



## Effects of Oral Maternal Administration of Caffeine on Reproductive Functions of Male Offspring of Wistar Rats

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**Summary:** Caffeine was investigated for its possible fetal programming effects on reproductive function of male offspring. Sixty-five pregnant Wistar rats were grouped into four. Group 1 was control and received distilled water. Groups 2, 3 and 4 were treated orally with 1.14, 3.42 and 5.70 mg/kg body weight of caffeine respectively. Each group was subdivided into four based on gestation days (GD) 1-7, 8-14, 15-21 and 1-21. The day of parturition was taken as postnatal day zero (0). Male offspring were sacrificed on postnatal day 70. Parameters determined were: weight at birth, body weight at postnatal day 21 and 70, anogenital distance (AGD) index, sperm parameters, reproductive organ weight, histology and hormonal profile (testosterone, FSH and LH). Data were analyzed using Analysis of Variance. Level of significance was taken at P<0.05. Male offspring belonging to caffeine treated dams showed dose dependent significant decreases in birth weight. Male offspring from dams treated with caffeine during GD 1-7 and GD 1-21 had a significant increase in their AGD index. Also, male offspring from dams treated with 1.14 and 5.70 mg/kg body weight of caffeine during GD 8-14 had a significant increase in AGD index. Dams treated with 3.42 mg/kg body weight of caffeine during GD 15-21, had a significant increase in the AGD index of their male offspring. The sperm motility of offspring from dams treated with 5.70 mg/kg body weight of caffeine during GD 1-7 and GD 1-21 were significantly increased. Offspring of GD 8-14 and GD 15-21 dams treated with 3.42 and 5.70 mg/kg body weight of caffeine respectively, showed significantly reduced serum testosterone level. There was a significant decrease in the weight of testes of offspring from dams treated with caffeine during GD 8-14. Histological sections of testes of offspring from caffeine treated dams showed interstitial congestions, edema, reduced germinal epithelial height and detached basal membrane. Maternal caffeine exposure during different gestational periods adversely affected birth weight and some reproductive indices in male offspring of Wistar rats.

**Keywords:** Caffeine, Anogenital distance index, Wistar rat, Sperm motility, Testosterone.

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### INTRODUCTION

The rise in occurrence of reproductive disorders has raised concerns regarding the impact of endocrine disrupting chemicals on reproductive health especially when such exposures occur during fetal life (Savabieasfahani *et al.*, 2006). Exposure to environmental and occupational toxicants and progressive changes in many aspects of lifestyle, including dietary habits, has been shown to deteriorate reproductive health, thus, affecting the ability of couples to conceive and maintain a healthy pregnancy. Among dietary factors affecting reproduction is caffeine consumption (Dlugosz and Bracken 1992; Kumar *et al.*, 2009). Data from *in vitro* studies suggested that caffeine has variable, dose-related effects on human sperm motility, number and structure (Dlugosz and Bracken, 1992).

Caffeine is a naturally occurring alkaloid found in

the seeds, leaves and fruits of more than 60 plants such as coffee and cocoa beans, kola nuts, and tea leaves. It is also present in a wide variety of foods and beverages including coffee, tea, cocoa, chocolate, colas and energy drinks also in a number of prescription drugs including cold and flu remedies, headache treatments, diet pills, diuretics and stimulants (Al-Shoshan, 2007). Average caffeine consumption by adult human varies among different cultures and nations from 80 to 400 mg/70kg person per day (Knight *et al.*, 2004; Mitchell *et al.*, 2014). Caffeine is the most widely consumed xenobiotic in pregnancy, with a potential to adversely affect the developing fetus (James, 1991). About 98% of women in reproductive age regularly consume caffeine in the form of caffeinated beverages or in caffeine containing medications while about 75% of them continue to do so during pregnancy (Sengpiel *et al.*, 2013). Caffeine has been reported to be capable of crossing the placenta barrier (Dlugosz and Bracken,

1992). If consumption of caffeine continues during pregnancy, the developing fetus is exposed to caffeine and its metabolites during the critical periods of development and this may alter growth and development which could affect physiology during adult life. Epidemiological studies suggested that caffeine consumption may affect skeletal growth (Bakker *et al.*, 2010) and cause low birth weight (Bracken *et al.*, 2003). Experimental studies also showed that caffeine consumption affected fetal growth and altered reproductive functions (Dorostgoal, 2012). However, these experimental studies used very high doses of caffeine which are not obtainable in humans. Therefore, the aim of the present study was to determine the effects of maternal caffeine consumption during different gestation periods on reproductive functions of male Wistar rats. It is worth noting that the dosages used here are within the average human caffeine consumption.

## MATERIALS AND METHODS

### Experimental Design

Sixty-five female Wistar rats weighing 180-200g obtained from the animal house, College of Medicine, University of Ibadan, were used for this study. The animals were housed in well ventilated wire mesh cages with constant 12-h light 12-h dark cycle. They were fed standard rat feed and clean water *ad libitum* and were allowed to acclimatize for two weeks, before the commencement of the study. The study was performed according to the recommendations from declaration of Helsinki on guiding principles for the care and use of laboratory animals (World Medical Association, American physiological society, 2002).

Female Wistar rats were mated with proven male breeders in the ratio 2:1 (female to male) during proestrous phase. The presence of spermatozoa in vaginal lavage confirmed mating on the morning after pairing and the day of confirmation of mating was taken as gestation day (GD) one. The pregnant rats were then divided into four major groups; the control group consisting of 5 animals, groups 2, 3 and 4 consisted of 20 animals each. The groups 2, 3 and 4 were then subdivided into four equal groups according to gestation days (GD), a) 1-7, b) 8-14, c) 15-21 and d) 1-21. Group one (control) received distilled water while groups 2, 3 and 4 were orally administered with caffeine at 1.14, 3.42 and 5.70 mg/kg body weight respectively. Subgroups a, b, c and d received caffeine on gestation days 1-7, 8-14, 15-21 and 1-21 respectively. Caffeine (Aesar Johnson Matthew Company, USA) was freshly prepared by dissolving it in distilled water daily before use. The day of parturition was taken as day zero of life. On postnatal day 1, the birth weight of the male pups was recorded. Also, the anogenital distances were measured

according to method used by Sathyanarayana *et al.*, (2010). At postnatal day (PND) 21 (weaning) and 70 (just before sacrifice) the body weight of the offspring was also measured. Five male offspring were randomly selected from each subgroup and sacrificed at PND 70, bringing the total number of male offspring used to 65.

### Blood Collection

Blood was collected from the medial canthus vein into plain sample bottles and centrifuged at 3500rmp for 10 minutes. Animals were thereafter sacrificed via cervical dislocation.

### Hormonal Assay

Serum concentrations of luteinizing hormone, follicle stimulating hormone and testosterone were determined using ELISA kits (Inteco Diagnostics, UK).

### Histology of the testes

The testes, epididymes and seminal vesicles were collected and weighed. Testes were fixed in Bouin fluid in preparation for histological sections. Tissue processing included tissue embedding, microtomy, trimming and nicking to expose the embedded tissue and orientate the tissue block. Staining was done using haematoxylin and eosin. The slides were then viewed with the microscope.

### Sperm Analysis

Caudal epididymal fluid was used for sperm analysis. Sperm count was determined using the microscope with the aid of the improved Neubauer hemocytometer. Count was done in five large Thoma square as described by Raji and Bolarinwa (1997). The result was expressed as count  $\times 10^6$  ml $^{-1}$ . Sperm motility was done as described by Zemjanis (1970).

### Statistical analysis

Data were expressed as mean  $\pm$  standard error of the mean (Mean  $\pm$  SEM). Analysis of variance was used in comparison of data followed by Tukey post-hoc test using SPSS (Version 17.0). P<0.05 was considered significant.

## RESULTS

### Birth weight and anogenital distance Index (AGD)

The birth weight and anogenital distance index (AGD) of offspring are shown in table 1 and figure 1 respectively. There was a significant decrease ( $P < 0.05$ ) in the birth weight of all pups from dams that received caffeine throughout gestation period (GD 1-21). Also, birth weight of pups from dams treated with 3.42 mg/kg body weight of caffeine during GD 1-7 and GD 15-21 decreased significantly. Furthermore, birth weight of pups from all dams treated with 5.70 mg/kg body weight of caffeine, irrespective of the gestation day significantly reduced (Table 1). All pups from GD

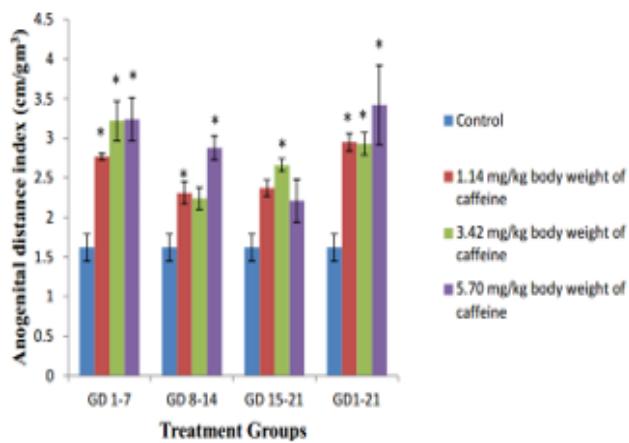
1-7 and GD 1-21 treated dams showed significant increase AGD index ( $P<0.05$ ) (Figure 1). Pups from dams treated during GD 8-14 with 1.14 and 5.70 mg/kg body weight of caffeine had increased AGD

index (Figure 1). There was also a significant increase in AGD index of pups from GD 15-21 treated dams that had 3.42 mg/kg body weight of caffeine (Figure 1).

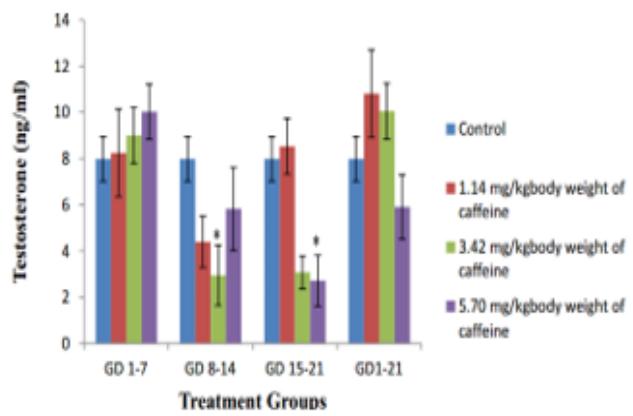
**Table 1.** Effect of maternal caffeine exposure on the body weight of offspring.

Dosage of Caffeine	Gestation Day (GD)	Birth Weight(g)	Weight(g) at PND 21	Weight(g) at PND 70
<b>Control</b>	-	5.50±.13	27.24±3.3	108±6.7
<b>1.14 mg/kg</b>	1-7	5.44±.51	27.61±.73	122±6.0
	8-14	4.98±.17	24.63±.63	116±3.26
	15-21	5.39±.05	17.20±.73*	109.7±4.4
	1-21	4.56±.04*	26.70±1.8	123±8.2
<b>3.42 mg/kg</b>	1-7	4.26±.10*	21.27±.73	135±6.8*
	8-14	5.30±.26	27.00±00	120±.34
	15-21	4.33±.12*	20.20±2.0	114±7.1
	1-21	3.18±.15*	25.15±.26	108±.40
<b>5.70 mg/kg</b>	1-7	3.32±.09*	20.93±.97	116.8±9.6
	8-14	3.16±.09*	20.68±.72	110±1.6
	15-21	3.04±.05*	21.02±.48	100±2.0
	1-21	3.03±.08*	27.24±.28	111.5±.22

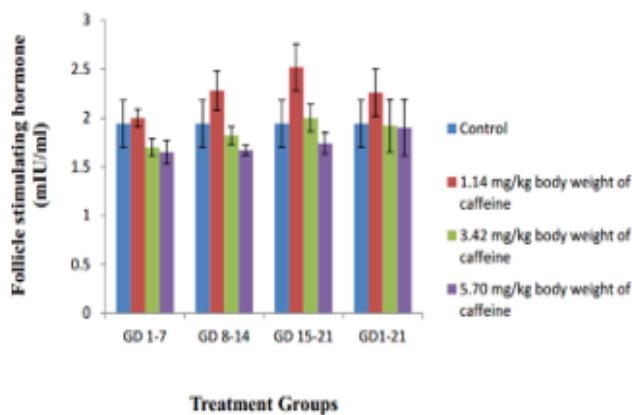
Values expressed as mean ± standard error of mean, n=5, \*significantly different from control at  $P<0.05$



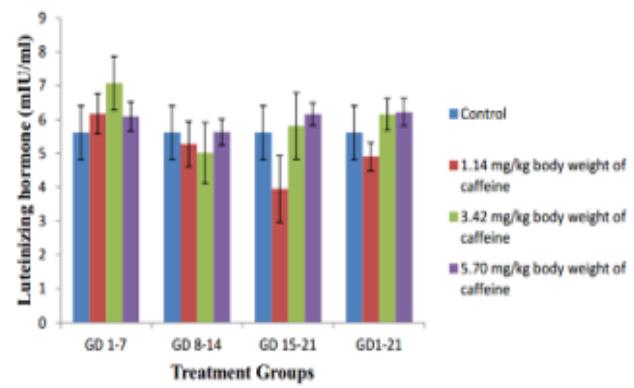
**Fig 1.** Effect of maternal caffeine exposure on anogenital distance index of male pups at birth. Values expressed as mean ± standard error of mean, n=5, \* $P<0.05$



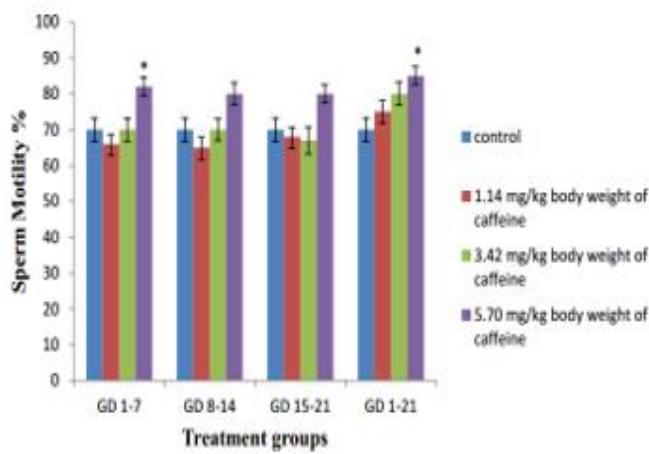
**Fig 2.** Effect of maternal caffeine exposure on serum level of testosterone. Values expressed as mean ± standard error of mean, n=5, \* $P<0.05$



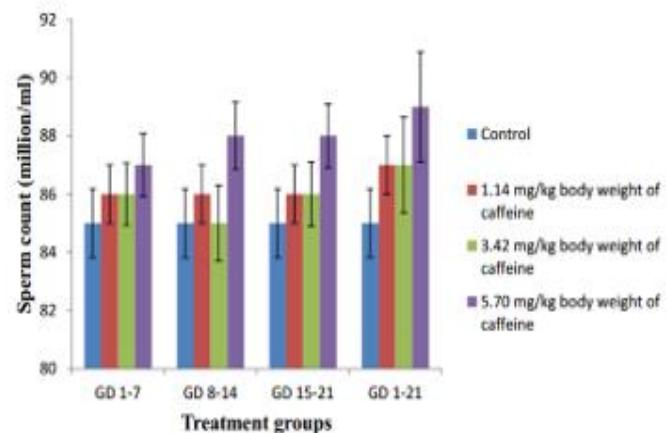
**Fig 3.** Effect of maternal caffeine exposure on serum level of follicle stimulating hormone. Values expressed as mean ± standard error of mean, n=5



**Fig 4.** Effect of maternal caffeine exposure on serum level of Luteinizing hormone. Values expressed as mean ± standard error of mean, n=5



**Fig 5.** Effect of maternal caffeine exposure on caudal epididymal sperm motility. Values expressed as mean  $\pm$  standard error of mean, n=5, \*P<0.05



**Fig 6.** Effect of maternal caffeine exposure on caudal epididymal sperm counts. Values expressed as mean  $\pm$  standard error of mean, n=5, \*P<0.05

Table 2. Effect of maternal caffeine exposure on weight of organs of offspring

Dosage of Caffeine	Gestation Day	Testis (g)	Epididymis (g)	Seminal Vesicle (g)
<b>Control</b>	-	1.08 $\pm$ 0.01	0.37 $\pm$ 0.04	0.33 $\pm$ 0.01
<b>1.14 mg/kg</b>	1-7	0.78 $\pm$ 0.16	0.24 $\pm$ 0.05	0.23 $\pm$ 0.04
	8-14	0.67 $\pm$ 0.07*	0.27 $\pm$ 0.04	0.17 $\pm$ 0.04*
	15-21	0.60 $\pm$ 0.14	0.16 $\pm$ 0.04*	0.17 $\pm$ 0.04*
	1-21	1.00 $\pm$ 0.17	0.27 $\pm$ 0.04	0.30 $\pm$ 0.07
<b>3.42 mg/kg</b>	1-7	0.85 $\pm$ 0.15	0.30 $\pm$ 0.07	0.40 $\pm$ 0.07
	8-14	0.73 $\pm$ 0.09*	0.24 $\pm$ 0.03	0.20 $\pm$ 0.00
	15-21	0.68 $\pm$ 0.02*	0.24 $\pm$ 0.04	0.27 $\pm$ 0.04
	1-21	0.83 $\pm$ 0.12	0.22 $\pm$ 0.05	0.23 $\pm$ 0.04
<b>5.70 mg/kg</b>	1-7	0.77 $\pm$ 0.11	0.22 $\pm$ 0.09	0.24 $\pm$ 0.04
	8-14	0.68 $\pm$ 0.04*	0.24 $\pm$ 0.04	0.27 $\pm$ 0.04
	15-21	0.38 $\pm$ 0.09*	0.27 $\pm$ 0.04	0.23 $\pm$ 0.08
	1-21	0.72 $\pm$ 0.25	0.20 $\pm$ 0.07	0.17 $\pm$ 0.04*

Values are express as mean  $\pm$  standard error of mean, n=5, \*significantly different from control at P<0.05

### Hormonal Study

Offspring of dams treated during GD 8-14 and GD 15-21 with 3.42 and 5.70 mg/kg body weight of caffeine respectively showed significant reduction (P<0.05) in serum testosterone level (Figure 2). However, there was no significant difference in the serum concentrations of follicle stimulating hormone and luteinizing hormone of adult offspring when compared with that of the control offspring (Figure 3 and 4).

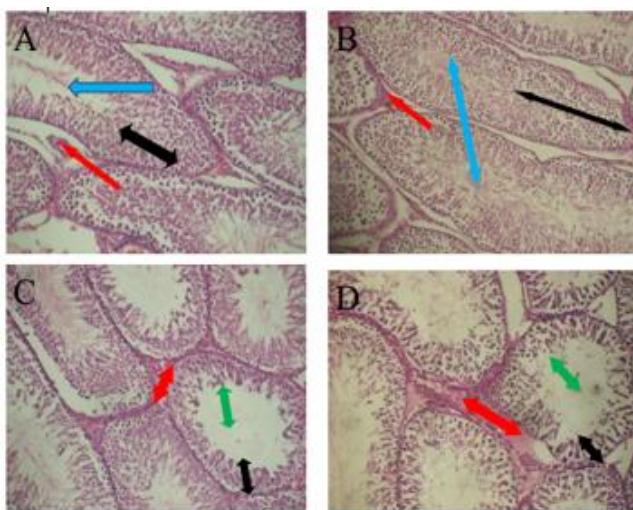
### Sperm Characteristics

The sperm motility of offspring from dams treated with 5.70 mg/kg body weight of caffeine during GD 1-7 and GD 1-21 was significantly increased (P<0.05) (figure 5). However, sperm count showed no significant difference across all groups (Figure 6).

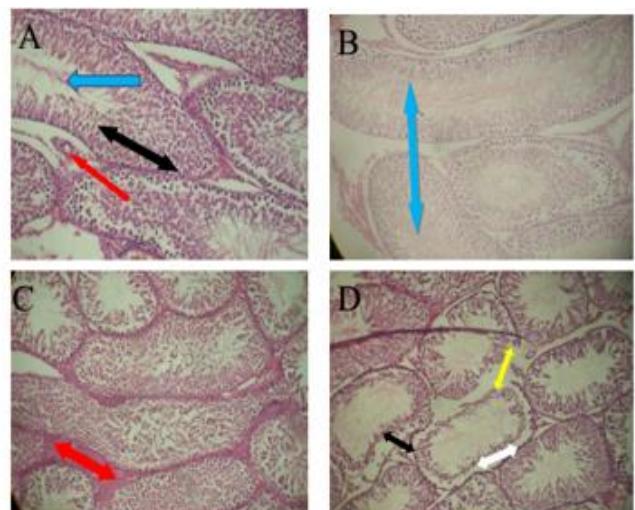
### Body weight and weight of organs

There was a significant decrease (P<0.05) in the body weight of offspring from dams treated with 1.14 mg/kg

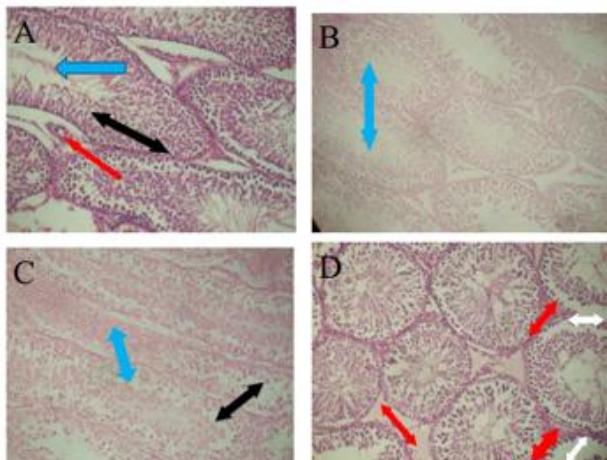
body weight of caffeine during GD 15-21 at PND 21 (Table 1). There was a significant increase (P<0.05) in weight of offspring from dams treated with 3.42 mg/kg body weight of caffeine during GD 1-7 at PND 70 (Table 1). However, there was a significant decrease (P<0.05) in the weight of the testes of offspring from all dams treated during GD 8-14 irrespective of the dose of caffeine administered. Offspring from dams treated during GD 15-21 with 3.42 and 5.70 mg/kg body weight of caffeine, showed significant decrease (P<0.05) in weight of testes (Table 2). The epididymal weight of offspring from dams treated during GD 15-21 with 1.14 mg/kg body weight of caffeine significantly decreased (P<0.05) (Table 2). Similarly, there was a significant decrease (P<0.05) in the weight of the seminal vesicle of offspring from dams treated with 1.14 mg/kg body weight of caffeine during GD 8-14, GD 15-21 and 5.70 mg/kg body weight of caffeine during GD 1-21 (Table 2).



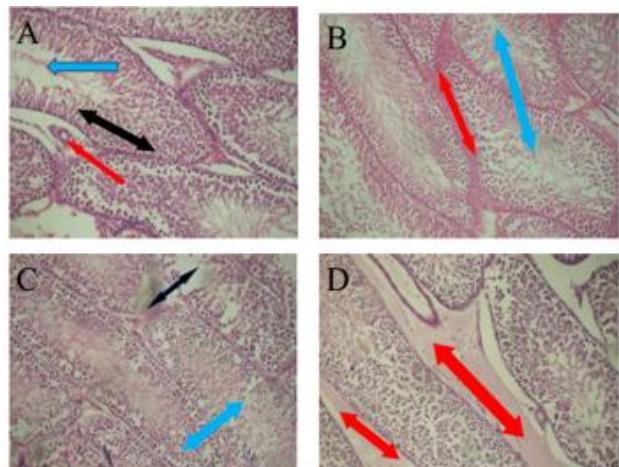
**Plate 1:** Transverse section through the testes of offspring from control (**A**), 1.14 (**B**), 3.42 (**C**) and 5.70 mg/kg body weight (**D**) caffeine treated dams during GD 1-7. Note the normal architectural layout with no visible lesion (**A** and **B**) and reduced germinal epithelial height (black arrow), empty lumen (green arrow), congestion and edema in parts of the interstitium (red arrow) in **C** and **D**. Stain: H&E. Magnification: X100



**Plate 2:** Transverse section through the testes of offspring from control (**A**), 1.14 (**B**), 3.42 (**C**) and 5.70 mg/kg body weight (**D**) caffeine treated dams during GD 8-14. Note the normal architectural layout with no visible lesion (**A** and **B**), Interstitial congestion (red arrow) in **C**, Epithelial cells detached from basal membrane (white arrow), absence of basal lamina (yellow arrow) and reduced germinal epithelium (black arrow) in **D**. Stain: H&E. Magnification: X100.



**Plate 3:** Transverse section through the testes of offspring from control (**A**), 1.14 (**B**), 3.42 (**C**) and 5.70 mg/kg body weight (**D**) caffeine treated dams during GD 15-21. Note the normal architectural layout with no visible lesion (**A** and **B**), Scanty germ cell (black arrow) and disorganized cytoarchitecture of the seminiferous tubules (blue arrow) in **C** and Interstitial edema (red arrows) and detached germinal cells (white arrows) in **D**. Stain: H&E. Magnification: X100



**Plate 4:** Transverse section through the testes of offspring from control (**A**), 1.14 (**B**), 3.42 (**C**) and 5.70 mg/kg body weight (**D**) caffeine treated dams during GD 1-21. Note the normal architectural layout with no visible lesion (**A**), congestion in parts of the interstitium (red arrow) in **B**, detached germinal cells (black arrow) in **C** and interstitial edema fluid accumulation (red arrows) in **D**. Stain: H&E. Magnification: X100

### Histology of testes of offspring from control and caffeine treated dams

There were visible lesions in the testes of offspring from caffeine treated dams when compared with their control counterparts. Offspring from dams that received 3.42 and 5.70 mg/kg body weight of caffeine at the different gestation days and throughout gestation had visible lesions. These were characterized by disorganized cytoarchitecture of the seminiferous tubules, empty lumen, vascular congestion, interstitial

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edema and scanty germ cells with reduced germinal epithelial height. Offspring from dams that received 1.14 mg/kg body weight of caffeine throughout gestation showed congestion in parts of the interstitium. However, those from dams that received caffeine at the same dose but at the different gestation days had no visible lesion (Plates 1-4).

## DISCUSSION

The results from this study showed that maternal caffeine exposure caused reduction in birth weight of offspring from dams that were treated with the highest dose of caffeine and offspring of dams treated with caffeine throughout gestation period. Low birth weight has been reported to be a marker of intrauterine growth restriction (Harding and Johnson, 1995). The results also showed that birth weight was affected by the highest dose as well as by the duration of administration. This was inferred from the fact that only those that received the highest doses and those that received caffeine all through pregnancy gave birth to pups with low birth weight.

Gestation day (GD) 1-7 in rats encompasses the preimplantation period and beginning of implantation (Witschi, 1962) and the developmental changes arising before implantation are likely to affect cell lineages (Fowden and Forhead, 2001). GD 8-14 in rats is the period of organogenesis and maximum fetal growth during which environmental insults may cause discrete structural defects that permanently reduce the functional capacity of the organ (Rhind *et al.*, 2001). During the phase of rapid growth, insults which alter the supply, uptake and utilization of nutrients will influence tissue growth and may switch the cell cycle from proliferation to differentiation with adverse consequences for total cell number (Fowden *et al.*, 1998).

Aldridge *et al.*, (1981) noted that caffeine enters the intracellular tissue water and is found in all body fluids and plasma. Caffeine increases metabolic rate and also speeds the rate of oxidative phosphorylation in the mitochondria so that the energy rich compound ATP is formed. It may cause a rise in the absorption of Iron and Calcium from food by a mother during pregnancy thereby depriving the fetus. These may affect fetal nutrition, causing a negative effect on growth and hence intrauterine growth restriction. Therefore, the low birth weight of the pups in this study may be attributed to the aforementioned factors, which correlate with the observation of Bracken *et al.*, (2003). However, there was an eventual catch up growth of the male offspring of caffeine treated dams at adulthood suggesting that decrease in birth weight in early postnatal life were transient. The association between low birth weights to adult disproportionate phenotype has been linked to poor nutrition and

oxygenation during early life because of placental compromise (Harding and Johnson, 1995).

Anogenital distance is the distance from the upper rim of the anus to the caudal rim of the genitalia, i.e base of the penis or vagina. The anogenital distance has been used to determine the sex of animals, since males have longer lengths than females (Hsieh *et al.*, 2008). Moreover, human studies in infants have also established that boys have longer perineal length than girls (Thankamony *et al.*, 2009). Measuring the anogenital distance in neonatal humans has been suggested as a noninvasive method to determine male feminization and thereby predict neonatal and adult reproductive disorders (Welsh *et al.*, 2008). If insult occurs during gametogenesis, reproductive potential of the next generation may be impaired (Rhind *et al.*, 2001). In the present study, all male pups of dams treated during GD 1-7 and GD 1-21 showed significantly increased AGD index. Also male pups of dams treated with 1.14 mg/kg and 5.70 mg/kg body weight of caffeine during GD 8-14 showed a significant increase in AGD index and pups from dams treated with 3.42 mg/kg body weight of caffeine during GD 15-21 showed significant increase in AGD index. Anogenital distance index shows a relationship between the AGD and body size. It is a ratio of anogenital distance and cube root of body weight which gives a normal index (Vandenberg and Huggett, 1995). Michael *et al.*, (2011) reported that AGD varies based on the integrity of androgen pathways. Sertoli cells contain aromatase or *CYP19* which is the key enzyme responsible for the conversion of androgens to estrogens. The action of caffeine may be by inhibiting this *CYP19* or aromatase, preventing the conversion of testosterone to estradiol. Hence it is suggested that this may be the reason for the longer AGD that was noted in this study which supports the findings of Clark *et al.*, (1990) who reported that a longer AGD is controlled by dihydrotestosterone, which is responsible for masculinization of external genitalia.

There were no significant differences in the serum concentrations of follicle stimulating hormone and luteinizing hormone of offspring from all treated dams as compared with that of the control offspring. Offspring of dams treated during GD 8-14 and GD 15-21 with 3.42 mg/kg and 5.70 mg/kg body weight of caffeine respectively, showed significantly reduced serum testosterone level. Testosterone is required in the normal development of the male reproductive organs. Its decrease in this study corresponds with the significant decrease in weight of reproductive organ of the offspring from the caffeine treated dams that were observed particularly the testes, epididymis and seminal vesicles as compared to the control offspring.

There was a significant increase in sperm motility of offspring from dams treated with 5.70 mg/kg body weight of caffeine during GD 1-7 and GD 1-21, while

sperm count showed no significant difference in the offspring from all treated dams compared to control offspring. As the spermatozoa leave the testes they are not fully motile, they continue their maturation and acquire progressive motility during their passage through the epididymis. Caffeine is a cyclic nucleotide phosphodiesterase inhibitor, which may act directly by affecting cellular metabolism, though its effect depends on the concentration of calcium ion. Meanwhile sperm maturation process in the epididymis involves activation of a unique protein called CatSper which is localized in the principal piece of the sperm tail. This protein is a calcium ion channel and it permits the cyclic AMP-generalized calcium ion influx. El-menoufy *et al.*, (1986) stated that the effect of inhibiting phosphodiesterase as a result of caffeine activity may result in an increase in intracellular cyclic AMP thereby potentiating the permission of the cyclic AMP-generalized calcium ion influx. Cyclic AMP initiates the changes which takes place during the process of sperm capacitation which includes increase in sperm motility among other changes. The increased sperm motility that was shown in the present study can be related to the findings of El-Gaafary (1994), where he noted that caffeine markedly increased and maintained the respiration and motility of ejaculated bovine spermatozoa.

Histology results showed that administration of caffeine to the dams throughout the period of pregnancy caused dose dependent alterations in the cytoarchitecture of the testes of the offspring. Vigezzi *et al.*, (2006) reported that the extent of testicular damage is closely related to the duration of drug consumption. The offspring of dams treated with 3.42 and 5.70 mg/kg body weight of caffeine throughout gestation and during the different gestation periods showed varying degrees of testicular abnormalities; GD 8-14 showed the most severe alteration especially in the basal lamina, germinal epithelium and interstitium. Rhind *et al.*, (2001) showed that GD 8-14 in rats is the period of organogenesis and maximum fetal growth during which environmental insults may cause discrete structural defects that can permanently reduce the functional capacity of the organ. This may be a cause of the alteration in functional capacity of the cells of the testes to secrete the male reproductive hormones as observed in this study. Also, the accumulation of fluid in the interstitium which is often an indication of other health problems may have impaired the normal Leydig cell function, thus affecting the amount of testosterone secreted. Alteration in basement membrane could impair testicular metabolism, thus causing germinal cell hypoplasia and tubular atrophy (Reuhla *et al.*, 2001) which may have resulted in reduced germinal epithelial height thereby affecting the number of spermatogenic cells.

## CONCLUSION

This study has shown that maternal exposure to caffeine had adverse effects on the birth weight, cytoarchitecture of the testes and serum testosterone level of male offspring of Wistar rats. Maternal administration of caffeine during gestation day 8-14 caused the most severe alterations.

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