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Morphometric and Histopathological Evaluation of the Testes and Accessory Sex Organs of Male Wistar Pups Following Perinatal Exposure to Chronic Cigarette Smoking

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ABSTRACT

Exposure to chronic cigarette smoking during pregnancy into infancy results in numerous morphological and behavioral consequences in infants, children, and adolescents. Cigarette smoke is a reproductive toxicant, demonstrating a strong relationship between smoking and impaired fertility. This study aimed to determine the morphometric and histopathological changes that may result in the testes and external genitalia following perinatal exposure to chronic cigarette smoking in Wistar pups. Male pups of dams exposed to two Rothmans cigarettes (nicotine= 0.9 mg, Tar= 7 mg) twice daily for 3 weeks post establishment of vaginal plug to 3 weeks postpartum delivery of dams (n=10), were compared to pups exposed to normal ventilation (n=10; Control). At postnatal day 49, the testes and epididymis of all male pups in both groups were excised, weighed, and processed for histopathological and histomorphometric analysis. Data collected were expressed as mean ± SEM and analyzed using the student's Newman-Kleus test at $p<0.05$. Pups in both groups increased in weight steadily with age. Photomicrographs and stereological analyses revealed that there were more tubules with generalized degeneration of germinal epithelium, depletion of elongating spermatids, and increased luminal diameter in the testes of pups exposed to cigarette smoke. Also, increased sloughed cell debris and apoptotic epithelial cells were observed in the ducts of the caput epididymis of the pups exposed to cigarette smoke. These results suggest that exposure to chronic cigarette smoking during pregnancy and early infancy can alter tissue structure and disrupt reproductive function in the testes and epididymis of rats, even when exposed in minute quantities.

Keywords: cigarette smoke, reproductive development, testis, epididymis, rat

INTRODUCTION

The initial period of reproductive development is an indifferent stage of sexual development in mammals. It coincides with the development of the urinary system and begins in essentially the same manner in both sexes. This process of development is programmed to develop as females. To become a phenotypic male, this developmental process must be modified. This modification begins with the activation of the Sry gene followed by total masculinization of the body. Activation of the Sry gene results in the formation of the fetal testis. The developing testis then produces and secretes testosterone and other androgens which drive the masculinization process. Disruption of this chain of events causes sexual differentiation disorders or, in rare cases, feminization of the male fetus. Common disorders of the male reproductive development constitute the testicular dysgenesis syndrome (TDS) which includes cryptorchidism and hypospadias, which are milder and are predisposing factors of severe disorders that present in young adulthood like poor sperm quality, azoospermia, and testicular cancer. [1,2]

The use of tobacco is a behavioral process that stimulates psychological and physiologic addictive moods among users. It is highly addictive due to nicotine, its active ingredient. Tobacco use includes cigarettes, cigars, cigarillos, small cigars, hookahs, pipes, electronic cigarettes, snuff, etc. However, cigarette smoking is still the most common form of tobacco use among adults and youths.[3] Cigarette smoke contains up to 7,000 toxic chemicals, including

nicotine, benzo(a)pyrene, and carbon monoxide, at least 70 of these are carcinogenic. Some of these carcinogenic substances include arsenic, carbon monoxide, tar, methane, nicotine, ammonia, and cadmium, amongst others.[4,5] Yet cigarettes remain one of the most frequently used drugs in pregnancy. Apart from direct prenatal primary exposure to cigarette smoke, there is also the danger of second-hand smoke exposure whose effects are indistinguishable from direct smoking.[5] Reports from recent research links maternal smoke exposure, whether mainstream or sidestream, with disturbance in the in-utero growth and development of the fetus.[6-9]

Reports from epidemiological studies performed during the last five decades have shown that cigarette smoke is a reproductive toxicant, demonstrating a strong relationship between smoking and impaired fertility.[10,11] Cigarette smoke toxins affect all stages of reproductive functions in both sexes. Smoking causes genetic damage to germinal cells, inhibits embryo fragmentation, and increases the risk of chromosomal errors and childhood cancers. Several constituents of tobacco smoke can cross the blood-testes barrier, thereby inducing significant alteration in sperm quality, such as a decrease in concentration, morphology, and motility of spermatozoa.[12, 13]

This study sought to investigate the morphometric and histopathological changes that may result in the testes and external genitalia from maternal exposure to chronic cigarette smoking in dams.

MATERIALS AND METHODS

Animal procurement, care, and handling

Animal experiments were conducted per the guidelines of the University of Ibadan Animal Care and Use Research Ethics Committee (Approval Number: UI-ACUREC/022/-1122/16). 20 adult female Wistar rats weighing about 120-150 g and 5 male rats weighing about 200-250 g were procured from the animal housing facility of the Faculty of Basic Medical Sciences, University of Ibadan. The animals were housed in standard-sized plastic cages at 23 ± 10C, maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h) with wood shavings as beddings.[14] Bedding were changed and cages were cleaned and sterilized weekly. The animals were given *ad libitum* with standard rodent feed and distilled water in drinking bottles. They were allowed to acclimatize for 7 days before the commencement of the experiment.

Procurement of cigarettes

Rothmans King Size cigarette packs manufactured by British American Tobacco; 1, Tobacco Road, Oluyole, Ibadan, Nigeria, were sourced from the local market around the school. Each cigarette stick contained 7 mg of tar and 0.9 mg of nicotine.

Pre-exposure procedure

Female rats were mated with adult male rats over 21 days (3 weeks) for copulation in the proportion of two female animals to one male animal with 2 rest days weekly. Pregnancy was confirmed with the detection of the coital plug in the vagina of the female rats. Once the vaginal plug was detected, the day was marked as gestational day 0 (D0). Female rats with vaginal plugs were assigned to 2 groups: 1 control and 1 exposure group based on randomization of their body weights (10 females in each group) and transferred into separate cages, labeled according to their groups.

Exposure protocol

An improvised inhalation chamber was constructed from locally available materials with slight modifications made to the model described by Aprioku & Ugwu.[15] The animals (two at a time) were introduced into the superior chamber and the cigarette was lit at the base of the inferior chamber using a lighter. After lighting the cigarette, a rubber puffing device was attached to the non-combustive end of the cigarette and pumped to generate the release of mainstream smoke, and the sticks were left to smolder for the rest of its burning time (roughly 15 minutes) generating side-stream smoke. After that, the procedure was repeated with one (1) hour interval of rest, and so all the animals in the exposure group received environmental tobacco smoke comprising mainstream and side-stream smoke generated from about four (4) cigarettes per day.[15]

Cigarette smoke exposure was carried out as follows: the female rats were exposed via whole-body exposure to smoke from two standard regular cigarettes (Rothmans King, nicotine = 0.9 mg, tar =7 mg, British American Tobacco, Nigeria) twice/day, five times per week, for 3 weeks of gestation and 3 weeks postpartum delivery (Figure 2). Each exposure lasted 20 minutes. Control rats were sham-exposed in a similar condition to room air only. This protocol emulated a passive smoking environment with the apparatus allowing possible confounders caused by side-stream cigarette smoke exposure.[15,16]

Post-exposure protocol

On postnatal day 1 (PND 1), live pups were counted and examined for clinical signs of toxicity (craniofacial malformations, low birth weights, prematurity, microcephaly, and limb, tail, and abdominal wall abnormalities). During the lactation period, pups were weighed weekly in groups (by sex and litter) and examined for gross morphological abnormalities. Mortality was recorded.

At weaning (PND 21), pups were given identifying marks on their tails and housed in groups of 3 to 5 animals according to the treatments of the dams. Individual pups' body weights were recorded weekly. When pups were weaned, dams were euthanized using a 100–2 mg/kg cocktail of Ketamine/Xylazine i.p.[17]. Male offspring were examined for preputial separation from the PND 38 to PND 48. They were also examined for scrotal testes and hypospadias.

Tissue collection

On PND 48, all male offspring were sacrificed and post-mortem examinations were conducted. The animals were euthanized using a cocktail of ketamine-xylazine i.p.[17]. Once euthanized, they were dissected quickly. The position of testes and gross morphology of internal and external genitalia were also noted. Testes were excised and their weights were recorded. The testes and epididymis of all the male offspring from the control and exposed groups were grouped in twos for histological and immunohistochemical analyses.

The tissues for histological analyses were fixed in Bouin's fluid for 24 hours and processed, embedded in paraffin blocks, sectioned at 5 microns, and stained using routine hematoxylin and eosin staining techniques. After tissue sections were dewaxed in xylene, they were rehydrated by decreasing grades of alcohol (absolute, 90%, and 70%) to water. The slides were placed in Harris hematoxylin solution for 8 minutes. After staining, the slides were washed in running tap water for 5 minutes. Next, the slides were differentiated in 1% acid alcohol for 30 seconds and washed in running tap water for 1 minute. The slides were blued in 0.2% ammonia water solution for 30 seconds and washed in running tap water for 5 minutes. The slides were rinsed in 95% alcohol and counterstain in eosin solution for 1 minute. After counterstaining, the slides were dehydrated through 95% alcohol and 2 changes of absolute alcohol for 5 minutes each. The slides were cleared using xylene and mounted using Di-*n*-butyl Phthalate in Xylene (DPX). The prepared slides were viewed using a light microscope and images were mounted on a Leica DM6000 Nikon Upright at 100x magnification and captured using the Leica application suite software (LAS V3.8, Leica Microsystems Ltd., Heerbrugg, Switzerland) by an experienced pathologist. Tissue slides were evaluated using the Image J software. Testicular histomorphometry was carried out by taking the following measurements and graded using staging criteria from cellular classification[18]:

- Tubular diameter: The diameter of 10 seminiferous tubules selected randomly from each slide.
- Height of the germinal epithelium: the height of the germinal epithelium from the ad luminal cells to the basement membrane of each tubule.

Statistical analysis

Data collected were analyzed using GraphPad Prism 8.0.1.244 for Windows (GraphPad Software, San Diego, CA, USA) Results were expressed as Mean ±SEM.[19] Student's t-test with Welch's correction was carried out to identify the differences between each mean. The confidence interval was placed at 95% and considered a p-value less than 0.05 as statistically significant.

RESULTS

A total number of 20 adult female Wistar rats were used in this study grouped into two (n = 2). Of these, 18 were confirmed pregnant and 14 carried to term and delivered a total of 119 pups. The pregnancies of the remaining 4 rats were aborted at different stages of gestation. Of the 119 pups, 20 died during the experiment at different postnatal days. Of the 99 left, there were 35 male pups, 62 female pups, and 2 pups with no sexual differentiation. All the male pups were grouped according to the grouping of their dams.

Physical Observations

Dams in the control group had more litter size (8.8±0.3742) than dams in exposed groups (6.88±0.9899) but it was not significant as shown in Figure 1.

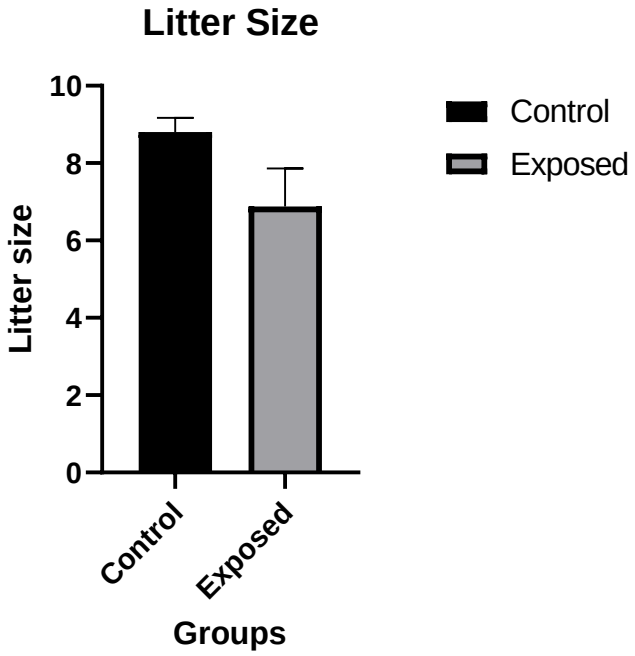

Figure 1: The litter size of the pups across the groups. Values were expressed as Mean ±SEM.

A few physical abnormalities were observed across the groups. Table 1 shows the number of animals that had physical alterations in the control and exposed groups.

Table 1: General Observations across the Groups

Observation	**Control**		**Exposed**		**Total no of animals with alterations**	**Percentage of Total Sample Size**
	No of animals	**Percentage of sample**	**No of animals**	**Percentage of sample**		
Growth retardation	2	(3.70%)	3	(6.67%)	5	5.05%
Fur/skin discoloration	-	-	20	(44.44%)	20	20.20%
Undescended Testis	1 (partial)	(1.85%)	-	-	1	1.01%
Craniofacial malformations	-	-	5	(11.11%)	5	5.05%
Abdominal wall defects	-	-	1	(2.22%)	1	1.01%
Sexual agenesis	1	(1.85%)	1	(2.22%)	2	2.02%

Abdominal wall defects and cryptorchidism were the least occurring alterations whereas fur/skin discolorations were the most occurring alterations. These features, however, were not a significant percentage of the total sample size. These alterations (Figure 2A-D) included retarded growth (both groups), fur/skin discoloration (exposed group only), craniofacial malformations (exposed groups), and sexual agenesis (exposed group).

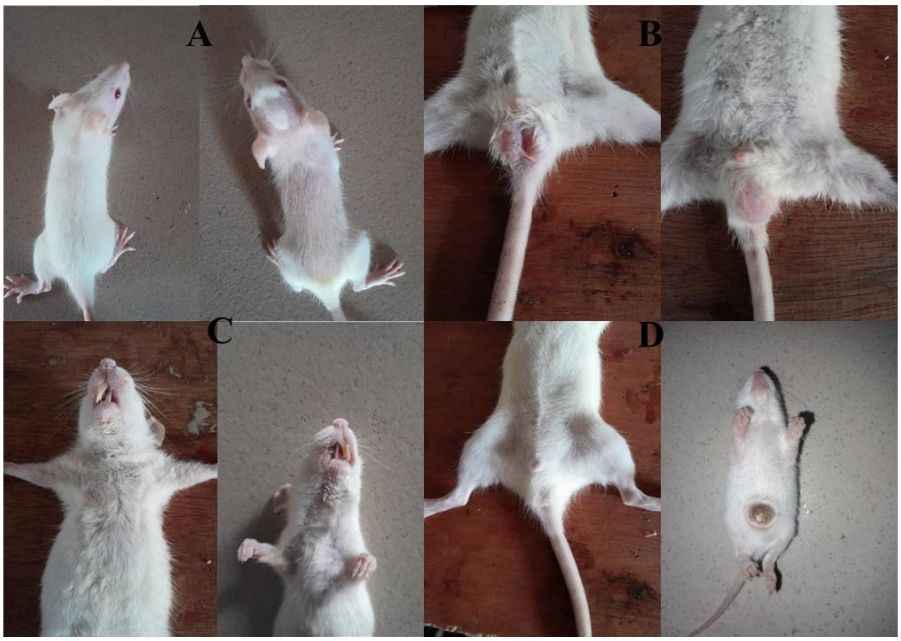

Figure 2A-D: Images of physical observations of the animals. A: Dorsum [L-R: Normal white fur/skin (control group), fur/skin discoloration (exposed group)]; B: genital region of the animals. [L-R: Complete/fully descended testes, partially descended testes]; C: oral region of the animals (black arrow). [L-R: long maxillary teeth]; D: the abdominal wall of the animals. [L-R: normal abdomen. Abdominal wall with a defect].

Growth retardation was assessed by weekly evaluation of their weights and other alterations were observed from their physical appearances from post-natal day (PND) 1 to PND 49 (Figure 3).

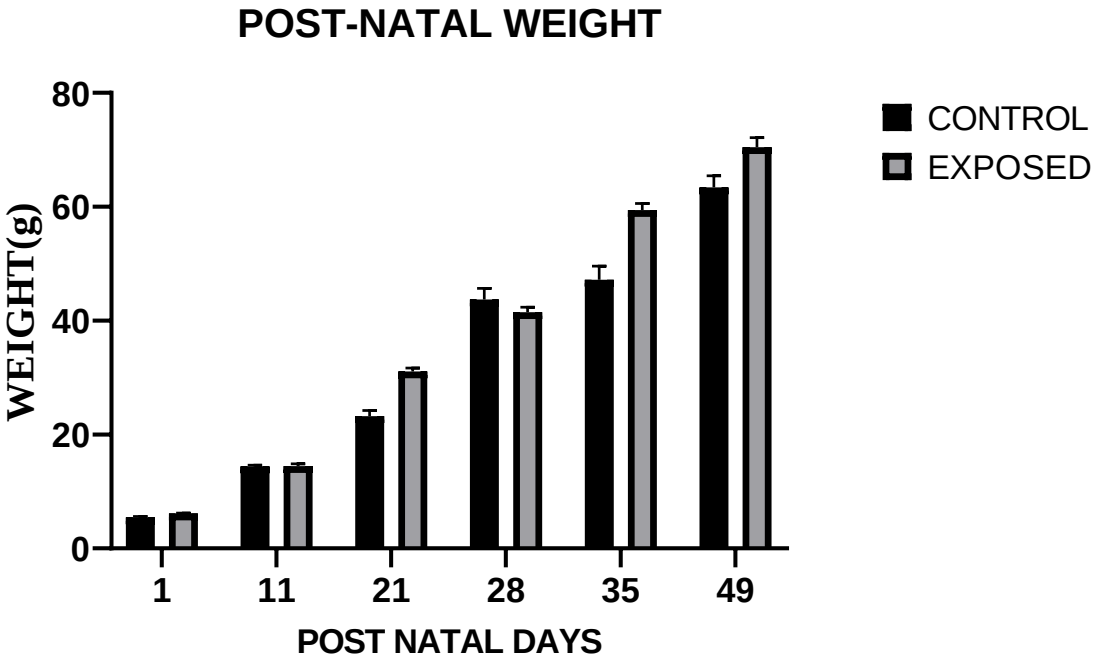

Figure 3: The body weights (g) of the pups across the groups. Values were expressed as Mean ±SEM.

Table 2 shows the body weights of the pups across the groups at birth (PND 1), weaning (PND 21), and final (before euthanasia: PND 48).

Table 2: Body Weights of Animals from Birth to Sacrifice

Period	**Weight (g) (**Mean ±SEM)	
	Control	**Exposed**
Birth	5.45±0.1319	6.12±0.0974
Weaning	23.23±0.9802	31.06±0.6001
Final	63.39±2.0543	70.45±1.6613

Male offspring were examined for scrotal testes, preputial separation, and hypospadias from PND 38. All male pups had fully descended testes by PND 48 except one pup which had only one testis in its scrotal sac at PND 49. All male offspring also had complete preputial separation by PND 48. Hypospadias was not observed in any of the animals. The testicular weights of male pups in the exposed group (0.28±0.02) were significantly ($p<0.05$) more than those in the control group (0.16±0.02) for both the left and right testes (Figure 4).

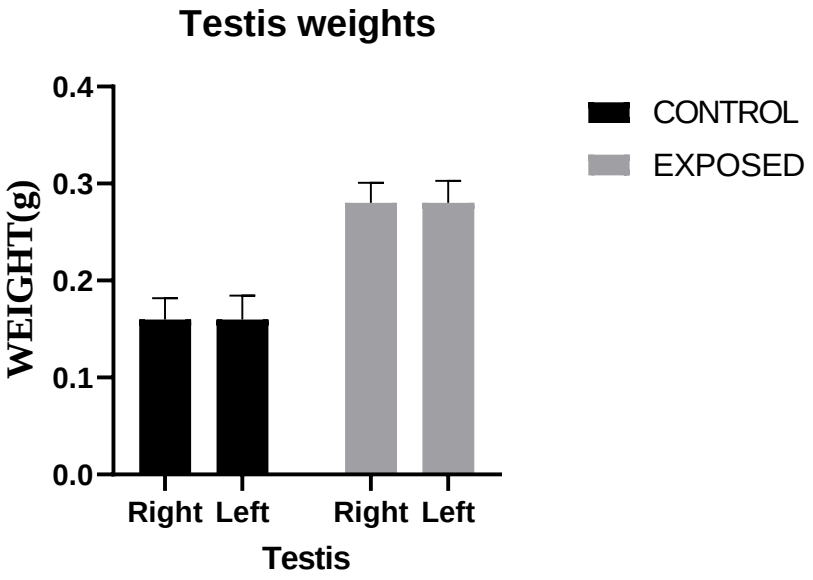

Figure 4: Effect of Perinatal Exposure to Chronic Cigarette Smoking on the testis weight in male Wistar pups. Data are expressed as mean ± SEM and analyzed by t-test followed by the Student Neuman-Keuls test.

Histopathological assessments

We used routine hematoxylin and eosin staining techniques to evaluate the histoarchitecture and morphometry of the testes and epididymis following maternal exposure to chronic cigarette smoking during gestation and the pre-weaning period. With the hematoxylin and eosin stains, we observed well-arranged seminiferous tubules in the testes of the pups in both groups with defined tubules and interstitium (Figure 5).

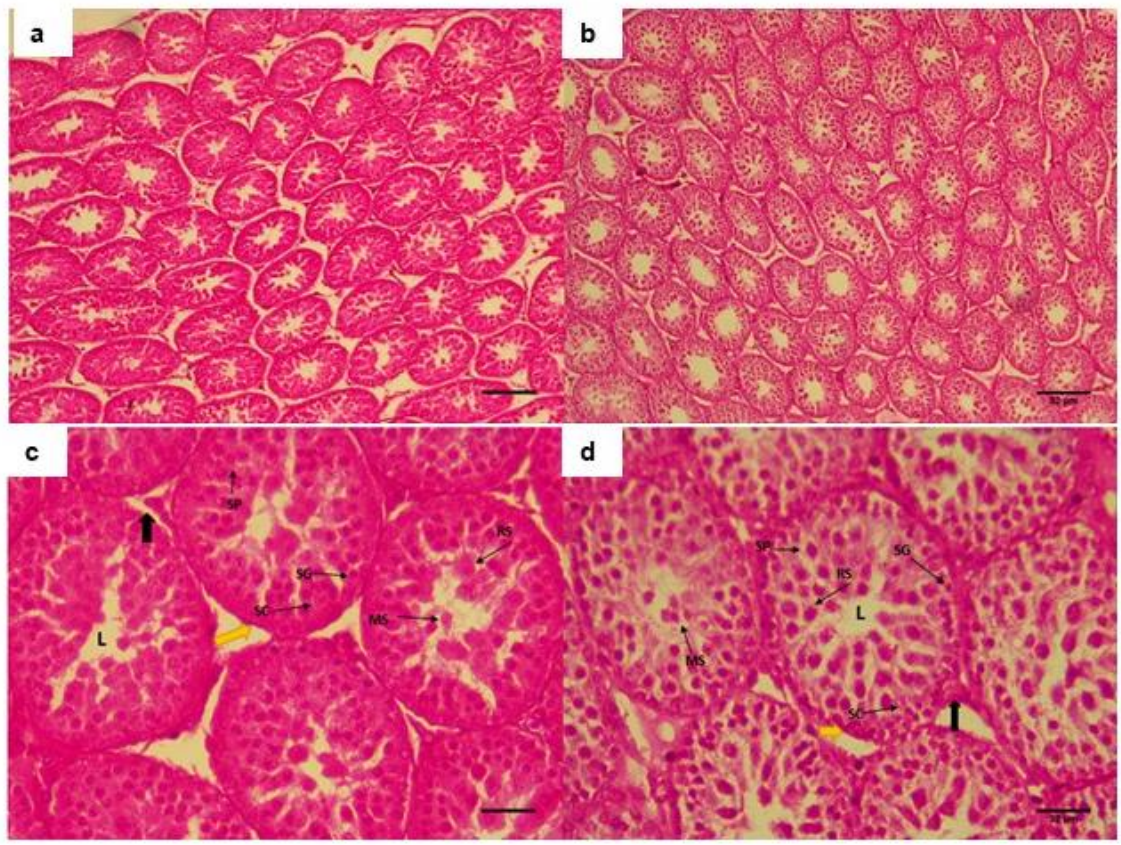

Figure 5: Photomicrographs showing the seminiferous tubules of the testes; H & E stain Scale bar=32µm (a & c): control group, (b & d): exposed group. Both groups feature well-maintained closely packed seminiferous tubules and interstitial spaces. However, tubules in the exposed group at low magnification (b) feature generalized degeneration of germinal epithelium and increased luminal diameter. At higher magnification, the cell groups were better identified and observed in the tubules: the proliferating spermatocytes (SP), mature elongating spermatids (MS) and round spermatids (RS) forming the ad luminal compartment, the Lumen (L), the prominent spermatogonia (SG) and Sertoli cells (SC) close to the basement membrane (Yellow arrow), and Leydig cells in the interstitium (Bold black arrow).

We measured the diameters of these tubules (Figure 6a) and found a slight reduction in the diameters of the exposed pups compared to the control; however, this difference was not significant. We also measured the height of the germinal epithelium (Figure 6b) in both groups and observed a significant ($p < 0.05$) reduction in the height of the tubules' germinal epithelia of the exposed pups.

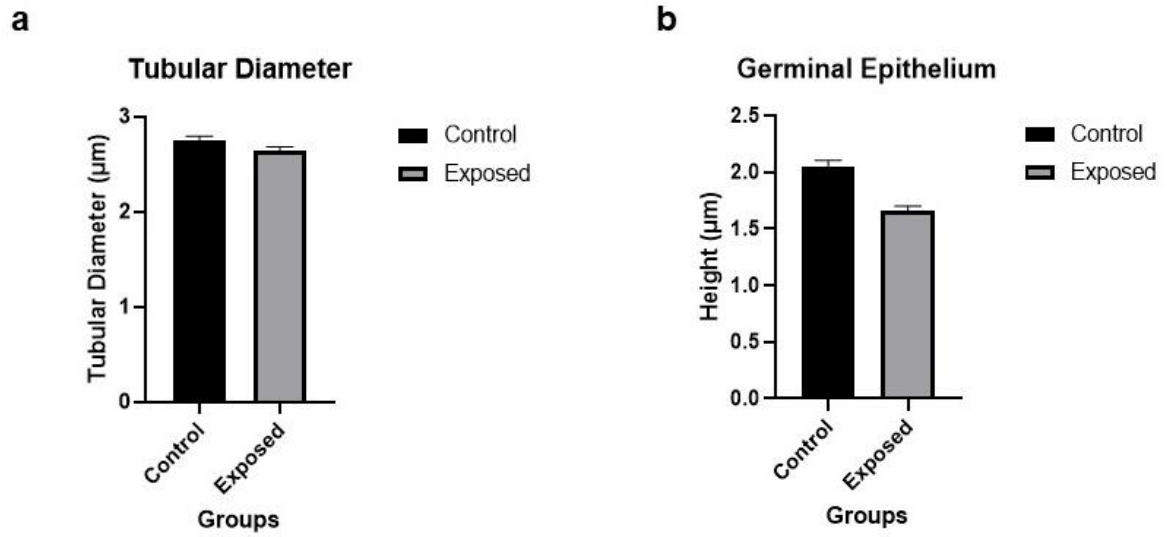

Figure 6: Bar Chart showing (a) the diameter (µm) of the seminiferous tubules and (b) the height (µm) of the germinal epithelium of the tubules in Figure 5. Values expressed as Mean ±SEM. At $p>0.05$, there was no significant difference between the tubular diameters in Control (2.75±0.0400) and Exposed groups (2.64±0.0360). However, the height of the germinal epithelium in the control group (2.75±0.0400) was significantly higher than that of the exposed group (1.67±0.0360) at $p>0.05$.

The photomicrographs from the exposed pups also showed degeneration of the developing sperm cells in the adluminal compartment (Figure 10). Histological evaluations of the epididymis also revealed a well-defined duct system in both groups (Figure 7). However, there was increased sloughed cell debris and apoptotic epithelial cells in the ducts of the epididymis of the exposed pups.

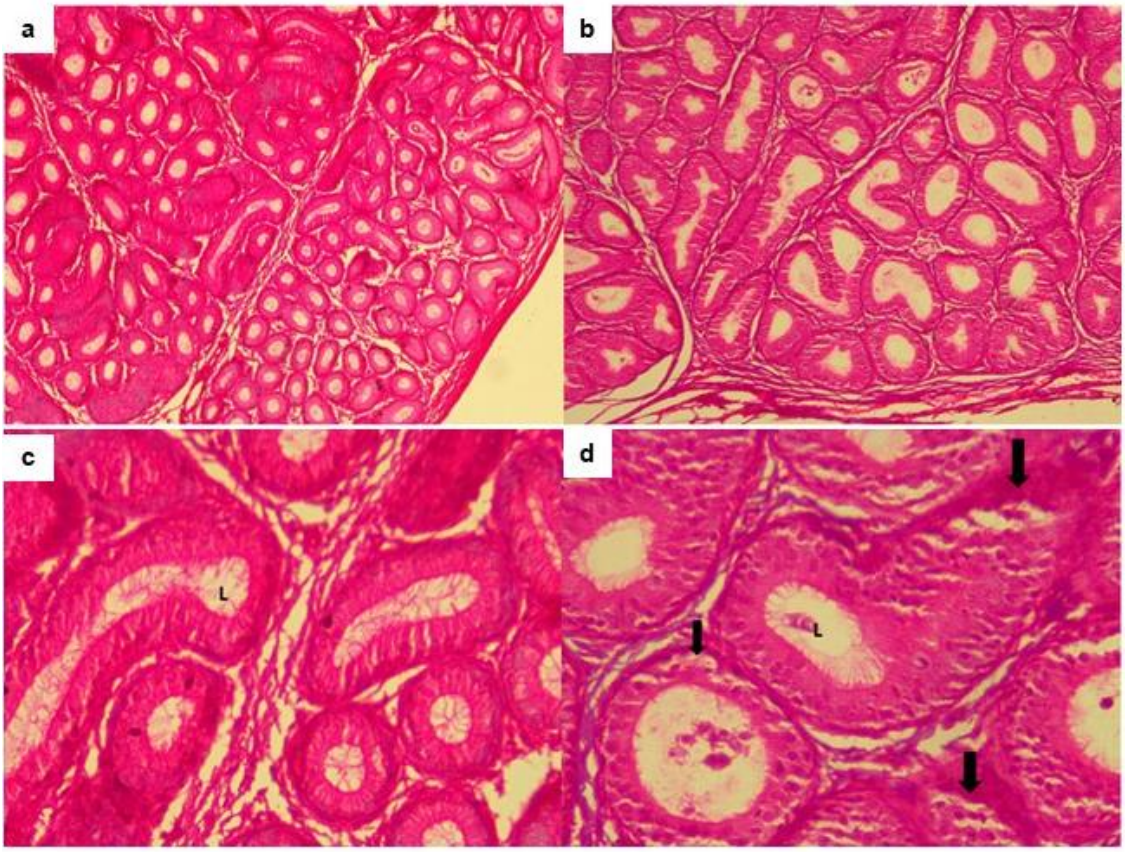

Figure 7: Photomicrographs showing the ducts in a small region of the proximal caput of the epididymis; H & E stain. (a-b: X100), (c-d: X400). Both groups at low magnification (a) and (b) feature normal epididymis architecture with clear lumens. However, cell debris is present in most of the ducts in the caput epididymis of pups exposed to ETS (b). The exposed group at increased magnification (d) showing sloughed cell debris in the Lumen (L) and apoptosis of epithelial cells (black arrow) compared to (c).

DISCUSSION

This study investigated the effects of maternal exposure to environmental tobacco smoke (ETS) over a prolonged period on male reproductive development in Wistar rats replicating exposure to environmental tobacco smoke in humans. The organs of interest were the testes and epididymis. Pregnant mothers and developing fetuses are particularly sensitive to the effects of exposure to environmental tobacco smoke. The physical anomalies observed in the pups could be attributed to the complex interplay between genetic and environmental influences. Previous studies have established a modest causal association between maternal exposure to cigarette smoke and the risk of craniofacial malformations, gastrointestinal defects, cryptorchidism, and neurological defects.[20,21] Exposure to cigarette smoking was reported to reduce the risk of hypospadias and skin defects according to Hackshaw, *et al*.[20] Hypospadias was not observed in any of the pups; consistent with reports from their review. However, in this study, pups of mothers exposed to cigarette smoke had an increased incidence of skin and fur discolorations.

Control animals averagely have more litter size than animals exposed to ETS. While this difference was not significant, it was consistent with findings from Klein and colleagues.[22] The difference in litter size accounted for the low birth weight observed in some pups in the control and exposed groups. Findings from previous research associate small litter sizes with increased body weights due to increased access to nutrients and high nurturing behavior.[23] However, the differences in the weight were not significant at PND 1.

The average weights per litter were similar in the early period but grew different towards the later period of the experiment. However, these differences were not significant. Thus, exposure to ETS during gestation and lactation could not have been solely responsible for the variation in body weights and growth rate observed in this study. The differences in weight could

be attributed to other factors such as litter size and maternal nurturing behavior. This is consistent with reports from Mammel and colleagues [7]. They also didn't find a significant association between prenatal exposure to cigarette smoke and weight differences in affected pups.

Histopathological evaluations have proved helpful in identifying features that are hallmarks of alterations caused by environmental influences, metabolic conditions, and genetic factors. Photomicrographs obtained from the results of the testes from pups in both groups revealed well-maintained closely packed seminiferous tubules and interstitial spaces. However, there were more tubules with generalized degeneration of germinal epithelium, depletion of elongating spermatids, and increased luminal diameter in the testes of pups exposed to ETS. This was depicted in the difference in the heights of the germinal epithelium measured, although the diameters of the tubules were almost similar. These findings suggest that ETS exposure may not distort the general arrangement of the tubules and their size when exposed in minute quantities and for a short duration but may deplete the cells that constitute the germinal epithelium and so increase the diameter of the tubules' lumen. Also, photomicrographs of regions of the head of the epididymis across both groups revealed normal epididymis architecture. However, an increase in sloughed cell debris and apoptotic epithelial cells was seen in the ducts of the caput epididymis of the pups exposed to ETS. This observation indicates that the spermatogenic process in the animals was disturbed and correlates with the disruptive signs observed in the testes. These findings agreed with the results observed by Omotoso and his colleagues.[24] They reported that exposure to varying concentrations of cigarette smoke distorted the testis and caudal epididymis of adult male rats in the experimental groups with signs of reduction in testicular and epididymis cell population, sloughing of epithelium, and degeneration of cells.

CONCLUSION

The results of this study show that perinatal ETS exposure affected the histoarchitecture and morphometry of seminiferous tubules; all of which are essential markers of normal reproductive function. It can be argued that exposure to environmental tobacco smoke (ETS) during pregnancy and early years of postnatal life can alter tissue structure and disrupt reproductive function in the testes and epididymis of rats; predisposing the exposed to infertility even when exposed in minute quantities.

DECLARATIONS

Acknowledgment

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Financial disclosure

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Conflict of interest

The authors have no conflicts of interest to declare.

Data availability and sharing statement

The corresponding author can make available, upon reasonable request, the data sets created and/or used in this study.

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