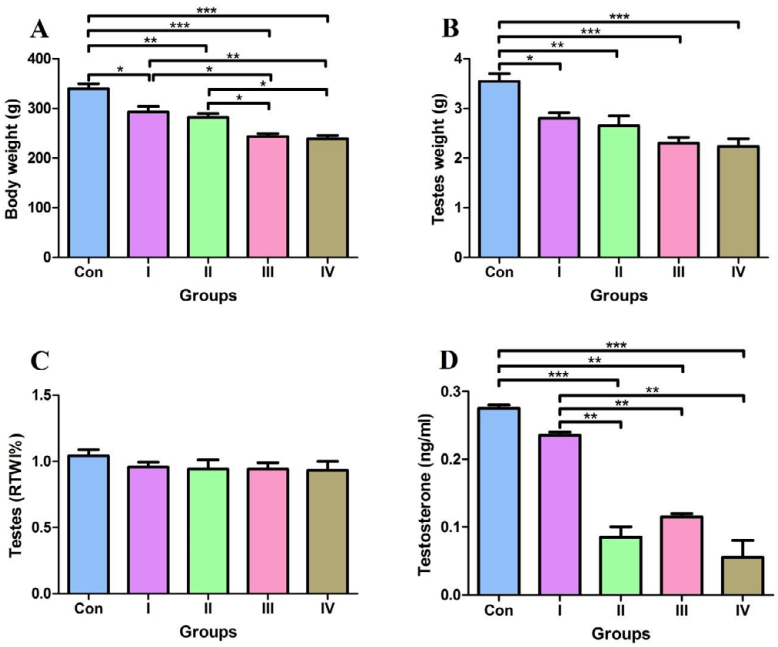

Fig. 1. *Alterations in body weight (**A**), testes weight (**B**), testes RTWI% (**C**) and serum testosterone level (**D**) of control group and BPA-treated groups; Group I and III (10mg/Kg/BW for six and twelve weeks, respectively) and Group II and IV (25mg/Kg/BW for six and twelve weeks, respectively). Values are mean ± S.E.M, standard error of mean represents by error bar. *P≤0.05, **P≤0.01, ***P≤0.001.*

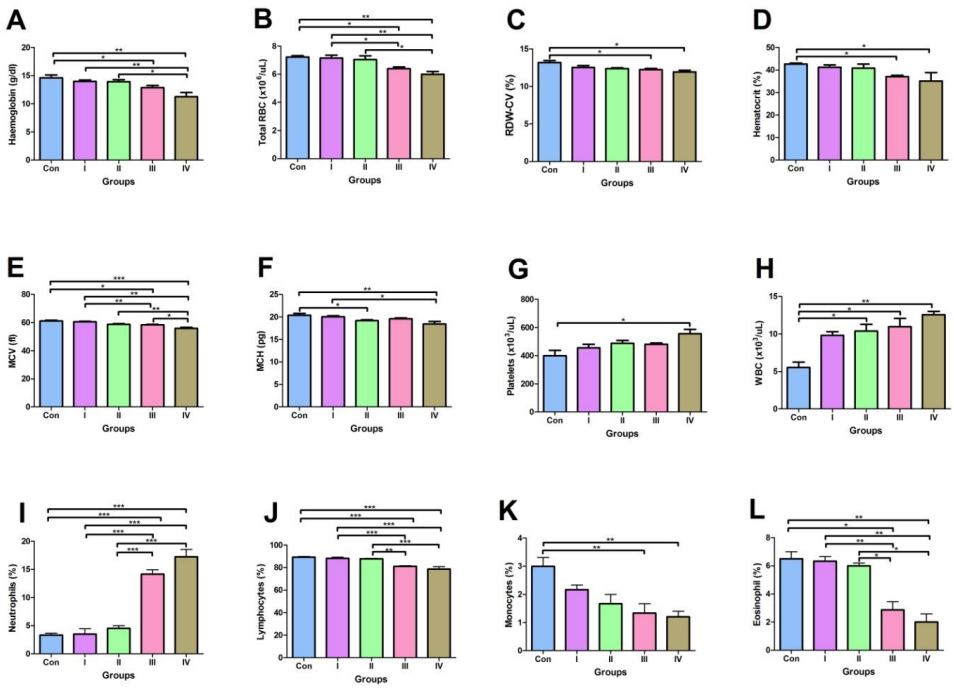

Fig. 2. *Chronic effect of BPA exposure on hematological parameters (**A**) Haemoglobin, (**B**) Total RBC, (**C**) RDW-CV, (**D**) Hematocrit, (**E**) MCV, (**F**) MCH, (**G**) Platelets, (**H**) WBC, (**I**) Neutrophils, (**J**) Lymphocytes, (**K**) Monocytes and (**L**) Eosinophils in adult male rats. Group I and II (10mg/Kg/BW for six and twelve weeks, respectively) and Group II and IV (25mg/Kg/BW for six and twelve weeks, respectively). Values are mean±S.E.M, standard error of mean represents by error bar.*P≤0.05, **P≤0.01, ***P≤0.001.*

DISCUSSION

The present study was outlined to evaluate BPA toxic effects on testes. Any disruption in spermatogenesis and steroidogenesis processes performed by testes will affect the reproductive system of males. BPA affected these processes and induced toxicity of the reproductive system. The body weight declined due to BPA oral administration showed changes in metabolic activity of rats. The present finding was supported by a previous study that showed rat body weight decreased after 1, 5 and 100mg/Kg/BW of BPA exposure (10). Paired and relative weight of testes declined significantly because of BPA chronic exposure and was associated with the decline in testosterone level and minute amount of germ cells. This suggests that maintenance and development of testes depends

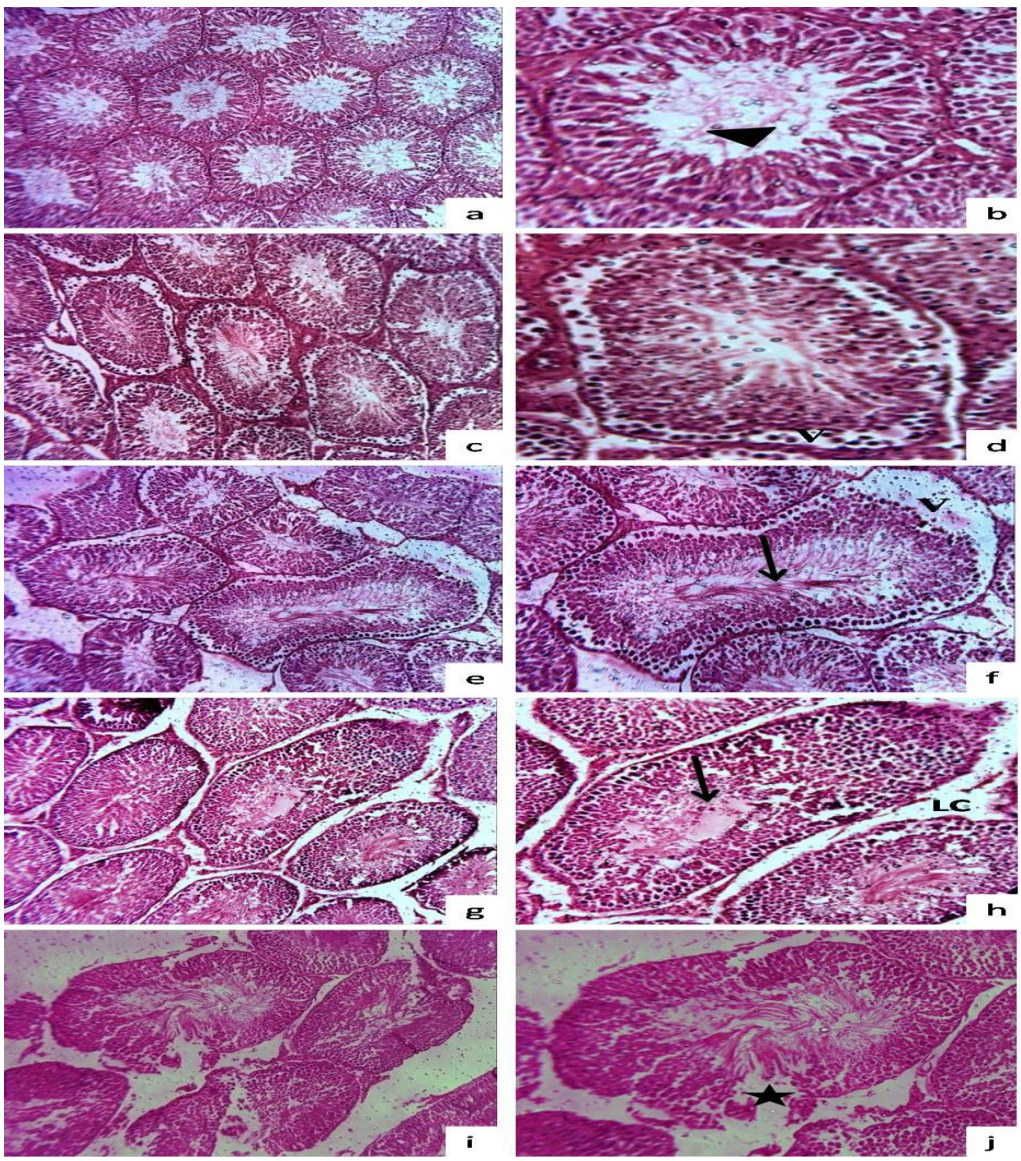

Fig. 3. *Hematoxylin and eosin staining of testes sections of BPA induced toxicity in Group I and III (10mg/Kg/BW for six and twelve weeks respectively) on 10X* ***(c, d, g, h)****. Group II and IV (25mg/Kg/BW for six and twelve weeks respectively) on 10X* ***(e, f, i, j)*** *and control sections* ***(a, b)*** *on 10X. Arrow head showing normal seminiferous tubules with active spermatogenesis, thin arrow showing lumen filled with cellular debris and immature germ cells, star showing sloughing of spermatogenic cells and ruptured basement membrane and LC showing poorly developed Leydig cells.*

on androgen. The same findings were found in the current investigation that testes absolute and relative weight declined significantly due to BPA low-dose exposure (11). The current findings agreed with another study in which testes weight decreased due to 20µg/Kg BW BPA exposure (12).

There was a significant decrease in the level of testosterone in all BPA-treated groups when compared to the control group. These results agree with findings of Gurmeet et al. (12) in which production of testosterone was dramatically suppressed. Testosterone plays an indispensable role to initiate the function of spermatogonia. There was a very low testosterone level in BPA-treated animals that caused the failure of spermatogenesis. This decrease might be linked with reduction in the number of Leydig cells because of oxidative damage and decreased activity of cytochrome P450 in the liver. Oxygen reactive species increased as a response to decline in the male specific cytochrome and destroyed the function of sperm (12). The decrease in hematological parameters of rats due to BPA chronic exposure may be due to hemolysis and shrinkage of RBCs by BPA, leading to a significant decrease in hematocrit value which causes anemia. The decrease rate of formation and increase destruction rate of RBCs might also be responsible for the decline in RBC count. The present findings are in agreement with previous studies (4). There was an increase in WBC count after chronic exposure of BPA that may be explained on the basis of the role of BPA in inflammation, or due to increased neutrophil percentage. This increase may be due to BPA-induced stress and activation of the immune system. This finding is in accordance with a previous study (13).

The decreased testosterone level and number of Leydig cells were also supported by histopathological study. The histopathological alterations in the current study indicated disruption in function and morphology of testes. It was noted that the process of spermatogenesis was affected in all the BPA-treated groups. Immature germ cells as well as degenerated germ cells were found in the lumen of seminiferous tubules. Vacuolization and ruptured epithelium were also observed. These results agree with the findings of Yousaf et al. (10). Significant decline in Leydig cell numbers might be the reason for these deformities. Testosterone is necessary for spermatogenesis and is also involved in further processing of spermatogenesis. As the spermatogenesis is suppressed due to decreased level of testosterone, the process of sperm formation is disrupted and causes infertility (14).

It can be concluded from the present investigations that BPA oral administration to adult male rats resulted in anemia and adverse effects on several hematological and hormonal parameters that indicates testicular injuries, which is confirmed by the histopathological changes.

ACKNOWLEDGEMENT

The authors acknowledge and are thankful to the Vice Chancellor, University of the Punjab, Lahore, Pakistan for providing funds for this study.

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