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Bisphenol S Exposure Perturbs Epididymis Function of Adult Male Golden Hamster, *Mesocricetus auratus*

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Abstract

Bisphenols are widely used in industrial and commercial products that exhibit endocrine-disrupting properties. Bisphenol S (BPS) has been reported to show adverse impact on human health. The objective of the present study was to examine the effect of BPS on epididymal function in the adult male golden hamster *Mesocricetus auratus*. Different doses of BPS (25, 50 and 75mg/kg BW/day) were orally administered for 28 days. BPS administration caused a reduction in body and epididymis weight, sperm count and sperm viability. BPS exposure also caused a reduction in the serum testosterone levels, suggesting its impact on testicular steroidogenesis. Further, the activities of antioxidant enzymes (SOD and catalase) in the epididymis were markedly decreased, while the levels of lipid peroxidation increased significantly in epididymis of BPS-treated hamsters. Epididymides obtained from BPS treated hamsters showed degenerative changes in the caput, corpus and cauda along with a decreased sperm count in the lumen. In conclusion, we demonstrate that exposure to BPS caused oxidative stress in the epididymis, which may lead to impaired reproductive function.

Keywords: BPS, Epididymis, Oxidative Stress, Semen Quality, Subfertility/Infertility.

1. Introduction

The environment is constantly being contaminated by various anthropogenic compounds. The industrial production of bisphenol S (BPS), one of the BPA substitutes, is increasing steadily on a global scale[1,2]. It has been used in multiple products such as epoxy resins, baby feeding bottles, coatings of beverage bottles and paper products[3]. Thus, humans are constantly exposed to BPS through several routes. Widespread exposure to BPS by the general population has been demonstrated in various countries, with the detection of BPS levels ranging from 0.02 to 21 ng/ml in urine samples[4]. BPS was introduced to the market as a possible safer substitute of BPA. The use of BPS surmounted due to the ban on the production of BPA[2]. BPS, a structural analougue of BPA, contains a sulfonyl group (with strong electron-absorbing ability) and two hydroxyl groups, is more stable to heat and sun light; thereby more resistant to degradation than BPA[2,5]. Widespread exposure of BPS to general population has been demonstrated in various countries, with the detection of BPS levels ranging from 0.02 to 21 ng/ml (0.8–84 nM) in urine samples[3]. Majority of the previous studies has focused mainly on the endocrine-disrupting activity of BPA, and less attention has been paid to the toxicity of the other bisphenols including BPS[6,7]. However, its production is not officially regulated and the tolerable daily intake (TDI) of BPS has not yet been established[8-10]. Studies on the potential health impacts of these BPA analogues revealed a number of negative side effects such as cytotoxicity, neurotoxicity and endocrine disruption[6,11,12]. Bisphenols have been detected in human breast milk, blood and urine[13]. BPS is also regarded as reprotoxic due to its potential to disrupt hormone signalling and thereby affecting male and female reproductive system. However, there is little evidence available on its impact on the male accessory reproductive organs.

According to World Health Organization (WHO) approximately 15% of couples globally are experiencing

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infertility, with roughly 50% of cases involving a male component as the primary or contributory issue[14]. Spermatogenesis is a complex process of germ cell division and differentiation that involves regular communication between the somatic Sertoli cell and developing germ cells. Germ cell development and their maturation is impacted by the absence of endocrine hormones and by faulty endocrine signalling in the testes[15]. Bisphenols can bind with estrogen and androgen receptors and impair reproductive functions[16-18]. BPS acts as an ERα agonist, thereby influencing the proliferation and migration of cancer cells[19] .

Studies have shown that BPS has an inhibitory effect on steroidogenesis[5,9,20]. BPS exposure suppresses GnRH transcript expression in the hypothalamus and decreases testosterone concentration[21-25]. BPS also induces ROS production, lipid peroxidation, protein and DNA damage along with compromised antioxidant enzyme activities in the testes[26-28]. Exposure to BPS modifies the antioxidant enzyme activities by interacting with Gly residue of CAT enzymes by hydrogen bond and hydrophobic forces that change the CAT secondary structure as displayed by various spectral methods and molecular docking investigations[29]. Recently, BPS was found to be genotoxic, cytotoxic as well as inducing apoptosis[20,30,31]. Recent evidence suggests that the BPS toxicity is equal or even may be greater than BPA[9,32].

Epididymis is an important accessory sex organ that provides a favourable microenvironment for sperm maturation, motility, transport and gaining fertilizing competence[33,34]. Further, epididymis stores the sperm until they get ejaculated as well as protects them from immunogenic reactions with the help of blood-epididymis barrier. The harmful effects of BPS on several organs, including the testes have been demonstrated in previous investigations; however, the evidences in regard to epididymis is still scanty[27-35]. Epididymis can also be a potential target organ for various environmental toxicants like BPS and this can lead to deteriotiation of sperm quality, thereby leading to male infertility/subfertility. Therefore, the present study was designed to investigate the possible effects of BPS exposure on the epididymal histoarchitecture and antioxidant potential.

2. Methods

2.1 Chemicals

BPS (CAS Number: 80-09-1) was purchased from Sigma Aldrich Chemicals, St. Louis, MO, USA. The investigation used only analytical-grade substances, all of which were obtained from HiMedia Laboratories and E. Merck India Ltd.

2.2 Animal Care and Maintenance

Adult male golden hamsters were maintained at the departmental animal house (Reg. NO.1802/Re/S/15/CPCSEA) BHU/DoZ/IAEC/2018-19/035 of Banaras Hindu University, Varanasi, India. The animals were housed in polypropylene cages with bedding of dry rice husk fed with commercial available rat food pellets (Amrut Laboratory Animal Feeds, Pune, India), and given access to water *ad libitum* at room temperature of 25 °C±2 with 12.5 h light/11.5 h dark cycle.

2.3 Experimental Design

Adult male golden hamsters (average body weight 130 ± 5 g) were randomly divided into four groups, each group containing five animals (n = 5).

Group I: Control (vehicle treated).

Group II: Low dose (25 mg BPS kg^{-1} BW day^{1})

Group III: Medium dose (50 mg BPS kg^{-1} BW day^{1})

Group IV: High dose (75mg BPS kg^{-1} BW day^{1})

BPS was dissolved in corn oil and administered through oral route daily for 28 days. BPS is lipophilic in nature and soluble in corn oil. Animals in the Group I were treated with corn oil to serve as control group.

2.4 Tissue Collection and Processing

Twenty-four hours after the final dose of BPS, animals were weighed and sacrificed by deep ether anaesthesia. Epididymides and testes were removed, blotted dry and weighed. Epididymides and testes from one side were then quickly fixed in Bouin's fixative for histological analysis while the other side were stored at -20°C for until further analysis.

2.5 Sperm Count and Viability

The cauda epididymis was used for sperm viability and sperm count analysis as per the guidelines of World Health Organization (2010) laboratory manual[36]. For sperm viability, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed according to Kumar *et al.*, 2019[37]. Briefly, sperm suspension was quickly diluted, and the germ cells were counted. 100µL of diluted sperm suspension (concentration of 4 ×

10^6 spermatozoa/ml) was placed in each of the 96 wells of the plate. 20 µl of MTT working reagent (stock solution 5 mg/mL of MTT in phosphate-buffered saline) was added and incubated at 37°C with 5% CO_2 for 1 h. Formazan that was formed was then dissolved by the addition of 100 µL of acidic propanol to each well. After 30 minutes, absorbance was read at 570 nm, in a microplate reader.

2.6 Histopathological Examination

The epididymal and testes tissues were fixed in Bouin's fixative for 24 h. After fixation, tissues were then dehydrated in different grades of alcohol (30%-50%) and placed in 70% alcohol for 3 days. Then tissues were then placed in 90% alcohol for 1 h and after two changes in 90% alcohol, tissues were transferred in absolute alcohol for 1 h. After two changes in absolute alcohol tissues were placed in benzene for overnight. On the next day, tissues were placed in paraffin wax. After three changes in wax, tissues were removed from wax, and paraffin blocking of tissues was done with the help of L piece. After block preparation, labelling was done with the help of paper and pencil and left it for one day. The sectioning was done with the help of semi-automatic microtome (RM2235) machine Leica, Germany. The tissue sections were spread on gelatin-coated glass slides. After complete spreading, tissue sections were further proceed for staining. The dewaxing of the sectioning was done in xylene for 20 minutes. After dewaxing, slides were proceeded for rehydration process in different grades of alcohols (100% – 90% - 70% - 50% - 30%). After rehydration, slides were placed in a hematoxylin stain for 20 min. The sections were then placed under running water for 30 min. After that, the slides were placed in eosin stain. After that, tissue sections on the slides were subjected to further dehydration in 90% and 100% alcohol. Then slides were placed in xylene for 5 min and mounted on DPX.

2.7 Testosterone Assay

Testosterone was estimated using an ELISA kit according to the procedure provided with the kit (Diametra: DKO02). The concentration of testosterone in serum was expressed as ng/mL. Briefly, 25µL of standard solutions of testosterone (provided in the kit) and serum sample were added into appropriate wells in triplicates. 100µL of testosterone-HRP conjugate was added to each well and the plate was incubated at 37°C for 60 min. The microwells in the plate were washed three times with wash buffer. After washing, 100µL of TMB substrate was added to each well. The plate was incubated at room temperature for 20 min in dark. After 20 minutes, stop solution was added to each well and the absorbance was recorded at 450nm. A standard curve was plotted to calculate the testosterone levels in the serum sample.

2.8 Antioxidant Enzyme Activity

Epididymis was homogenized in ice-cold PBS (50 mmol, pH 7.4) and centrifuged at 12,000×g for 30 min[37]. The supernatants were collected, and protein levels estimated using Bradford assay[38]. The rest of the supernatants were used to assay antioxidant enzyme activity.

2.8.1 Superoxide Dismutase

Superoxide dismutase (SOD) activity was spectrophotometrically estimated as per the published protocol[37]. Briefly, 0.5 mL of supernatant was added to the reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), 20 mM L-Methionine, 1% Triton X-100, 10 mM hydroxylamine hydrochloride, 50 mM EDTA and incubated for 5 min at 37 °C. 50 µM of riboflavin was added to each sample and illuminated under red light. After 10 min of exposure, 1ml of freshly prepared Griess reagent (1% sulphanilamide, 5% orthophosphoric acid, 0.1% N-1-napthylethylenediamine dihydrochloride) was added, absorbance recorded at 543 nm. SOD activity was calculated and expressed as units (U)/mg of protein. Appropriate controls that contained phosphate buffer in place of sample was maintained.

2.8.2 Catalase Activity

Catalase (CAT) activity was evaluated as per the methodology described earlier[37,39]. Briefly, 1 ml of tissue supernatant was mixed with 5 ml PBS and 4 ml of H_2O_2 (0.8mM). After 1 min of incubation, 1 ml of this solution was taken into a fresh tube and mixed properly with 2 ml of acidic potassium dichromate ($K_2Cr_2O_7$) solution. Then the mixture was boiled for 10 min. Tubes were cooled at room temp. and absorbance was recorded at 570 nm. The amount of H_2O_2, depleted/min/mg of protein, was used to express the enzyme activity of catalase.

2.9 Lipid Peroxidation

Lipid peroxidation (LPO) was estimated according to the protocol described in Kumar *et al.*, 2020[40]. 0.2 ml

of supernatant was mixed with 3.3 ml of thiobarbituric acid (TBA) reagent, which contained 0.8% TBA, 0.8% butylatedhydroxytoluene, 0.8% sodium dodecyl sulphate and 1.5 mL of 20% acetic acid. This mixture was incubated at 95 °C for 60 minutes in a water bath. The reaction mixture was centrifuged at 500 g for 10 min at 4 °C after being cooled to room temperature. The absorbance of malonaldehyde (MDA)-TBA by-product was read at 532 nm. Levels of lipid peroxidation was expressed as nmol MDA/mg protein.

2.10 Statistical Analysis

Data analyses were performed using Graph Pad Prism 8 (USA) software with one-way ANOVA and Dunnett's multiple comparisons test. The differences were considered statistically significant when $p< 0.05$.

3. Results

3.1 Body Weight and Relative Epididymis Weight

A significant decline in body weight was observed in 75mg BPS-treated hamsters compared to control. No change in the body weight in 25mg and 50mg BPS-treated hamsters was observed when compared to the control (Figure 1a). A significant decrease in the relative epididymis weight was observed in 75mg BPS-treated hamsters while such an effect was not observed in the 25mg and 50mg BPS treated hamsters (Figure 1b).

3.2 Serum Testosterone Level

A significant decrease in serum testosterone was observed in the 50mg and 75mg BPS treated hamsters when compared with control. However, 25mg dose of BPS had no significant effect on serum testosterone level when compared with control hamsters (Figure 1c).

3.3 Sperm Count and Viability

A significant reduction in sperm count was observed in 50mg and 75mg BPS treated hamsters as compared to control hamsters, while 25mg BPS treated hamsters showed no significant effect on sperm count when compared with control (Figure 2a). Significant decrease in sperm viability was observed in 50mg and 75mg BPS treated hamsters as compared to controls. However, sperm viability was not affected in hamsters treated with 25mg BPS (Figure 2b).

3.4 Histological Alterations in the Epididymis

Control (vehicle-treated) hamsters showed normal histoarchitecture in all three regions (caput, corpus and cauda) characterized by normal tubules, cellular arrangement, and lumen filled with sperm (Figure 3a, 3b, 3c). 25mg BPS-treated animals showed normal histoarchitecture of caput, corpus and cauda epididymides (Figure 3d, 3e and 3f). The caput of 50mg and 75mg BPS treated animals showed degenerative changes such as widening of the intercellular spaces and occurrence of cellular debris and less sperm in the lumen (Figure 3g

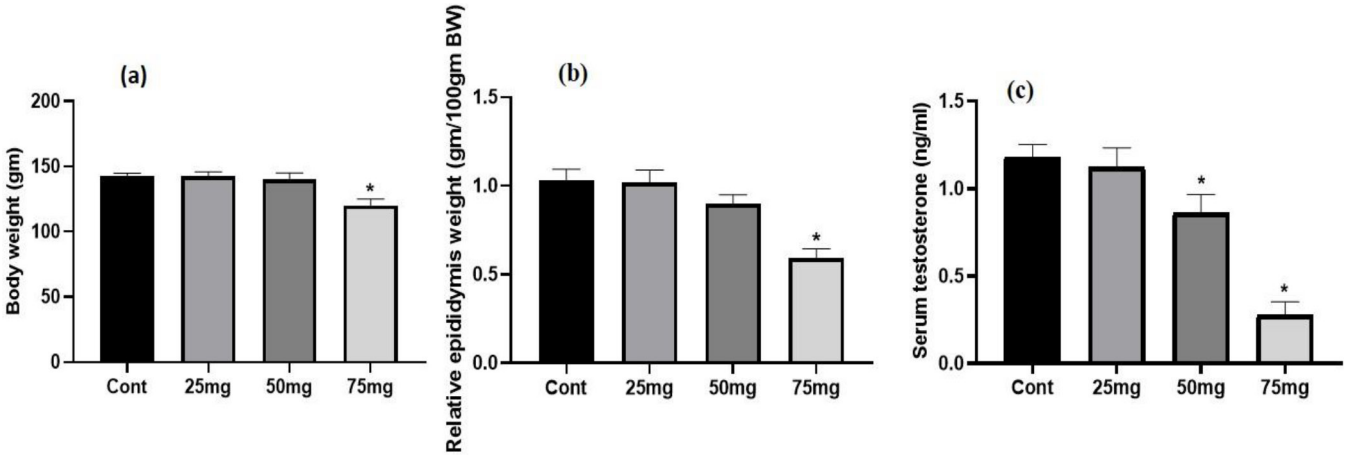

Figure 1. Effect of BPS on weight parameters and testosterone level. (a) Body weight (b) relative epididymis weight. (c) serum testosterone. Values are presented as mean ±SEM (n=5). Significance of difference * $p< 0.05$ compared to control.

and 3j). The corpus tubules of 50 mg treated hamsters showed less sperm in the lumen (Figure 3h), while 75mg BPS treated hamsters showed degenerative changes along with less sperm in the lumen (Figure 3k). The cauda epididymis of 50mg and 75mg BPS treated hamsters showed degenerative changes such as occurrence of vacuolated cells, with reduced spermatozoa in the lumen (Figure 3i and 3l).

3.5 Epididymal Antioxidant Enzyme Activities

A significant decrease was observed in the SOD activity in the 50mg and 75mg BPS-exposed hamsters when compared to control animals, whereas the 25mg BPS treated group showed no significant change in SOD enzyme activity when compared with control (Figure 4a). A significant decrease in catalase activity was observed in the 75mg BPS

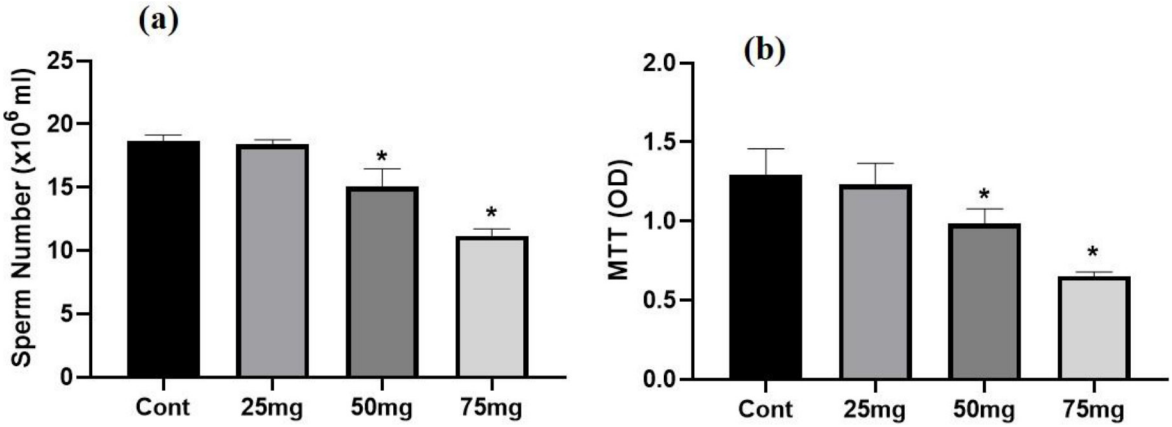

Figure 2. Effect of BPS on sperm parameters. (a) Sperm count, (b) Sperm viability. Values are presented as mean ±SEM (n=5). Significance of difference * $p < 0.05$ compared to control.

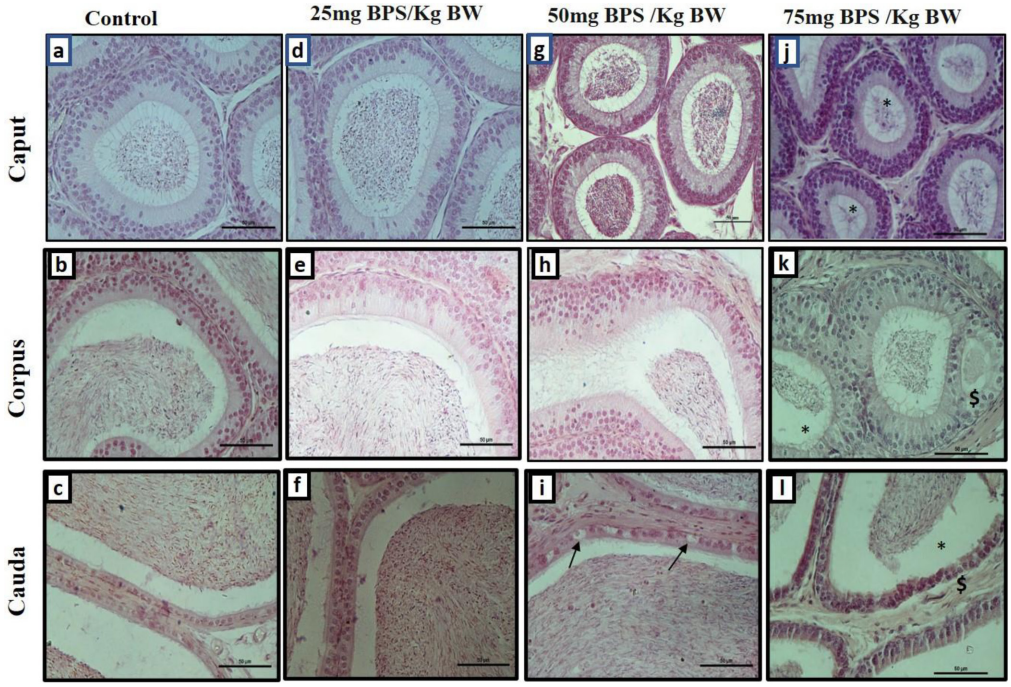

Figure 3. Photomicrographs showing histological changes in the epididymis of control and BPS treated hamsters. Arrows indicate vacuolization, * indicate decline in sperm number in the lumen, $ indicate degenerative changes in epithelial layer. All the images shown were photographed at 40X magnification.

treated hamsters when compared with control. In contrast, 25mg and 50 mg of BPS had no significant effect on catalase enzyme activity compared with control (Figure 4b).

3.6 Epididymal Lipid Peroxidation (MDA level)

A significant increase in lipid peroxidation was observed in the 50mg and 75mg BPS-treated hamsters compared with control, while 25mg BPS had no significant effect on lipid peroxidation compared with control (Figure 4c).

3.7 Histological Alterations in the Testes

Testis obtained from the control group showed the presence of normal seminiferous tubules as represented by sequentially arranged germ cells and the presence of sperm in the lumen (Figure 5a and 5b). BPS 25mg treated group showed normal testicular histoarchitecture similar to the control (Figure 5c and 5d). BPS 50mg and 75mg treated hamsters revealed prominent degenerative changes in the testicular histoarchitecture as evident by the appearance of vacuoles, presence of giant cell, Leydig cell atrophy, depletion of germ cell number, and absence of sperm in the lumen of seminiferous tubule (Figure 5e-5h).

4. Discussion

The alarming rise in environmental contamination by various pollutants is a matter of deep concern for human health. Over the last few decades, there has been a decline in male fertility; potential reasons for this phenomenon include environmental risk factors[41]. Male reproductive organs may get overexposed to environmental contaminants, which might be linked to reproductive disorders[41]. The toxicants can modify the mechanisms involved in sperm maturation by interfering with sperm membrane. Toxic substances have the ability to change the amount and quality of sperm generated during spermatogenesis, which in turn can change the quality of sperm that cross the epididymis[41,42]. All the substances that inhibit the synthesis of testosterone in the Leydig cell may also have a negative effect on the epididymis. A common synthetic chemical found in many industrial items is bisphenol S (BPS), a structural analogue of Bisphenol A. BPS is used in the production of industrial products such as feeding bottles, paper products, food containers, and kitchen utensils[1,43,44]. Because of its increased commercialization, the rise in BPS concentration in industrial items has now reached an alarming level[45]. Humans are exposed to the BPS by various routes[19,44].

In recent years, human mean sperm concentrations have significantly decreased from 113 to 61 million/ml. Consequently, the percentage of infertile couples globally has risen to 15%[46]. The decrease in fertility and the deteioraton of reproductive health prompted to investigate the causes and management of infertility. The epididymis is a crucial reproductive organ that controls sperm concentration, maturation, storage and protection. The spermatozoa are immature in the testes and acquire motility and fertilization ability while passing through the epididymis. Epididymis is a duct-like structure that joins the testis to the vas deferens and consists of three-part caput, corpus, and cauda. When sperm cells interact with the distinct luminal environment of each epididymal part, then sperm maturation takes place[47]. Epididymis is an important organ that contributes to fertility. Therefore, exploring whether BPS affects epididymis function and reproductive health is required. In the present study, we investigated the dependent effects of BPS on the status

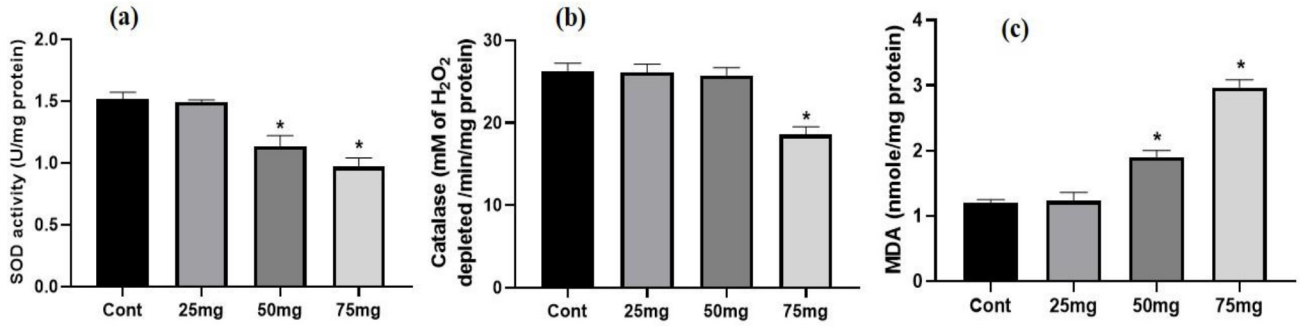

Figure 4. Effect of BPS on epididymal antioxidant enzyme activity. (a) SOD activity, (b) Catalase activity, (c) Lipid peroxidation. Values are presented are mean ±SEM (N=5). * p< 0.05 compared to control.

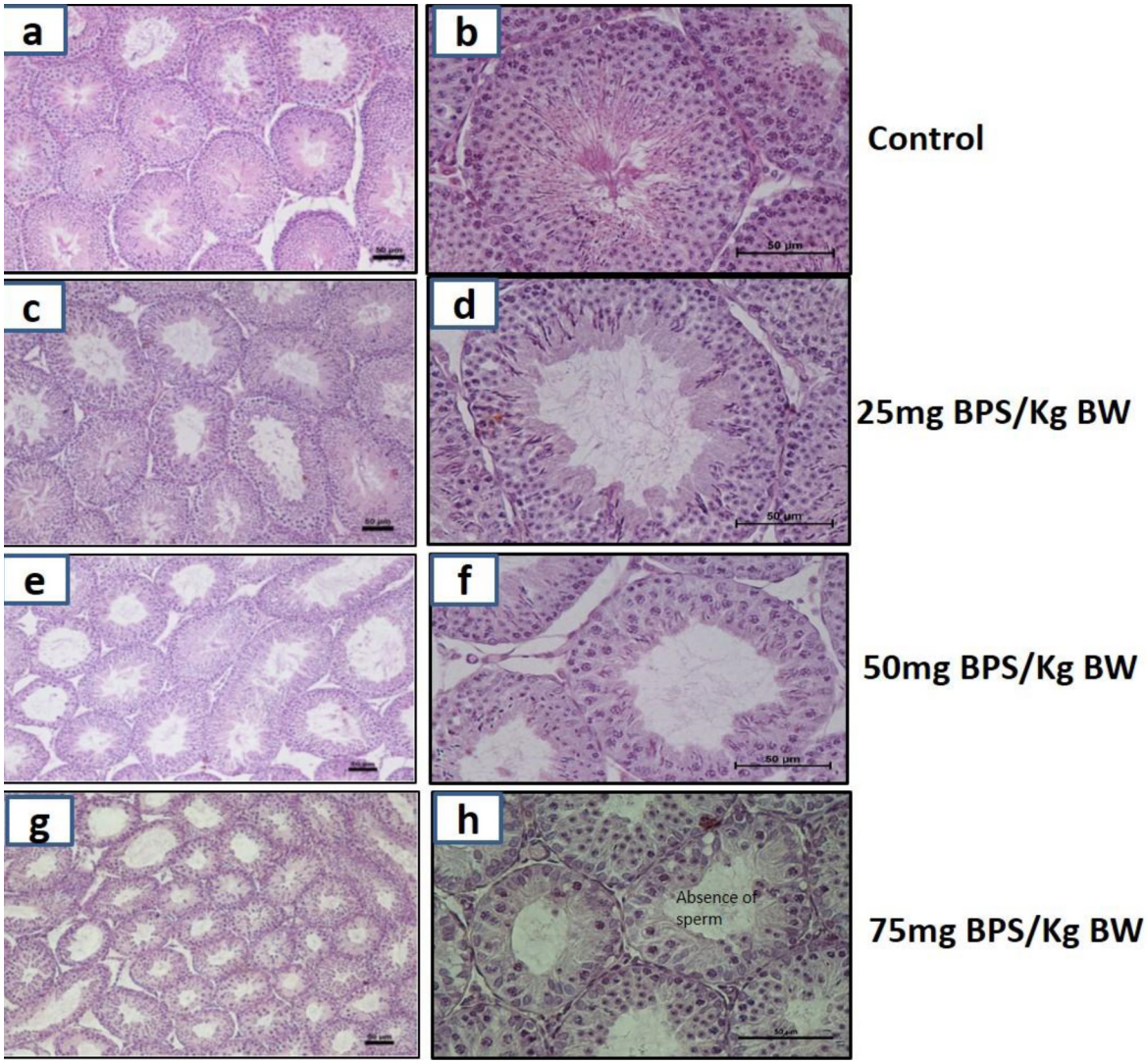

Figure 5. Photomicrograph showing histological changes in the testes of control and BPS-treated hamsters. The images shown were photographed at 10X and 40X magnification.

of antioxidant enzymes, lipid peroxidation, hormonal alteration, and histoarchitecture of epididymis along with semen quality. We observed a significant reduction in body weight of hamsters treated with 75mg BPS when compared with the control, suggesting BPS is toxic. These results are consistent with the earlier findings[37,40,48,49]. In the present study, the reduction in the relative epididymis weight was recorded following the high dose of BPS administration while lower dose had insignificant effects on the relative epididymis weight. Further, it is well established that the sperm undergoes maturation and acquires motility in the epididymis. BPS treatment at a dose of 50mg and 75mg showed a marked decrease in sperm number and viability as compared with control. This decrease in the sperm parameters in BPS-treated animals might have resulted from the adverse effect of the BPS and that affected testicular spermatogenesis epididymal function[49,50]. Testosterone is produced by Leydig cells and is responsible for the development of the accessory reproductive organs, including epididymis and contribute to the maintenance of male fertility and growth of the reproductive system[51,52]. In the present study, a marked decline in serum testosterone was observed in the BPS-treated hamsters, suggesting the inhibitory effect of BPS on testosterone biosynthesis. Testosterone deprivation causes epithelial cell apoptosis and a decrease in the diameters of tubule in epididymis[53].The histological alterations in the epididymis and androgen depriviation

were also observed previously on exposure of other toxicants such as cadmium, ethane dimethanesulphonate (EDS), dibutylphthalate[53,54]. Reactive oxygen species (ROS) are the by-products of oxygen metabolism produced during cellular processes. This metabolism is highly activated during spermatogenesis[55], indicating that the male reproductive system generates high levels of ROS, leading to oxidative stress. Oxidative stress is also caused due to any imbalance between ROS generation and efficient removal by antioxidant enzymes[55]. Antioxidant enzymes are highly efficient against ROS, and among which SOD, catalase, and glutathione peroxidase (GPX) are the major antioxidant enzymes noted in male genital tract[56.57]. SOD, reduces the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), while catalase enzyme catalyses the conversion of H_2O_2 to H_2O and GST catalyses conjugation of reduced glutathione to a variety of substrate for detoxification[58].

The amount of lipid peroxidation in the cell membrane is increased due to the persistence of reactive oxygen species (ROS). Lipid peroxidation is the oxidative degradation of lipids, which is main integral component of cell membrane. In the present study, we observed that BPS at a dose of 25mg had no effect on antioxidant enzyme activities, whereas at other doses, i.e. 50mg and 75mg, a significant increase in the lipid peroxidationand decreased antioxidant enzyme activities was observed, suggesting that the BPS exposure induces oxidative stress in the epididymis and impaired antioxidant system. It is now recognised that the deleterious effects of environmental toxicants on the spermatogenesis and steroidogenesis in the gonad are caused due to induction of oxidative stress[27,58]. Additionally, it is well established that oxidative stress causes germ cell apoptosis and faulty spermatogenesis in the testes[49]. These findings were further supported by the histological changes in the epididymis. We noted that epididymis had degenerative changes such as the appearance of vacuoles, necrotic changes in some cells and reduction in the sperm concentration in the lumen following the administration of 50mg and 75mg BPS. Testes is the primary target of toxic insult via disruption of cell junctions and germ cell loss, which results in inadequate sperm production[59]. Further, histopathological evaluation of the testes is thought to be a more sensitive sign in the assessment of male reproductive toxicity. Therefore, we extended the study to identify histological changes in the testes. Testicular degenerative changes, including atrophied Leydig cells, the emergence of vacuoles, a decrease in the number of germ cells, a reduction in the diameter of the seminiferous tubule, an increase in the height of the germinal epithelium, and the absence of sperm in the lumen of the tubule were observed in hamsters exposed to the 50mg and 75 mg BPS. Thus, it is suggested that besides androgen deficiency, oxidative stress plays crucial role in BPS-induced testicular toxicity and associated histological damages. The results are in line with other studies that found the cadmium-mediated histological alterations in the epididymis, testes, and accessory sex organs[54]. Further, several experimental investigations have shown that exposure to environmental toxicants causes a considerable atrophy of the epididymis, a decrease in the lumen's width, and the number of sperm[42,59,60].

In conclusion, the present results suggest that exposure to BPS resulted reduced antioxidant enzymes activities, thereby increased lipid peroxidation in the epididymis in goldem hamster. BPS treatment also interfered with testosterone biosynthesis by induction of oxidative stress. Further, a decrease in testosterone production and increased accumulation of cellular ROS may contribute to the histological damages in the testes and epididymis. Taken together, this study suggests that exposure of BPS may have a potential risk to male reproductive health/ fertility.

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