Histomorphological and histomorphometric changes in the testes of male Wistar rats following prepubertal exposure to caffeine

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SUMMARY

This experiment was designed to study the effects of caffeine on the histomorphology of the testes of prepubertal male Wistar rats. Prepubertal Wistar rats (25-28 days old), weighing between 50-60 g were randomly divided into three treatment groups (B, C and D) and control (A) of ten rats each. Group A rats received distilled water while Groups B, C, and D rats were respectively given daily dose of 50 mg/kg, 100 mg/kg, and 200 mg/kg of caffeine dissolved in distilled water for 14 days by gavage. Five rats from each group were sacrificed 24 hours after the last caffeine administration while the remaining rats were monitored for another three weeks for them to attain sexual maturity. The rats were sacrificed under diethyl ether anesthesia and the testes were fixed by immersion in Bouin's fluid. The tissues were processed for histomorphological and histomorphometric assessment. Data obtained were analyzed using one way ANOVA followed by Dunnett post hoc test for comparison with control. Alpha level was set at 0.05. Results showed that treatment with caffeine caused a significant decrease in testicular weight and a marked cellular degeneration and depletion of germ cells in the testes of rats in group D. The height of the germinal epithelium was significantly reduced when compared with control. This study concluded that prepubertal exposure of Wistar rats to caffeine reduced testicular growth and deleteriously alters testicular morphology.

Key words: Caffeine – Testes – Germinal epithelium – Histomorphology – Histomorphometry – Prepubertal Wistar rats

INTRODUCTION

Caffeine is a pharmacologically active component of many foods, beverages, dietary supplements and drugs (Wikoff et al., 2017). Being the most widely consumed psychoactive drug in the world, its consumption remains largely unregulated and legal in nearly all parts of the world unlike most other psychoactive substances. Consumption of high caffeine-containing energy drinks by the adolescent population has increased markedly in recent years due to the risk-taking behavior of adolescents and their lack of education on the negative effects of caffeine (Reissig et al.,

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2009; Seifert et al., 2011; Dorostghoal et al., 2012). There is growing concern about a decrease in male reproductive health, because a number of laboratory animal studies and human cases have pointed to toxic effects of caffeine on the male reproductive system (Weinberger et al., 1978; Pollard et al., 1990; Ramlau-Hansen et al., 2008; Dorostghoal et al., 2012; Tinwell et al., 2013). For instance, several studies on effects of caffeine on testicular morphology and function in men and adult animals have been documented (Weinberger et al., 1978; Nawrot et al., 2003; Park et al., 2015; Bae et al., 2017). In addition, a number of studies suggest that prenatal and lactational caffeine exposure impairs male gonadal development and thus later gonadal function (Ramlau-Hansen et al., 2008; Dorostghoal et al., 2012). Since the adolescent gonad is not fully mature either anatomically or histochemically (August, 1972), it would not be surprising if chronic exposure to caffeine during this vulnerable period were to interfere with normal gonad maturation. Although detrimental effects of caffeine on the development of the reproductive system during prenatal period have been widely demonstrated, data on the effect on the reproductive system during the prepubertal period are relatively sparse and conflicting. Considering that puberty is a period of rapid development of reproductive capacity, its vulnerability to insults seems to be greater than that in adults (Sharpe, 1988). This study therefore aimed to investigate the effects of caffeine on morphology, morphometry as well as the parameters of growth and maturation of the testis in prepubertal male rats throughout most of their rapid growth period.

MATERIALS AND METHODS

Animals and Treatment

Prepubertal male (4 weeks) Wistar rats (50 - 65 g) were obtained from the Animal House of the College of Health Sciences, Obafemi Awolowo University, Ile–Ife, Nigeria. They were maintained under the condition of natural light and dark cycles, temperature and humidity. Standard laboratory rat chow (Ace feed, Osogbo) and clean water were provided daily to the rats throughout the period of experiment. Ethical clearance was obtained from Health Research and Ethics Committee (HREC) of Institute of Public Health, Obafemi Awolowo University and the guidelines for care and use of animals were strictly followed.

Experimental Design

The rats were randomly assigned into four groups of ten rats each (n = 10). The four groups are groups A, B, C and D. Rats in Group A were the normal control and they received distilled water orally for 14 days while Groups B, C, and D received 50 mg/kg, 100 mg/kg and 200 mg/kg graded doses of caffeine (Sigma-Aldrich, USA) respectively, administered orally for a period of 14 days. Twenty-four hours after the last administration, five rats from each group were anaesthetized under diethyl ether and their testes were excised. The remaining rats were monitored for another three weeks to attain sexual maturity after which they were also sacrificed.

Organ weight

At the end of the experiment, testes from each rat were excised, trimmed free of fats and weighed on a top loader digital weighing balance (Camry). Relative testicular weight was defined as percentage ratio of testes weight to final body weight. The formula below was used to calculate the organ weight.

$$\text{Relative organ weight} = \frac{\text{organ weight}}{\text{final body weight}} \times 100\%$$

Histological procedure

The testes were fixed in fresh Bouin's fluid and tissue processing was done via paraffin wax embedding method of Drury and Wallington (1980). The embedded tissues were sectioned on a rotary microtome (Leica RM 2125 RTS) and 5 µm thick sections were obtained and stained with Hematoxylin and Eosin for the demonstration of general histoarchitecture. The stained sections were examined under a Leica DM 750 microscope connected to a computerized digital camera system (Leica ICC50) and digital photomicrographs were taken.

Histomorphometry

All histomorphometric evaluations were performed by the same trained, calibrated, and blinded examiner using an image analysis system (Image J Version 1.4.3.67) to analyze digital photomicrograph taken at a final magnification of 100x. Four serial sections were traced for each testis, and eight measurements per section were made of the number of seminiferous tubules within two defined regions at 100-fold magnification; these measurements were computed to obtain a mean value per animal. The seminiferous tubular epithelia height, seminiferous tubular diameter and seminiferous luminal diameter were measured while the cross-sectional area and volume of the seminiferous tubules were calculated. For analyzing the diameters of the seminiferous tubules, we defined regions that had round or nearly round cross sections of tubules as possible. Then, the longest diameter (D_1) and the perpendicular diameter (D_2) of each cross section of tubules were measured; each seminiferous tubular diameter D was calculated as $\sqrt{D_1D_2}$.

Statistical Analysis

Results were expressed as Mean ± S.E.M and data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett post hoc test for comparison with control. Alpha level was set at 0.05. All statistical analysis was done using GraphPad Prism5 (5.0.3.0, GraphPad Inc.).

RESULTS

Organ Weight

There was a significant decrease in relative testicular weight of treated rats in group D ($p < 0.05$) when compared with control (Fig. 1). There was no difference in testicular weight between groups A, B, C and D after the recovery period (Fig. 2).

Histopathological results

Histological examination of haematoxylin-eosin-stained sections revealed that the rats which received the highest dose (group D), had morphological features of histopathological damages, and these are presented by cellular degeneration resulting in depleted germ cells, a disorganized seminiferous tubular epithelium and presence of pyknotic nuclei (Figs. 3 and 4). However, these features observed in group D rats were reversed after the 3-week recovery period (Figs. 5 and 6). Histological examination of the testes of treated groups B and C rats showed no obvious injury to the seminiferous tubules, spermatogonia cells and the Leydig cells scattered in-between them in the interstitial spaces. They reveal instead seminiferous tubules having normal testicular morphology, primary spermatocytes showing no degenerative changes which showed spermatogenesis was not disturbed (Figs. 5 and 6).

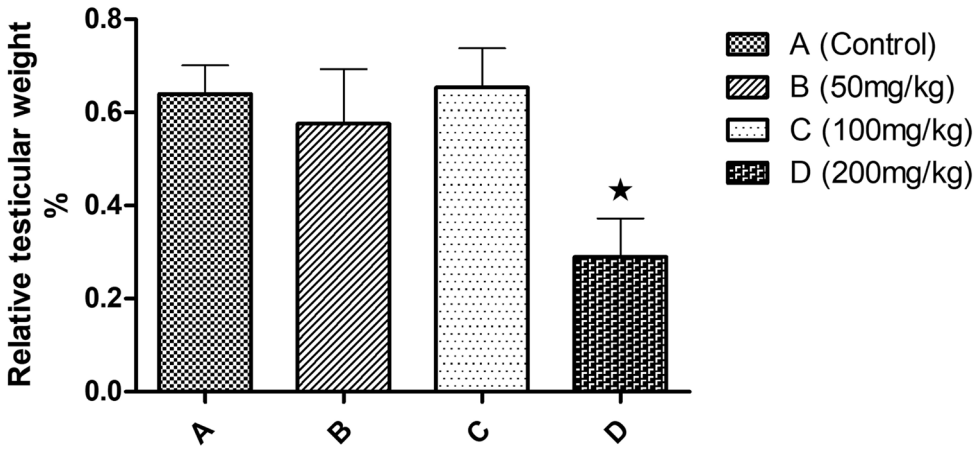

Fig. 1.- Effects of caffeine on the relative weights of the testes. Values are expressed as the mean ± SEM. * P<0.05 vs control.

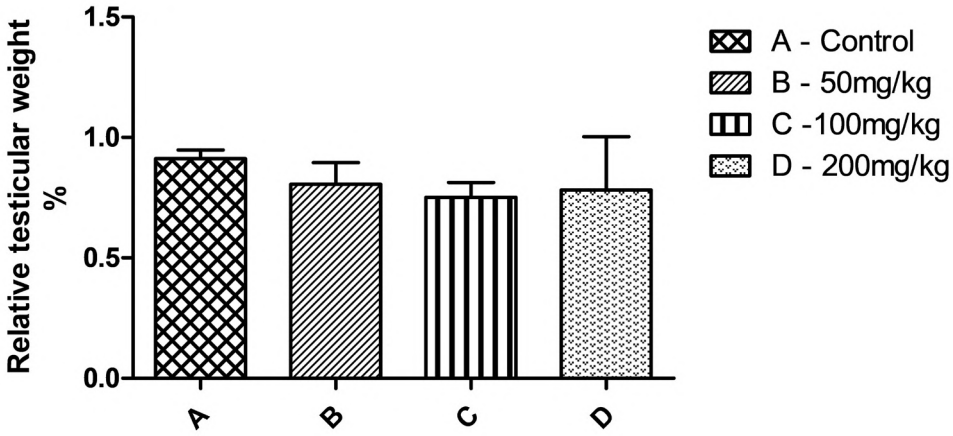

Fig. 2.- Effects of caffeine on the relative weights of the testes after 21 days recovery. Values are expressed as the mean ± SEM.

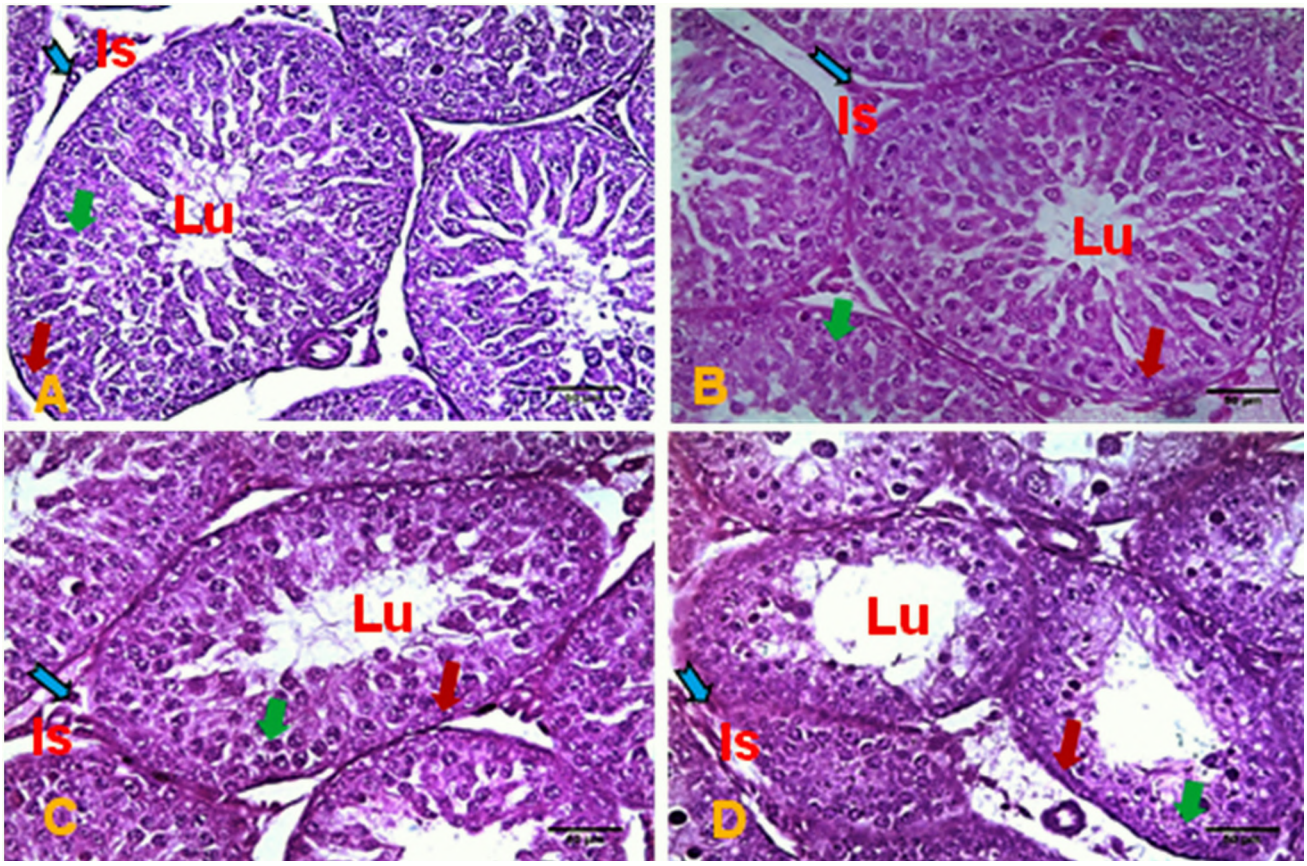

Fig. 3.- Sections of testes of prepubertal Wistar rats subjected to H&E stain after treatment. Note in slides A, B, C intact seminiferous tubules showing its lumen (Lu), interstitial cells of Leydig (blue arrows) within the interstitial space (Is) spermatogonia (red arrows), primary spermatocytes (green arrows). Also note D with smaller sizes of seminiferous tubules. Scale bars = 50 µm.

Histomorphometry

Histomorphometric analysis of the seminiferous tubular diameter presented nearly no differences ($p > 0.05$) between control group (A) and each of the other treated groups (B and C), while group D showed a significant reduction ($p < 0.05$) in seminiferous tubular diameter in comparison to group A and a significant reduction ($p < 0.05$) in seminiferous epithelial height in comparison to groups A and B (Table 1). The seminiferous luminal diameter was significantly increased in group D when compared with the other groups (Table 1). At recovery, significant decrease was observed in seminiferous epithelia height of

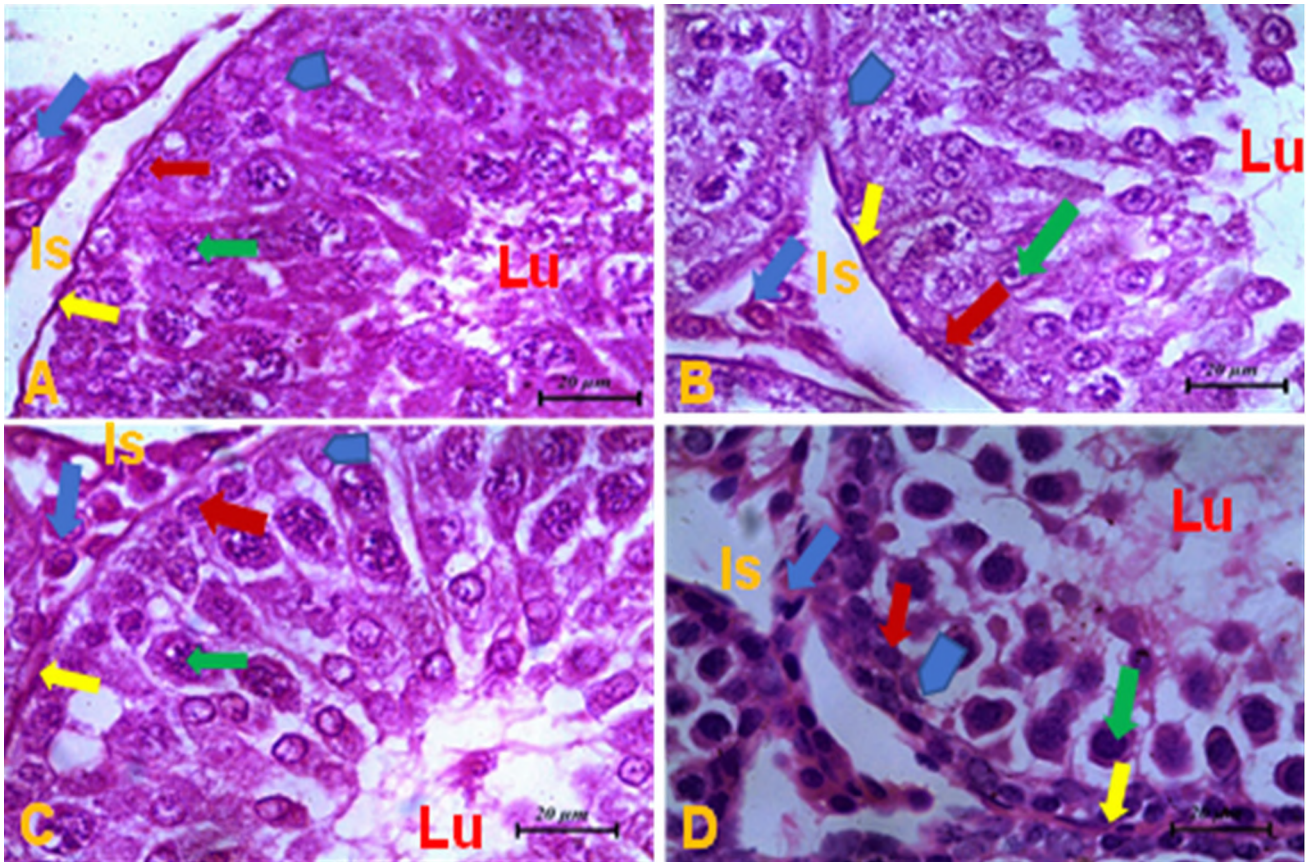

Fig. 4.- Sections of testes of prepubertal Wistar rats subjected to H&E stain. In slides A, B, and C, note the seminiferous tubule with a regular and thin basement membrane (yellow arrow), lumen (Lu), interstitial spaces (Is) containing interstitial cells (blue arrows), spermatogonia (red arrows), primary spermatocytes (green arrows), Sertoli cells (block arrows). Also note in D germinal epithelium with scanty immature spermatogenic cells. Scale bar = 20 µm.

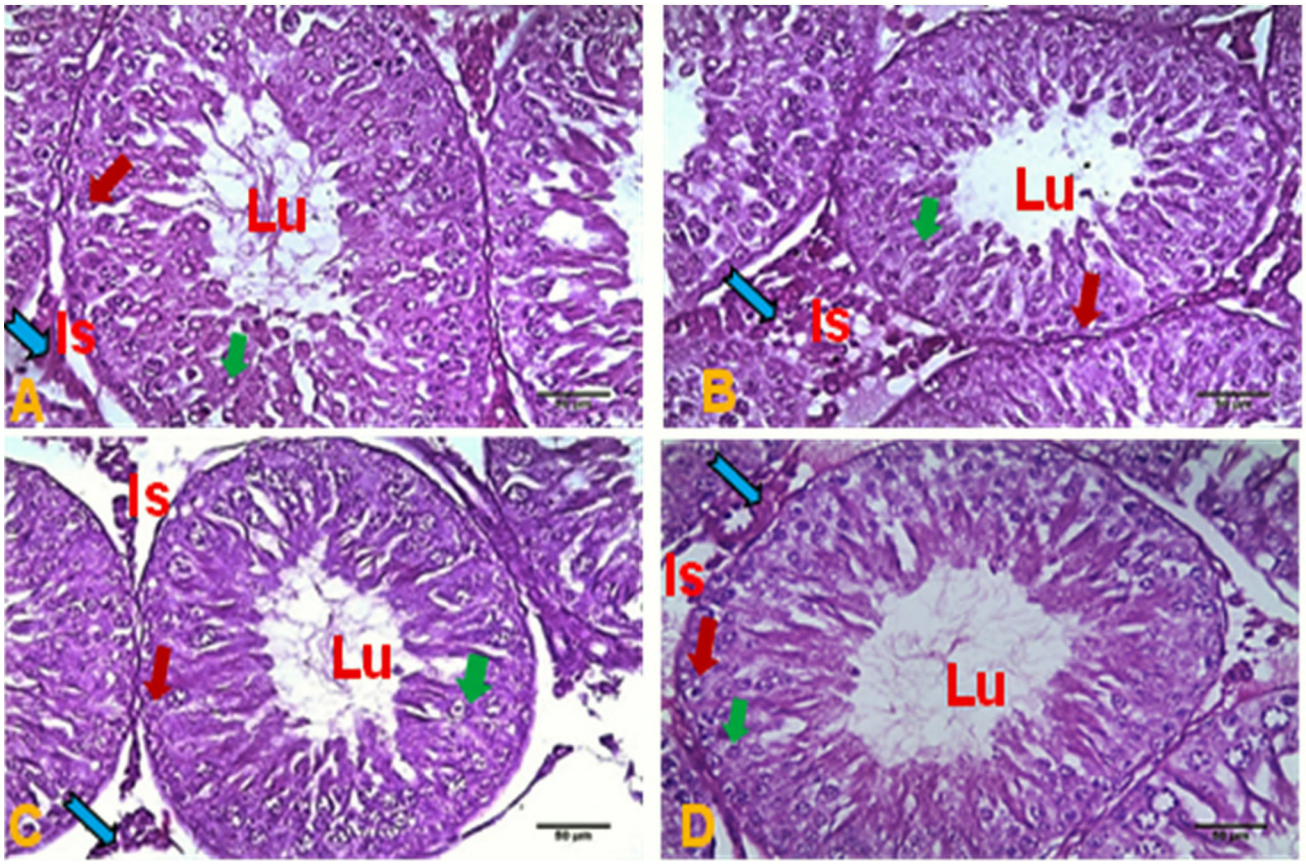

Fig. 5.- Sections of testes of prepubertal Wistar rats subjected to H&E stain following recovery. In slides A, B, C, D note the seminiferous tubules showing lumen (Lu), Interstitial cells of Leydig (blue arrows) within the interstitial space (Is), spermatogonia (red arrows) and primary spermatocytes (green arrows). Also note the marked recovery in D. Scale bars = 50 µm.

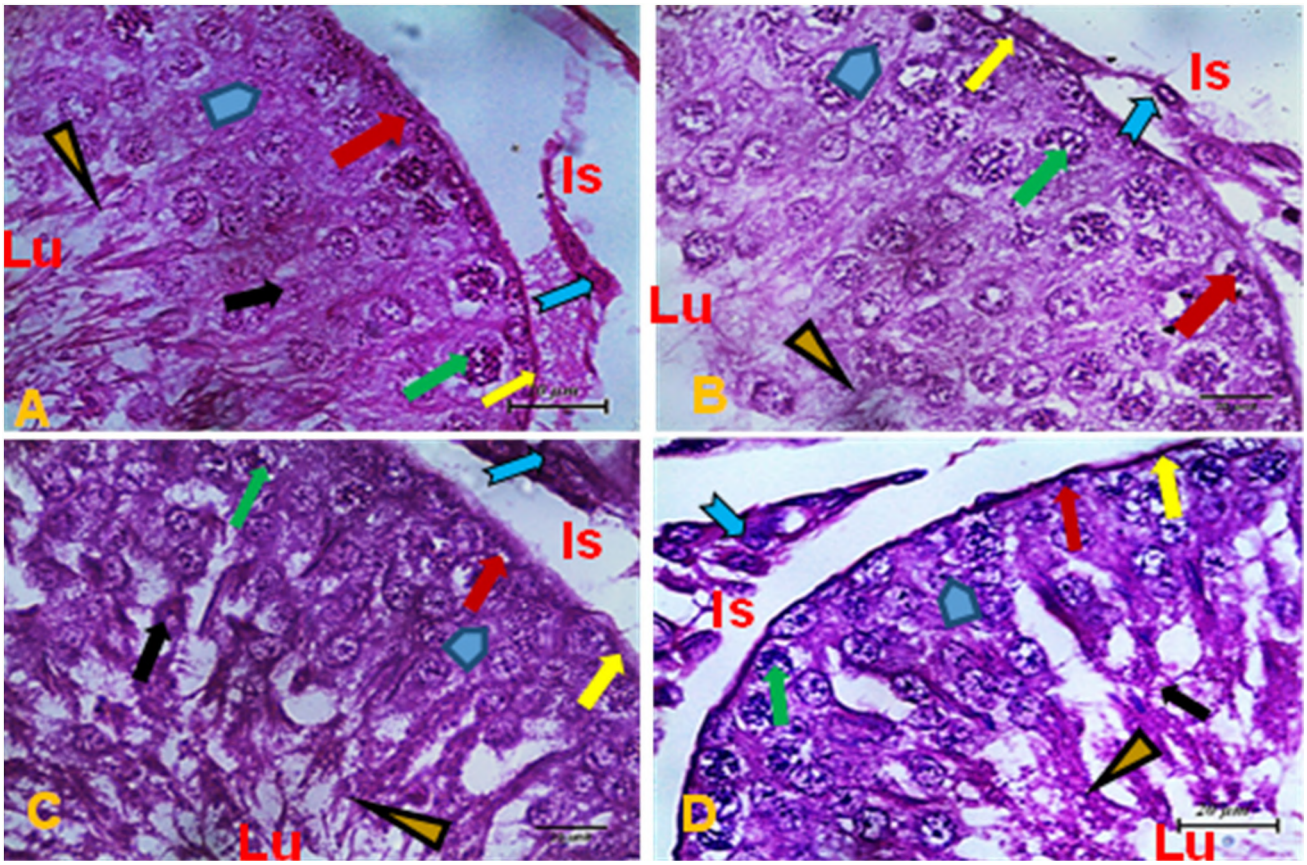

Fig. 6.- Sections of testes of prepubertal Wistar rats subjected to H&E stain following recovery. In slides A, B, C, and D note the seminiferous tubule with regular and thin basement membrane (yellow arrow), lumen (Lu) containing mature spermatozoa, interstitial spaces (Is) consisting of interstitial cells of Leydig (blue arrows), spermatogonia (red arrows), spermatocytes (green arrows), Sertoli cells (block arrows), round spermatids (black arrows), flagellum of elongated spermatids within the lumen (arrow heads). Scale bar = 20 µm.

Table 1. Effects of caffeine on the morphometry of the testis following treatment with caffeine.

Groups	Seminiferous tubular diameter (µm)	Seminiferous tubular epithelia height (µm)	Seminiferous luminal diameter (µm)	Cross-sectional area of seminiferous tubule (mm^2)	Volume of seminiferous tubules (μm^3)
Gp $1A_1$	249.8 ± 10.92	0.229 ± 0.019	76.02 ± 3.377	$49.02 \times 10^3 \pm 9.4 \times 10^1$	$8.16 \times 10^6 \pm 6.8 \times 10^3$
Gp $1B_1$	222.8 ± 19.62	0.179 ± 0.013	90.6 ± 9.027	$38.99 \times 10^3 \pm 30.2 \times 10^1$	$5.79 \times 10^6 \pm 3.9 \times 10^4$
Gp $1C_1$	221.1 ±11.13	0.180 ± 0.003	86.6 ± 5.086	$34.58 \times 10^3 \pm 30.5 \times 10^1$	$4.84 \times 10^6 \pm 4.0 \times 10^4$
Gp $1D_1$	209.8 ± 19.72*	0.155 ± 0.025*	102.3 ± 8.421*	$38.40 \times 10^3 \pm 9.7 \times 10^1$	$5.66 \times 10^6 \pm 7.2 \times 10^3$

Values are presented as Mean ± SEM (n=5).* = Significantly different from Control ($p<0.05$)

groups C and D when compared with control. A significant decrease in the seminiferous tubular diameter of groups B, C and D was also observed when compared with control while seminiferous luminal diameter of group D increased significantly ($p < 0.05$) when compared with control (Table 2). In addition, the cross-sectional area of the seminiferous tubule and the volume of the seminiferous tubules significantly decreased ($p < 0.05$) in groups B, C, and D compared to the control group A.

Table 2. Effects of caffeine on the morphometry of the testis following recovery from treatment with caffeine.

Groups	Seminiferous tubular diameter (µm)	Seminiferous tubular epithelia height (µm)	Seminiferous luminal diameter (µm)	Cross-sectional area of seminiferous tubule (mm^2)	Volume of seminiferous tubules (μm^3)
Gp $1A_2$	320.8 ± 22.43	0.281 ± 0.009	122.2 ± 5.975	$80.84 \times 10^3 \pm 4.0 \times 10^2$	$17.29 \times 10^6 \pm 5.9 \times 10^4$
Gp $1B_2$	274.0 ± 10.89*	0.239 ± 0.005	115.8 ± 4.102	$58.97 \times 10^3 \pm 9.3 \times 10^{1*}$	$10.77 \times 10^6 \pm 6.8 \times 10^{3*}$
Gp $1C_2$	250.1 ± 6.912*	0.194 ± 0.007*	121.7 ± 4.344	$49.13 \times 10^3 \pm 3.8 \times 10^{1*}$	$8.19 \times 10^6 \pm 1.7 \times 10^{3*}$
Gp $1D_2$	252.5 ± 13.41*	0.157 ± 0.028*	144.6 ± 12.820*	$50.08 \times 10^3 \pm 1.4 \times 10^{2*}$	$8.43 \times 10^6 \pm 1.3 \times 10^{4*}$

Values are presented as mean ± SEM (n=5). * = Significantly different from Control ($p<0.05$)

DISCUSSION

Morphological assessment of the testes of rats exposed to caffeine in this study revealed that caffeine intake caused a significant reduction on in testicular weight, a marked cellular degeneration and depletion of germ cells, a significant decrease in height of the germinal epithelium, seminiferous luminal diameter, seminiferous tubular diameter, cross sectional area and volume of seminiferous tubules of Wistar rats. This observation is in line with results of previous studies in which caffeine toxicity was reported to be associated with a decrease in germ cell proliferation, hence a reduced number of differentiating germ cells (Bae et al., 2017), as the weight of the testis is directly proportional to the mass of the differentiated spermatogenic cells (Boockfor and Blake, 1997). However, this morphological disruption appears to be reversible after the period of recovery. Oluwole et al., (2016) opined that the decrease in organ weight caused by caffeine could be due to the ability of caffeine to cause lipolysis. This resulted from inhibition of cyclic nucleotide phosphodiesterase that leads to accumulation of cyclic AMP in tissues which then activates hormone-sensitive lipase to promotes lypolysis (Hursel and Wester-Plantega, 2010). The decrease in organ weight, may have also resulted from diuresis which hinders reabsorption of salts and water, or increased metabolism (Huang et al., 2005). This study is also in line with the observations of Dorostghoal et al. (2012), who reported that prenatal caffeine exposure brought about a dose-related reduction in the testis weight of male offspring.

The examination of the histological sections of group D showed that caffeine induced deleterious effects on testicular architecture which could have resulted from interference with the testicular micro-environment thereby resulting in the disruption of the initiation and maintenance of the spermatogenesis. A reduction was observed in seminiferous tubular epithelia height of the group administered with the highest dose reveals shows caffeine has negative effect on testicular morphology. Pollard et al. (1990) had earlier reported that exposure of male Wistar rats to 30 mg/kg per day of caffeine, given by gavage for 15 to 38 consecutive days led to breakdown of the germinal epithelium. The seminiferous tubular diameter was observed to be decreased in all the animals after the recovery period. The decrease in seminiferous tubular diameter may be due to the fact that caffeine interferes with cell division which leads to a reduced number of spermatogenic cells. Dorostghoal et al. (2012), reported a decrease in the diameter of seminiferous tubules of the offspring of rats given caffeine during the prepubertal, postpubertal and adulthood periods. Pollard et al. (1990) also showed that caffeine when given prenatally, inhibited differentiation of the seminiferous cords. Daily administration of 30 or 60 mg/kg caffeine to mature male rabbits for four consecutive weeks caused a decrease in the sizes of the seminiferous tubules and inhibited spermatogenesis (Ezzat and el-Gohary, 1994).

CONCLUSION

This study concluded that prepubertal exposure of Wistar rats to caffeine reduced testicular growth and predisposed the seminiferous tubules of growing male Wistar rats to deleterious structural changes which were reversible over a recovery period.

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