Original Research Article

Role of Bisphenol A in regulation of arrival of sexual maturity and fertility in male albino rats

Abstract

Reproductive toxicity during prepubertal and pubertal period causes significant alterations in sexual maturation and fertility. Bisphenol A has been associated with decreased sperm production, damaged testicular cells, perturbed hormone levels and reduced fertility. Its role in sexual maturity and duration before first successful mating is still elusive. The present study aimed to investigate BPA induced alteration in testicular development and fertility at critical period of peri-adolescence, adolescence and adulthood. Male albino rats were administered with 5, 25, and 50 mg/kg body weight/day (Group II, III and IV) for three separate time intervals 42-63, 42-91, and 42-105 PND. Body and testicular weight were measured and polynomial regression was applied to predict associations between variables. Fertility test was observed at every 7 days interval for each dose at investigated time intervals. Histological observations were carried out to examine parallels between results of fertility test. Results of each parameter was compared with respective sham control (Group I). A consistent decline in testicular weight gain was observed in BPA treated groups. Strong relatedness between body and testicular weight variables was evident. Initial successful mating was delayed significantly in BPA administered rats. Group IV showed maximum delay in first successful mating. Histological observations indicated interferences at various stages during spermatogenesis along with presence of tubular vacuolization, indicative of cellular toxicity. Higher doses of BPA inflicted more damage, likewise, longer duration amplified severity of adverse activity. BPA administration at critical period of reproductive development in male rats can adversely affect testicular functions leading to delayed and reduced fertility.

Key words: Male fertility, Bisphenol A, Spermatogenesis, Sexual maturity

1. Introduction

Age is the determining factor in assessing long-term influence of chronic toxicity. Specific age at which toxic exposure was noted, plays important role in determining longevity and eventuality of induced adverse effects. In a life time adolescence is considered as one of the most critical transitions of elements such as; biological growth, and social development. Biological processes such as pubertal transition from childhood to adulthood causes intense psychological pressure. Periadolescence in rats have shown enhanced performance in active avoidance learning task due to hyperactive and conspecific play behaviour, nonetheless, perform poorly in more complex appetitive and avoidance learning task [41]. Sexual maturity is also one of the complex transitions that takes place during this period of time. Biological, genetic and neuro-endocrinal factors combinedly affect the sexual maturity. Gonadal hormones, cortisol, and cascades of other hormone play significant role in successful onset of puberty. According to the U.S. Census Bureau, human reach adolescence at age between 10-19 years [43]. Toxic stress and related adversities during pubertal age have been associated with mental and physical health disparities later in life [13,19]

Bisphenol A, a potent endocrine disrupting chemical, has been associated with precocious puberty in girls, indicating direct or indirect influence in modulation of ovaries and uterus [30,39]. Essentially, during periadolescence and pre-pubertal age level of oestrogens and androgens are extremely low, nonetheless, receptors of these steroids are expressed very early [26]. It explains how BPA exposure during early life changes child hormones, leading to several pubertal changes [27]. Male reproductive system has been one of the most debated topics in BPA induced infertility. Toxic effects of BPA have been demonstrated by alterations in foetal, pubertal and adult life of rodents [23,24,28]. It is now widely accepted that BPA cause alteration in testicular structure, function and semen parameters [3,22,34,37,45,48]. As per earlier studies androgen augments pubertal growth in male, however, growth spurt kinetics of pubertal development have been strongly associated with normal testicular functions [12,32]. Despite, wide-range of research in early life exposure of BPA, its specific role in arrival of sexual maturity was loosely attended. This study estimated age specific differential role of BPA exposure in alteration of male reproductive organ, sexual maturity and fertility.

2. Methodology

2.1 Test compound

Bisphenol A [2,2-bis (4-hydroxyphenyl) propane or $C_{15}H_{16}O_2$] of 99% purity was purchased commercially (Sigma Aldrich, MO, USA).

2.2 Experimental animals

Wistar albino rats (*Rattus norvegicus*) of age 42 PND (postnatal days), weighing in range of 100-110 g were used in this study. An appropriate day and night schedule (12 h: 12 h) was provided to these animals in the departmental facility. Polypropylene cages of size 43×27×15 cm were labelled and subsequently housed with randomly selected animals. Rats were fed with pellet diet and drinking water was provided *ad libitum*.

2.3 Ethical approval:

Animals were maintained in the departmental facility under appropriated supervision of veterinary expert. All experiments were strictly carried out under guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2010) and Indian National Science Academy (INSA), for Care and Use of Animals. Experiments and protocols were also approved by the Institutional Animal Ethics Committee (IAEC) [6]

2.4 Experiment design

Animals were divided in three groups based on daily doses of BPA administration i.e., 5 mg, 25 mg, and 50 mg/kg body weight. According to Long and Evans (1920) rats reach puberty at around 50 PND, this is a significant event, during which first wave of spermatogenesis in testis and first functional sperm in the epididymis appear [21]. These groups were further divided in three sub-groups based on age i.e., peri-adolescent (42-63 PND), adolescent (42-91 PND), and adult (42-105 PND). Therefore, BPA was administered daily from 42 PND to 63 PND, 91 PND, and 105 PND. Table 1 shows the doses of BPA and age-wise distribution of animals in the groups and sub-groups. A parallel sham control group was used, animals of these groups were administered with vehicle only. At termination of each schedule animals from each group were sacrificed for further investigation.

Table 1: Details of investigated groups with respective specifications (n=15)

Group Name	**Specifications**	
Group I	a.	Sham control - Vehicle treated for 3 weeks from 42-63 PND
	b.	Sham control - Vehicle treated for 7 weeks from 42-91 PND

	c.	Sham control - Vehicle treated for 9 weeks from 42-105 PND
Group II	a.	Oral administration of 5 mg BPA/kg/body weight/day for 3 weeks from 42-63 PND
	b.	Oral administration of 5 mg BPA/kg/body weight/day for 7 weeks from 42-91 PND
	c.	Oral administration of 5 mg BPA/kg/body weight/day for 9 weeks 42-105 PND
Group III	a.	Oral administration of 25 mg BPA/kg/body weight/day for 3 weeks from 42-63 PND
	b.	Oral administration of 25 mg BPA/kg/body weight/day for 7 weeks from 42-91 PND
	c.	Oral administration of 25 mg BPA/kg/body weight/day for 9 weeks 42-105 PND
Group IV	a.	Oral administration of 50 mg BPA/kg/body weight/day for 3 weeks from 42-63 PND
	b.	Oral administration of 50 mg BPA/kg/body weight/day for 7 weeks from 42-91 PND
	c.	Oral administration of 50 mg BPA/kg/body weight/day for 9 weeks 42-105 PND

3. Parameters

3.1 Body and testicular weight

Body weight was recorded at the initial day of experiment and on the final day before termination of schedule. Following termination of experiment animals were sacrificed and testes were collected for weight measurement.

3.2 Fertility test

Periodical fertility tests were conducted every 7th day from commencement of experiment by cohabitating individual male with fertile females in 1:2 ratio. Success of mating was confirmed by vaginal plug/appearances of spermatozoa in the vaginal smear. Fertility was established by number of pregnant females following each successful mating.

3.3 Histopathological analysis

Testicular tissues were taken following scheduled euthanization. Tissues were then fixed in 4% paraformaldehyde for 24 hours, later dehydrated in ethanol. This was followed by clearing xylene and embedding tissues in paraffin wax. Further to this 5 µm thin sections were cut and fixed on glass slides. Later, staining with Harris's haematoxylin and eosin for light microscopic observations was carried out.

3.4 Statistical analysis

Mean of numeric observation was presented with respective standard error (SE). One way Analysis of variance (ANOVA) (MINITAB, LLC, PA) was applied for assessment of treatment related significant change. Student's t-Test was applied for paired type data for associated probabilities. For all quantitative variation $P<0.05$, 0.01, and 0.001 were considered significant, highly significant and extremely significant. Polynomial regression analysis was carried out to evaluate turns and end behaviour within variables of testicular weight and body weight.

4. Results and Discussion

4.1 Body and testicular weight

Initial body weight of test animals in all groups was within range of 105-110 g. Final weight was observed at 63, 91, and 105 PND. A constant increase in body weight of control rats was observed, revealing weight gain of 60, 90.80, and 150 g at observed postnatal days, respectively. Though, constant increase was also observed in test groups, nonetheless, the weight gain was significantly lower in comparison to parallel control (Figure 1A-1C). Lowest weight gain in comparison to control was observed in Group IV, which was recorded as 39.20, 60.60, and 97.80 g at observed postnatal days, respectively (Figure 1C).

Similarly, age wise increase in testicular weight was observed in all groups. Nonetheless, significant decline in testis weight was apparent when compared with parallel controls (Figure 2). Testicular weight of control animals showed maximum elevation at 105 PND, the elevation was exponentially high in comparison to 63 PND to 91 PND. The same varied significantly in rats exposed to 5, 25, and 50 mg BPA (Groups II-IV).

4.2 Polynomial regression analysis of variation in body weight and testicular weight

Second order polynomial expression indicated flattened curve in dose-wise distribution of weights, which implicated complete relatedness (R^2=1) between both testicular weight and body weight (Figure 3A). On the other hand, age-wise distribution of variables indicated differential end behaviour comparing to unique trend of turns in dose-wise distribution. Despite differential 3rd order polynomial expression, near-perfect relatedness (R^2=0.999) and increasing end behaviour was evident in weight variations following 105 PND with respect to 63 PND. On the contrary, with same credentials expression of terms in 42-91 PND variables indicated a negative end behaviour against 42-63 PND variables.

4.3 Fertility record

BPA significantly altered mating successes, causing reduction in fertility of BPA exposed males. Unlike control males successful mating was evident from 49 PND onwards, however, pregnancy was only confirmed following 63 PND. At 63 PND, only 60% fertility was observed in control males (Table 2). No sperm was observed in the vaginal smear of females cohabitated with BPA administered male rats during 42-63 PND, specifically groups III and IV. Sperm was visible in vaginal smear of females mated with male rated of Group II of age 63 PND, nonetheless, no pregnancy was observed. Groups of males observed for 42-91 PND indicated similar pattern as 42-63 PND, nonetheless, sperm begin to appear in cohabitated females in groups II and III following 63 PND. Despite, successful mating was confirmed, groups II and III showed no pregnancy until 70 PND. Pregnancy was observed in females mated with groups II and III following 77 PND. Interestingly, no sperm was observed in vaginal smear of females cohabitated with Group IV males until 77 PND. Initial observation of pregnancy in mated female was only apparent from 84 PND. Groups of animals exposed to BPA for 9 weeks between 42-105 PND showed similar trend as if groups observed during 42-91 PND. However, significantly lower fertility was observed in males exposed to BPA. Noticeably, a gradual increase in fertility was observed in Group II, maximum fertility was noted on 105 PND, amounting 40%. Groups III and IV, indicated slight improvement in fertility by 91 and 98 PND, but eventually declined on 105 PND by almost 10% (Table 2).

4.4 Histological observations

Histology of testicular tissues of control rats (Group I) indicated normal architecture, including round seminiferous tubules surrounded by thick basal lamina and interstitial cells of Leydig. Sertoli cells and germ cells appeared normal in all age groups of control. However, number of sperms in the lumen of seminiferous tubules increased significantly in 91 and 105 PND rats when compared with 63 PND control (Figure 4). BPA exposed Group II also indicated normal testicular histology following 63 PND, which was comparable to control. However, following 42-91 PND and 42-105 PND of BPA exposure, Group II showed tubular vacuolization and germ cells degeneration, respectively (Figure 4). Higher number of vacuolization and degeneration of germ cells were noted in Group III. Degree of degeneration increased significantly as duration of BPA exposure increased (i.e., 42-63<42-91<42-105). Higher disorientation in the germ cell proliferation was noted in Group IV, loss of basal lamina and Leydig cells was observed in animals exposed for 63 PND. Whereas, Group IV exposed for 42-

91 and 42-105 PND indicated severe disorder in progression of spermatogenic cells. Seminiferous tubules were unorganized with thin and disrupted basal lamina. Large number of germ cells undergone degeneration. Tubular vacuolization was evident in all Group IV animals regardless of duration of BPA exposure (Figure 4). Cessation of spermatogenesis was not witnessed in any BPA exposed groups irrespective of duration of exposure. Lumen of seminiferous tubules were partially filled in all Group III and IV animals. Group II animals showed adequate quantity of sperm in lumen regardless of duration of exposure, however, unlike Group I, no elevation in the number of sperm was apparent between 63 PND and 105 PND.

4.5 Discussion

Delayed sexual maturity (DSM) is considered when boys and girls of age 13-14 years does not show signs of gonadal development [40]. Pubertal signs such as breast development and testicular enlargement are considered as first sign of gonadal development. Various conditions have been associated with delayed sexual maturity in males and females, such as; chronic illness, nutritional deficiency, social and psychological situations. Nonetheless, overall deficiency and/or imbalance in growth hormone led to DSM in adolescents [17]. Bisphenol A has been associated with reduced testicular weight by multiple studies [1,16,18]. Earlier study has noted gonadal tissues to be sensitive target for reprogramming effect of key hormones [31]. Woldemeskel (2017) explained that testicular weight is sensitive to toxicity, specifically due to perturbation in rapidly dividing germ cells [47]. Explicit effects of BPA on male reproductive system indicate that early life exposure may interrupt sexual maturation during adolescence. The present study aimed to evaluate alteration in fertility of BPA exposed male rats at critical period of first successful mating. These alterations were further verified with testicular weight and histological observations.

In general, exponential weight gain in Wistar male rats have been noted following 28 PND, which continues incessantly until 77 PND. Following 77 PND, weight growth curve begins to flatten although gain in weight still continues [25]. Present study noted 55%, 83%, and 138% growth in weight of control rats following 63, 91 and 105 PND, respectively. Based on earlier reports on weight growth in Wistar male rats under controlled diet, the present growth rate was expected and in accordance with standard growth curve. However, daily administration of BPA indicated dose dependent decline in body weight. Through investigated doses and period of exposure a decline of 10%-46% in overall weight gain was observed when compared to control. Maximum decline in weight

gain was observed in rats administered with 50 mg/kg BPA whereas, minimum decline was observed in 5 mg/kg BPA administered rats. There are three types of circumstantial responses reported on alterations in body weight following BPA exposure, 1) BPA is an obesogenic chemical [20], 2) Conjugated BPA is related to reduction in body to mass index (BMI) [8], and 3) Reduction in body weight during weaning period followed by an increase during post-weaning [42]. Results of this study showed reduction in weight gain invariably in all rats administered with BPA, which indicated potential increase in conjugated BPA, previously associated with interferences in glucose homeostasis [42].

With respect to body weight, testicular weight was also expected to follow the trending influence of BPA. Comparing to parallel control, extremely significant decline in testicular weight was observed in 105 PND rats, irrespective of doses of BPA administered. Dose related deflection in testicular weight was distinctly observed during each period of investigation, nonetheless, maximum deflection was observed in 91 PND, followed by 63 PND and 105 PND. It appeared that BPA had a complete control on testicular development, as decline in testicular weight gain following 42-63, 42-91, and 42-105 PND of administration was certain and highly consistent. Although, higher doses did show higher decline in testicular weight gain comparing to control. It also emerged that higher doses of BPA (i.e. 25 and 50 mg/kg) had minimum impact on reduction in testicular weight gain when compared with low dose (i.e. 5 mg/kg). One of the possible reasons for this could be low bioavailability of BPA. A study by Pottenger et al. (2000) explained that BPA follows route dependency, where relative bioavailability of oral administration was lower than intraperitoneal or subcutaneous administration [29].

To establish robust relatedness between body and testicular weight under influence of BPA exposure, polynomial regression was applied. Under dose-wise alterations a complete relatedness (R^2=1) was observed, however, a slightly negative end behaviour was evident for animals exposed to 5 mg/kg BPA (R^2=0.999). It reveals that there is a probability of loss in relatedness between body weight and testicular weight, nonetheless, its chances are highly unlikely at this particular dose. Various earlier studies have noted increase in body weight while reduction in testicular weight following low dose exposure of BPA [2,5,7,15]. Thus, it is possible to assume an inverse proportionality between body and testicular weight. Similarly, under age-wise distribution of body and testicular weight, a positive end behaviour was observed in 42-105 PND. Which implicates probability of strong dependence between both variables of weights. Comparatively, lower dependency within

variables were present during period of 42-91 PND. It was important to note that regardless of presence of turning points in polynomial trendline of 42-105 PND variables, the R^2 values remain close to perfect '1', indicative of strong relatedness between testicular and body weight. Likewise, variables of 42-91 PND were strongly related under influence of BPA. Notably, both age-wise distribution of variables (i.e. 42-91 and 42-105 PND) were compared against 42-63 PND variables, thus, the trendline only predicts long-term dependency and relatedness between body and testicular weights based on initial alterations in weights strictly under influence of BPA. The present study predicts that continuation of daily doses of BPA for longer term may alter body and testicular weights independently.

A study by Robb et al. (1987) reported that Wistar rats first round of sperms was only visible in cauda epididymis at the age of 50 PND, which increases to full strength by 75 PND [33]. The present study evaluated fertility in rats commencing from 42 PND, the observations of Robb et al. were found in accordance to estimated results of fertility. Since no observation of sperm in the vaginal smear was considered 'no successful mating', presence of sperm without pregnancy was considered 'no fertility'. This study indicated presence of sperm in vaginal smear from as early as 49 PND which continued with no fertility until 56 PND days in control rats. Whereas, fertility was first recorded in control rats at age of 63 PND. There was clear sign of dose dependent delay in successful mating of animals exposed to BPA. In an interesting study by Farabollini et al. reported that male sexual performance towards stimulus female rats was impaired due to BPA exposure [11]. This study also reported depotentiation of male behaviour in male rats. This study noted no fertility in 5 and 25 mg/kg BPA exposed males until 70 PND, whereas, no fertility was noted on 77 PND in rats exposed to 50 mg/kg BPA. Interestingly, no successful mating was observed in rats exposed to highest dose until 70 PND, which indicates significant alteration in potentiation of sexual behaviour in male rat. There are multiple studies that indicated impairment in the fertility of male rats following BPA exposure [9,36,46]. The present study confirmed dose dependent reduction in fertility of exposed male rats. No successful mating beyond 49 PND days affirmatively indicate delay in sexual maturation of exposed rats.

Histological observations reaffirmed findings of fertility test. With increasing dose and duration of exposure an escalating trend in disorganization of germ cell progression was witnessed. Various earlier studies have noted similar histological attributes following BPA exposure [10,14,44]. Tubular vacuolization in seminiferous tubules was commonly present in all BPA exposed rats. Vacuolization

and atrophy in testicular tissues are signs of sever toxicity [4]. A study by Alboghobeish et al. reported that 50 mg/kg BPA treated rats exhibited vacuoles and atrophy in seminiferous tubules [1]., which went in accordance to the current study. This study indicated that periadolescent exposure of higher doses of BPA posed greater threat to fertility later in life than lower dose. However, this does not corroborate to safety at lower doses. This study noted multiple degeneration of germ cells in seminiferous tubules of BPA exposed animals. Number of cells undergone degeneration increased substantially based on duration and dose of BPA administration. By that it means that animals administered with 50 mg/kg BPA daily for 42-105 PND contained highest number of germ cell degeneration. It is to be noted that during normal spermatogenesis several germ cells undergo degeneration at critical key check points, such as; type A spermatogonia, midpachytene spermatocytes, 1° and 2° spermatocytes [35]. Germ cell degeneration is also high during early pubertal age as suitable physiological microenvironment is not prepared under crucial hormonal deficiency [38]. Excessive degeneration of germ cells is indicative of major interference in spermatogenesis or deficiency of critical hormonal homeostasis. Based on this, it is more likely to observe functional failure of testis, if it is exposed to BPA during period of pubertal testicular growth.

5. Conclusion

Conclusively, daily doses of BPA investigated in this study indicated significant alterations in achieving sexual maturation and length of peri-adolescence in male Wistar rats. Testicular weight which is an important marker for evidential pubertal changes, showed substantial decline in growth during critical 42-63 PND period. Rats exposed to 50 mg/kg BPA showed no successful mating until 70 PND which comparatively delayed first successful mating by nearly 4 weeks when compared with control. The present study additionally confirmed that BPA administration at critical period of reproductive development in male rats can adversely affect testicular functions leading to delayed and reduced fertility.

6. Ethical approval:

Animals were maintained in the departmental facility under appropriated supervision of veterinary expert. All experiments were strictly carried out under guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2010) and Indian National Science Academy (INSA), for Care and Use of Animals. Experiments and protocols were also approved by the Institutional Animal Ethics Committee (IAEC).

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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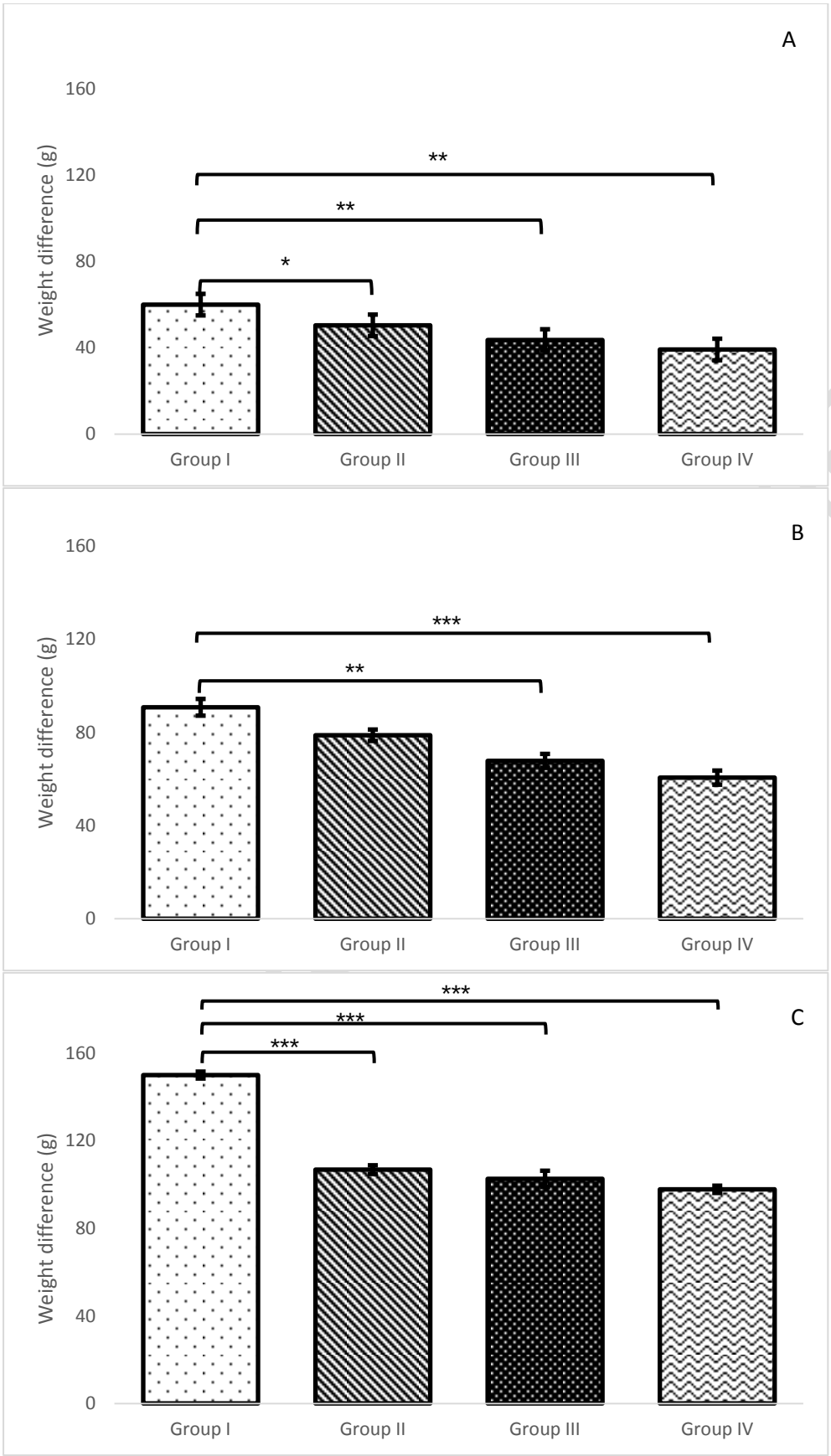

Figure 1: Body weight difference between initial and final weight of BPA exposed groups along with respective parallel control. A. 42-63 PND, B. 42-91 PND, and C. 42-105 PND.

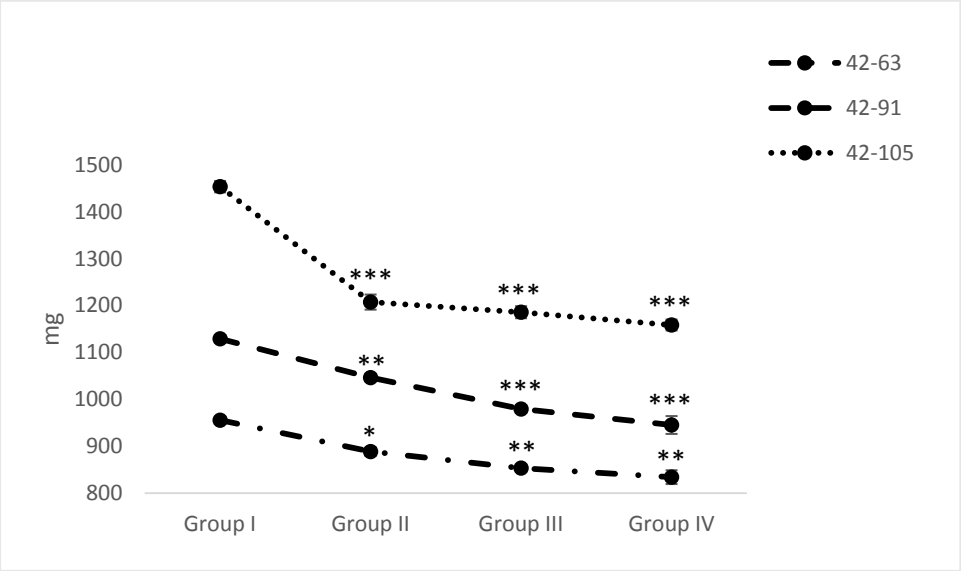

Figure 2: Testis weight of animals administered with daily doses of BPA against sham treated animals.

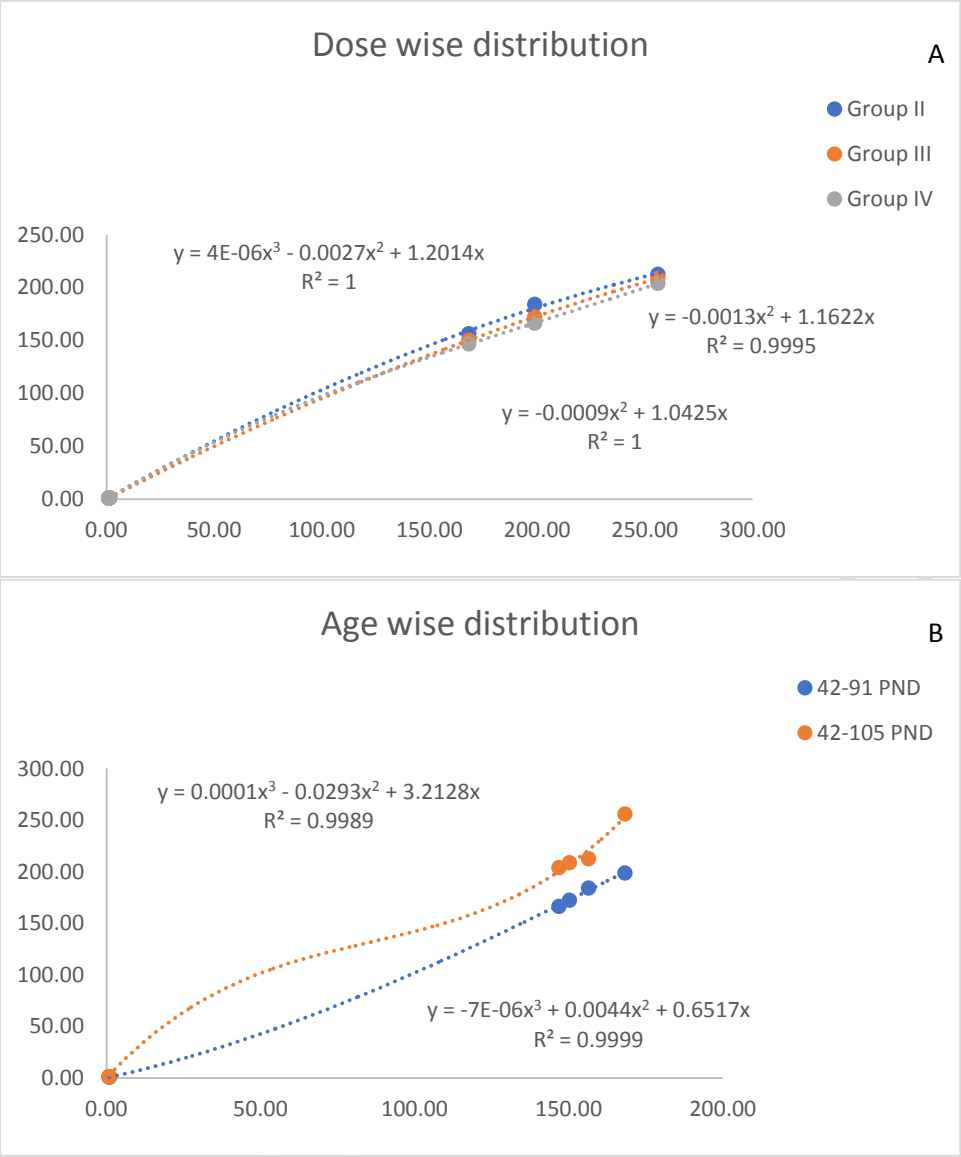

Figure 3: Polynomial regression analysis of body weight and testicular weight with respect to A. dose dependent and B. age dependent variables. Best fit scenario was observed at 2nd order polynomial in dose wise distribution, whereas, 3rd order was applied for age wise distribution.

Table 2: Fertility record of animals exposed to daily doses of BPA in comparison with sham control.

42-63 PND				
Mating days	Group I	Group II	Group III	Group IV
42PND	NS	NS	NS	NS
49PND	0	NS	NS	NS
56PND	0	NS	NS	NS
63PND	60	0	NS	NS
42-91 PND				
	Group I	Group II	Group III	Group IV
42PND	NS	NS	NS	NS
49PND	0	NS	NS	NS
56PND	0	NS	NS	NS
63PND	60	0	0	NS
70PND	100	0	0	NS
77PND	100	20	10	0
84PND	100	20	30	10
91PND	100	20	30	20
42-105 PND				
	Group I	Group II	Group III	Group IV
42PND	NS	NS	NS	NS
49PND	0	NS	NS	NS
56PND	0	0	NS	NS
63PND	60	0	0	NS
70PND	100	10	0	NS
77PND	100	20	10	0
84PND	100	20	10	10
91PND	100	20	30	20
98PND	100	30	30	10
105PND	100	40	20	10

NS: No sperm in the vaginal smear

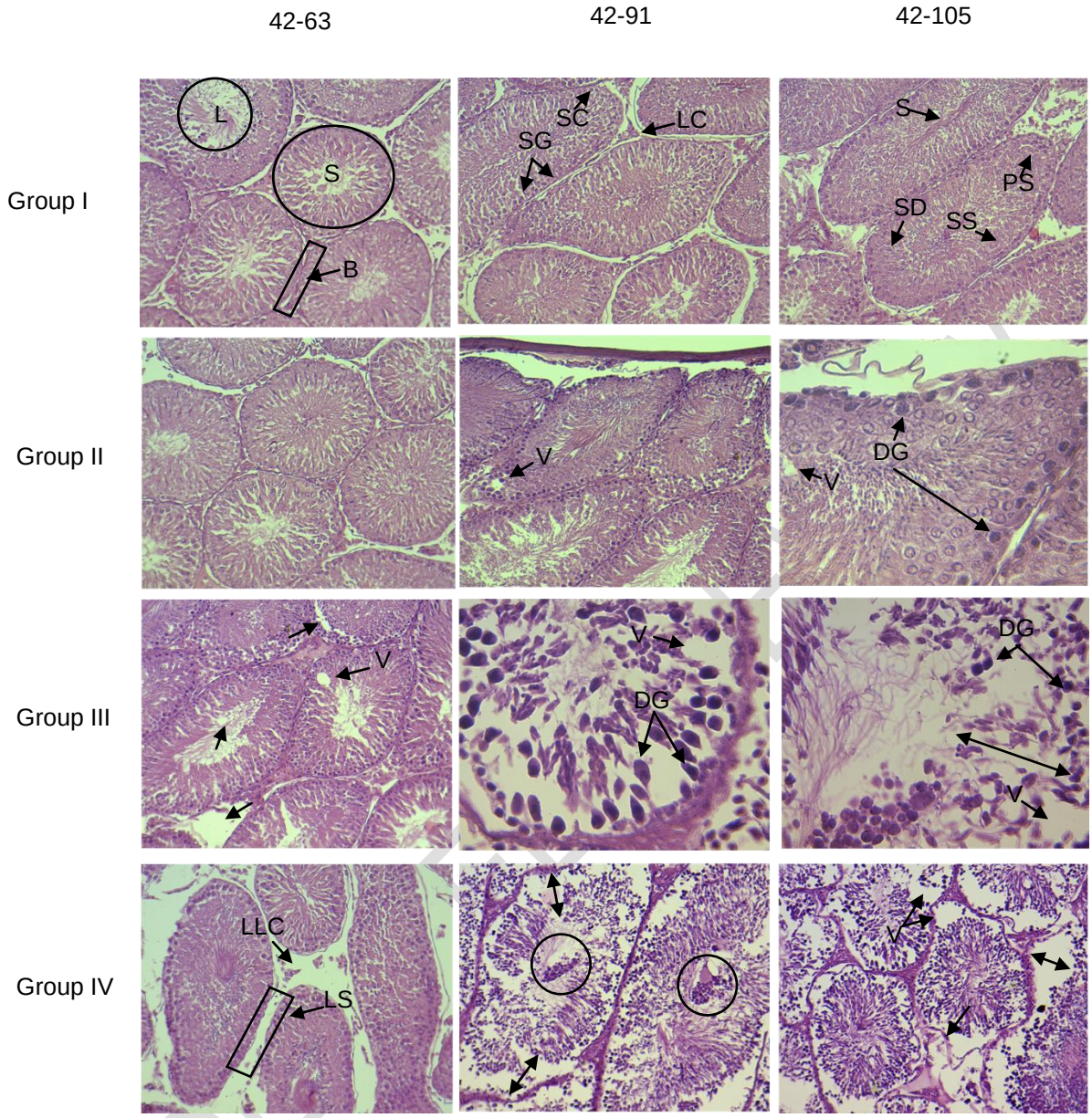

Figure 4: Histological observations of testicular tissues of groups I-IV at 63, 91 and 105 PND. Group II animal of age 63 PND showed tightly packed round shaped seminiferous tubules (ST) separated with interconnecting basal lamina (BL). Appearances of Leydig cells (LC) and Sertoli cells (SC) were normal, propagation of germ cells including spermatogonia (SG), primary (PS) and secondary spermatocytes (SS), spermatids (SD) also appeared normal when compared with control. While testicular architecture of Group II rats of age 63 PND appeared normal, 91 PND rats indicated BPA appearance of vacuolization (V) in the seminiferous tubules. Through 105 PND Group II rats indicated degeneration of germ cells

(DGC) along with vacuolization. Tubular vacuolization was visible in Group III rats as early as 63 PND. Though similar abnormalities carried through 91 and 105 PND, number of germ cells undergone degeneration increased substantially. Group IV rats additionally showed loss of smooth muscles in between seminiferous tubules and loss of Leydig cells at age of 63 PND. Whereas, disorientated germ cell development (shown with double sided arrows) was normal in 91 PND and 105 PND rats of Group IV. Germ cells appeared to have fallen into the lumen (shown with circles). An increase in number of sperms in the lumen of sham control rats were observed through 63, 91 and 105 PND, whereas, the same was not observed in groups II-IV. Nevertheless, sperms were distinctly present in the lumen of seminiferous tubules of all BPA exposed rats.