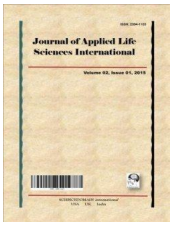

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Comparison of the Histomorphology of Testes in Albino Wistar Rats Following Separate and Co-Administration of *Citrus aurantifolia* and Caffeine

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Authors' contributions

This work was carried out in collaboration between all authors. Author CMC designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JLB and DEG managed the analyses of the study, and also managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study was an attempt to evaluate the effects of *Citrus aurantifolia* (*CA*) and caffeine on the testes of adult male albino *Wistar* rats. No doubt, *CA* and caffeine have health benefits; however, there is a scarcity of data about their effects on the testes. Thirty-five adult male albino *Wistar* rats weighing between 200g and 230g were divided into seven equal groups. 1ml and 2mls of *CA* were given to groups B and C, respectively, using insulin syringe, via oral ingestion. A mixture of 0.5 ml of *CA* and 0.5 ml of 50% caffeine was given to group D, while a mixture of 1 ml of *CA* and 1 ml of 50% caffeine was given to group E. 1 ml of 5% caffeine solution and 2mls of 5% caffeine solution were given to groups F and G, respectively. None was given to group A being the control. They were sacrificed after 21 days; the testes were harvested, weighed and subjected to routine laboratory

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method. Micro architecture of the testes was normal in the control group A. Testicular and intercellular constriction were observed in B, C, and D. These features were moderate in B compared to C and D. C, had in addition, vascular degeneration, germinal cellular hypertrophy and tubular atrophy; while D, had in addition, myoid and Ledyg cells degeneration. All the abnormality seen in B, C, and D, were present in E, F, and G. which had, in addition, tubular necrosis. These showed that consumption of a high dose of *CA* and caffeine, either separately or combined is harmful to the testes.

Keywords: Testes; adult male Albino Wistar rats; Citrus aurantifolia; caffeine.

1. INTRODUCTION

1.1 Caffeine

Caffeine is a substance that may boost your mood, metabolism and mental and physical performance [1]. Caffeine (1,3,7 – trimethylxanthine) is found in beverages such as coffee, energetic and soft drinks; in some medications for pain and headache and in some over the counter stimulants. The rate at which caffeine is consumed has resulted in it being regarded as one of the most widely consumed stimulants in the world. Its consumption cut across homes and corporate offices [2,3].

Caffeine provides no nutritional value on its own. Studies have also found that people who drink coffee regularly have a lower risk of developing Alzheimer and dementia, and cut suicide risk by 45 percent. These benefits are limited to people who drink high-octane coffee, not decaf. Some people consider coffee to be a health drink, but like most foods, over indulging can cause side effects [4].

Caffeine is now regarded by so many individuals as a wonder drug; the consumption of caffeine is socially accepted as a legal drug. It is reputed to possess diuretic properties and it is implicated in increased mental activity and promotion of wakefulness. In the pharmaceutical domain, caffeine is most times used alongside aspirin or codeine in analgesic preparation.

Until 2004, the International Olympic Committee (IOC) [5] restricted caffeine use during competition as it was believed to be ergogenic. A review of the World Anti-doping Code in 2003 reversed the ban (performance-enhancing) compound (World Anti-doping Agency 2003) [6] although caffeine is still considered to be ergogenic even at levels below the former IOC threshold [7,8].

1.2 *Citrus aurantifolia* (Lime)

The common names of *Citrus aurantifolia*: include lime, acid lime, green lime, Mexican lime or sour lime. *Citrus aurantifolia* is chiefly processed and used as refreshing drinks, tasty deserts and for seasoning meals and sauces.

***Citrus aurantifolia* (lime)**

The fruit rind and the leaves, even the juice can be processed into tea and may act as an expectorant. It is implicated in the relief of catarrh resulting from flu and cold [9,10]. Consumption of *Citrus aurantifolia* fruit is associated with a reduced risk of stomach cancer [11]. Amelioration of some types of kidney stones has been associated with the intake of juices from citrus fruits such as lemon, orange and lime [12].

Due to the abundance of health-giving secondary metabolites, *C. aurantifolia* is also used for the treatment of several ailments such as anxiety [13], lung and prostate cancers [14] and gastrointestinal disorders and obesity [15]. The chemical composition of *C. aurantifolia* is responsible for its health-promoting effects. The chemical composition includes vitamin C, and terpenes, which includes flavonoids and limonoids [16,17,18]. Besides its scientifically proven antimicrobial, antioxidant, cytotoxic, anxiolytic, and antidiabetic effects, *C. aurantfolia* extract has been commonly utilized for weight

loss and as sports performance enhancer, in dietary supplements [19,20,21].

Citrus aurantifolia is reputed to have sperm-immobilizing properties, and as such has been investigated as a potential topical vaginal contraceptive [22].

1.3 Testes

The testes, commonly known as the testicles, are a pair of ovoid glandular organs that are central to the function of the male reproductive system. Each testis is ovoid in shape, approximately 4.5cm long, 2.5 cm wide and 3cm in depth, and is covered with the slick, visceral portion of the tunica albuginea [23]. Weighing 10–15 g each, the testes are suspended outside the body in a fleshy sac called the scrotum. The scrotum attaches to the body between the base of the penis and anus [24]. In males, the testes continually produce sperm that accumulates in the epididymis and vas deferens until they and seminal fluid from accessory glands are ejaculated through the penis. The testes are also responsible for the production of male sex hormone testosterone. Autonomic reflexes stimulate waves of contraction in walls of the vas deferens. These contractions sweep sperm back through the inguinal canal to the prostatic urethra, buried within the prostate gland beneath the bladder, where the sperm mix with seminal fluid from the seminal vesicles and prostate. The membranous urethra carries the semen through the floor of the pelvis into the base of the penis, where bulbourethral glands add their products, and finally to the outside [23]. Some 200 million sperm develop daily. Typical ejaculates contain at least 20 million sperm/ml, the minimum considered necessary for fertility. of this sperm, at least 50% must be motile and 60% must appear normal for an adequate chance that fertilization will occur [23].

The rat's testes are contained in scrotal sacs. In a young rat, they descend between 4-6 weeks of age. The rats have an open inguinal canal, as a result, throughout its life span, the testes have the capacity to move up into the abdominal cavity. The rats have a polygamous mating habit, this results in its possession of larger testes [25].

Due to the rate at which caffeine and *Citrus aurantifolia* are consumed in the society with their purported health benefits, there is the need to carry out research as regards their effects on male fertility by first scrutinizing the histology of the testes as a prelude to proper research on its effect on fertility.

2. METHODOLOGY

The *Citrus aurantifolia*, fruits, were bought from Item market, Uyo, Akwa Ibom state, Nigeria. The manual extractor was used for the compression of the fruit. The juices were squeezed out of the fruit gently into a clean beaker and filtered using Whatman filter paper. The filtered juice was collected and stored in a properly covered container in a refrigerator. The total quantity of juice in mLs to be administered in a day to all the rats concerned was calculated and noted. It was freshly prepared on daily basis every time the rats were administered. However, on the days set aside for weighing the rats; we first prepared, stored in the refrigerator and weighed the rats. The administration took place after weighing, which normally did not take up to an hour. The coffee used was bought from a reputable supermarket along Ikot Ekpene road, Uyo, Akwa Ibom state, Nigeria. The 5% coffee solution was prepared by pouring 95 mLs of warm distilled water into a calibrated measuring glass cylinder followed by the addition of 5 grams coffee. It was neither dissolved directly in juice nor prepared first with water and mixed with juice. It was administered separately on its own after the preparation and shacking manually with hand.

A total of thirty five adult male albino *Wistar* rats were used for the research. Body weight ranging from 200g to 230g was one of the main criteria used in selection. The rats were sourced from the animal house of the Faculty of Basic Medical Sciences, University of Uyo, Akwa Ibom state, Nigeria. The rats were housed in clean cages in a standard animal room and at room temperature. They were fed normal rat pellets ad libitum and had free access to good drinking water and were acclimatized for two weeks. The rats were divided into seven groups, namely: control group A and experimental groups B, C, D, E, F, and G. Each group comprising of male rats. Using the insulin syringe fitted with canular for feeding rats, 1 ml and 2mls of *Citrus aurantifolia* were given to groups B and C, respectively. A mixture of 0.5 ml of *Citrus aurantifolia* and 0.5 ml of 5% coffee containing caffeine was given to group D, while a mixture of 1 ml of *Citrus aurantifolia* and 1 ml of 5% coffee containing caffeine was given to group E. 1 ml of 5% coffee containing caffeine solution and 2mls of 5% coffee containing caffeine solution were given to groups F and G, respectively. Group A (control)

was administered distilled water. The rats were administered on daily basis throughout the three weeks period before the harvesting of testes. The rats were weighed at seven days interval for three weeks and were anaesthetized on day 22. Cotton wool soaked in ethyl ether was placed in a glass desiccator and the rats were gently placed inside and the lid covered for a few minutes to make the rats unconscious. The testes were harvested and weighed prior to subjection to routine laboratory method. The histological procedure described by Drury and Wallington [26] was adopted.in assessing morphological alterations in the cytoarchitecture of the testes using H & E technique.

All procedures involving animals in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

2.1 Statistical Analysis

Data were analyzed using the descriptive statistical tool, Primer, version 3.0, and were expressed as a mean ± standard error of the mean (M ± SEM) and subjected to one-way analysis of variance. A significant difference between means was assessed by Student - Newman-Keuls post hoc test. 95% level of significance (P = 0.05) was used for the statistical analysis. Microsoft Excel 2010 package was used for graphs and error bars.

3. RESULTS

Contrary to expected behaviours like increase mobility, instead the rats in the test/ experimental groups were calm but excited.

3.1 Comparison of Changes in Body Weights of Albino *Wistar* Rats in Grams

There was an initial decrease in the body weight of rats and a gradual increase in their body weight following administration of *Citrus aurantifolia* and caffeine, as shown in Table 1.

3.2 Comparison of the Average Weight of Testes of Rats in Grams

The weight of testes of rats given a high dose of caffeine was the lowest compared to others, hence, associating high dose of caffeine with weight loss in testes. There was also marked weight loss in the testes of rats given a high dose of *Citrus aurantifolia* and testes of rats given a combination of a low dose of *Citrus aurantifolia* and caffeine. This revealed that high dose of *Citrus aurantifolia* is implicated in weight loss in testes but does not cause weight loss in testes at the same rate as caffeine given in high dose, as shown in Table 2.

Hematoxylin and Eosin (H&E) Method for General Demonstration of testes both for the control and experimental groups are shown in Fig. 1 through 7, representing groups A to G.

Table 1. Comparison of body weight in grams of rats in control group A and experimental groups B, C, D, E, F. and G

Groups	Day 1	Day 7	Day 14	Day 21
A	213.80±16.98	185.40±17.77	195.80±14.75	218.33±27.50
B	206.80±12.29	181.40±11.99	210.00±13.90	223.00±11.00
C	210.00±6.85	182.60±10.02	205.00±13.56	197.00±7.50
D	210.00±13.23	174.00±10.78	203.40±11.34	231.50±26.50
E	214.20±9.76	184.00±13.47	225.00±10.30	234.00±3.50
F	219.20±14.49	189.00±13.92	220.20±15.30	253.50±13.50
G	212.00±7.05	187.40±10.17	199.00±13.44	218.50±19.50
	P = 0.995	*P* = 0.988	*P* = 0.684	*P* = 0.429

Values are expressed as Mean ± Standard Error of Mean (M ± SEM).
Key: A = distilled water
B = 1 ml of Citrus aurantifolia
C = 2 mls of Citrus aurantifolia
D = A mixture of 0.5 ml of Citrus aurantifolia and 0.5 ml of 5% coffee
E = A mixture of 1 ml of Citrus aurantifolia and 1 ml of 5% coffee
F = 1 ml of 5% coffee
G = 2 mls of 5% coffee

Table 2. Comparison of the weight of testes in grams of rats in control group A and experimental groups B, C, D, E, F. and G

Groups	Weight
A	1.40±0.00
B	1.40±0.00
C	1.30±0.00
D	1.30±0.00
E	1.70±0.04
F	1.50±0.04
G	1.16±0.02

Values are expressed as Mean ± Standard Error of Mean (M ± SEM)

Source of variation	SS	DF	Variance est.
Between groups	0.89	6	0.15
Within groups	0.09	28	0.00
Total	0.98	34	

3.3 Histopathological Analysis

Results from microscopic examination of the various groups showed that testes from GROUP A revealed the normal cellular architecture of seminiferous tubules with a distinct area of interstitium containing Leydig cell, the tubules enclosed the germinal cell lining with primary and secondary spermatogonium, spermatocytes, spermatids, spermatocytes radiating towards the luminal semen. There is no evidence of cellular abnormality seen.

Rats from experimental GROUP B treated with low dose of *C. aurantifolia* (1ml) revealed moderate cellular abnormality, with interstitial constriction, tubular constriction and atrophy as compared to control group while GROUP C treated with High dose of *C. aurantifolia* (2ml) revealed severe cellular abnormality, with spermatogenic lining cell degeneration and hypertrophy, vascular degeneration, germinal cell hyperplasia and slugging, interstitial constriction, tubular atrophy as compared to control group.

In GROUP D, the rats were treated with a low dose of a combination of *C. aurantifolia* (0.5ml) and coffee (0.5ml). The findings revealed severe cellular abnormality, with spermatogenic lining cell degeneration and hypertrophy, vascular degeneration, myoid cell degeneration, germinal cell hyperplasia and disappearance of Sertoli cells within the atrophied tubules as compared to control group, while GROUP E, treated with high dose of combination of *C. aurantifolia* (1ml) and coffee (1ml) revealed severe cellular abnormality, with spermatogenic lining cell degeneration and hypertrophy, vascular degeneration, myoid cell degeneration, and tubular necrosis as compared to control group.

GROUP F was treated with a low dose, 1 ml of 5% coffee, the result revealed a severe cellular abnormality, with spermatogenic lining cell degeneration and hypertrophy, vascular degeneration, myoid cell degeneration, and tubular necrosis as compared to control group. However, GROUP G treated with high dose, 2 ml of 5% coffee, revealed a severe cellular abnormality, with spermatogenic lining cell degeneration and hypertrophy, vascular degeneration, myoid cell degeneration, and tubular necrosis as compared to control group.

4. DISCUSSION

Our findings suggest that consumption of high dose of *Citrus aurantifolia* (CA) and coffee, either separately or combined is harmful to the testes. When consumed alone, the harmful effect *CA* had on the testes was not as severe as the harmful effect caffeine had on them when caffeine was also consumed alone. However, in both the separate consumption of *CA* and caffeine, the severity of the effects was dose-dependent. Deduction made from the findings suggest that the harmful effect resulting from combined administration of *CA* and caffeine was also dose-dependent but did not parallel caffeine when administered alone dose-dependently. Comparison of the harmful effect of combined administration of *CA* and caffeine on the testes resulting from gradual increase in their dosage with the harmful effect of caffeine resulting from the gradual increase in its dosage, suggest that the degree of severity resulting from a gradual increase in dosage in caffeine was higher than the degree of severity resulting from a gradual increase in the combined administration of *CA* and caffeine.

The study by Spritzler investigated the effect of different doses of caffeine. Light-to-moderate caffeine intake seems to provide impressive health benefits in many people. On the other hand, very high dosages may lead to side effects that interfere with day-to-day living and might even cause serious health issues. Although responses vary from person to person, the effects of high intake demonstrate that more isn't necessarily better [27].

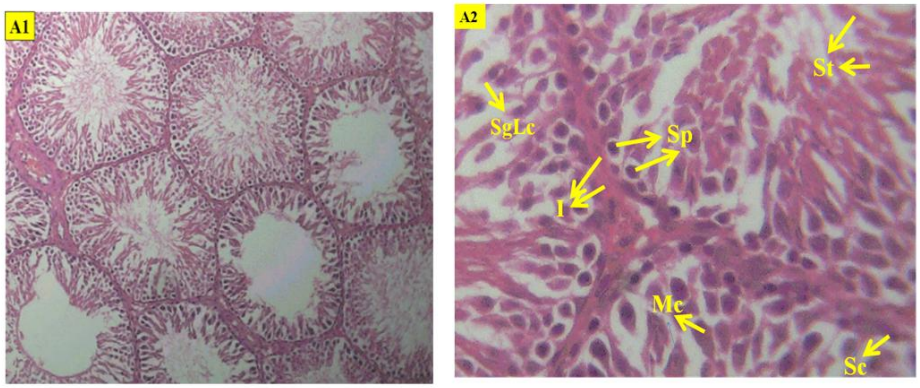

Fig. 1. Histologic section through the control group A testes without treatment at magnification A1 (X100) and A2 (X400) stained with H and E technique

Keys: Sglc –Spermatogenic lining cells, I – Interstitium, Mc – Myoid cells, Sp – spermatocytes, ST – Seminiferous tubules,Sc –sertolic cells.

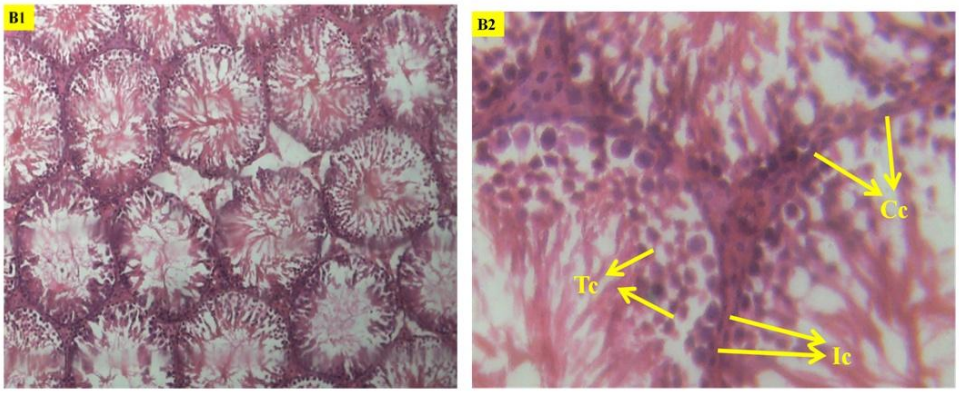

Fig. 2. Histologic section through the experimental group B testes treated with 1ml *Citrus aurantifolia* at magnification B1 (X100) and B2 (X400) stained with H and E technique

Keys: Cc- cell clumping, TC- Tubular constriction and IC- Interstitial constriction

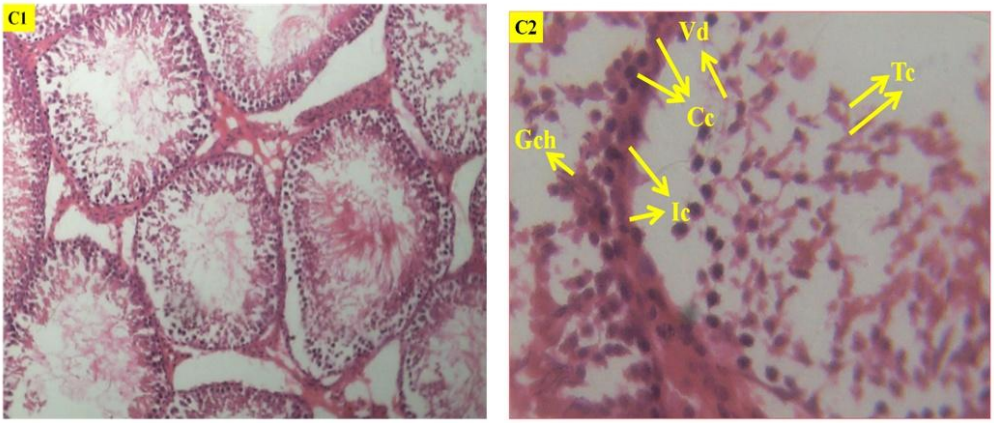

Fig. 3. Histologic section through the experimental group C testes treated with 2mls *Citrus aurantifolia* at magnification C1 (X100) and C2 (X400) stained ith H and E technique

Keys: Cc- cell clumping, Tc- Tubular constriction and Ic- Interstitial constriction, Vd – vascular degeneration, Gch – germinal cellular hypertrophy

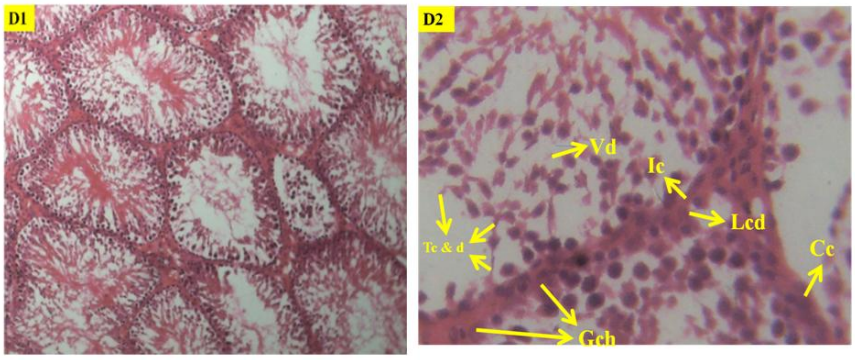

Fig. 4. Histologic section through the experimental group D testes treated with a mixture of 0.5 ml of *Citrus aurantifolia* and 0.5 ml of 5% coffee at magnification D1 (X100) and D2 (X400) stained with H and E technique

Keys: Cc - cell clumping, Ic- Intercellular constriction, Vd – vascular degeneration, Gch – germinal cellular hypertrophy, Tubular constriction & degeneration, Lcd-ledyg cell degeneration

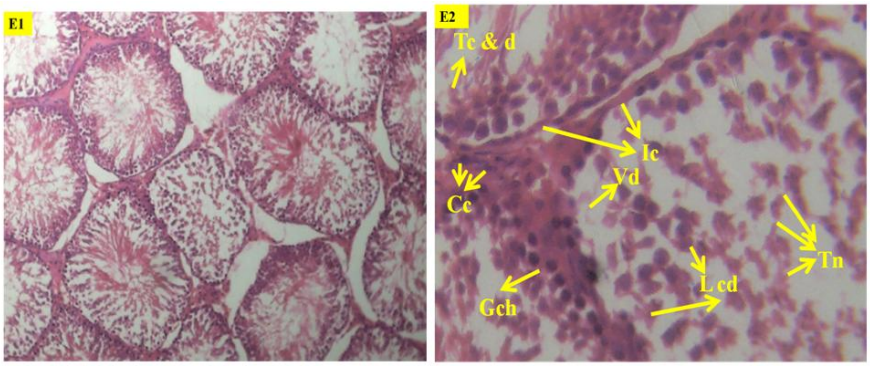

Fig. 5. Histologic section through the experimental group E testes treated with a mixture of 1ml of *Citrus aurantifolia* and 1 ml of 5% coffee at magnification E1 (X100) and E2 (X400) stained with H and E technique

Keys: Cc - cell clumping, Tc & d - Tubular constriction & degeneration, Ic-Interstitial constriction, Vd – vascular degeneration, Gch – germinal cellular hypertrophy, Lcd- ledyg cell degeneration and Tn - Tubular necrosis

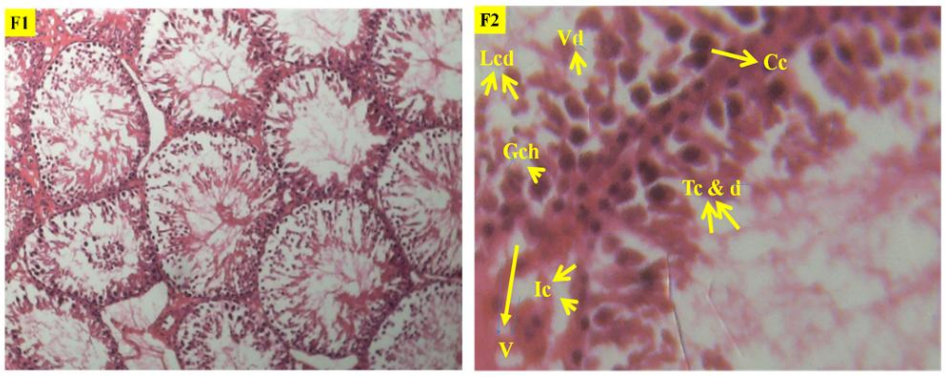

Fig. 6. Histologic section through the experimental group F testes treated with1ml of 5% the coffee solution at magnification F1 (X100) and F2 (X400) stained with H and E technique

Keys: Cc - cell clumping, Tc & d - Tubular constriction & degeneration, Ic-Interstitial constriction, Vd – vascular degeneration, Gch – germinal cellularhypertrophy, Lcd- ledyg cell degeneration, Tn - Tubular necrosis and V - vacuolation

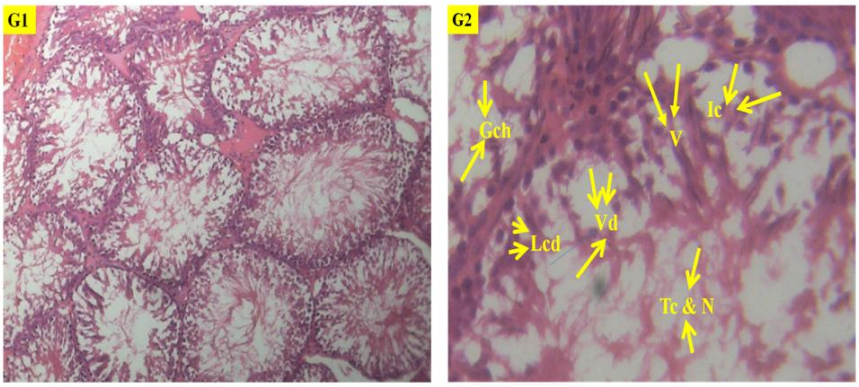

Fig. 7. Histologic section through the experimental group Gtestes treated with 2 ml of 5% the coffee solution at magnification G1 (X100) and G2 (X400) stained with H and E technique

Keys: Tc & d - Tubular constriction & degeneration, Ic-Interstitial constriction, Vd – vascular degeneration, Gch – germinal cellularhypertrophy, Lcd- ledyg cell Degeneration,Tn - Tubular necrosis.and V- vacuolation.

Besides antioxidants, *Citrus aurantifolia* juice also contains high amounts of organic acids like citric acid, coumaric and ferulic acids [28,29,8], Patil *et al,* from their research work, concluded that *Citrus aurantifolia* juice may independently cause adverse effects on testicular function and indeed have antifertility potential in the male. As testicular milieu is highly sensitive to most chemicals, the testicular effects of *Citrus aurantifolia* juice may be attributed partly to the acid constituents of the plant [27].

An in vitro study has shown that lime juice destroys both human immunodeficiency virus and sperm cells. The high acidity of the lime is suspected to be responsible for the destruction of the HIV and sperm cells [30,31]. Another study showed that lime juice reduces the numbers of ova shredded and causes irregularity in the histology of the ovaries and uterus in female rats and may possibly affect fertility [32].

Caffeine administered to male rats at 50 mg/kg/day subcutaneously for 4 days prior to mating with untreated females, caused decreased male reproductive performance in addition to causing embryotoxicity. In addition, long-term exposure to high oral doses of caffeine (3 g over 7 weeks) was toxic to rat testes as manifested by spermatogenic cell degeneration [33].

5. CONCLUSION

The findings from the study show that irrespective of the innumerable health benefits associated with *Citrus aurantifolia* and coffee, they might still have negative effects and consequences on the male reproductive capability. To better clarify the harmful effect of *C. aurantifolia* and coffee on male reproductive capability, further work should be carried out employing immunohistochemical techniques and TUNEL or AnnexinV assay.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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