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Pre-Pubertal Caffeine Exposure Interfered with Sperm Development in Wistar Rats without Deleterious Effects on Reproductive Hormones

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ABSTRACT

This study investigated the effects of caffeine, a constituent of widely consumed energy drinks and beverages, on the sperm characteristics and level of reproductive hormones in developing male rats. Forty pre-pubertal Wistar rats (25 – 28 days old), weighing between 50 – 60 g were assigned into 4 groups (n = 10). Three test groups were administered daily with 50 $mgkg^{-1}$, 100 $mgkg^{-1}$ and 200 $mgkg^{-1}$ of caffeine for 14 days by gavage while the controls received equivalent volumes of distilled water used in dissolving caffeine. Five rats from each group were sacrificed 24 hours after the last administration of caffeine while the remaining five were monitored for further three weeks till they were all sexually matured. Caudal part of the epididymis were excised and the sperm count, morphology, motility and viability were determined. The concentration of testosterone and luteinizing hormone were determined in the serum using enzyme-linked immunosorbent assay. 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.

The result showed a significant decrease in epididymal sperm motility and count at treatment doses of 50 mg/kg, 100 mg/kg and 200 mg/kg. The 200 mg/kg dose also showed a decrease in sperm viability. There was also an increase in the number of abnormal sperm at treatment doses of 50 mg/kg, 100 mg/kg and 200 mg/kg compared to the control. Serum testosterone and luteinizing hormone levels were not significantly altered following treatment; however, the luteinizing hormone level was significantly increased in the group which received 200 mg/kg of caffeine after recovery. The study concluded that prepubertal caffeine exposure interfered with epididymal sperm development in a dose dependent manner without significant effect on the synthesis of reproductive hormones

Key words: caffeine, pre-pubertal Wistar rats, sperm count, sperm motility, sperm morphology, sperm viability, testosterone, luteinizing hormone.

INTRODUCTION

Caffeine (1, 3, 7 – trimethylxanthine), is a component of several natural plants such as tea leaves, cocoa beans, kola nuts [1], and also present as an additive in energy drinks and beverages [2]. It is used in medications as pain relievers [3], diuretics, muscle relaxants and in the treatment of Parkinson's disease [4]. Caffeine can be artificially synthesized. This artificially synthesized caffeine has no chemical difference from the natural caffeine [5]. Caffeine is one of the world's most consumed psychoactive drug [6] and caffeine-containing substances are consumed by about 80% of the world's population on a daily basis [7]. Of this population, it has been reported that children and adolescents are regular consumers of energy drinks containing high amounts of caffeine [8] thereby arousing interest in its probability in involvement with current decrease in male reproductive health. Although, there are existing literatures which state that caffeine has no distinct/clear effect on semen quality [9], several studies suggested its consumption has been found to be associated with a dose-dependent significant decrease in testicular size in mice [10], reproductive and developmental toxicities [11], increase in sperm motility [12] and increase in the frequency of sperm abnormalities [13].

Puberty is the period of sexual maturation and at which reproductive capacity is attained. The period between birth and puberty is known as pre-puberty and this phase provides the opportunity to evaluate toxicity [14]. Adequate bioavailability of testosterone plays an important role in the structural and functional integrity of the testes [15]. Bae *et al.* [16] reported exposure of rats to caffeine caused a decrease in serum levels of testosterone, while Ezzat and el-Gohary [17] reported an increase in plasma testosterone and a decrease in luteinizing hormone levels in rabbits. Our literature survey reveals that there is dearth of literature on the effects of caffeine exposure on testicular function at prepuberty. This study aimed at bridging this gap in knowledge.

MATERIALS AND METHODS

Chemicals: Caffeine was procured from Sigma-Aldrich (St. Louis, MO, USA). ELISA kits for testosterone and luteinizing hormone (Accu Bind, ELISA Microwells) were obtained from Monobind Inc., Lakeforest, CA 92630, USA.

Animal management and experimental design: Forty prepubertal male Wistar rats aged four weeks, and bred at the Animal House of the College of Health Sciences, Obafemi Awolowo University, Ile–Ife, Nigeria, were used for the study. The rats were housed in plastic cages under natural light and dark cycle, temperature and humidity and allowed access to standard laboratory rat chow and clean water. Ethical approval for this study was obtained from Health Research and Ethics Committee (HREC) of Institute of Public Health, Obafemi Awolowo University. The rats received humane care according to the National Institutes of Health Guide for Care and Use of laboratory Animals [18]**.** The animals were randomly divided into four groups (1, 2, 3 and 4) of ten rats each (n = 10) and treated as follows: Rats in Group A served as vehicle treated control and they received distilled water daily for fourteen days while groups B, C, and D were given 50mg/kg, 100 mg/kg and 200mg/kg of caffeine respectively, orally using an oral cannula for a period of 14 days. Weekly body weight was determined with the aid of a digital weighing balance

Sacrifice: Twenty-four hours after the last administration, five rats from each group (control and treated) were anaesthetized with diethyl ether and sacrificed. The remaining rats in each groups were sacrificed on day 21 after the last administration to study the effect of the

treatment on the characteristics of spermatozoa after the animals had reached puberty.

Sample collection and sperm analysis: The testicles and the epididymis were excised through a midline pre-scrotal incision and the caudal epididymis was separated. Epididymal sperms were collected by slicing the caudal epididymis a glass petri dish containing physiological saline (1:10 w/v) and incubated at room temperature for 5 minutes to allow sperm to swim out of the epididymal tubules. The supernatant (sperm suspension) was used for the analysis of sperm count, morphology, motility and viability.

Sperm motility: About 5.0 µL of supernatant containing the sperm was placed between the slide and cover slip and observed at 100x magnification under a Leica DM 750 research microscope. Progressive sperm motility was evaluated in the middle fields and sperm motility was expressed as percentage of motile sperm in the total sperm counted.

Sperm morphology: For the analysis of sperm morphology, a drop of about 20 µL of sperm suspension was placed on the clean slide to produce a smear. Sperm smears were dried and stained with eosin-nigrosin stain (1% eosin Y and 5% nigrosine) and examined on a Leica DM750 research microscope at 400x. About 200 spermatozoa per slide were evaluated for head and/or flagellar defects and the results were expressed as percentage of abnormal cells.

Sperm viability: An aliquot of 20 µL of the sperm suspension was diluted with an equal volume of nigrosine-eosin (1% eosin Y and 5% nigrosine). Then, a smear of content was carried out on the microscope slide and after drying, the preparations were observed under microscope (Leica DM750) at 400x. About 200 spermatozoa were examined for viability. Unstained sperm (with intact membrane) were said to be viable while stained sperm (with non-intact membrane) were classed as non-viable. Results were expressed as percentage of viable spermatozoa in total sperm counted.

Sperm count: Sperm count was evaluated using the Neubauer haemocytometer chamber. An aliquot of 10 µL of the sperm suspension was diluted in 990 µL of semen-diluting fluid (containing 5 g sodium bicarbonate, 1 mL paraformaldehyde and 99 mL distilled water) and mixed by vigorous pipetting. About 10 µL of diluted contents was loaded into the hemocytometer (Neubauer chamber) and the sperm were allowed to settle by keeping the hemocytometer in a humid chamber for 10 minutes. The number of spermatozoa in the squares of the hemocytometer was counted under microscope at 400x. Sperm concentration expressed in millions of sperm per mL was determined as follows:

$$Sperm\ count\ (10^6/\text{mL}) = \frac{Number\ of\ sperm\ cells\ x\ dilution\ factor\ x\ depth\ factor}{Number\ of\ areas\ counted}$$

Hormonal assay: Blood samples were obtained by cardiac puncture under anaesthesia into separate plain bottles. These were, thereafter, left for 30 minutes to clot and then centrifuged at 3000 rpm for 5 minutes to obtain serum. The serum levels of testosterone and luteinizing hormones were determined using enzyme-linked immunosorbent assay method.

Statistical analysis: GraphPad prism 5.03 was used to carry out all statistical analyses. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett post hoc test for comparison with control. Results were expressed as Mean ± S.E.M. and $p < 0.05$ was considered significant.

RESULTS

Effects of Caffeine on the Body Weight: Administration of caffeine at 50 mg/kg, 100

mg/kg and 200 mg/kg to prepubertal rats did not cause any significant effect on the body weight of the treated animals (Fig. 1). Following withdrawal of treatment, the body weights were also not significantly different amongst the groups.

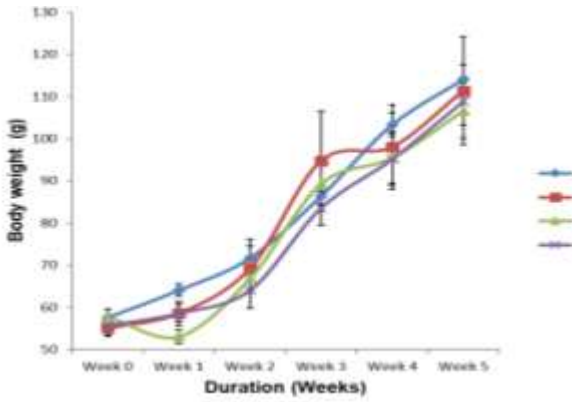

Figure 1: Effects of caffeine of body weight gain. Each bar represents Mean ± SEM,

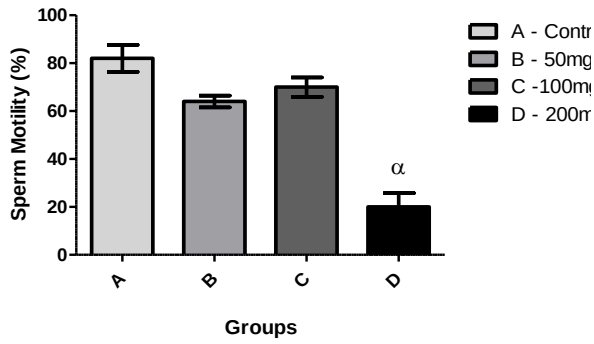

Figure 2: Effects of caffeine on the sperm motility of prepubertal Wistar rats. Each bar represents Mean ± SEM, (α) significantly different from control.

Effects of caffeine on sperm parameters: Administration of caffeine to Wistar rats significantly inhibited the rate of sperm motility of treated rats in group 4 when compared with group 1 (Fig. 2). Also, when compared with control rats, group 4 rats showed a marked decrease in sperm viability, (Fig. 3). Groups 2, 3 and 4 rats showed a significant decline in sperm count when compared with group 1 (Fig. 5) while a significant increase was also noted in abnormal sperm morphology of groups 2, 3 and 4 when compared with group 1 (Fig. 4).

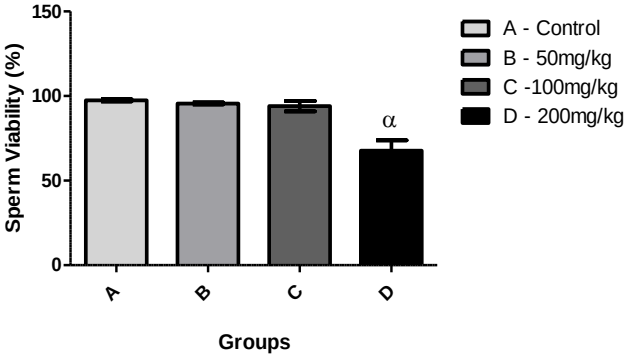

Figure 3: Effects of caffeine on the sperm viability of prepubertal Wistar rats. Each bar represents Mean ± SEM, (α) significantly different from control.

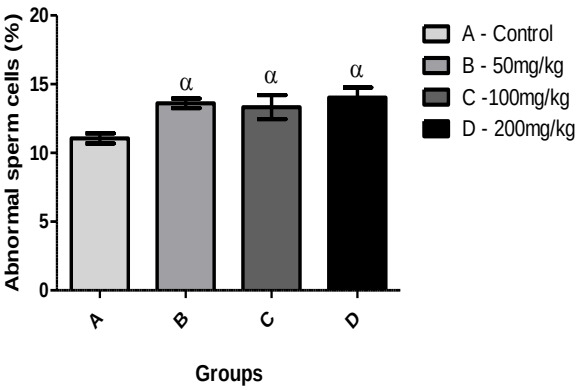

Figure 4: Effects of caffeine on the sperm morphology of prepubertal Wistar rats. Each bar represents Mean ± SEM, (α) significantly different from control.

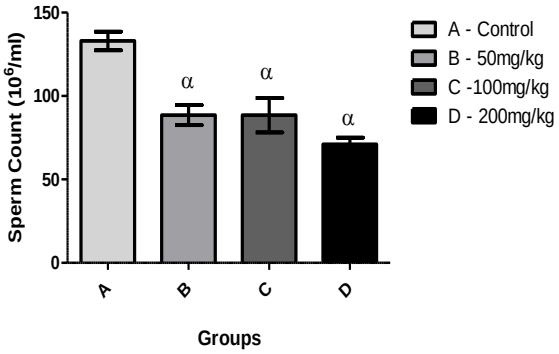

Figure 5: Effects of caffeine on the sperm count of prepubertal Wistar rats. Each bar represents Mean ± SEM, (α) significantly different from control.

Effect of Caffeine on Testosterone and Luteinizing Hormone Levels: Following administration with caffeine, at 50 mg/kg, 100 mg/kg and 200 mg/kg there was no significant effect ($p>0.05$) on serum testosterone concentration in treated groups when compared with control. The concentration of testosterone increased both in caffeine-treated and recovery groups but the increase was not significant (Tables 1 and 2). Similarly, caffeine administered at 50 mg/kg, 100 mg/kg and 200 mg/kg did not cause any significant effect ($p>0.05$) on the luteinizing hormone concentration in rats in the treated groups when compared with rats in control group. However, withdrawal of the treatments led to a significantly ($p<0.05$) increased serum luteinizing hormone concentration in male Wistar rats in the recovery group which received the highest dose of caffeine when compared with control.

Table 1: Effects of caffeine on hormone concentration of prepubertal Wistar rats following treatment.

GROUPS	TESTOSTERONE LEVEL (ng/ml)	LUTEINIZING HORMONE (mIU/ml)
Control	0.076 ± 0.010	18.74 ± 1.315
50 mg/kg	0.083 ± 0.011	18.01 ± 0.853
100 mg/kg	0.195 ± 0.047	21.52 ± 2.618
200 mg/kg	0.088 ± 0.033	23.76 ± 2.299

Values are presented as Mean ± SEM, n=5. Gp A, control; Gp B, 50 mg caffeine, Gp C, 100mg caffeine, Gp D, 200 mg caffeine. $P<0.05$.

Table 2: Effects of caffeine on the hormone concentration of prepubertal Wistar rats 21 days after caffeine treatment.

GROUPS	TESTOSTERONE LEVEL (ng/ml)	LUTEINIZING HORMONE (mIU/ml)
Control	1.331 ± 0.282	18.38 ± 3.450
50 mg/kg	0.713 ± 0.139	19.71 ± 4.780
100 mg/kg	0.926 ± 0.211	22.48 ± 4.368
200 mg/kg	1.134 ± 0.212	30.54 ± 3.498*

Values are presented as Mean ± SEM, n=5. Gp A, control; Gp B, 50 mg caffeine, Gp C, 100mg caffeine, Gp D, 200 mg caffeine. * = Significantly different from Control ($p<0.05$)

DISCUSSION

The results showed that administration of caffeine at the different doses for 14 days had deleterious effect on the sperm but did not interfere with body weight and concentration of luteinizing and testosterone hormones. The data from previous studies suggest that caffeine may have an effect on body weight either in young, adult rats or pregnant rats and those exposed during the period of lactation [19]. Bae et al. [16], reported caffeine fed rats had lower body weight gain which was dose dependent. It was observed in this study that the body weights of the prepubertal rats were not affected by exposure to caffeine during the 14 days' administration period.

In this study, 14 days' administration of 50, 100 and 200 mg/kg of caffeine to prepubertal rats did not produce significant effect in testosterone concentration of the control and treated groups of prepubertal and pubertal rats. In addition, no significant effect was observed in the level luteinizing hormone across the different groups of prepubertal rats. This finding suggests that caffeine did not interfere with the synthesis of testosterone and luteinizing hormone following treatment as there was availability of luteinizing hormone by the anterior pituitary lobe at levels required to initiate synthesis of enough amounts of testosterone through the hypothalamo-pituitary-testicular axis. A significant ($p<0.05$) increase in the serum luteinizing hormone concentrations was observed in rats given 200 mg/kg, 21 days after caffeine administration. This increase might have resulted from increased secretion of gonadotropin releasing hormone from the hypothalamus causing an increased secretion of luteinizing hormone from the pituitary gland. Adequate bioavailability of the hormone testosterone

is a key to the structural and functional integrity of the testes [15]. Contrary to the findings of this study, Ezzat and el-Gohary [17] reported decreased luteinizing hormone and increased testosterone hormone levels in rabbits which were treated with caffeine while Uno *et al.* [20] reported a decrease in serum levels of testosterone and luteinzing hormones in all caffeine treated rats when compared with control. Bae *et al.* [16] also reported increased serum levels of testosterone in rats treated with 60mg/kg caffeine and no significant difference in the rats which received 120mg/kg of caffeine when compared with control. These contrasting reports explains the views of Dias *et al.* [21] that caffeine is a cellular modulator which is capable of acting indirectly on both fetal and adult life, either by impacting the hypothalamo-pituitary-gonadal-system or by having a direct toxic effect on the germinal epithelium.

In this study, sperm analysis, of the four sperm parameters, revealed significant difference between the treated rats and the control rats. A remarkable decrease was seen in sperm motility, sperm viability and sperm count, while an increase was seen in the abnormal morphology. These irregularities may be due to alterations in the biosynthetic process underlying spermatogenesis in the testes [20], reduced quantity of acrosin content in the spermatozoan or alterations in sperm maturation in the epididymis. This observation has been reported in offsprings of rats which were given caffeine during gestation [19]. The decrease in motility may have resulted from small quantity of acrosin content in the spermatozoan, as it has been reported that the prerequisites for sperm movement towards the oocyte is optimal motility and acrosin content [11]. Contrary to our observation, increased epididymal sperm motility from acute treatment and in vitro administration of caffeine in the rat was reported by Grima and Cheng, [22].

CONCLUSION

This study concluded that caffeine produced deleterious effects in epididymal sperm parameters in Wistar rats without altering testosterone and luteinizing hormone concentrations.

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