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Effect of Bisphenol A and Exogenous Sex Hormones on the Male Reproductive System of Wistar Rat

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ABSTRACT
Background: Bisphenol A (BPA) is as an endocrine disrupter with effect on semen parameters and sex hormone regulation.
Aim/Objectives: This study was done to investigate the effects of BPA and Synthetic Sex Hormones on male Wistar rat's reproductive system.
Methodology: Twenty pubertal male Wistar rats weighing between 50g – 100g were randomly assigned to four groups (I-IV); Group 1: control rats were given distilled water. Group 2: rats were administered with plastic BPA 200mg ,Group 3: rats were given 0.625 mg of oestrogen once daily, Group 4: rats were given intramuscularly 15mg of testosterone for six weeks to cover seminiferous cycles in Wistar rats.
Results: showed a significant mean weight gain at 6th week in BPA group compared with the and control (98.35 + 32.8 vs 84.63 +25.3)(P=0.017).There was a decrease in the total sperm count of the BPA exposed group compared to control (177.80±33.3 vs 298.40± 58.2) (P=0.006).The oestrogen exposed group showed an increase in sperm cells with no head when compared to control (57.80±13.1 vs 29.40±13.0)(P=0.009). Sperm vitality decreased in all the three experimental groups compared to the control (42.4%vs 57%)(P=0.000).There was an observed increase in the oestrogen hormone levels in BPA group compared to control (177.5ng/ml vs 103.2ng/ml) and slight increase in Testosterone hormone level among the BPA exposed of wistar rats compared to control (24.39ng/ml vs 10.41 ng/ml). Histology showed that BPA caused disoriented elongated spermatid, leydig cell degeneration with increment in intertubular space, degenerated germinal layer of the seminiferous tubules; thinned out with aspermic lumen, it remained entirely benign.
BPA and Synthetic Sex Hormones cause significant changes in the male reproductive system.
Key words: Bisphenol A, Synthetic Sex hormones, male reproductive system changes, Spermatogenesis.

I. INTRODUCTION

Endocrine disrupting chemicals (EDCs) are chemical compounds that interfere with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis and the regulation of developmental processes.[1,2] This may have adverse outcome on the hormonal regulation of the male reproductive system.[1] EDC affect gamete cell which predisposes an organism to subfertility.[2] Several studies had shown sperm parameters are declining globally in bothhumans and animals[.2.3]

Bisphenol A (BPA) is an organic molecule belonging to Alkyl phenol group.[4] BPA is a chemical produced in large quantities for use primarily in the production of polycarbonate plastics and epoxy resins.[5] It is one of the most likely common EDCs found in our environment. Recent animal study has shown that BPA exposure lowers sperm parameter and sex hormones male rats.[6] Studies had shown BPA causes a sperm death and had toxic effect on male reproduction.[7] Therefore, the aim and objective study was to compare the effect of Bisphenol A and synthetic sex hormones on the male reproductive system of Wistar rats at Bayero University Kano.

II. Materials and Methods

Study design: A randomized controlled trial.

The experiment was performed in the Physiology Laboratory of Bayero University, Kano, over a period of 6 weeks after obtaining approval from the university ethical committee (BUK/CHS/REC/01/16). A total of 20 sexually mature male Wistar strain albino rats, 90 days of age weighing 50-100kg were purchased from the Animal House of Department of Physiology, Bayero University, Kano. The principles of laboratory animal care were followed throughout the experiment. The animals were provided with sufficient space and housed at room temperature, with a 12:12 h light–dark cycle throughout the

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experiment and were allowed one week for acclimatization prior to dosing. Their cages were cleaned twice a week and were fed with Starter feeds purchased from.

The twenty (20) male wistar rats were randomly divided into four (4) groups of five (5) rats. The animals were recruited using a systemic randomized sampling at a sampling interval of 4 representing the four 4 groups (Group A, B, C and D). The allotted numbered animal was marked, grouped and housed in several cages.

Oral Bisphenol A 4mg/kg/Dose (Germany, CAS No.80-06-7) was purchased from Bristol Scientific company. The oestrogen used was oral Oestrogen 3.125ug/dose (ETTK1-002,10/2018. Premarine) and Testosterone propionate injection USP 15mg/dose (India MB-04-87) were used for the study groups B, C and D, while placebo was given to group A ie the control group.

Thereafter, their sperm parameter, hormone profile and testicular histology were measured and observed.

All animals were weighed on a daily basis during dosing and then weighed on weekly basis until the termination of experiment (after 6weeks duration) using a digital weighing scale (ACCULAB VI1200, USA) to evaluate for body weight change. Experiment was terminated 6weeks after the start of dosing and animals were humanely sacrificed by an overdose of halothane followed by cervical dislocation.

Approximately 3ml of blood was collected by intra-cardiac puncture meticulously and dropped into ethylene diamine tetra-acetate (EDTA) bottles appropriately labelled A1-A5, B1-B5, C1-C5 and D1-D5 C1 to C5, B1 to B5, O1 to O5 and T1 to T5. Intracardiac puncture was done for each group meticulously to avoid contamination. Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (27˚C) and 2 drops of warm 2.9% sodium citrate was added. Liver, kidney, testes, epididymis, seminal vesicles, and ventral prostate were removed and weighed for histology, unique identification numbers were assigned to each specimen bottle. The samples were kept in a cold chain and subsequently transferred to the central research laboratory for analysis.

The blood samples for hormone assay were centrifuged at 3,000 rpm for 10 minutes in an automatic refrigerated centrifuge to separate the serum. The serum FSH, LH, Oestrogen and testosterone levels were determined in the samples using commercially available ELISA Kit. Seminal analysis was performed with haemocytometer and microscope to get the concentration of the spermatozoa, sperm motility and the percentage of live spermatozoa. They were compared to determine the statistically significant effect on Sperm count, Sperm morphology. The tissues removed were stained with hematoxylin and eosin (H&E) to examine histological and morphological changes in the testes of the oestrogen, testosterone and BPA treated animal tissues respectively. Gonadosomatic Index by weight was calculated as described by (Caldeira*et al*,2010) and Silva *et al* ,2014) by dividing the average weight of the right and left testicles by the live weight before sacrifice. The result was then multiplied by one hundred GSI = (average weight of testes + live weight before sacrifice) x 100.

The data obtained were analyzed using Statistical Package for Social Scientist (SPSS version 21). The results were presented with the aid of tables and figures. Levene test was applied for equality/homogeneity of variance calculated for the absolute weight, weight gain and semen parameters and indices. Significant differences among means of the groups were determined using oneway analysis of variance (ANOVA) followed by a post hoc test (Turkey pairwise Test) for multiple group comparison. A p-value of <0.05 was considered as significant.

III. RESULTS

Table 1: Mean Body Weight (kg) of Experimental Groups During the Experiment.

		MEAN ± SEM					
GROUPS	Number of animal(N)	week1	week2	week3	week4	week 5	week6
Control	5	56.80 ± 1.924	67.80 ± 4.324	66.60 ± 1.140	71.20 ± .837	92.00 ± 13.491	105.00 ± 15.890
Bisphenol A	5	54.60 ±	62.80 ±	64.40 ±	68.60 ±	87.40 ±	106.40 ±

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		7.436	9.731	8.385	9.864	21.663	4.336
Oestrogen	5	97.40	109.80	114.00	122.60	137.00	145.60
		±	±	±	±	±	±
		18.379	19.942	18.641	15.582	16.583	17.573
Testosterone	5	88.00	99.80	107.40	115.60	124.60	131.60
		±	±	±	±	±	±
		21.954	21.603	25.265	25.851	26.614	33.269

Mean Weight Gain of Experimental Animals

The Table shows the Weight gain of each experimental group calculated for each week (1 to 6). There was no statistically significant difference in weight gain between all the experimental groups from week 1 to 5. There was no statistically significant difference in weight gain across all the groups P>0.05. Except in week 6 which showed significant difference when the control group was compared with the BPA group over the duration of 6 weeks as depicted on the Table 4.2. The other experimental groups; oestrogen and testosterone showed statistical significance difference (P<0.05) when compared to BPA group at week 6.

Groups	control	BPA	Estrogen	Testosterone	F	p
			Mean ± SEM			
Week1 (kg)	0.00+0.000	0.00+0.000	0.00+0.000	0.00+0.000	-	-
Week2 (kg)	19.30+4.813	14.78+5.779	12.88+2.430	14.43+7.589	1.270	0.318
Week3 (kg)	17.36+4.377	18.08+5.093	17.69+7.630	22.87+9.604	0.690	0.571
Week4 (kg)	25.46+4.069	25.74+9.866	27.34+12.919	32.74+14.145	0.476	0.703
Week5 (kg)	61.78+21.436	58.26+26.743	42.33+13.581	43.58+16	1.209	0.889
Week6 (kg)	84.63+25.322	98.35+32.833[a]	51.41+16.242[b]	51.23+22.604[b]	4.565	0.017

Table 2: Mean Weight Gain of Wistar Rats Weekly Among Experimental Groups.

a Statistically significant difference (P < 0.05) when compared to control group
b Statistically significant difference when other groups compared to BPA

Total Sperm Count and Morphology of The Different Experimental Groups

There was observed reduction in the total number of sperm cell count ($X10^6$/ml) values in all the three experimental groups as compared to the control group. However, the difference was statistically significant at (p<0.05) when BPA and oestrogen exposed experimental animals were compared with the control group

Normal cells ($X10^6$/ml) values were reduced in all three experimental groups as compared to the control group, and the difference was statistically significant at (p<0.05)

Cells that had no heads ($X10^6$/ml) values though statistically significant at p<0.05 showed statistically significant difference in the oestrogen group when it was compared to the control group.

Cells with no tail (X 10^6/ml) values on the table 4.4 showed no statistically significant difference p>0.05 when compared to the control group.

Coiled tailed sperm cell values in all the experimental groups showed no statistically significant difference (p>0.05)

Values of dead sperm cells count ($X10^6$) in different experimental group showed statistically significant difference at P<0.05. Dead cells were significantly reduced in the BPA exposed group compared to control group (Table 4.4) while the oestrogen and testosterone groups showed statistically significant increase in dead sperm cells when compared to BPA group.

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Table 4: Total Sperm Count and Morphology of Wistar Rats Among Experimental Groups

Groups	Control	Bisphenol A	Estrogen	Testosterone	F	P
			Mean±SEM			
Total no. of Sperm cells count (x106/ml)	298.40± 58.205	177.80±33.252[a]	203.80±34.867[a]	220.40±56.136	6.084	0.006
Normal cells(x 10^6)	170.20±36.044	75.00±18.385[a]	49.60±11.546[a]	58.80±18.820[a]	29.031	0.000
Cells with no head(x106/ml)	29.40±12.97	37.80±7.46	57.80±13.10[a]	47.60±12.38	5.49	0.009
Cells with no tails(x106/ml)	15.60±5.367	19.40±4.722	21.20±8.927	20.80±9.445	0.592	0.629
Cells with coiled tails(x106/ml)	10.00±3.082	12.00±6.205	12.20±6.058	8.60±5.459	0.515	0.679
Dead cells(x106/ml)	73.20±16.300	33.60±12.157[a]	63.00±13.910[b]	84.60±23.586[b]	8.215	0.002

a Statistical significant difference (P < 0.05) when compared to control group
b Statistical significant difference when other groups compared to BPA
a,b mean difference significant at P<0.05

Sperm Vitality, Gonadosomatic Index and Live Dead Ratio.

Sperm Vitality in the different experimental groups showed statistical significance at p<0.05 with reduction in values of sperm vitality seen in BPA, oestrogen and testosterone exposed Wistar rat. BPA groups had sperm vitality of 42.4% as compared to control with a value of 57%.

There was Statistically significant Reduction in Gonadosomatic Index of the oestrogen and testosterone group when compared with control and BPA group.

Live dead ratio values in the different experimental groups was statistically significant difference (p<0.05) in the ratio observed in the oestrogen and testosterone experimental groups when compared to that of the control group and BPA group (Table 4.5).

Table 5: Sperm Vitality, Gonadosomatic Index and Live Dead Ratio Among Experimental Groups

Groups	Control	Bisphenol A	Estrogen	Testosterone	F	P
Sperm vitality %	57%	42.4%[a]	24.04%[a]	26.67%[a]	54.697	0.000
Gonadosomatic index	1.94±0.317	1.88±0.753	1.38±0.148[a,b]	0.79±0.081[a,b]	34.996	0.000
Live dead ratio	2.39±0.570	2.73±2.004	0.81±0.212[a,b]	0.71±0.152[a,b]	5.001	0.012

a Statistical significant difference (P < 0.05) when compared to control group
b Statistical significant difference when other groups compared to BPA
a , b mean difference significant at P<0.05

4.6 Serum Levels of Oestrogen, Progesterone and Testosterone Among Experimental Groups

FSH, LH values of whose quantity were not detectable as machine was not sensitive enough to detect their levels and was estimated as < 0.01mIU /ml .

Oestrogen hormone values was observed to be highest amongst the oestrogen exposed group with an absolute value of 211ug/ml. While control group has the lowest value 103.2ug/ml.

Testosterone hormone values was highest amongst the testosterone exposed group with a value of 52.5ng/ml.While the BPA exposed group had a testosterone hormone value of 24.39 ng/ml which is elevated as compared with the control testosterone hormone value of 10.41ng/ml.

The highest level of Progesterone was observed in the control group (9.55ng/ml) while the lowest was found in the testosterone group. The Table 4.6 was however not subjected to any statistical analysis.

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Table 4.6: Hormonal Analysis of Wistar Rats Among Experimental Groups After Experiment.

GROUPS	CONTROL	BPA	OESTROGEN	TESTOSTERONE
HORMONES				
FSH (mIU/ml)	0.1	0.1	0.1	0.1
LH (mIU/ml)	0.1	0.1	0.1	0.1
OESTROGEN (ug/ml)	103.2	177.5	211	134.7
PROGESTERONE (ng/ml)	9.55	3.53	3.86	2.7
TESTOSTERONE (ng/ml)	10.41	24.39	13.26	52.05

4.7 Histological Examination of The Testes and Expressional Change H and E Picture Of The Testes In Experimental Groups

Photomicrograph of histological prepared testicular tissue from wistar rats showing normal spermatogenesis with spermatogonia, Primary spermatocyte and spermatozoa as in plate I.

The Bisphenol A exposed experimental group showed disoriented elongated spermatic cells and Aspermic Lumen as in plate II.

Spermatogenic activity in oestrogen exposed group with Sertoli cells SC ,primary spermatocyte PS and spermatozoa within the Lumen (plate III) with increase thickness of cell layers.

Photomicrograph showing histologic picture of testes stained with H and E showing aspermic lumen and thinned out cell layers (plate IV).

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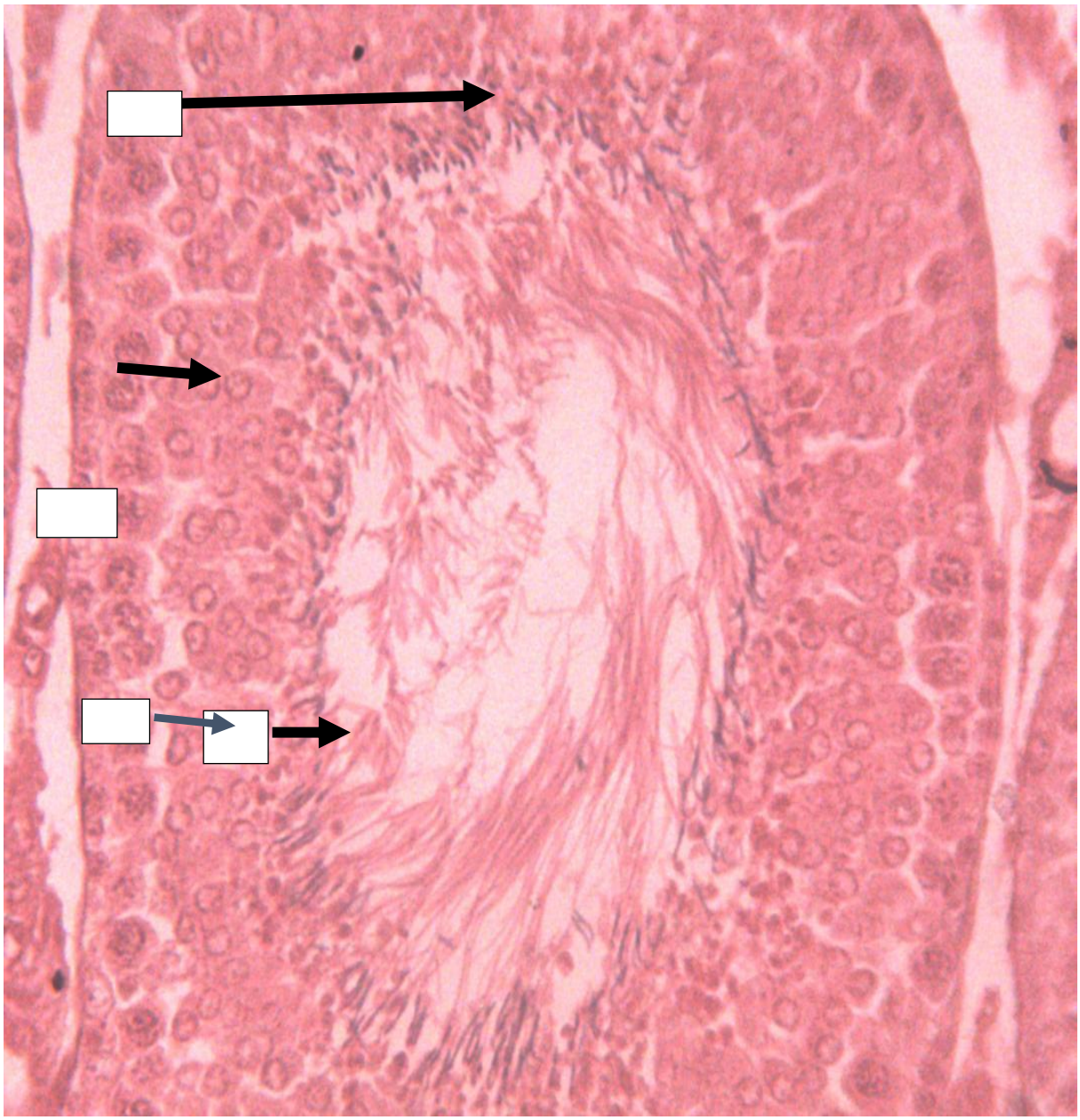

Plate I: Photomicrograph of testis from the control group Showing normal spermatogenesis with Sertoli cells (SC),spermatogonia (SG),primary spermatocyte (PS) and spermatozoa (SP) seen in the seminiferous tubules (H&E x 250).

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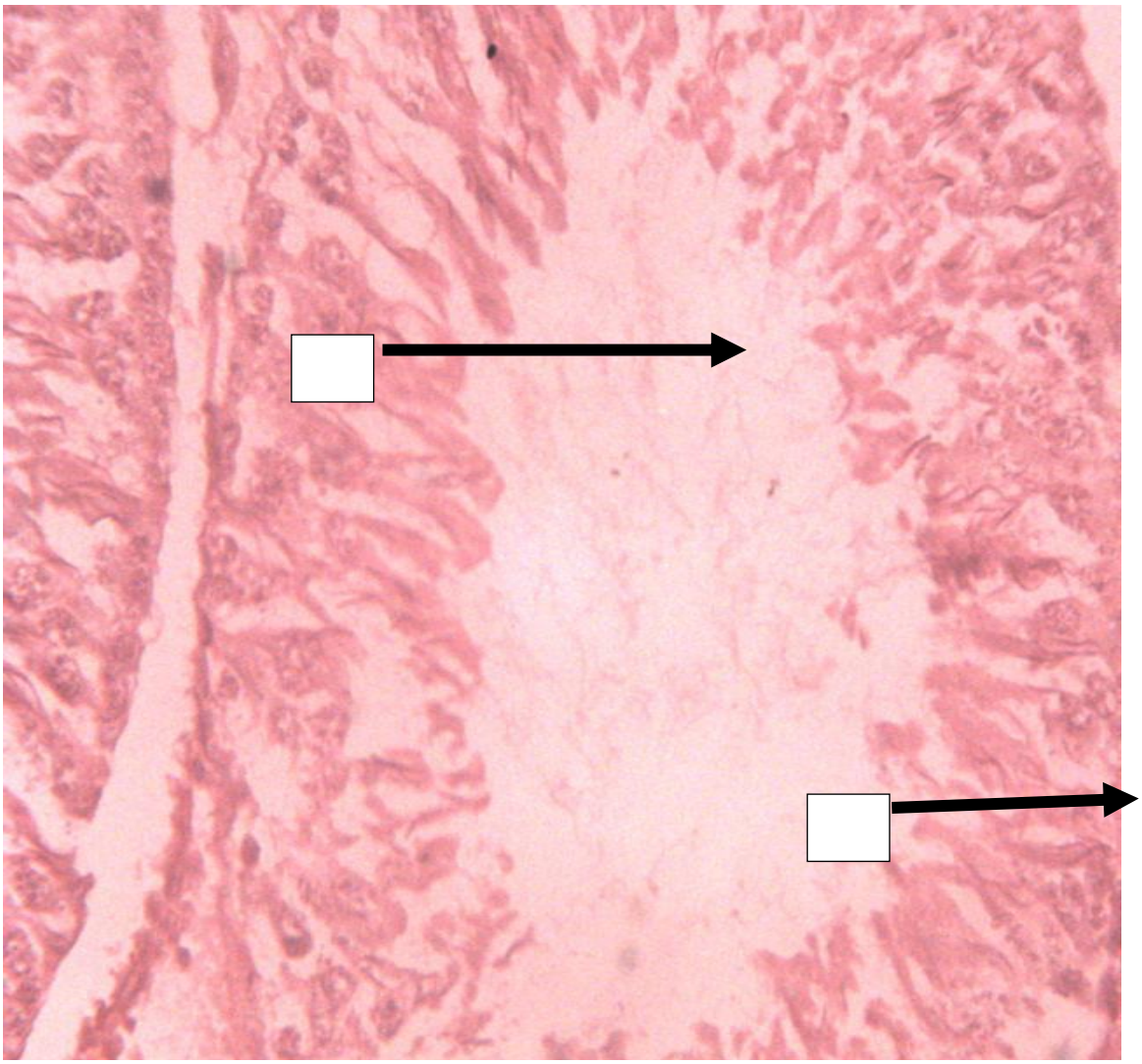

Plate II: Photomicrograph of the testis from Wistar rats given Bisphenol A with aspermic lumen (AL) and, disoriented elongated spermatids (DE) (H&E x 250).

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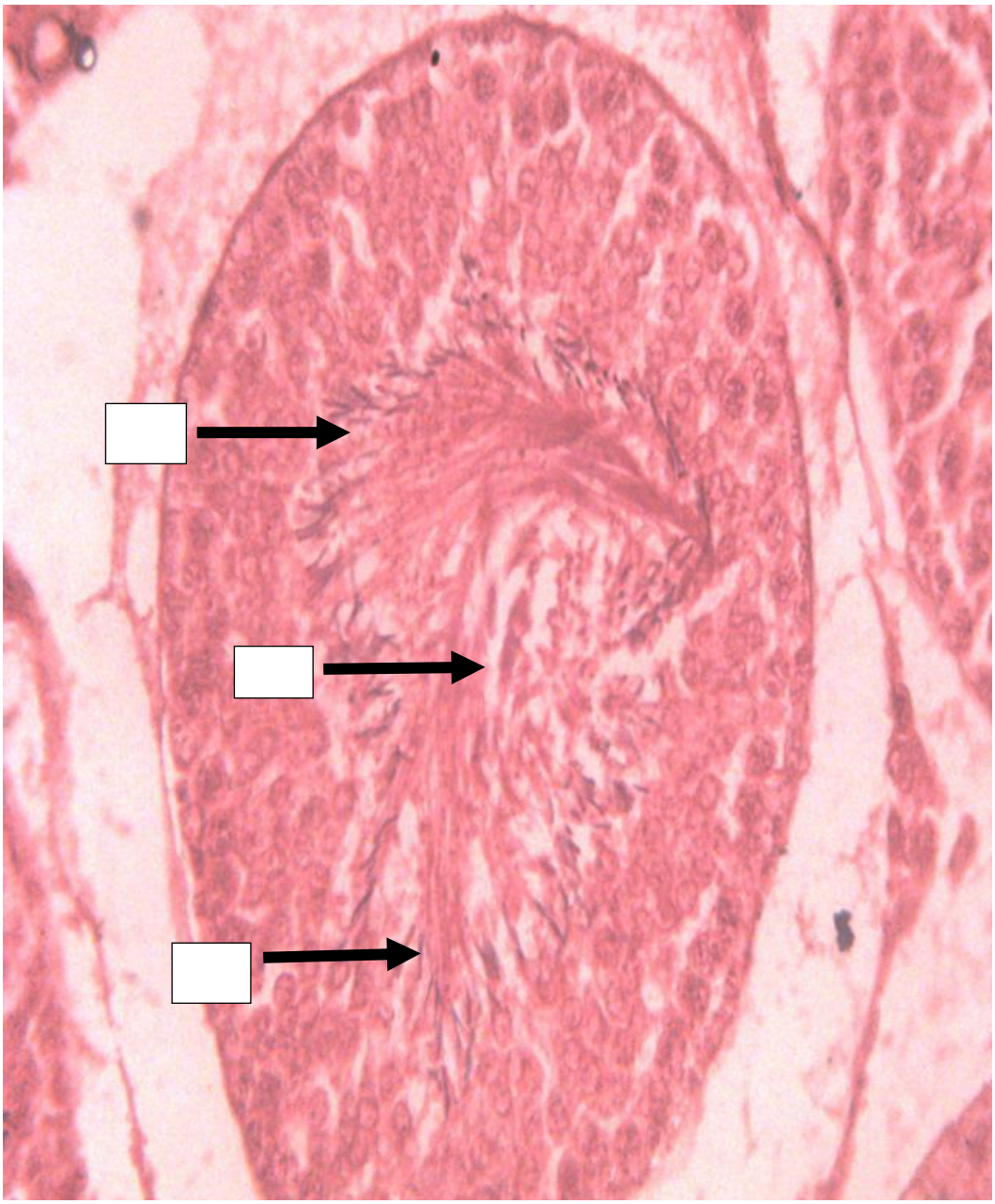

Plate III: Photomicrograph of the testis of Wistar rat given oestrogen showing of spermatogenic activity with Sertoli cells (SC), primary spermatocyte (PS) and spermatozoa (SP) seen in seminiferous tubule lumen (H&Ex 250).

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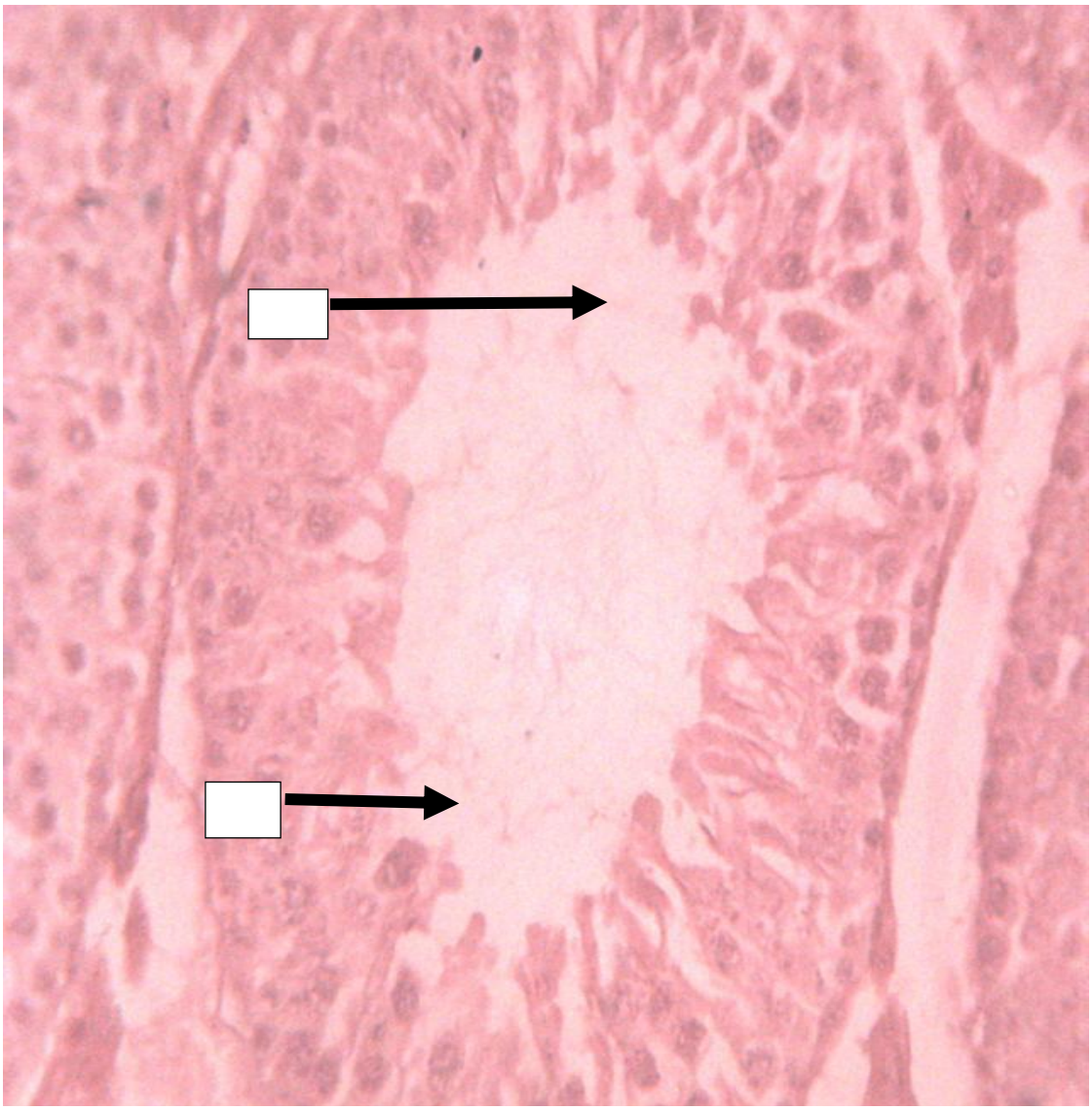

Plate IV: Histological examination of the testes and expressional change H and E picture of the Testosterone group. Photomicrograph of the testis of wistar rat given Testosterone showing spermatogonia (SG) and aspermic lumen (AL)(H&E x 250).

IV. DISCUSSION

The study found an increase in the weekly weight gain among the Wistar rats in all groups, however the difference in weight gain was found to be statistically significant in the 6th week in favour of BPA group compared to oestrogen and testosterone groups, despite being on similar diet. BPA, oestrogen and testosterone are anabolic.[7,8] A study among Chinese workers found higher serum and urinary BPA in those with high body mass index.[9] It also compares with another study that showed BPA is associated with significant weight gain among experimental animals.[10]BPA enhances adipocyte differentiation and lipid accumulation in target cells in a dose dependent manner. [5] Various mechanisms have been associated with BPA and obesity and diabetogenicity.[11,12,13,14,15]

There is an observed decrease in the mean value of the total sperm cell count ($X10^6$/ml) across the experimental groups when compared to the control. These may give an explanation to other studies that showed how common plastics are killing sperm cells in Laboratory wistar rats, who struggle to carry out meiosis.[16] A study that compared various doses of BPA and Vit E showed decrease in sperm count in the BPA group though not statistically significant.[19] Another study using previously considered nontoxic dosage of BPA

(5mg or 25mg BPA/Kg/day) showed compromised total sperm production and specific functional parameters.[23] Many studies showed significant reduction in total sperm cell count compared to control which is in keeping with this current study.[15,17,22] The WHO criteria for sperm parameters provides sensitive comparative values capable of identifying the most true abnormalities of male semen.[19] In assessing semen quality in this study there was also an observed lower total sperm count observed among exogenous sex steroid exposed groups. The reduction was statistically significant in the oestrogen group compared to the control. The reduction in the testosterone group was not statistically significant similar to the study by Klinifelters and Saurez, 2007. Most studies did not prove a direct cause-and-effect relationship between exogenous testosterone and lowered sperm count.[20] Low sperm count or quality is found to be the only cause of infertility in about 20% of couples, and is a contributory factor in a further 25% of couples.[19] It is estimated that in between 30% and 50% of men with poor semen quality have noknown cause for the identified decreased numbers,

In this study normal morphology of sperm cells ($X10^6$/ml) values were reduced in all three experimental groups compared to the control group which is similar to a previous study that showed significantly decreased sperm morphology in male adult rat that were exposed to Bisphenol A and testosterone.[23] In contrast a study that explored mechanism of BPA and oestrogen effect on spermatogenesis and semen parameters showed no effect on normal sperm morphology but apoptotic sperm cell numbers were low in BPA and oestrogen exposed groups.[22]The observed effects by the experimental group on sperm parameters could be due to ability of BPA, oestrogen and testosterone to permeate the blood-testis barrier with resultant alteration in the micro-environment of seminiferous tubules.[22,23] It is still not clear if BPA is playing a role in men's continued infertility today but their mechanism of metabolism and excretion though clearly illustrated still leaves BPA in the body system at certain concetrations.[23] This study also showed that oestrogen exposed group had the highest number of abnormal sperm cell morphology. This observation is in accordance with the work which showed oestrogen to be ineffective in improving sperm qualities.[25]

The Gonado-somatic index in this study was similar in the BPA group and control; it is however decreased in the estrogen and testosterone groups compared to the control and even the BPA group. In this study, The GonadoSomatic Index showed no change for the BPA exposed group but the estrogen and testosterone groups showed a decrease when compared to both the BPA and control groups. Similar result was seen in the live dead ratio. Live Dead Ratio was highest in the oestrogen exposed group similar to another study that studied Gonadosomatic index and live dead ratio in oestrogen exposed animal models.[23]

LH and FSH levels were not detectable as their concentration was below the detectable range for the ELISA kit used in the study. Thusit can be inferred that the higher centres producing FSH and LH produced low levels that could not be detected. A Similar study using dosages of BPA that were thought to be non-toxic to the reproductive system showed FSH and LH concentrations decreased significantly at 5mg/kg and 25mg/kg with observed attempts by pituitary to re-establish normal levels of LH, FSH and testosterone serum concentrations with increased Gonadotropin Releasing hormone receptors, LH receptors, FSH receptors, oestrogen receptor ESR2 and ESR1 in hypothalamus. BPA disrupts the hypo-thalamo-gonadal axis causing a state of hypogonadotropic hypogonadism.[23]The effects of BPA on steroidogenesis in Leydig cells was analyzed by measuring the LH, FSH, oestrogen, testosterone and progesterone synthesis with high levels of oestrogen and testosterone.[21] In a similar study FSH levels remained same for the control group, BPA 200 and estrogen while LH hormone levels increased slightly in BPA 200 as compared to control group and estrogen.[23] The level of testosterone in the testosterone exposed group was 5 folds higher than the control group. It is also higher in the BPA group when compared to control. High dose BPA affects steroidogenesis as confirmed in in-vivostudies ofprepubertal mice which resulted in induction and increased concentrations of testicular testosterone.[20] A Similar study with BPA 200 showed a slight increase in testosterone levels and a more significant reduction in testosterone in oestrogen group.[23] However in another study there was a significantly decreased serum level of testosterone in Wistar Albino rats treated with BPA at a dose of 50ug/100g BW and 100ug/100g BW with no statistical significant decrease at 5ug/100g BW when compared to control and vitamin E intervention groups.[19] Our findings is also contrary to the finding of Mansir et al. which showed the levels of testosterone was lower in rats treated with BPA when compared with the control groups.[24] There is no clear explanation to justify the different results at this point.[22] Some studies suggest that BPA can induce gene expression, which

subsequently alter steroidogenesis in testicular Leydig cells.[24]

Endogenous oestrogen was increased in the BPA and oestrogen exposed groups indicating that BPA contributes significantly to oestrogen synthesis. BPA increased serum Oestradiol levels by increasing Leydig cell aromatase activity. BPA was as well an oestrogen hormone mimicker from other studies. A study comparing BPA 50ug/kg BW/day and 17 B oestradiol E2 20ug/kg BW/day at the 15 to 30 postnatal days showed delayed spermatogenesis establishment in pre-pubertal rats.[25] The effect of BPA when compared to oestrogen seems to be more than through estrogen receptors.[25] Results obtained from this study of hormones showed progesterone still remains of value and of biochemical significance and can be explored in further research in female wistar rats.

There were disoriented elongated spermatic cells with aspermic lumen, thinned out cell layers the BPA group in contrast to the control group which had several matured spermatozoa within the lumen. The finding is Similar to a study of BPA at 5ug/kg, testicular weight decreased significantly it was postulated that histology remained entirely benign with disoriented elongated cells.[19] Wistar rats treated with BPA at 1ug/kg showed no effect. Other well controlled studies concluded that weak environmental oestrogen mimickers; BPA in general may at low doses not pose an effect to male reproductive system even when administered over 2 generations.[22,23] Another study showed that at 200ug/kg BPA caused significant compound related changes in the reproductive parameters and histology in albino rats.[19] Srivastava *et al* study used BPA 100ug/kg/day also observed disoriented elongated sperm cells and thinned out cell layer the epididymis showed spermatozoa.[19]Histological examination of testicular tissue showed significant histopathologic changes characterized by degenerative changes in the germinal layer of the seminiferous tubules, leydig cell degeneration, increment in intertubular space as compared to controls and vitamin E intervention group and apoptosis was evident in the epididymis.[19] Human testicular biopsy specimens in azoospermic condition showed primary testicular defects in half of the subjects investigated.[22] Sperm reserves in caput, corpus and cauda of the epidydimis decreased significantly with increased transit time, other abnormalities detected were spermatogenic arrest, testicular atrophy, and hypo spermatogenesis.[23]

In this study spermatogenic activity in oestrogen exposed group within sertoli cells SC, primary spermatocyte PS and spermatozoa within the lumen appeared comparatively similar to the control group with an increase thickness of cell layers. This finding was similar to a study using oestradiol benzoate administered comparatively and reported no clear effects in male adult reproductive structures.[25,26] The testosterone group showed spermatogonia, thinned out cell layers and aspermic lumen at the same time. Similar in findings to other studies that postulated that prolonged and uncontrolled use of testosterone cause various histological and morphological abnormalities in the testis including reduction of testicular volume and seminiferous tubule length, germ and Sertoli cells' sloughing, and severe depletion of Leydig cells in the interstitial compartment. In another study, it is well recognized that a long-term use of nandrolone frequently results in male infertility, as a predominant side effect.[26] Moreover, treatment with relatively high doses of testosterone leads to decrease of testis and epididymal weights, sperm number, and sperm motility. In the testis, even though clear morphological change on Leydig cells was not observed, sloughing of Sertoli and germ cells was frequently found with the high dose treatment of testosterone.[23] Similar phenomenon was commonly observed in the testis by testosterone treatment. Testosterone at high dose could affect histology of the testis.[26]

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