UNDER PEER REVIEW

Coffee and Caffeine Consumption in Reproductive Functions of Adult Wistar Rats

Abstract:

Research interests in Coffee have expanded with the discovery of the antioxidant properties of this *Coffea Arabica.* Coffee from *Coffea Arabica* is a popular beverage consumed worldwide. Its effect on health has been a global puzzle. In this study, the effect of coffee consumption on some reproductive structures and functions of Wistar rats were investigated A hundred and seventy-five (175) Wistar rats bred in the Animal house of the Faculty of Basic Medical Science of Delta State University were used for this experimental research. Thirty (30) rats were used for toxicity test. The study proper was divided into two units. (Units one (1) and two (2)). Seventy (70) rats used in Unit one (1) were randomly selected into groups of ten (10) rats of seven (7) groups each. All animals were fed with normal rat chow and water. All the experimental rats were treated for four (4) weeks period. Group 1, control, received food and water only, groups 2, 3 and 4 received 40mg/kg, 60mg/kg and 80mg/kg, doses of Coffee respectively while Groups 5, 6 and 7 received 30mg/kg, 45mg/kg and 60mg/kg doses of Caffeine respectively. After administrations of test substances, animals were sacrificed accordingly and serum samples collected. The testes, hypothalamus and hippocampus were also harvested for histological studies while serum samples were analyzed for specific parameters. In Unit two (2) Seventy-five (75) rats of both sexes were mated and treated in varying mating and treatment groups accordingly until after gestation then, the pub weight, litter size, survival rate and gestation length were measured. Both Caffeine and Coffee treatments showed a dose- dependent effect on most parameters measured. Coffee was found to increase antioxidant enzymes, decrease liver enzymes and also affect reproductive outcome negatively. All comparisons were done at (P>0.05).

Keywords: Coffee, Caffeine, *Coffea Arabica,* Reproduction

1. INTRODUCTION

Infertility is an age-long world health concern. It affects approximately 15% of all couples with Male factor infertility contributing approximately half of these cases[1]. The drive for solutions to this problem has produced various causative findings including: environmental, dietary and genetic factors. While a host of these factors have been put forward as causes, a large number of scientists have implicated dietary life style as a main area of concern. Changes in physiological status probably due to dietary factors resulting in oxidative stress has been implicated as a potent predisposing factor in infertility[1 & 2].

In recent years, evidences have shown that oxidative stress may play a role in the pathogenesis of idiopathic male factor infertility[3] Oxidative stress results

UNDER PEER REVIEW

from free radicals, reactive oxygen species and imbalances in antioxidant and oxidants status but can be reduced by consumption of antioxidant supplementation such as honey tea, coffee, vegetables, wine, juice, sprouted grains and other food[4 & 5].

The increasing worldwide resort to coffee consumption may not be unconnected with recent findings that it contains ingredients that promote health in both male and female sexes. Coffea, a popular beverage consumed worldwide has been extensively studied as a protective food, able to produce physiological or metabolic effect in human organism[6]. This beverage is consumed by more than 75% of women of reproductive age in the U.S.[7]. Caffeine is the active ingredient in coffee[8] and caffeine is widely consumed in different food drinks and drugs.

A May 2012 study published in New England Journal of medicine showed that coffee drinkers who drink at least two or three cups a day were about 10% or 15% less likely to die for any reason during the 13 years of the study[9]. The history of coffee as a drink is ladened with controversies. Over the ages, some of the world's greatest composers, thinkers and statesmen have extolled coffee's virtues while others have denounced it as a poisonous mind corrupting drug[10].

Though reports abound on the effect of coffee on human subjects especially the negative effects of coffee on maternal health particularly in the temperate regions, however, not much investigation has been carried out on the effect of coffee on Testosterone FSH and LH in males and Africans generally and whether the anti-oxidant status of coffee could boost the reproductive functions of male hormones. This study wishes to investigate the interplay between the Humoral and hormonal factors on different doses of Caffeine and Coffee on the hormone levels and sperm functions in albino rats.

1.1 Aim of Study

The aim of this study is to determine the effects of Coffee on the reproductive functions of Wistar rats. Specifically, the study attempted to investigate:

i. The effect of Coffee and Caffeine on general body and weights of the testis of Wistar rats
ii. The effect of Coffee and Caffeine on semen quality of Wistar rats.
iii. The effect of Coffee and Caffeine on the basal levels of reproductive hormones (testosterone, FSH, LH) of Wistar rats

UNDER PEER REVIEW

iv. The effect of Coffee on the fertility of the male rats: Pregnancy rate of female rats mated by the male rats; litter size, average pup weight, survival rate of the pubs within the 1st and 2nd week of delivery.

2. METHODOLOGY

2.1 Research design

One hundred and seventy-five (175) Wistar rats bred in the Animal house of the Faculty of Basic Medical Science of Delta State University were used for this experimental research. Thirty (30) rats were used for toxicity test. Two series of pilot studies were done before commencement of the final experiments. The study proper was divided into two units. Units one (1) and two (2). Seventy (70) rats used in Unit one (1) were randomly selected into groups of ten (10) rats of seven (7) groups each. All animals were fed with normal rat chow and water. All the experimental rats were treated for four (4) weeks period. Group 1, control, received food and water only, groups 2, 3 and 4 received 40mg/kg, 60mg/kg and 80mg/kg, doses of Coffee respectively while Groups 5, 6 and 7 received 30mg/kg, 45mg/kg and 60mg/kg, doses of Caffeine respectively. After administrations of test solutions, animals were sacrificed by cervical dislocation and serum samples collected. The testes, liver and kidneys were also harvested for histological studies. In Unit two (2) Seventy-five (75) rats of both sexes were mated and treated in varying mating and treatment groups; Normal male + Normal Female (control) Coffee Treated female + Normal male Caffeine treated female + Normal male Coffee treated male +Normal female Caffeine treated male + Normal female until after gestation then, the pub weight, litter size, survival rate and gestation length were studied. The experiments in this unit (2) were done in two phases; first phase were simply treated and results taken while in second phase, treatments were stopped for withdrawal period of two weeks before allowing the experiment to continue or stop in different cases. All results were expressed as Mean ± Standard deviation. The evaluation of data for significance was done, using One-way Analysis of Variance (ANOVA). The statistical data were analyzed using the SPSS 20 Statistical software. A p-value of ($P>0.05$) was considered statistically significant.

2.2 Study Area

This study will be carried out at the College of Health Sciences, Delta State University, Abraka, Delta State. Abraka is a 536 km² area of land in Ethiope East Local Government

UNDER PEER REVIEW

Area of delta state. The said Local government area (Ethiope east) has a population strength of about 202,712 (NPC, 2016) it is located at about latitude 5^0 53' North and longitude 5^0 40' East (Encarta map, 2009). Geopolitically, the town (Abraka) is part of Delta state, which is located in the south-south geopolitical zone of Nigeria.

2.6 Ethical Considerations

Approval for the use of animals was granted by Medical Ethical Committee, Delta State University, Abraka, Delta State, Nigeria.

2.7 Procedure

3.7.1 **Preparation of stock solution of caffeine**

High dose (60mg/kg)

1200mg (1.2g) of Caffeine was weighed with an electronic weighing balance and dissolved in 200ml of distilled water. This gave stock solutions of 1200mg/200ml (6mg/ml).

Medium dose (45mg/kg)

900mg (0.9g) of Caffeine was weighed with an electronic weighing balance and dissolved in 200ml of distilled water. This gave stock solutions of 900mg/200ml (4.5mg/ml).

Low dose (30mg/kg)

600mg (0.6g) of Caffeine was weighed with an electronic weighing balance and dissolved in 200ml of distilled water. This gave stock solutions of 600mg/200ml (3mg/ml).

3.7.2 Preparation of Stock Solutions of Coffee

Low dose (40mg/kg)

800mg (0.8g) of coffee was weighed with electronic weighing balance and constituted in 200ml of distilled water. This gave stock solutions of 800mg/200ml (4mg/ml).

Medium dose (60mg/kg)

1200mg (1.2g) 0f c0ffee was weighed with electronic weighing balance and constituted in 200ml of distilled water. This gave stock solutions of 1200mg/200ml (6mg/ml).

UNDER PEER REVIEW

High dose (80mg/kg)

1600mg (1.6g) of coffee was weighed with electronic weighing balance and constituted in 200ml of distilled water. This gave stock solutions of 1600mg/200ml (8mg/ml).

3.7.3 Administration of Coffee Solution

High dose (80mg/kg), Medium dose (60mg/kg) and low dose (40mg/kg) were estimated from the lethal dose of coffee (192mg/kg). For high dose, medium and low dose of coffee, 1.6g, 1.2g and 0.8g were dissolved in 200ml of distilled water making the stock concentration to be (8mg/ml), (6mg/ml) and (4mg/ml) respectively.

The body weight of male Wistar rats were taken and the dose of test drugs in milliliter to be administer were calculated.

3.7.4 Administration of Caffeine Solution

Caffeine was administered to experimental animals according to their body weight, such that animal weighing 200g, 150g, 170g received 2ml, 1.5ml and 1.7ml respectively. Caffeine was administered orally using oral canola

3.7.5 Sample Collection

At the end of experimental administrations, the wistar rats were anesthetized in a dessicator containing cotton wool soaked with chloroform. After they had attained deep anesthesia, they were brought out of the dessicator and a laparotomy was carried out (by making a V-shape incision in the abdominal region with the aid of a surgical scissors) and the visceral organs were exposed. The epidydimis was ruptured and semen analysis was done immediately while the testes collected into a well labeled plain bottle containing 10% formal saline awaiting histological analysis.

3.7.6 Determination of Body and Organ Weight

The body weight of the experimental male wistar rats were initially taken after procurement, (before administration). The final weights of the wistar rats were determined before sacrificing and sample collection. Percentage weight gain was later calculated using the formula:

$$\text{Percentage weight gain (\%)} = \frac{\text{Final body weight} - \text{Initial body weight (g)}}{} \times 100$$

UNDER PEER REVIEW

Final body weight (g) 1

The testes were harvested and trimmed of adherent tissues. They were placed in filter paper and weighed. Relative organ weights were also determined thus:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)} \times 100}{\text{Body weight of rat on sacrificing (g)}}$$

3.7.7 Estimation of semen count:

Semen fliud was diluted 1 in 20. Sodium bicarbonate formalin diluting reagent was used to kill the cells to ensure proper counting. Using a Pasteur pipette, the improved Neubauer counting chamber was filled with well mixed diluted semen. It was allowed to settle for about 3-5 minutes. Using 10x objective, the number of semen was counted in an area of 2 sq mm.The number of permatozoa in 1ml was calculated by multiplying the number counted by 100 000.

3.7.8 Estimation of semen morphology:

A thin smear of liquefied well mixed semen was made on a slide. While still wet, the smear was fixed with 95% ethanol for 5-10 minutes and was allowed to dry. The smear was washed with sodium bicarbonate-formalin solution to remove any mucus which may be present. The smear was rinsed with several changes of water. The smear was covered with diluted methylene blue and allowed to dry. The proportion of normal and abnormal spermatozoa was examined using 40x objective.

3.7.9 Hormonal Assay

Determination of Follicle Stimulating Hormone (FSH)

Principle: The FSH quantitative test was based on a solid phase enzyme-linked iimmunosorbent assay (ELISA). The assay system utilizes a mouse monoclonal anti-a.-FSH antibody for solid phase (microtite wells) immobilization and another mouse monoclonal anti-~-FSH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample' is allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between the solid phase and enzyme-linked antibodies.

Procedure: The desired number of coated wells in the holder was secured. 50 ul of standard, specimens, and controls were dispensed into appropriate wells. 100 ul of Enzyme Conjugate

UNDER PEER REVIEW

Reagent was added into each well. This was mixed thoroughly for 30 minutes and was further incubated at room temperature (18 -250C for 45 minutes. The incubation mixture was removed by flicking plate contents into a waste container. 100 ul of TBM reagent was dispensed into each well and then mixed gently for 10 seconds. The optical density was read at 450 nm with a microtiter plate reader within 15 minute.

Determination of Luteinizing Hormone (LH)

Principle: In this test, the targeted antigen, LH, in the sample is immobilized through binding between the streptavidin coated onto the walls of the microplate and the biotin molecule that is part of the biotinylated monoclonal antibody added in the enzyme conjugate. Together with another specific enzyme-conjugated antibody, the target antigen and biotinylated antibody forms a sandwich complex on the walls of the microplate after a period of incubation.

Procedure : The desired number of coated wells in the holder was secured. The data sheet was prepared with sample identification. 50 ul of standard, sample and control were pipette into each well. This was followed by 100 ul of conjugate reagent. This was mixed thoroughly for 30 seconds and was incubated at room temperature for 60 minutes. The content of the wells was discarded by decantation or aspiration. 30 ul of working wash solution was decanted or aspirated. This was repeated for two (2) additional times for a total of 3 washes. 100 ul od TBM reagent was added to each well. This was gently mixed for 10 seconds. This was incubated at room temperature for 15 minutes without shaking. 50 ul of stop solution was pipette and mixed gently for 10 to 20 seconds. Then the absorbance of each well was read at 450 nm.

Determination of Testosterone

Principle: Testosterone EIA Is based on the principle of competitive binding between Testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of rabbit anti Testosterone. In the incubation, goat anti-rabbit lgG-coated weds are incubated with 10 JIl of Testosterone standil'ds, controls, patient ' samples, 100 J.LI Testosterone-HRP conjugate reagent and 50 JIl rabbit anti- Testosterone reagent at 37°C for 90 minutes.

Assay procedure:

The desired number of coated well was secured in the holder. 1 ul of standard, sample and control were dispensed in appropriate wells. 100 ul of testosterone- HRP conjugate reagent

UNDER PEER REVIEW

was dispensed into each well. This was mixed thoroughly for 30 seconds and incubated for 90 minutes at 370C.

The microwells were rinsed and flick 5 times with distilled water. 100 ul of TMB reagent was dispensed into each well and gently mixed for 10 seconds. This was incubated at room temperature for 20 minutes at 18 to 250C. The reaction was stopped by adding 100 ul of stop solution to each well. This was gently mixed and absorbance at 450 nm was read with microliter reader.

Determination of Estrogen

Principle: The E2 EIA is based on the principle of competitive binding between E2 in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-Estradiol. In the incubation, goat anti-rabbit IgG-Coated wells are incubated with 25 ul E2 standard, control and sample. 100 ul Estradiol-HRP Conjugate Reagent and 50 ul rabbit anti-Estradiol reagent at room temperature (18-250C) for 90 minutes.

Procedure: The desired number of coated well in the holder was secured. 25 ul of standard, sample and control were dispensed into appropriate wells. 100 ul of Estradiol-HRP conjugate reagent was dispensed into each well. 50 ul of rabbit anti-Estradiol (E2) reagent was was dispensed in each well. This was thoroughly mixed for 30 seconds and further incubated at room temperature (18-250C) for 90 minutes. The microwell was rinsed with distilled water and flicked 5 times. 100 ul of TMB reagent was dispensed into each well and was gently mixed for 10 seconds. This was further incubated for 20 minutes at room temperature (18-250C). 100 ul of stop solution was added to stop the reaction. This was mixed gently and absorbance of mixture was read at 450 nm with a microtitre well reader within 15 minutes.

3.7.10 Fixation

The tissue was carefully harvested and fixed in 10% formal saline for 48 hours by immersion in a beaker. The 10% formal saline was prepared through the following processes; 900ml of distilled water were measured out using an adequately calibrated measuring cylinder, thereafter 100ml of formalin was measured out with another calibrated cylinder, the 900ml of water was then added to the 100ml of formalin and finally 8.5g of Nacl salt was measured with an electronic weighing device, the 8.5g of Nacl salt was added to the mixture of formalin and water to give the 10% formalin required to fix the sample.

UNDER PEER REVIEW

Tissues Processing

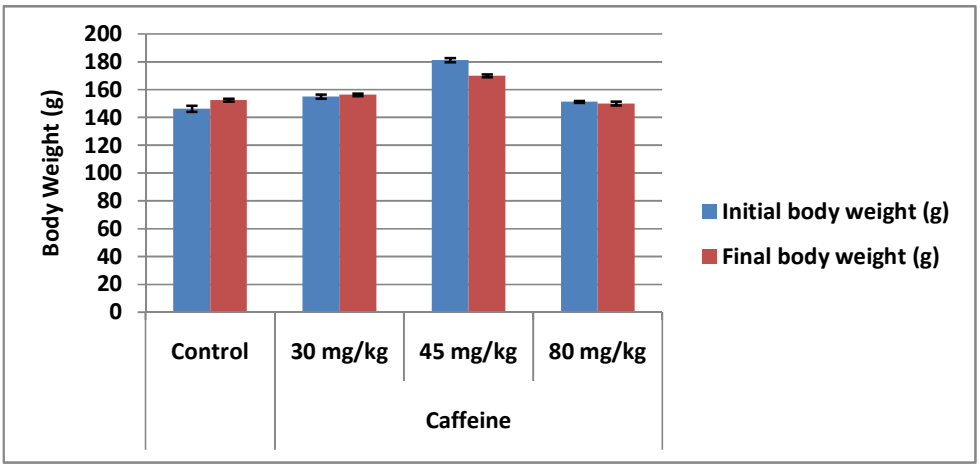
The sample was processed using the paraffin wax method with an automated tissue processor

3.7.11 Statistical Analysis

The evaluation of data for significance was done, using one-way Analysis of Variance (ANOVA). The statistical data were analyzed using the SPSS 20 statistical software. A p-value of less than 0.05 was considered statistically significant.

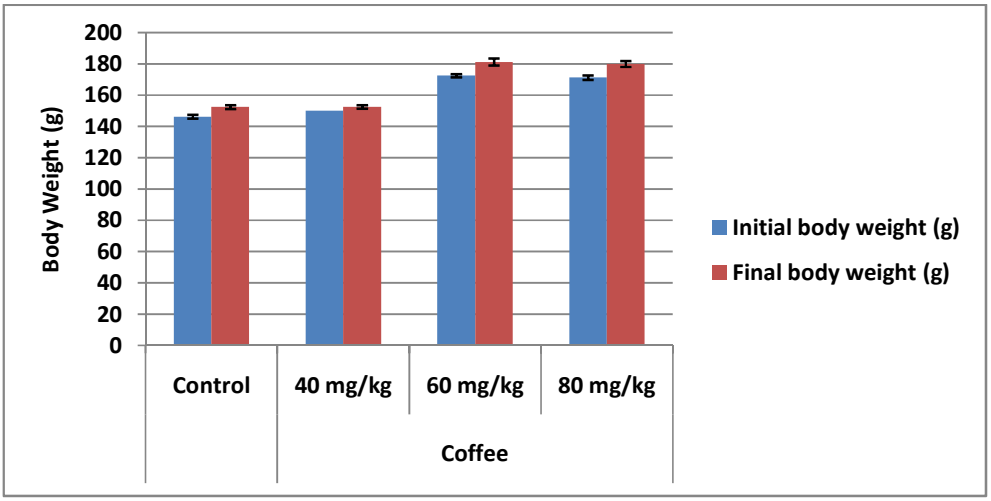
4. RESULTS

Figure 4.1 Body weight test carried out with respect to treatment with Caffeine.



From figure 4.1 above, administration of caffeine low dose caused weight gain of 4.10%. However, administration of caffeine high and medium dose caused weight reduction of -6.62% and -8.33% respectively when compared to control (with weight gain of 0.80%).

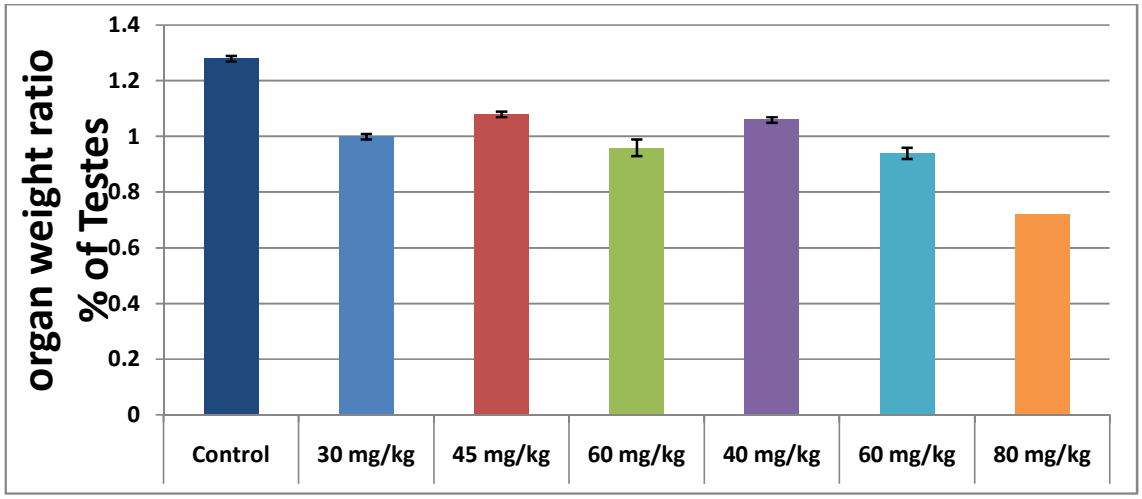
Figure 4.2 Body weight test carried out with respect to treatment with Coffee.



UNDER PEER REVIEW

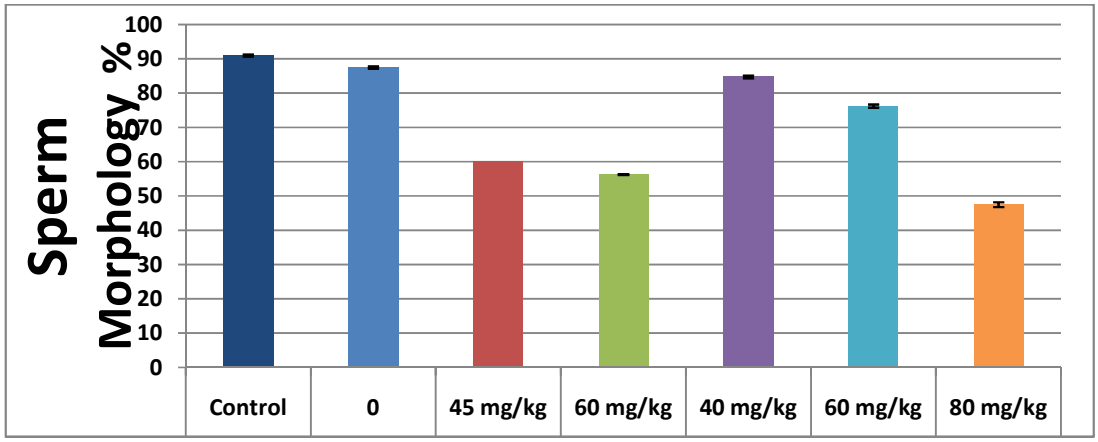
From Fig 4.2 above, there was percentage weight gain of 4.83%, 1.48% and 4.86% in high dose, medium dose and low dose respectively when compared to control group (0.80%).

Figure 4.3 Results of organ weight ratio of the Testis carried out with respect to treatment with Coffee and Caffeine.



From Fig. 4.3 above, there was significant difference (P>0.05) in relative testes weight among groups (control, high dose, medium dose and high dose) means.

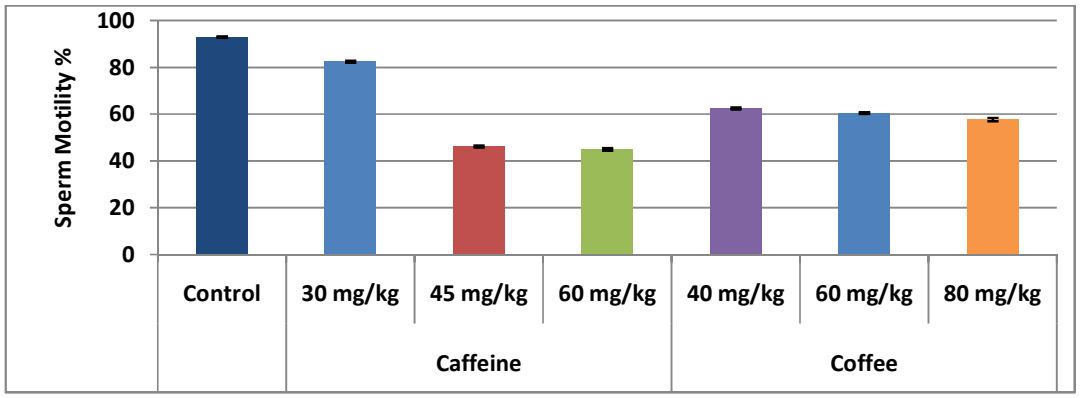
Figure 4.4 Results of sperm morphology carried out with respect to treatment with Coffee and Caffeine.



From Fig. 4.4 above, there was significant (P>0.05) decrease in semen morphology among groups only in higher doses. Lowest dose for both solutions showed a non-significant decrease.

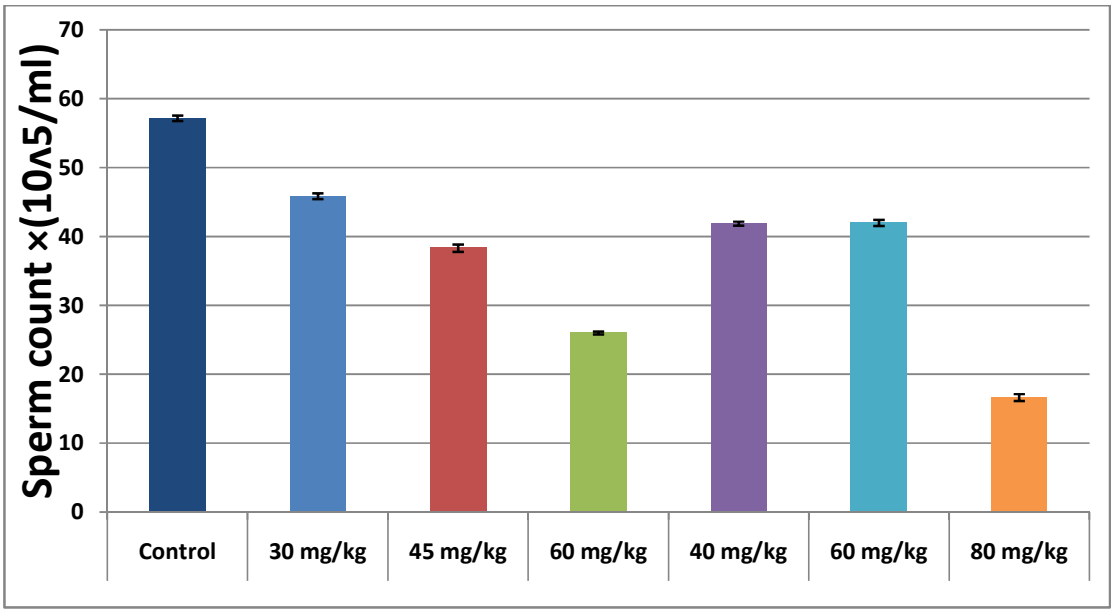
UNDER PEER REVIEW

Figure 4.5 Results of sperm motility carried out with respect to treatment with Coffee and Caffeine.



From Fig. 4.5 above, there was significant (P>0.05) decrease in semen progressive motility among groups except at lowest dose Caffeine treatment where there was non-significant decrease

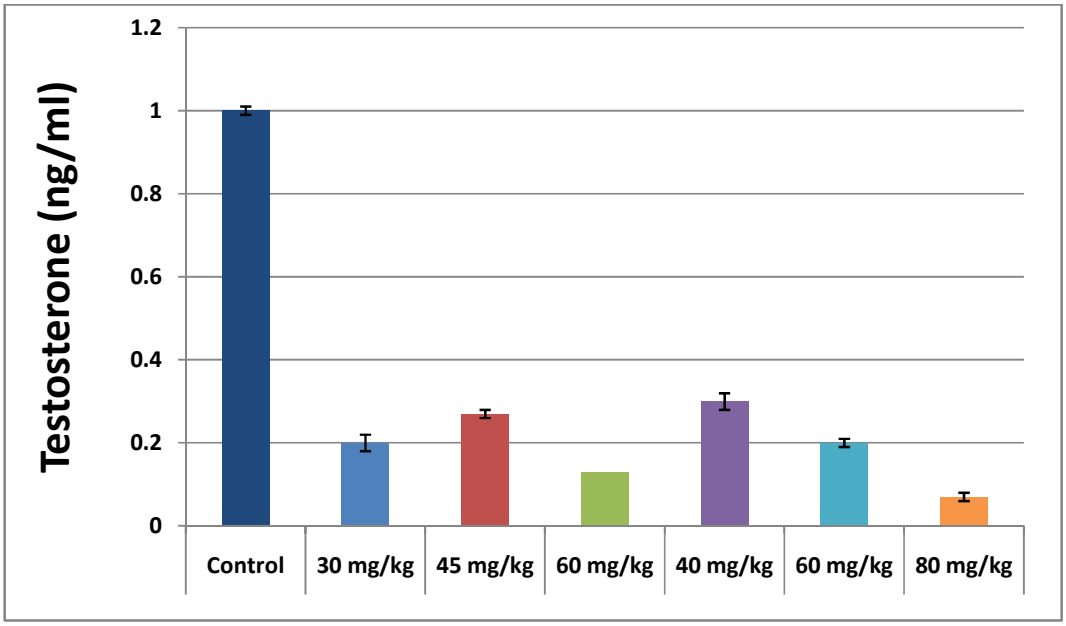
4.6 Results of sperm count carried out with respect to treatment with Coffee and Caffeine.



From Fig. 4.6 above, there was significant decrease (P>0.05) in sperm count following the administration of Coffee and Caffeine at all doses. Values in all doses showed significantly (P<0.05) decrease when compared to value in control group in a dose dependent manner, i.e. The lowest values are seen in the highest doses.

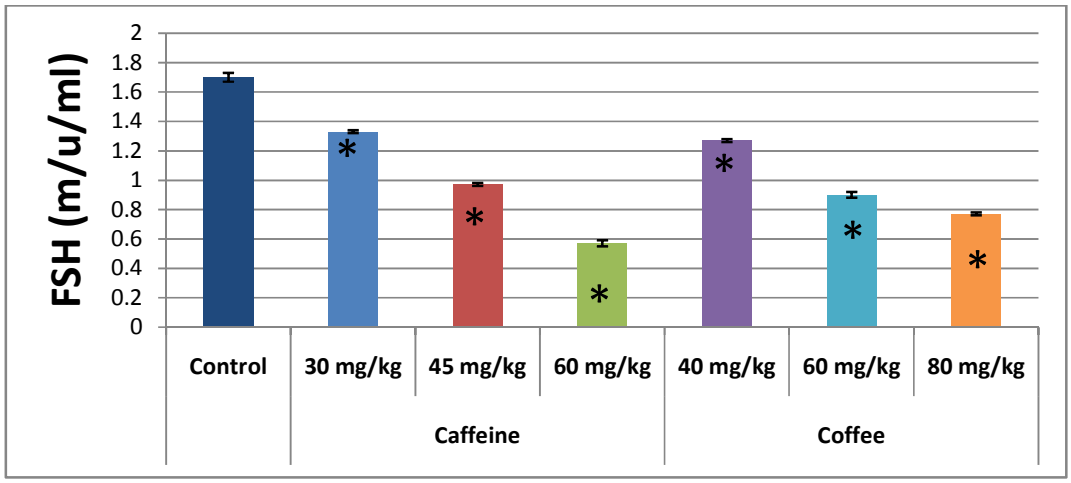
UNDER PEER REVIEW

Figure 4.7 Results of testosterone levels carried out with respect to treatment with Coffee and Caffeine.



From Fig 4.7 above, There was significant decrease (P>0.05) in serum Testosterone levels following the administration of caffeine and coffee at all doses. Testosterone values in all doses significantly (P<0.05) decreased when compared to value in control group in a dose dependent manner, i.e. The highest values are seen in the lowest doses followed by medium and highest coffee, showing dose dependence. This pattern was similar except caffeine at lowest dose treatment.

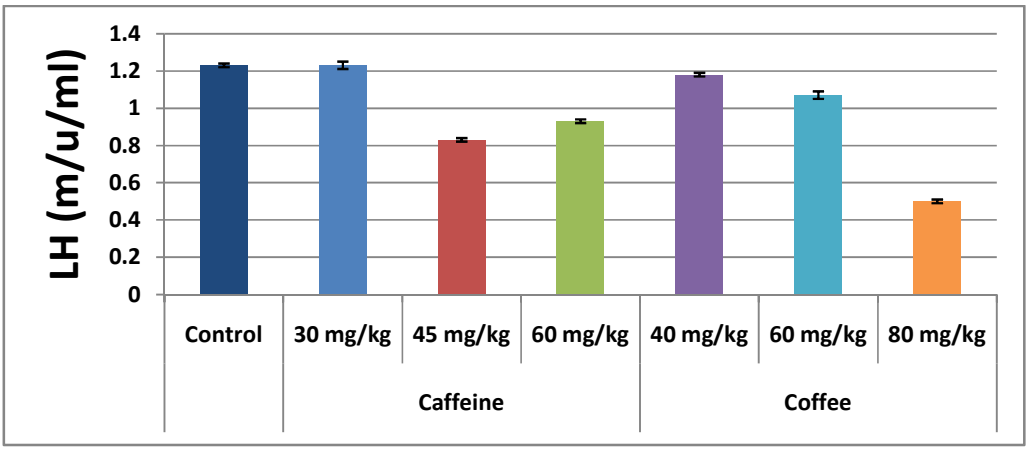
Figure 4.8 Results of FSH levels carried out with respect to treatment with Coffee and Caffeine.



UNDER PEER REVIEW

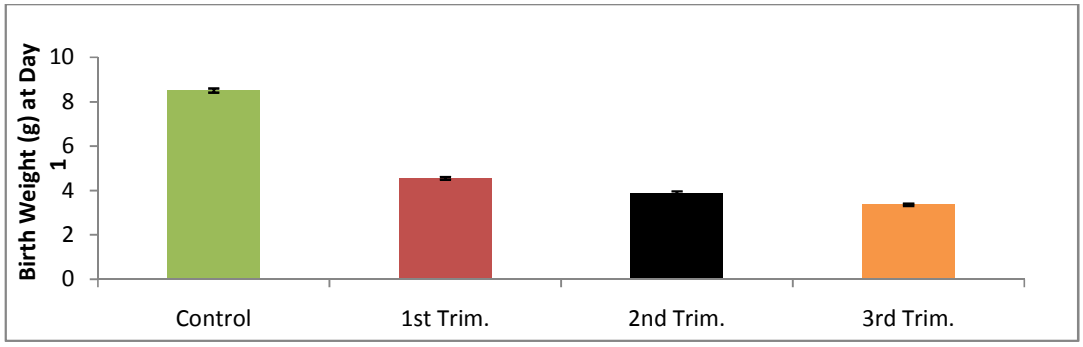
From Fig 4.8 above, There was significant decrease (P>0.05) in serum FSH levels following the administration of caffeine and coffee at all doses. FSH values in all doses significantly (P<0.05) decreased when compared to value in control group in a dose dependent manner, i.e. The highest values are seen in the lowest doses followed by medium and highest coffee, showing dose dependence.

Figure 4.9 Results of LH levels carried out with respect to treatment with Coffee and Caffeine.



From Fig 4.9 above, There was significant decrease (P>0.05) in serum LH levels following the administration of caffeine and coffee at moderate and high doses for coffee but only high dose of Caffeine showed significant decrease. The effect is dose dependence curve but in caffeine administration there was an anomalous dose effect on LH levels.

Figure 4.10 Results of birth weight changes recorded as a result of treatment at different trimesters with Coffee and Caffeine in females.



1st Trim – Group treated with coffee only at First trimesters of pregnancy

2nd Trim – Group treated with coffee only at second trimesters of pregnancy

3rd Trim – Group treated with coffee only at second trimesters of pregnancy

UNDER PEER REVIEW

From Fig 4.10 above, There was significant decrease (P>0.05) in birth Weight following the administration of coffee at all trimesters but most at the 3rd trimester as compared with control group.

4. DISCUSSION

Controversies on coffee consumption are ranging, because coffee has also been found to produce some negative (undesirable) effects. Regarding the conflicting results of epidemiological studies on caffeine and its effects on reproductive outcomes, caffeine-containing foods and beverages still remain one of the most consumed by most human populations of the world, its health effects have been and are still being studied extensively[11] Coffee is known to have beneficial effects as a result of its antioxidant properties however its harmful effects is mainly due to its caffeine content[12].

Caffeine is the World's most widely consumed psycho-active substance, but unlike most other psychoactive substances, it is legal and unregulated in nearly all jurisdictions[11]. An estimated 80% of the world's population consume a caffeine-containing substance daily[13]. Given this widespread use, the potential health effects of coffee are important for public health as well as for helping an individual make an informed choice regarding coffee consumption.

In the present study, the effects of coffee consumption on various reproductive parameters were studied. The histology of testes and semen quality, testicular weight and animal body weight were evaluated.

Effect of coffee on general and organ weight

The findings from this study demonstrated that consumption of coffee may have the potentials of decreasing body weight. The was no significant change in weight (P< 0.05), showing that the weight decreased due to treatment must have been counterbalanced by weight gain due to growth and adequate feeding over the duration of experiment. This closely agrees with that reported that coffee reduces body weight and also with that reported that increase in the intakes of coffee were inversely associated with weight gain [14]. More so this agreed with who opined that the significant loss in body weight could be attributed to the diuretic effect of Caffeine and its role in enhancing fat metabolism[15].

UNDER PEER REVIEW

Effect on reproductive hormones

The results showed that coffee treatment on rats decreases both male and female hormones, though in varying proportion. In experiment to demonstrate the effects of chronic administration of caffeine and stress on feeding behavior of rats, found out that caffeine elevates testosterone level. From the study carried out there was decrease in testosterone level[16]. This decrease could be due to effects of caffeine, probably through acting on the hypothalamic-pituitary gonadal axis.

The result also showed that administration of high dose and low dose of coffee caused significant reduction in serum testosterone when compared to control group. Testosterone is known to be critically involved in the development of sperm cells (spermatogenesis) and therefore, derangement in testosterone level results widely from leydig cell dysfunction and testicular steroidogenic disorder[17].

The reduced level of serum LH, FSH and Testosterone could be as a result of the inhibitory effect of caffeine on the anterior pituitary gland. The anterior pituitary gland secretes FSH and LH which helps in the secretion of Testosterone and these hormones are necessary for spermatogenesis. Inhibition of the pituitary gland could lead to reduction in FSH and LH level and eventually Testosterone level[18].

Also, the result of the study showed that there was significant difference in serum FSH among high dose, medium dose and low dose following administration of coffee to wistar rats. LH level in medium dose was significantly reduced when compared to serum LH level in control group and high dose. Serum LH in low dose was also significantly decreased when compared to serum LH level in medium dose. There is no previous literature to support this finding.

Recent studies show that coffee increases blood antioxidant capacity following acute and chronic intakes, Although, all types of coffee contain relatively high concentration of antioxidant, caffeine coffee may possess even greater antioxidant activity [18 & 19]. Depletion of each of the antioxidant system increases the vulnerability of various tissues and cellular component of oxygen species. Moreover, other research reported that interfering with free radical metabolism with antioxidant supplement may prevent useful activities of the body, Recently, coffee has aroused scientific interest because it is rich in source of a number of phenol compounds with antioxidant effect in vitro[20].

UNDER PEER REVIEW

5. CONCLUSION

In the various investigations carried out in the study, Coffee was seen to rival the deleterious effects of caffeine in almost all parameters measured suggesting that caffeine content of coffee is up to the level at which pure caffeine exerts it effect. Both Caffeine and Coffee treatment showed a dose- dependent effect on most parameters measured. Findings from this study established for the first time that Coffee consumption affects both male and female in the outcome of their reproductive activities and that the most dangerous period to consume this food drink is in the third trimester of gestation period.

Recommendations

The results from this study have necessitated recommendations for further studies to be carried out on the following:

i. Effects of Coffee on the neuro-endocrine system (eg dopamine, noradrenaline) in the hypothalamus and other sexual behaviour regulatory centres in the brain.

ii. An electron microscopic evaluation of any ultrastructural changes in the testes, epididymis, anterior pituitary gland and the hypothalamus.

REFERENCES

1. Agarwal A, Desai N.R, Makker K (2009). Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: an in vitro pilot study. Fertility and Sterility 92 (4): 1318–25.

2. Auger J, Kunstmann J.M, Czyglik F, Jouannet P (1995). Decline in semen quality among fertile men in Paris during the past 20 years. The New England Journal of Medicine 332 (5): 281–5.

3. Damayanthin, D., Janayagam, A. Agirwal, A. Chleo C., and Pallavi, P. (2014). Lycopene and male inferfility, Asia Journal of Andrology Epub A Head of Print.

4. Matkovics A (2009).” A new strategy of antioxidant therapy” Oru Hetil. 147:747-754

5. Shanin Y, Shanin V. and Zinoviev E (2009) “Antioxidant theraphy in clinical practices”Albee publishing house Moscow Russia, p.128

6. Abreu, R. V., Eliane Moretto, Silvia-Oliverag Marcio Flavw, and Dutra, M. (2011). Chronic caffeine ingestion effect on the brain pharmacology biochemistry. 99:659-664.

UNDER PEER REVIEW

7. Frary C.D, Johnson R, Wang M.Q (2005). Food sources and intakes of caffeine in the diets of persons in the United States. JADA. Vol. 105, Issue 1, Pages 110-113.

8. Funk GD (2009). "Losing sleep over the caffeinationof prematurity". J. Physiol. (Lond.) 587 (Pt 22): 5299–300.

9. Friedman L, Weinberger M.A, Farber T.M, Moreland F.M, Peters E.L, Gilmore C.E (1979). Testicular atrophy and impaired spermatogen¬esis in rats fed high levels of the methylxanthines caffeine, theo¬bromine, or theophylline. J Environ Pathol Toxicol; 2: 687-706.

10. Brugnon F, Janny L, Communal Y (2010). Apoptosis and meiotic segregation in ejaculated sperm fromRobertsonian translocation carrier patients.Hum Reprod 25 (7): 1631–42.

11. Grosso L.M and Bracken M.B (2005). Caffeine metabolism, genetics, and perinatal outcomes: a review of exposure assessment considerations during pregnancy. Ann Epidemiol; 15:460.

12. Fredholm B.B, Bättig K, Holmén J, Nehlig A, and Zvartau E.E (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacological Reviews, 51(1), 83-133.

13. Bellisle F, Thornton SN, Hebel P, Tahiri M (2010) A study of fluid intake from beverages in a sample. EJCN 64: 350–355.

14. Greenberg J.A, Axen K.V, Schnoll R, Boozer C.N (2005). Coffee, tea and diabetes: the role of weight loss and caffeine. Int J Obes Relat Metab Disord; 29:1121-9.

15. Marieb, E. N (2000). Human anatomy and physiology, 5th ed. Menlo Park, CA Benjamin Cumming.

16. Phillip, William translating Jan Huygen van LinschotenasDiscours of voyages into Easte& West Indies, 46. 1598. Oxford English Dictionary, 1st ed. "coffee, n."Oxford University Press (Oxford), 1891.

17. Zhang Z, Hu G, Caballero B, Appel L, Chen L (2011). "Habitual coffee consumption and risk ofhypertension: a systematic review and meta-analysis ofprospective observional studies". Am. J. Clin. Nutr. 93(6): 1212–9.

18. Tomlinson M.J, Pooley K, and Simpson T (April 2010). Testing of Accubead in: Validation of a novel computer-assisted sperm analysis (CASA) system using multitarget-tracking algorithms". Fertil. Steril. 93 (6): 1911–20.

19. Schell C, Alberchi M, Mayer C, Schwarzer J; Frungieri M and Meyerhofer A (2008) “Exploring human testicular peritubular cells, identification of sensory product and regular”. Endocrinology, 149:1678-168.

UNDER PEER REVIEW

20. Canan Demritas, Ebru ofluoghu, A limed Hussein, Hatice pasaoglu (2012). Effect of caffeine on oxidant antioxidant mechanisms in the rat liver Gaz med. J; 23:13-18.