**Bisphenol S: A replacement equally critical to male reproducibility**

Abstract

There is a sense of relief among consumers finding BPA-Free tag on various plastic products. But is it true, that a BPA-Free plastic is safe? Various studies suggest that very low dose of BPA can lead to multiple health adversities, including obesity, behavioural problems, reproductive anomalies, breast and prostate cancer. Despite regulations of BPA use in plastic products, the banning of bisphenols is facing serious defence from industries, noting unavailability of replacement. Nonetheless, analogue compounds such as BPS and BPF were introduced to the market and applied without rigours toxicological examination. In the present study role of BPS in male fertility and spermatogenesis was evaluated. Wistar albino rats were orally administered with 100, 500, and 1000 µg/kg body weight/day of BPS for 45 days. An additional group of animals were treated with simultaneous 1000 µg/kg body weight/day of BPS and 80 mg/kg body weight/day of Vitamin E for 45 days. Result of the study showed significant decline in fertility of male rat among all test groups. Hormonal assays confirmed dose dependent decline in serum testosterone, FSH and LH. The consequential impact was visible in stereological analysis which revealed significant alteration in density and volumetric count of germ cells and Sertoli cell. Histological architecture of testis indicated vacuolization in seminiferous tubules and presence of pyknotic spermatogonia. Conclusively, the results indicated BPS induced reproductive toxicity in testicular cells leading to decline in fertility. The study also confirms that concurrent dose of Vitamin E limited toxic impact of BPS.

Key words: BPS, Spermatogenesis, Male fertility, Reproductive toxicity

Introduction

Bisphenol S (BPS) is structurally similar to Bisphenol A (BPA) having two phenolic group on either side of sulfonyl group[1]. Repetition of BPS atoms are collectively known as polyethersulfone (PES). For decades of use bisphenol compounds are omnipresent these products are found in bottles, food/drink cans, dental implants, paper products[2]. Many studies have acknowledged BPA as endocrine disruptor and linked it with various health adversities such as; developmental decline, metabolic disorder, obesity, endocrine disorder, infertility, DNA damage, breast cancer etc.[3-5]. Due to such wide-range adversities and consequent policy regulations industries turned to replacements with relatively lower or no side-effects. These chemicals were structurally and chemically similar to BPA and formed by adding formaldehyde (BPF), sulfur trioxide (BPS), hexafluoroacetone (BPAF) etc. Most plastic products claiming ‘BPA free’ contain BPS[6-7].

A study by Frenzilli et al.[8] exposed *Salmo trutta* with 2 mg/kg fish and estimated for estrogenic disrupting marker. The result showed increase in vitellogenin and thyroid hormone triiodothyronine (T3) in plasma. Author also reported increase in number micronucleated cells. Due to extraordinary similarities in properties, BPS is equally hormonally active as BPA and thus, has endocrine disrupting effect (Rochester and Bolden, 2015). Since BPS is likely to interfere with endocrine system, its role in spermatogenesis and oogenesis can confirm its potential disrupting ability. A study by Roelofs et al.[9] reported that Leydig cells exposed to BPS revealed significant alterations in testicular steroidogenesis.

BPS is present in food products, a study by Cao et al.[10] reported detection of BPS in 11 composite samples of meat and meat products. Likewise, in a Canadian survey out of 140 food samples 79 showed presence of BPS and other bisphenols transferred through packaging materials to food products[11]. It is worrisome that BPS can be found in most of the individuals due to its ubiquitous. According to a study by Ghayda et al.[12] out of 338 paired samples of urine and semen 76% were positive for detectable of BPS concentration. Despite immense health risks upon ingestion there are insufficient data on toxicity. Specifically, in the field of male reproductive system, where BPA has already proven to show targeted adversities, role of BPS is still either elusive or less robust. It is also to note that concentration of doses of bisphenols have huge disparities in actions and adversities[13-14]. Thus, in the present study, role of low-dose exposure of BPS on fertility, testis and testicular functions were investigated.

Materials and methods

Test materials

Bisphenol S or 4,4’-Sulfonyldiphenol, 4-Hydroxyphenyl sulfone, was purchased commercially from Sigma Aldrich (Merck, MO, USA).

Animal model and ethical clearance

Wistar albino male rats (*Rattus norvegicus*) of age 3 months weighing 150-200 g were used in the present study. All animals were maintained in the university departmental animal facility under 24 hrs expert observation. All animals were housed in polypropylene cages (43×27×15 cm) provided with drinking water *ad libitum*. Animal house was routinely monitored for 12:12h light and dark schedule. Approval from Institutional Animal Ethics Committee (IAEC) was procured and experiments carried out according to prescribed guidance of Committee for the Purpose of Control and Supervision of Experiments and Animals[15].

Experimental design

Animals were divided randomly based on doses of BPS. Control animals (Group A) were treated with distilled water of equal amount used to dissolve BPS doses. Animals treated with 100, 500, and 1000 µg/kg body weight of BPS were designated as Group B, Group C, and Group D, respectively. Control and test groups were treated daily for 45 days with respective doses of vehicle, and BPS, respectively. Group animals treated with 1000 µg/kg body weight of BPS and 80 mg/kg body weight/day of vitamin E for 45 days were labelled as Group E (Table 1). On the 46th day animals were withdrawn from treatment and euthanized for further investigations. Doses of BPS used in the present study were according to the OECD guidelines for the testing of chemicals[16] and referred from earlier studies[17-18].

Table 1: shows specification of each group and number of animals.

|  |  |
| --- | --- |
| Group | Specification |
| Group A | Control - Treated with equal amount of distilled water used to dissolve BPS for 45 days. |
| Group B | 100 µg/kg body weight/day of BPS for 45 days |
| Group C | 500 µg/kg body weight/day of BPS for 45 days |
| Group D | 1000 µg/kg body weight/day of BPS for 45 days |
| Group E | 1000 µg/kg body weight/day of BPS and 80 mg/kg body weight/day of Vitamin E for 45 days |

Body and organ weight

Body weight were measured on the day of commencement of experiments and on the final day of scheduled autopsy. Following euthanization reproductive organs (main and accessory) were removed and weighed for group wise distinctions.

Fertility test

Periodical fertility tests were conducted following commencement of experimental schedule by cohabitating the male rats with fertile female rats at 1:2 ratio. Success of mating was confirmed by vaginal plug/appearances of spermatozoa in the vaginal smear.

Serum hormone analysis

Levels of FSH, LH, and testosterone in serum were measured by ELISA kits purchased from ThermoFisher Scientific (MA, USA).

Stereological assay of testicular cells

Density of germ cells (spermatogonia, spermatocytes, and spermatids) and Sertoli cell in the testis of control and BPS treated animals were evaluated according to method explained by Zhengwei et al.[19] and Wreford[20]. Briefly, cells were counted based in nuclear number at 40× on an axioscopic microscope. Counting cross checked and confirmed by nuclear counting through Image J software (NIH, USA). Field counting was followed according to Systemic Uniform Random Sampling Scheme (SURSS) developed by Gundersen and Jensen[21]. For calculation of densities of cells, 30 frames of 100 µm2, which corresponds to 3000 µm2 were evaluated per animal. Numerical density (NV) and numerical count (NC) per testis were calculated as follows:

NV = number of cells counted/area of frame × number of frames × depth)

NC = NV × testis weight

Histological evaluation of testis

A section of testis was fixed in 4% paraformaldehyde later dehydrated in graded ethanol. Tissues were cleared by Xylene and embedded in paraffin wax. A 5 µm thin section was fixed on microscopic slide and stained with Harris’s haematoxylin and eosin. Slides were observed under microscope at 10×, 40× and 100× oil immersion.

Statistical analysis

Numerical values of parametric analysis were represented in Mean±SE. These values were further compared statistically by One-way Analysis of variance (ANOVA) in combination with Tukey’s and Dunnett’s multiple comparison test (MINITAB, PA, USA). Student *t*-test was applied for paired data analysis (MS-EXCEL, Microsoft, SV, USA), level of significance was set at confidence intervals (CIs) of 95%, 99%, and 99.99%.

Results

Response of BPS on body weight gain

The present study showed consistent decline in weight based on increase in dose of BPS. In comparison to control (Group A), significant decline in weight gain was observed in Group C and Group D (Figure 1). Minimum weight gain was observed in animals treated with 1000 µg/kg body weight/day of BPS (Group D). based on initial and final weight the percentage change in Group D rats were mare 9.31% comparing to 14.36% and 12.48% of Group B and C, while control animals were noted with 17.43% weight gain. Interestingly, despite exposure of 1000 µg/kg body weight/day of BPS, Group E showed normal weight gain comparing to control. Group E was recorded with 14.99% of weight gain on the final day of observation (Figure 1).

Impact of BPS on reproductive organ weight

Unlike body weight, reproductive organ weight showed complex variations. Maximum variation was noted in Group D, where testis, epididymis, ventral prostate, and seminal vesicle showed significant decline in weight (Table 2). Surprisingly, Group C showed only significant decline in testis, remaining investigated organs showed weights within control range. Furthermore, Group E animals which appeared to have better weight gain comparing to other test groups revealed significant decline in both testis and ventral prostate. Based on observations of Group D and E, it appeared that animals treated with 1000 µg/kg body weight/day of BPS had significant impact on the reproductive organs regardless of main or accessory (Table 2). Notably, ventral prostate appeared to have no significant change in weight comparing to control.

Fertility record

Fertility record showed cent percent fertility in Group A through all observational schedule, whereas, all test groups showed slight decline in fertility (Table 3). Group B showed brief decline in fertility on the 30th day of exposure, which resumed back to 100% on 45th day of observation. The Group C showed slight but consistent decline on 30th and 45th day of observation which showed 90%. Unlike, other test groups Group D showed constant decline in fertility based on observation recorded on days 30 (90%) and 45 (80%). Remarkably, Group E showed slight but consistent decline in fertility, which was recorded as 90% on both 30th and 45th days.

Role of BPS in modulation of primary gonadotropins and testosterone

There was significant decline noted in serum testosterone of animals treated with various doses of BPS. The decline was dose dependent. Levels of testosterone in test groups B-E were noted as 2.26±0.09, 1.83±0.12, 1.58±0.11, and 1.94±0.07 ng/ml, respectively, against control (Group A) which was noted as 2.95±0.09 ng/ml. Group E indicated slight increase in level of serum testosterone comparing to Group D, nonetheless, variation was extremely significant (Figure 2A). Unlike response of testosterone, level of oestrogen in serum was completely inverse. A robust constant increase in the level of oestrogen was noted in Group B-D. These were measured as 1.22±0.06, 1.63±0.09, 1.94±0.10, 2.01±0.10 and 1.53±0.14 ng/ml for Group A-E, respectively (Figure 2B). Surprisingly, striking toleration in level of oestrogen was recorded which was statistically fall into the control range.

Level of FSH in test animals were significantly low comparing to control (Figure 2C). Concentration of FSH in groups B-E were noted as 1.93±0.23, 1.74±0.18, 1.60±0.26 and 1.86±0.11 mIU/ml against control (Group A) which was recorded as 3.09±0.07 mIU/ml (Figure 2C). Similar pattern was noticed in test groups for the concentration of LH. A constant drop was observed in LH concentrations as the dose of BPS increased. There was slight increase in concentration was observed in Group E when compared with Group D, however, it remained significantly low in comparison to control (Figure 2D).

Stereological analysis of testicular cells

Number of cells per cubic centimetre revealed distinct disparity among test groups when compared with control. Administration of 100 μg/kg of BPS (Group B) led sharp decline in the numbers of spermatogonia and spermatids, while significant increase in the number of spermatocytes were observed. There was slight increase in number of Sertoli cells, nonetheless, it remained within control range. Animals treated with 500 μg/kg of BPS (Group C) showed further decline in spermatogonia and spermatids, where notably, decline in spermatogonia was intensely sharp comparing to Group B (Figure 3A). Number of spermatocytes in Group C were extremely elevated comparing to control, while remained similar to Group B. Slightly lower number of Sertoli cell was observed in this group comparing to control, however, it continued to be non-significant. Interestingly, 1000 μg/kg of BPS (Group D) revealed extraordinary decline in number of all investigated cells except for Sertoli cell. Significant decline in number of spermatocytes, spermatids and spermatogonia was witnessed, though no alteration in number of Sertoli cell was observed in Group D animals (Figure 3A). Group E showed clear toleration against BPS comparing to Group B-D. It appeared that though irregularities in the number of spermatocytes were still prominent, nonetheless, comparatively higher number of spermatids and spermatogonia in Group E against Group B-D showed better endurance against BPS.

The surface plot showed the volume of spermatids which was maximum in control (Group A) largely inclined to spermatocytes in Group B and C (Yellow shade) (Figure 3B). Group C showed low volume of Sertoli cell in Group C (Green shade) comparing to other groups including control. Volume of spermatids were note resembled control in any test groups, indicating severity in the spermatogenic events. Group D showed lowest volumetric count of spermatogonia, leading to which obvious decline in spermatocytes, and spermatids was witnessed (Blue shade) (Figure 3B). Surprisingly, number of spermatocytes in testis of Group E resembled Group B and C, indicating similar response despite exposure 1000 μg/kg of BPS. It appeared that Vitamin E has protective role against the BPS induced testicular adversities.

Histological evaluation

Control testis indicated tightly packed seminiferous tubules, lumen filled with newly formed sperms. The interstitial space was occupied by Leydig cells, basal lamina was intact and thick, hosting Sertoli cells properly. Adluminal compartment was accommodating spermatogenesis. Spermatogonia, primary and secondary spermatocytes were visible in abundance. BPS showed direct effect on the histological structure of testis. Animals treated with 100 μg/kg of BPS (Group B) indicated round oval shaped seminiferous tubules with clear stages of spermatogenesis. Nonetheless, interstitial spaces between seminiferous tubules were found to be disintegrated, basal lamina was thin and Leydig cells were present ununiformly. Despite, lumen contained newly formed sperm and all stages of spermatogenesis were present (Figure 4). Initial distortion in spermatogenic wave was apparent in Group C, animals treated with 500 μg/kg of BPS indicated decline in spermatogonial stem cells which appeared to have fallen in the lumen, presence of spermatogonia cells with pyknotic nuclei was evident (Figure 4). Though primary spermatocytes were in abundance comparing to secondary spermatocytes and spermatids. Lumen of seminiferous tubules were only partially filled indicating interference in spermatogenesis. Animals treated with 1000 μg/kg of BPS (Group D) revealed severe damage to histological structure of testis, including thin or disappearance of basal lamina, disintegrated interstitial space, loss of Leydig cells. Excessive loss of spermatogonial cells and presence of vacuoles in the seminiferous tubules were common. Although presence of sperms was witnessed in the lumen it was either very limited or appeared to have fallen germ cells. Germ cells were disproportionate indicative of severe interreferences in spermatogenic events (Figure 4). Better tolerance was apparent in histological architecture of testis in animals treated with 1000 μg/kg of BPS and simultaneous dose of Vitamin E (Group E) (Figure 4). Nevertheless, basal lamina were still thin and interstitial spaces were partially disintegrated, Leydig cells were also appeared to have been affected. Lumen of seminiferous tubules indicated presence of sperms though it was limited in comparison to control (Group A). All stages of spermatogenesis were present; however, number of spermatids were comparatively low comparing to control.

Discussion

It is well-known that BPA is toxic to human health, thanks to extraordinary research-based facts, global campaign, governmental policies and bans. Industries begin searching for a suitable replacement, leading to development of many analogues of BPA, those were quickly adopted for containing similar properties. Nonetheless, already established notion against BPA led to higher scrutiny and quality of analogues on the basis of its potential adversities. Despite, many analogues of BPA are being used as a replacement. As per report of Chem Analyst the global demand of BPS is growing with 4.2% and expected to continue growing for another one decade[22]. In such scenario, it becomes extremely important to evaluate both short-term and long-term implications of its use. Previous studies have noted its ability to mimic oestrogen and to act as anti-androgen[23-26]. Since it interferes with endocrine system, thus, it is highly likely to be involved in reproductive functioning of both male and female. There are number of studies on adversities induced by BPS, including, metabolic perturbation, glycolytic dysfunction[27], developmental anomalies of mammary gland[28], neurotoxicity[29], alteration in kidney functioning[30] etc. BPS role in spermatogenesis and oogenesis has been well documented. Previous study claimed BPS exposure impairs meiotic event leading to disruption of germ cell progression[31]. Regardless, details on its mode of action during spermatogenesis and functional modulation of principal regulatory hormones are not well understood. The present study uncovers role of BPS in cell-wise progression of spermatogenesis with parallel evaluation of hormonal constancy.

The present study noted consistent decline in body weight gain of animals administered with 500 and 1000 μg/kg of BPS. Although it is noteworthy that 100 μg/kg of BPS could only marginally affect weight gain. Many previous studies have noted decline in body weight of rats following oral administration of rat[32-33]. However, a study by Azevedo et al.[17] reported that administration of 500 μg/kg of BPS increased weight gain in Wistar rats, which went contradictory to the present observation. The study by Azevedo et al.[17] was carried out for longer duration (6 months), it is possible that following initial decline significant resumption may have occurred. The present study clearly showed non-significant weight gain comparing to control in animals administered with 1000 μg/kg of BPS in combination with Vitamin E. Also, the lowest dose in the study (100 μg/kg of BPS) indicated weight gain in control range. In multiple cases sudden metabolic shock induced by chemicals lead to low appetite[34]. Thus, it is likely to observe change in weight gain when the body acclimatized to dietary alterations.

Decline in body weight gain was reflected on reproductive organ weight. Multiple organs showed significant decline in animals treated with 500 and 1000 μg/kg of BPS. Testis, Epididymis, ventral prostate and seminal vesicle indicated significant decline in weight, specifically in animals treated with 1000 μg/kg of BPS. Impact on reproductive organ weight following administration BPS indicate targeted impact on reproductive organs. Reduction in organ weight is reflective of chemical toxicity[35], thus, it can be established that BPS is highly likely to induce toxic response on male reproductive system. The possible impact of reduction in reproductive organs was also witnessed in the fertility record. The 15 days mating observations clearly showed decline in overall fertility of all test animals. Although consistency and continuation of decline in fertility was recorded in animals treated with 1000 μg/kg of BPS. Strikingly, concurrent administration of Vitamin E also could not resume complete fertility by the end of experimental schedule. The observed fertility record robustly confirmed significant role of BPS in male fertility.

Hormonal assays confirmed that administration of BPS regardless of doses affect concentration of testosterone and oestrogen. A consistent decline in testosterone indicate dose dependent restrain in biosynthesis of steroid hormone. Leydig cells are primary source of testosterone[36], decline in testicular weight indicate possible adversity in cellular functions. Likewise, increase in oestrogen can be predicted as in case of male its concentration is inversely proportional to concentration of testosterone[37]. Interestingly, bisphenols have been associated with feminization of male foetuses, atrophy of testis and epididymis[38]. It can be assumed that BPS also encourage increased biosynthesis of oestrogen while depleting production of testosterone through testicular damage.

Similar observations were observed for both investigated gonadotropins, which declined dose dependently, indicating direct role of BPS on hypothalamic-pituitary-gonadal (HPG) axis. Notably, LH and FSH are responsible for control of testosterone production and spermatogenesis in testis, respectively[39]. The present study indicated sharp decline in concentration of LH which corresponds to earlier observation for level of testosterone. Low level of LH confirms presence of low serum testosterone in animals treated with BPS. This study also hypothesizes a direct impact of BPS on HPG based on dose dependent decline. Decline in level of FSH is indicative of potential disturbance in spermatogenesis, which was observed with various degree in animals treated with BPS.

The stereological analysis robustly confirmed that density and count of all three investigated germ cell (*viz*. spermatogonia, spermatocytes, and spermatids) declined sharply in animals treated with BPS. The most aggressive decline was noted in animals treated with 1000 μg/kg of BPS, although a better toleration was noted in animals treated with concurrent dose of Vitamin E. Regardless, interference in germ cell progression was apparent and degree of these interferences were hugely dependent on dose of BPS. The least affected testicular cell by BPS administration was Sertoli cells. It is to note that cell death of Sertoli cells is not common, most injury to Sertoli cells is primarily reflected in metabolic and regulatory pathways that would lead to germ cell degeneration[40].

The observations of stereological analysis were established in histological architecture of testis. Although minimum interference was observed in sperm cell progression of animals treated with 100 μg/kg of BPS, the waning of basal lamina indicated direct impact of BPS. It is to note that basal lamina is mainly comprised of collagen type IV and glycoprotein laminin[41-42]. A previous study has mentioned that proteotoxicity is associated with laminins[43] of basal membrane. Strikingly, a study by Atlas et al.[44] reported that BPS disrupt acini organization MCF cells which grown in basement membrane matrix. The present study recorded loss of basal lamina in all test groups despite varying doses. Thus, it can be hypothesized that BPS induces proteotoxicity in the testicular microenvironment causing depletion of basal lamina. Loss of interstitial spaces and Leydig cells were also common in all test groups. There are multiple studies on bisphenols exposures (including BPS) that confirmed destruction of development and functions of Leydig cells, causing testicular dysgenesis and delayed puberty[45-46]. With higher dose of BPS more obvious and expected interference of germ cell progression was witnessed. Presence of pyknotic spermatogonia was common in animals treated with doses ≥500 μg/kg of BPS, which is a manifestation of apoptosis[47]. Likewise, presence of vacuolization was observed in seminiferous tubules of animals exclusively treated with 1000 μg/kg of BPS. Which is again an expression of excessive toxicity[48], indicating toxic role of BPS at higher doses in reproductive organs. Formation of vacuole and fragmentation is well associated with disproportionate oxidative stress[49-50], thus, despite same dose of BPS in Group E, concurrent administration of Vitamin E led to no or minimum vacuolization in the seminiferous tubules. This explained that oxidative stress may be one of the modes of BPS toxicity in testicular cells.

Conclusion

The present study evidently indicated role of BPS in altering of sexual hormones, spermatogenic events, and overall fertility in male. Based on pyknotic spermatogonial cells it can be concluded that the progression of germ cells from top to bottom was affected though induced apoptosis in spermatogonia. This study hypothesizes BPS induced proteotoxicity in the basal membranes damaging interstitial tissues and structural integrity of seminiferous tubules. Loss of Leydig cells and low testosterone levels reasonably explain reduction in fertility of exposed animals. Oxidative stress is potentially the prominent actor in BPS induced damage of testicular structure and functions, which was confirmed by better toleration in group of animals concurrently administered with Vitamin E.

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Figure 1: Body weight gain in male albino rats during 45 days of treatment against control. Level of significance was tested against vehicle treated control. \*P<0.05

Table 2: Reproductive main and accessory organ weight in test groups and control group.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Testis (g) | Epididymis (mg) | Vas deferens (mg) | Ventral prostate (mg) | Seminal vesicle (mg) |
| Group A | 1.43±0.02 | 63.15±1.17 | 8.04±0.31 | 41.26±1.32 | 211.61±1.47 |
| Group B | 1.44±0.02 | 61.54±0.97 | 7.88±0.13 | 41.66±1.24 | 210.92±3.18 |
| Group C | 1.37±0.01\*\* | 60.54±1.58 | 7.92±0.13 | 40.21±1.59 | 208.40±3.12 |
| Group D | 1.26±0.02\*\* | 57.38±1.10\* | 7.86±0.13 | 38.00±1.28\* | 201.37±2.51\*\* |
| Group E | 1.38±0.01\*\* | 61.24±1.51 | 7.94±0.08 | 38.78±2.09\* | 205.87±2.28 |

Level of significance was evaluated against sham treated control. \*P<0.05, \*\*p<0.01

Table 3: Percentage fertility of control and BPS treated male rats, pre and during the treatment.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Group A | Group B | Group C | Group D | Group E |
| Pre-Treatment | 100 | 100 | 100 | 100 | 100 |
| Treatment Phase | | | | | |
| 15 days | 100 | 100 | 100 | 100 | 100 |
| 30 days | 100 | 90 | 90 | 90 | 90 |
| 45 days | 100 | 100 | 90 | 80 | 90 |

A

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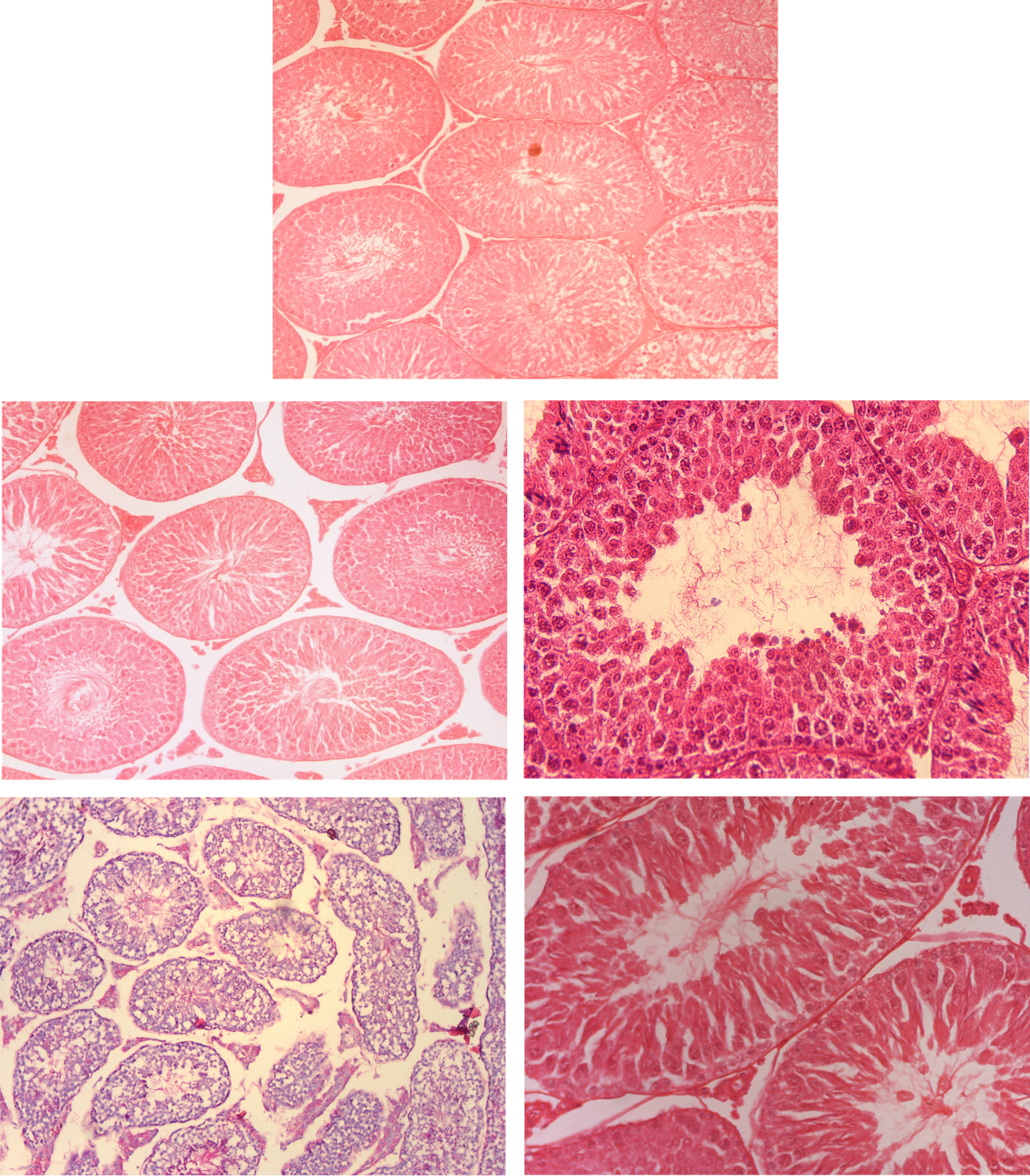
\*

Figure 2: Assay of A. testosterone, B. oestrogen, C. follicle stimulating hormone, and D. luteinizing hormone in testicular tissue of animals treated with various doses of BPS and control. Level of significance was examined against control. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

Stereological assay of testicular cells

Figure 3: Stereological evaluation of germ cells and Sertoli cell in the testis of vehicle treated control and BPS treated rats. A. testicular cell number of cells per cubic cm (NV) and volumetric count of cells/testis (in millions).

Group A



S

DIS

DIS

DIS

L

L

BL

SC

SD

ST

CT

L

LC

SG

Group E

Group D

Group C

Group B

Figure 4: Progression of germ cells in control (Group A) showed normal spermatogenic event consisting of abundant spermatogonia (SG), primary and secondary spermatocytes (S), and spermatids (SD). Lumen of seminiferous tubules (ST) in control group was filled with newly formed sperms. Interstitial spaces (IS) were tightly packed and Leydig cells (LC) normal. Group B showed normal seminiferous tubules and spermatogenic events (encircled lumen), though interstitial spaces were partially disintegrated (DIS), number of Leydig cells were also limited (shown in blue shade). Group C showed empty lumen and disturbed spermatogenic event. Pyknotic spermatogonia were visible (shown with arrows), progression of spermatogenic stages were not complete (shown with tow-sided arrow). Germ cells appeared to have fallen into the lumen (shown with arrow). Group D indicated pyknotic spermatogonia and vacuolization in seminiferous tubules at multiple occasions (Shown with arrow). In blue shade shown complete depletion of Leydig cells and interstitial tissues. Group E indicated sperms in the lumen (encircled lumen), spermatogenic events were also appeared to be normal. Though basal lamina was thin and interstitial spaces appeared disintegrated (shown with arrow).