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Adverse Effects of Excessive Soft Drinks Consumption on Male Fertility in Wistar Rat Models.

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ABSTRACT: This study examines the possible effects of prolonged consumption of soft drinks on sperm parameters in male wistar rats. Thirty-five (35) male wistar rats were divided into five groups with the control groups administered with distilled water only, and four other test groups administered with single dose (1.2ml) Coca-Cola, double dose (2.4ml) Coca-Cola (Cocacolax2), 0.6ml bullet drinks and 1.2ml sugar solution. Aside from the administered samples, food and water were regularly kept at their disposals. The experimental study lasted for forty (40) days. The weights before and after administration were measured to ascertain the effects of the test samples on the mean weight difference as well as their mean relative weight difference. Sperm parameters were taken to ascertain the effects of the test samples on fertility. The results from this study have shown that consumption of soft drinks have stimulating effects on weights, with corresponding adverse effects on spermatogenesis of test groups when compared with the control group

KEYWORDS: Coca Cola, Sperm, Testes, Spermatogenesis, Weight, soft drinks

INTROUCTION

It is quite agreeable that there is a remarkable degree of confidentiality in the exact composition of sugar-sweetened/caffeinated beverages particularly the Coca-Cola products. The main components of Coca-Cola beverage nevertheless known are water, phosphoric acid, caffeine and glucose/fructose or artificial sweeteners. Artificial sweeteners and caffeine content constitute the main distinguishing contents between the different types of Coca-Cola and other soft drinks/beverages. Glucose/fructose concentration in regular Coca-Cola is remarkably high. Sugar is substituted by artificial sweeteners like aspartame, cyclamate and acesulfame k, in other types/ brands of Coca-Cola beverages (Maes *et al.*, 2012). Prolonged intake of glucose may result to insulin resistance and may eventually lead to glucotoxicity arising from the subsequent high blood sugar concentration. High level of glucose in the blood reacts with proteins (but also bases of DNA with other carbonyl substances and oxidized lipids) in a milliard reaction that is non-enzymatic to form a latter product called advanced glycation end product (AGE) but proceeded by the formation of Amadori products (Klenovics *et al.*, 2013). In reproduction, a definite level of reactive oxygen specie is required for several Physiological processes such as hyper activation, normal sperm function and acrosome reaction (de Lamirande and Gagnon, 1995). Reactive oxygen specie overproduction in other instances results in damage into macromolecules and oxidative stress. Such macromolecules include lipid peroxidation, oxidation of DNA and protein oxidation (Aitken and Roman, 2008). Caffeine is another compound found in Coca-Cola and has been shown to possess protective effects in brain, liver, epididymis and kidneys through an upsurge in antioxidant enzymes. As was shown in a recent study by Vignoli *et al 2011*, the antioxidant capacity of caffeine appears to be dependent on dose focusing on the coffee bean's constituent amount of caffeine. In young male volunteers on the other hand, the anti-inflammatory cytokine interleukin – 10 decreases with caffeine consumption and elevates oxidative stress (Tauler *et al.*, 2013). The possible detrimental effects of Coca-Cola and other sugar-sweetened/caffeinated beverages on the reproductive organs of males have been examined and elucidated by only few studies. The effects of various types of Coca-Cola beverages were analyzed in vitro about some decades ago. In 1985, Umpierre *et al*, observed lowered motility of sperm after one minute of incubation with Coca-Cola drinks. Low P^H was found to convey the attributes for most of the effects, but since different Coca-Cola formulations displayed different actions. A separate study with the use of trans-membrane migration method, performed two years later, did not prove the

spermicidal effect (Hong *et al.*,1987). In view of the controversy in these studies, both viewed from in vitro experimental requirements and mostly focused on sperm motility and functions, the present study was programmed to unveil and explain the possible consequential effects of Coca-Cola and other sugary/caffeinated beverages on the testicular tissues oxidative status. Celec and Behuliak, in a previous study, indicated in male rats that ad libitum consumption of various cola-like beverages for three months slightly elevated both estradiol and testosterone in plasma when comparable with the control group that drink tap water (Celec and Behuliak, 2010). With group differences, a little above the level of statistical significance, the duration used for the study might have been minimal to show agreeable effects.

The testis is a highly dynamic organ, not only on the total stage, but also in post-natal development and adult life. It is made up of two broad compartments: The steroidogenic leydig cell with the interstitium, and the seminiferous tubules. Sertoli cells are of great importance in spermatogenesis due to their key roles. The sertoli cells are target cells for testosterone and Follicle Stimulating Hormone (FSH), and they also possess the responsibility of initiating and maintaining spermatogenesis. They provide structural and nutritional supports as well as forming tubules for developing germ cells (Russell L.D., 1980). The gonads appeared as an outgrowth and matured either as an ovary or a testis, based mainly on the SRY gene presence on the Y chromosome, (McElreavey & Fellous, 1999; Swain & Lovell-. 1999). Sertoli cells transform in function in response to SRY. They combine to form cords and they are capable of producing mullerian inhibiting substance together with the peritubular cells that arise from the mesonephric. Leydig cell develops subsequently in the interstitial milieu and begin to produce testosterone (Josso *et al.*, 1998, Yao *et al.*, 2002). Dynamic changes are related with development of the cord into tubule at puberty and spermatogenesis initiation, germ cells move from the bottom to the top of the tubule epithelium in adult life while transforming further the spermatids are finally released from the top of the seminiferous epithelium into the lumen of the tubule and subsequently become spermatozoa.

Proteases or protease inhibitions of cysteine, serine, metalloprotease family, according to past researcher were partakers of this spatiotemporal and highly organized process, either during the development of testis (Tohonen *et al.*, 1998; Grimmond *et al.*, 2000; Guyot *et al.*, 2003) or at precise spermatogenetic stages. (Fritz *et al.*, 1993; Charron *et al.*, 2005; Wong, 2005; Longin *et al.*, 2001).

Caffeinated Energy Drinks

The absence of regulatory supervision has resulted in over indulgence in the marketing of energy drinks. The effect of caffeine from an open background that occurs naturally (coffee, tea, cocoa and foods that contains these ingredients) had not been fully elucidated (Wesensten, 2014). Caffeine centralization of the caffeinated energy drinks possesses the range of 0-141.1mg/serving. That of the carbonated sodas gauged from no detection to 48.2mg/serving and caffeine contained in other beverages rose from less than 2.7 to 105.7mg/serving (McCusker, *et al.*, 2006).

Testicular Development and its Effects on Spermatogenesis

The interconnecting network of the various cellular activities during spermatogenesis is greatly complex and complicated. (Gnessi *et al.*, 1997; Jégou *et al.*, 1999). The tools development for high-through-put protein recognition has allowed a small number of laboratories to carry out the expression studies, for differential protein profiling and / or sequential determination of testicular proteomes from different species either based on the entire organ (Huang *et al.*, 2005) or on cells that are isolated (Com *et al.*, 2003; Essader *et al.*, 2005; Rolland *et al.*, 2007). The development of germ cells via spermatogenesis is probably an aspect of reproduction in males that are often times investigated in elaborated experiments. An intriguing cellular differentiation process called spermatogenesis leading to the production of millions of spermatozoa daily involves the coordinated expression of specified genes and specific gene product generation at each process step, coupled with the continuous communication linking development gene cells (Jegou, 1993).

Various groups have carried out complicated analyses based on dissimilate strategies, to identify gene of preferential proteins on expressed specifically at each spermatogenesis stage. Among these strategies, one involved comparing different categories of germ cells that have been purified, brought together with a serial analysis of gene (SAGE) experiment in mice, (*Wu et al.*, 2004), Gene chip micro assay mice experiments (Shima et al., 2004; Namekawa et al; 2006 Chalmel *et al.*, 2007) and rats (Schlecht *et al.*, 2004; Chalmel *et al.*, (2007), or differential proteomic rat analysis (Rolland *et al.*, 2007). Localization of protein and hypotheses formulation relating to protein functions are two preliminary processes for proteome analysis, a definition of Anderson. Anti-Sperm Antibodies (ASA) have been employed from seminal plasma for the identification of proteins that are potentially involved in immunological complications causing infertility. A similar approach was for the identification of seven testicular proteins known by sera from rats induced to experimental autoimmune orchitis (Fijak *et al.*, 2009).

Histological Examination of Testicular Tissues

Serious attention has been devoted recently to the possible unpromising radio frequency field health effects, which are used increasingly in telecommunications. Miscarriages and severe quality impairments have been detected apart from the most prevalent health problems associated with mobile phone users like memory impairment, sleep disturbance, headache, tiredness, depression, brain tumors and Neuroendocrine dysfunction (Fejes *et al.*, 2007; Sege C, 2000; Sege C, Carpenter D.O, 2009) as studies have revealed a wide spectrum of possible effects, the unfavorable outcomes of mobile phone use on male fertility have not still been fully established, ranging from insignificant ones to testicular damage of inconsistent degrees (Deepinder *et al.*, 2007). It should be

emphasized that results from laboratory studies that are controlled are often controversial, inconclusive and unrepeated, as for the effects of radioactive exposure on the testes; there are several consequences of realization in testicular tissues. Investigators of different groups have reported their chance shelved on specific effect of cell phones on testicular function in rats (Dasdag *et al*, 2003; Dasdag et al, 2008; Lebovitz R.T.M, Johnson L., 1983; Ribeiro et al., 2007). Disputably to this, Akdag *et al*, 1999 discovered that in chronic increase exposure, sperm counts are lowered while sperm morphology and weight changed.

MATERIALS AND METHODS

This is an experimental study to determine the effects of soft drinks consumption on the reproductive parameters of males using wistar rats. The rationale behind the use of albino wistar rats for human studies is that they are physiologically similar to humans and are small, sociable and easy to handle, (Barnett, 1963).

Ethical Considerations

International instructions for laboratory animals care and use in biomedical research as the Canadian council of animal care advised (CCAC, 1985) and the recommendation of the guiding principles in care and use of animal research (American physiological society 2002) were also adhered to in the course of this study.

To ensure the above and in accordance with the proposed regulatory guidelines, the research was duly approved by the University of port Harcourt ethical committee.

Procurement/Preparation of Test Substances

It is well noticed in our society today that people consume drinks without giving due considerations to the quantity taken, contents of drinks, the amount required by the body and the possible implications associated with over consumption.

Two soft drink types were used; they include Coca-Cola and bullet drinks. The others were glucose solution and distilled water.

Below are the steps on how the volumes of each of the experimental drinks required for daily administration were measured:

1. We considered the situation where a normal 70kilogram (kg) adult human being consumes a bottle of 50cl coke per day.
2. Humans in another instance consume 2 bottles of 50cl, summing up to 100cl of Plastic coke per day.
3. Humans who consume one tin of (25cl or 250ml) bullet energy drink per day.
4. We measured the glucose content of the plastic bottle (50cl) of Coca-Cola which was found to contain 54g of sugar. This sugar equivalent was dissolved in distilled water of about (50cl) just like the Coca-Cola solution.
5. Distilled water in bottle was also stored separately for use in the control rats.

Since the above amounts are considerations for a normal 70kg human, we then deduced each to the weight equivalent of rats which were measured to possess an average of 160g.

Experimental Design

After acclimatization, the 35 wistar rats were randomly divided into five (5) groups. Prior to administration each rat was weighed and classified in accordance with their individual groups. In accordance with the preliminary calculations carried out**,** rats in **group 1** (Control) were each given 1ml of distilled water orally once a day. Rats in test **group 2** were orally fed with 1.2ml (Single dose) of Coca-Cola daily; **Group 3** rats were given 2.4ml (Double dose) of Coca-Cola daily; **Group 4** rats were administered with 0.6ml of bullet drink daily while the **group 5**- rats were administered with 1.1ml (i. e123.424ml) of sugar dissolved in 50cl (500mg) of water daily. All drinks were administered orally each day for exactly forty (40) days after which the animals were duly sacrificed (5 from each group). Samples of sperm were collected from the epididymis for analysis of sperm parameters while the testes were extracted for Hormonal Parameters, Histopathological Changes and Biochemical Constituents.

Measurement of Body Weights

At the beginning of the study, the initial weights of each of the rats were taken using a weighing balance. Before each sacrifice on the 41st day, the rats were reweighed and the new weights recorded. After sacrificing each rat, the testes were removed by scrotal incision and also weighed using electronic weighing balance before being stored in sample bottles containing bougyen's reagent for subsequent histological studies.

Sample Collection and Analysis

Each one of the rats was then anesthetized in a desiccator containing cotton wool soaked with chloroform, laid in a supine position, on a dissecting board and with limbs fastened to the board with dissecting pins under this condition. In course of the dissection, pelvic incisions were made to expose the epididymis and scrotal incisions were also performed to expose the testes using a scalpel with sharp blade.

Assessment of Experimental Parameters

In this study, the parameters assessed were sperm analysis, histological changes in the testes of the rats.

Histopathological Assessment of the Testes

The histological technique adopted here is as outlined by Carleton (1967). The processes involved include:

- Testicular tissue processing
- Embedding of testicular tissues
- Sectioning on slides
- Dewaxing, staining and dehydration
- Examination of sections

Embedding Of Testicular Tissues

Two changes of paraffin wax at 55^0C, each lasting for two hours were used to embed the tissues. Thereafter, the tissues were blocked out using an L-shaped metal molder and then they were mounted on a wooden block trimmed to size.

Sectioning On Slides

The blocks were cut into ribbons of five using rotary microtone. Then sections were picked with horse brush into a slide that was floated in 20% alcohol and then in warm water bath for proper straightening. The sections were now mounted on albumenized slides and dried in oven at 37^0C overnight.

Dewaxing, Staining, Dehydration and Examination of Sections

The sections were dewaxed (removing the paraffin wax) using two changes in xylene for two minutes each and then dehydrated by passing through descending grades of alcohol from absolute to 70% alcohol for one minute in each grade.

The sections were then washed with water and stained with hematoxylin for 20 minutes and washed again with water, 1% acid and alcohol was used to differentiate the tissue for five (5) seconds and washed with water thereafter. The tissues were blued in ammonical water for two (2) minutes, washed with water and concentrated with 1% aqueous eosin for minutes, rinsed with water and dehydrated again in ascending grades of alcohol starting from 70%, 90% and then 100% for one (1) minute in each grade. The tissues were then cleaned in xylene covered with coverslip and finally mounted in DPX (distrene, triceresyl phospatexylene). The examinations of the slides were done under the microscope.

Sperm Analysis

The sperm analysis was performed at Divic specialist medical laboratory Rumuosi, Port Harcourt, Nigeria, following the outlines of the world health organization (WHO,1999) as contained in the WHO protocol MB-50 (1983) for method of the effect of oral administration of soft drinks on fertility of male wistar rats.

The sperm characteristics analyzed in this study are; Sperm motility, Sperm morphology, Sperm viability, Total sperm count.

Measurement of Sperm Motility

This analysis was performed by placing a drop of sperm – saline combination on a slide and covered with a cover slip made of glass. The slide was then positioned on the slide stage of a low powered microscope and viewed under x40 magnification. The motility was determined by observing and evaluating the percentage of sperm that are motile, actively motile, sluggish and dead.

Assessment of Sperm Morphology

A thin smear of liquefied well mixed semen was made and 95% ethanol was added while the smear was still wet for minutes; this was followed by 1ml of gimso stain and allowed to stand for 10 minutes. Therefore, it was mixed with Na_2C0_3 formal saline to remove any mucus which may be present. The mucus was then rinsed.

Determination of Sperm Viability

In the sperm viability determination, the percentage number of defective sperm cells (i.e. total percentage of defects formed in the Head, mid piece and tail) was recorded as the non-viable sperm while the percentage normal sperm cells from the total sperm count was recorded to be viable.

Assessment of Total Sperm Count

Following the removal of the testes, the vas deferens was identified and sperm was gently squeezed out from the epididymitis tail through the vas deferens into a petri dish, then 1ml of formal saline was added to enhance and prolong survival. Then one drop of the sperm and formal saline mixture was taken into a test tube and 19 drops of semen diluents was added (1 in 20) and mixed thoroughly for five (5) minutes. Pasture pipette was used to collect a drop of the mixture and placed on the improved Neubeuer counting chamber after charging it and allowed for the mixture to spread under the cover slip by capillary action. The counting chamber now containing the sperm was then mounted on the slide stage of the binocular light microscope using adjustable light source and viewed under x40 magnification. The sperms were then counted by counting the number of sperms in four (4) major squares on the top and bottom right to give a total of sixteen (16) squares. Then the number counted was multiplied by 106 and expressed per ml (/ml).

RESULTS

Table 1: Effects of Soft Drinks on Body Weights

Groups	Mean Weights before Administration (grams)	Mean Weights after Administration (grams)	Mean Weight Difference (grams)	Relative Weight Difference (%)
1 (control)	160.00 ± 0.00	220.00 ± 8.94	60.00 ± 8.94	0
2 (Coca cola® ×1)	160.00±0.00	244.00 ± 0.00	84.00 ± 4.00*	40.00 ± 2.50*
3 (Coca cola® ×2)	164.00 ± 4.00	248.00 ± 8.00	84.00 ± 4.00*	40.00 ± 1.11*
4 (Bullet drink)	166.00 ± 4.00	264.00 ± 9.80,	98.00 ± 10.20*	63.33 ± 6.79*
5(Sugar solution)	160.00 ± 0.00	234.00 ± 14.00	74.00 ± 14.00	23.33 ± 8.75

Mean ± sem.; * = Mean relative weight difference after administration is significantly different from that of the control group ($P<0.05$)

Table 2: Effects of Soft Drinks on Sperm Analysis

Group	Sperm Motility (%)	Sperm Count (million/ml)	Sperm Viability (%)	Abnormal Morphology (%)
1 (Control)	90.60±3.66	42.20±8.87	98.20±0.58	1.40±0.25
2 (Coca cola® ×1)	80.00±5.24	41.00±6.66	97.40±0.68	2.60±0.68
3 (Coca cola® ×2)	87.20±7.03	25.60±1.03*	97.00±0.45	3.00±0.45
4 (Bullet drink)	70.00±9.49	23.60±4.87*	96.20±2.06	2.00±0.32
5 (Sugar solution)	72.00±9.57	47.20±5.02	96.60±0.68	3.40±0.68

Values are presented in mean ± sem. n= 5. *means values are significantly different from the control ($P\leq0.05$)

Histological Slides of Testicular Tissues

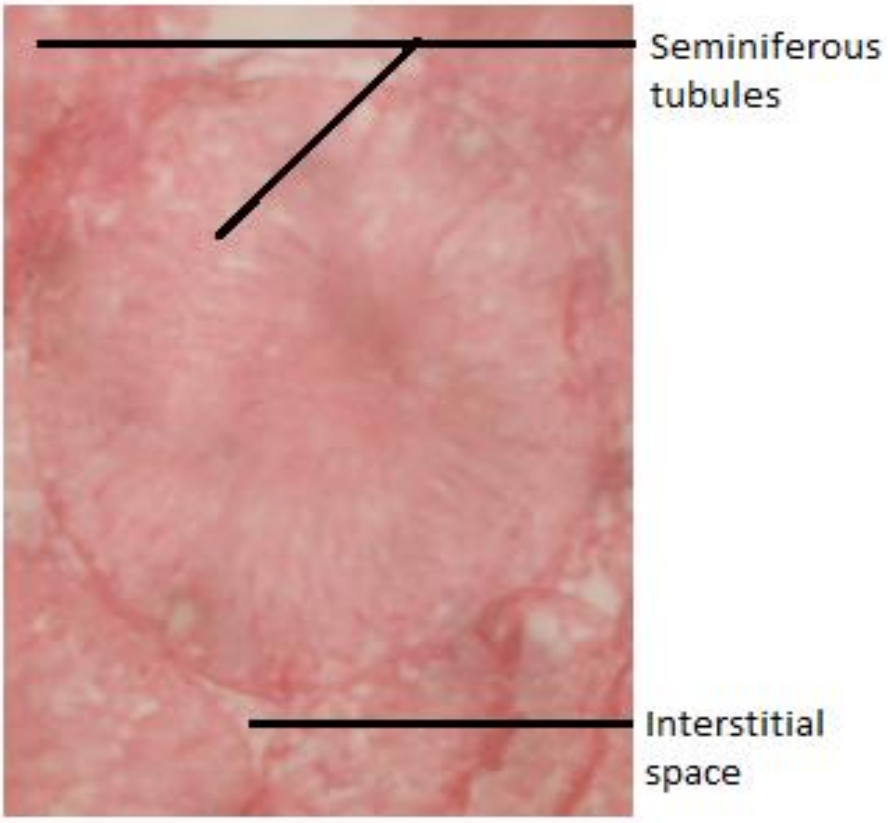

Plate .1: A Photomicrograph showing the Histological Variations of the various Testicular Tissues in the Control Group
Normal Seminiferous tubules containing;

(i) Spermatogonia at the basement membrane are histologically normal
(ii) Primary and secondary spermatocytes are also orderly lined and are in good histologic conditions
(iii) Spermatids and mature spermatozoa with their flagella pointing to the luminal border of the seminiferous tubules
(iv) Interstitial space contains normal interstitial (leydig) cells

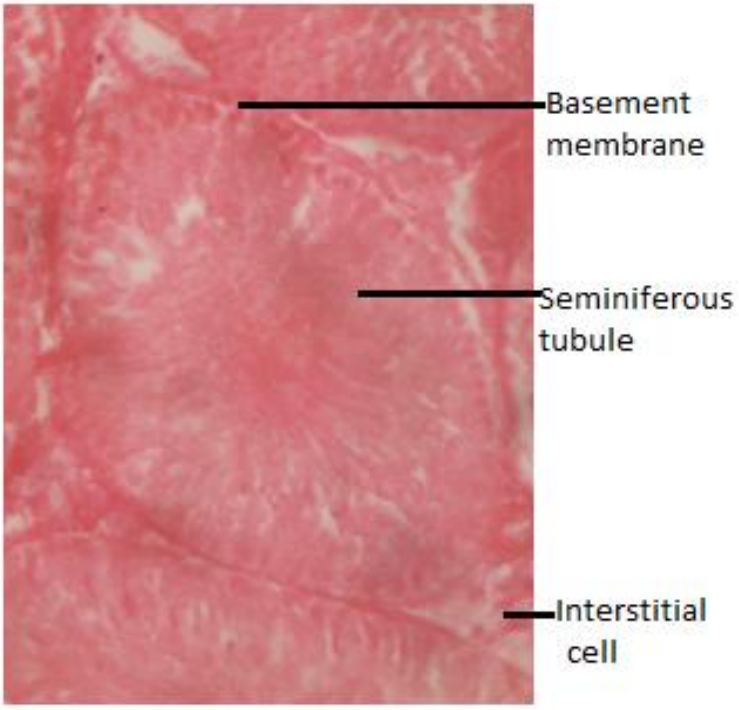

Plate.2: A Photomicrograph showing the Histological Variations of the various Testicular Tissues in the Coca-Cola x1 Group Rats

Normal Seminiferous Tubules Containing;

(i) Spermatogonia at the basement membrane are histologically normal and active. The tubules are well lined, brighter and they are neatly joined end to end with other cells.

(ii) Primary and secondary spermatocytes are also orderly lined and are in good histologic conditions

(iii) Spermatids and mature spermatozoa with their flagella pointing to the luminal border of the seminiferous tubules

(iv) Interstitial space contains normal interstitial (leydig) cells

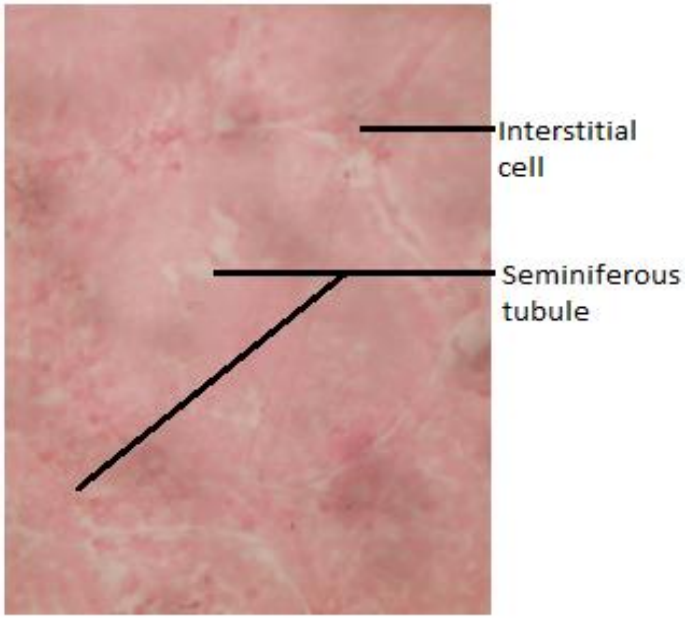

Plate 3: A Photomicrograph showing the Histological Variations of the various Testicular Tissues in the Coca-Cola x2 Group Rats

(i) The above seminiferous tubule contains normal Spermatogonia that are lined around the region of the basement membrane.

(ii) Interstitial (Leydig) cells are well mapped out and demarcated from the basement membrane on the right border of the seminiferous tubules but not the left.

(iii) Primary and secondary spermatocytes and even the spermatids are not well defined.

(iv) Interstitial spaces are not properly defined but cells still indicate normal histologic studies.

(v) Scattered pores are fairly observed in within the tubules.

DISCUSSION

The results on the effects of body weights of male wistar rats after forty (40) days administration of the test substances are presented above in Table 1. The result shows that there was an increase in the mean body weights difference of rats in all soft drink administered test groups, compared to how it was in the control group. Indeed, all except the sugar solution group were significantly ($P<0.05$) higher when compared to the control group. The mentioned sugar solution group was insignificant ($P>0.05$) even though it was still higher when compared to the control group. In relative terms, there were elevations in all relative body weights by 40.00%, 40.00%, 63.33% and 23.33% respectively, in single dose coca cola group, double dose coca cola group, bullet drink group and sugar solution group compared to the control.

The increase in mean weight difference and their corresponding relative weight differences noted among the test groups suggest the premise that all test substances generally, and the bullet drink specifically, contain ingredients that are either stimulatory or inhibitory to the appetite Centre's of the hypothalamus. That is, stimulation of the lateral hypothalamic nuclei and a corresponding inhibition of the ventromedial hypothalamic nuclei (satiety Centre) causes voracious feeding resulting in increased obesity and marked weight gain (Burbach *et al.,* 2001). Also lesions of the paraventricular nuclei and stimulation of the dorsomedial nuclei causes excessive eating with the similar growth enhancing effects (Lohmeier, 2003).

Effects of Soft Drinks on Sperm Function

The sperm count, motility, viability and morphology were used in this study to evaluate the effects of prolonged administration of soft drinks on the reproductive system of male wistar rats. These androgenic parameters are often evaluated to determine the fertility of male subjects. In this study, soft drinks administration insignificantly decreased the motility, count and viability with corresponding increase in percentage of sperm with abnormal morphology. The deleterious effects of the soft drinks on the sperm characteristics could be attributed to decreased and unfavorable spermatogenic activities in the testis and epididymis (Nassan *et al.*,2021). Reduced epididymal sperm count and elevated morphologically abnormal sperm, as discovered in this study, have similarly been attributed to decreased testosterone mobilization for use in the testis even though the testosterone production in this study was increased. Testosterone is known to be critically involved in sperm cell development (spermatogenesis) and the resulting leydig cell dysfunction and testicular steroidogenic disorder arises from derangement in testosterone level. This suggests that although the soft drinks possessed contents which were found to especially show significant decrease in the double Coca cola and bullet drink groups, such decreases did not interfere with the testicular steroidogenesis and hence, spermatogenesis. Hence, it does place any detrimental effect on the hypothalamo-pituitary-testicular axis since the results indicated increased testosterone levels but were not mobilized from their site of production to the sites of their targeted action due to the possible reduced synthesis of androgen binding globulin (ABG). Also decreased sperm count could be associated with the decreased glycogen content of the testis which causes a shortage in the energy source for spermatogenic activity after the administration of soft drinks.

In the present study, the caudal epididymal sperm motility is reduced. These reductions predict alteration in sperm production and sperm maturation. It is kept in awareness that the main function of the epididymis is sperm maturation which leads to the achievement of motility, viability and fertilizing ability of spermatozoa. Also the functioning of the epididymis is androgen dependent. Thus, the decreased sperm motility and viability in this study suggest alteration in sperm maturation in the epididymis due to the impact of soft drinks on the activities of the epididymis either by reduced testosterone level, or that the drinks components might have permeated the blood-testis barrier.

The reduced level of sperm motility, count and viability observed in this study are consistent with earlier reports (Eze 2012; Wood *et al.*, 2012) but however, proved considerable amount of contradiction with those of Oguike and Achibong (2011) who rather viewed an improvement in sperm quality.

It was observed that after the period of administration, progressive significant ($P<0.05$) increases were noticed in the rat's groups treated with single dose coca cola, double dose coca cola and bullet drinks which corresponds to the relative increases of 40.00%, 40.00% and 63.33% respectively when compared to the control group. However, an anomaly was observed in the sugar solution group which, although there was an increased mean weight difference, such increase was insignificant ($P>0.05$) with a corresponding relative difference of 23.33% when compared to the control group.

CONCLUSION

The results of this study indicated that consumption of soft drinks exert stimulatory effects on rat's weights and then exert adverse effects on all sperm parameters. Increased and prolonged consumption of various soft drinks types cause a generalized increase in body weight. Prolonged consumption of soft drinks, especially Coca-Cola, bullet drinks and sugar solution in relatively high amount will cause reduced sperm motility, count, and viability but increased abnormal morphology

CONFLICT OF INTEREST: The authors hereby declare that there is no conflict of interest.

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