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# Toxicological Effect of Prolonged Coca-Cola and Bullet Soft Drinks Consumption on Male Hormonal and Testicular Oxidative Profile in Wistar Rats

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**ABSTRACT**

This study examines the effects of prolonged consumption of soft and bullet drinks on different sex hormones i.e. luteinizing hormones (LH), Follicle Stimulating Hormone (FSH), Testosterone (T), testicular antioxidative parameters ie Malondialdehyde (MDA), superoxide dismutase (SOD)and the histopathological effects of soft drinks consumption on the testis. Thirty five (35) male wistar rats were employed in this study for seven weeks.Group1 (control) was giving normal feeds and water ad libitum, group2 was administered 1.2ml coca cola drinks,Group3 double dose (2.4ml) coca cola drinks,Gruop4 (0.6ml) bullet drinks while 1.2ml sugar solution was given to group5 including normal feeds and water ad libitum.The results from this study shows significant decrease in superoxide dismutase mean values among the test groups compared with control while in Malondialdehyde there was an increased significantly in group4 administered bullet drinks (13.60µg/mg), group2 mean values administered 1.2ml coca cola drinks was (7.50 µg/mg), group3 administered double dose of 2.4ml Coca Cola drinks daily was (6.60µg/mg) while gruop5 mean values administered 1.2ml sugar solution daily was (5.00 µg/mg) compared with the control group of (2.60 µg/mg) mean significant values of <0.05. There was a significant increase in the mean reproductive hormones mean values among the test groups compared with the control group. except the testosterone mean value in group5 administered sugar solution that has lower value than the control group. Findings from this study have shown the toxicological adverse effect of prolonged consumption of coca cola and bullet soft drinks on testicular pathophysiology and significant increase in male reproductive hormones signaling testicular damaged which can result in infertility among males.

**KEYWORDS:** Hormones, Antioxidants, Male, Soft Drinks, Testicular, Leydig. .

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## INTRODUCTION

Follicle Stimulating hormone (FSH) and inhibin B are said to link each other in grossly the same manner as Luteinizing Hormone (LH) and Testosterone (T). Follicle stimulating hormone is the product of the pituitary gland that affects the testis, and inhibin B is depicted to be the testicular products that feed back to inhibit via inhibition of the FSH. Follicle-stimulating hormone tends to exercise its greatest role during development on the sertoli cells which functions in matured animals by aiding the survival of germ cells (Kumur, 2005; Ruwanpura*al.*, 2008; Sairam and Krishnamurthy 2001). Additionally, it is quite seldom to find change in the level of gonadotropic hormone without a change in the other, therefore reports of selective change in follicles stimulating hormone are indeed unusual, often times, in toxicology studies follicle stimulating hormone is found to be elevated as a compensation response to injury.

**HEALTH IMPLICATION OF SOFT DRINKS CONSUMPTION:** Obesity and overweight have always

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been in constant relation with low sperm count (Sermondade., 2013) and fertility decrement in natural (Sallmen *et al.,* 2006) and aided conception (Bakos *et al.,* 2011). It has been suggested that alternations in hypothalamic –pituitary-gonadal axis could explain the effects of excess body weight on the production of sperm but increased scrotal temperature resulting from the deposition of abdominal and scrotal fat, or the accumulation of liposoluble endocrine disruptors in adipocyte clusters (Sermondade *et al*., 2013).

However, it seems possible that other metabolic effects of obesity, such as deregulated endocrine discharge, insulin resistance and increment in systemic inflammation (Hammoud *et al.,* 2008). Also, dietary influences have been related to some resulting metabolic effects of obesity, suggesting that through similar mechanisms, specific aspects of the diet may affect sperm production. Numerous studies (Malik *et al.,* 2010; Mozaffarian *et al.,* 2011), which include two randomized trials (de Ruyter *et al.,* 2012) have demonstrated that Sugar Sweetened Beverages (SSBs) induce weight gain and obesity, it is also a famous notion that sugar sweetened beverages can evoke in metabolism, some of the consequences of obesity (Stanhope *et al*., 2009). However, a very limited number of studies have examined how sugar sweetened beverages intake are related with semen qualities, levels of reproductive hormone or fertility in male (Chiu *et al.,* 2014). While a study performed recently in rodents discovered that sugary drinks impact negatively on male fertility (Ruff *et al.,* 2013) although this literature in human is not commonly found. An energy drink is a drink that provides our body with extra energy during times of increased physical stress or strain due to its exceptional composition. They are soft drinks popularly known for energy boosting. Energy obtainable from the sugar content of this drink are not often held in emphasis, (Nehligetal,1992) rather through a choice of substances added by the manufacturers. These could be disclosed by the energy drinks in colorful cans and bottles displayed alongside soft drinks and alcohols, sports drinks and juices in bars, stores, clubs, and sometime filling stations. The general belief is that the drink will be fruity and sweet since their cans are quite welcoming. In addition to the packaging being attractive and colorful, the manufacturers' assertion that they are energy boosters also lure many into its purchase (Kukuljan *et al.,* 1997). They also possess names that portrays strength, speed, sexuality, full throttle, power, such as bullet, power horse, monster, red bull, no fear, spark, lost venom, rock star, impulse, double shot, rip it, superman, tab energy, and they primarily set up for male targets, shakers, movers on the go, all-nighters, sportsmen, clubbers and those whose jobs compel them to spend lengthy hours in exerting brain or energy power. Young people are typically attracted to energy drinks (Wallimann *et al*., 1992). The main contents of bullet energy drinks are Natural mineral, Dextrose, Sugar, Acidifier (E330), Carbon dioxide, Taurine (0.38%), caffeine, etc. Taurine and Caffeine for instance possess health related problems. Caffeine binds and Beta-adrenergic receptors bind to themselves on the heart muscle cell surface, which leads the way to a rise in the cyclic AMP level inside the cells (by impeding the enzyme that depletes cyclic AMP), thus found to mimic the effects of epinephrine (which binds to receptors on the cells that speed up cyclic AMP reaction and production). This has the ultimate effect on elevating the glycolytic rate and increases the amount of ATP available for muscle relaxation and contraction. Based on research, a temporal increase in the stiffening of arterial walls can be caused by drinking caffeinated coffee (Howard and Marczinski, 2010). In this respect, taurine is necessary in the fetal development of the eye and consequently defective vision may occur in case of taurine shortage. Generally, on the fetal development of the hearing, taurine has marked effects (Van-Den *et al.,* 2008).

**EFFECTS ON CAPACITIES OF ANTIOXIDANTS:** In another research carried out on the capacity of antioxidant of some caramel-containing beverage drinks, Brenna et *al,* investigated in 2008 that antioxidant properties of food and beverages have been extensively studied, and that on the antioxidant capacity of these categories of drinks, few data have been derived. Apart from drink based on fruit juice, some of the soft drinks most common contain one of the four caramels colours allowed in food, as a colouring agent. Melanodin compounds are contained in caramels. Melanodin compounds have been reported to partake in the antioxidant potencies of some food stuff. They participate in the enhancement of antioxidant activity of some soft drinks that contain caramel, such as an original Italian soft drink called chinotto together with cola drink. Main parametric analysis was made on some commercial caramel colours for main parameters: 5-hydroxymethyl-2-furfural (HMF), fructose content and residual glucose, total reducing compounds by the Forlin - which constitute ciocalteau reagent, and the activity of antioxidants by this FRAP and DPPH methods. Analysis of similar types were performed on different soft drinks coloured with E150d (one of the four caramel colours contained in food). From the result, even when soft drinks have a lower antioxidant activity than other beverage products like tea, chocolate or coffee, they may add to the properties of antioxidants supposed with the diet since the antioxidant activity ranges from 0.2 for non-alcoholic drinks that are like cola to 1.0 mmol Trolox equivalent per liter for Chinotto drinks.

**ANDROGEN ANTAGONISM: -** When binding of endogenous testosterone and/or dihydrotestosterone to androgenic receptor (AR) are blocked by treatment (i.e. androgenic receptor antagonism) or otherwise inhibits androgenic receptor signaling, it results effectively and rapidly in reduced androgenic signaling, both centrally and peripherally. To this end, changes due to atrophy are

commonly seen in the accessory sexual organs, mammary gland and epididymis similar to those seen with reduced testosterone above. Nevertheless, the testicular spermatogenesis incurs no degenerative changes, and there is generally hyperplasia in the leydig cell rather than atrophy as the luteinizing hormone secretion serve as the channel through which the leydig cells are stimulated from the anterior pituitary gland to increase testosterone production, with normal or increased luteinizing hormone. (viguier-Martinez *et al.,* 1983; O'Connor, Cook *et al.,* 1998; O'Connor *et al.,* 1999; O'Connor *et al.,* 2002a).

**HYPERANDROGENISM**: -This is induced to prevent luteinizing hormone secretion and thereby causing the reduction of circulating luteinizing hormone and hence testosterone concentration in the general circulation and testis. However, these quantities of testosterone may not be enough to cause spermatogenesis, which seemingly depends on actual high levels. This reduction will produce the small testosterone effects in the testes state specific (stage VII/VIII and XIV) death of germ cell, retention of spermatids and reduced/degenerating elongated spermatids. Since accessory organs will be preserved by the exterior androgen stimulation, they will generally appear normal. Depending on the strength of the androgenic stimulation and the length of treatment time, leydig cell degeneration may also appear evident (O'Connor, Davis *et al.,* 2000b). This androgen will partly or wholly replace the internal androgen needed in testis for sperm formation as the concentration of exogenous androgen circulation increase will bring the changes to normal even at low doses (Sun *et al.,* 1989). As leydig cells are no longer being stimulated to produce testosterone, they should be atrophic and accessory sexual organ may actually prove larger due to greater degree of androgen stimulation (O'Connor, Davis *et al*., 2000b). Low amount of luteinizing hormone as well as low endogenous testosterone are the serum hormones expected here. To know if the administered androgen cross-reacts with the antibody measuring endogenous testosterone should be the task of the investigator; here, very high reading would be shown for cross reactivity.

**TESTOSTERONE BIOSYNTHESIS:** Since leydig cells possess an abundant smooth muscle endoplasmic reticulum and excessive mitochondria with tubular cristae, they have a steroid secreting cell cytoarchitecture. They are able to take up acetate for steroidogenesis from lipoproteins present in circulation or synthesize cholesterol from the said acetate (Liu *et al.,* 2005). The cytochrome p450 17α-hydroxylase/C17-20-lyase ($P450_{c17}$), that mediates the production of later steps, the biosynthesis of testosterone may be stimulated in cholesterol synthesis, since it has been shown recently to exhibit squalene epoxidase activity (Liu *et al.,* 2005). In this process, steroidogenic acute regulatory (STAR) protein has been observed to be responsible (Stocco 1999). In response to agents that enhances steroid production, STAR is expressed in steroidogenic tissues, and steroidogenic failures such as takes place in the complication of congenital lipoid adrenal hyperplasia are the results of mutations in the STAR gene. Star mediating cholesterol transfer mechanism in the mitochondria has not attained full characterization (Stocco 1999). Already shown to be included in intra mitochondrial trans-cholesterol is another mitochondrial protein called Peripheral-type Benzodiazepine Receptor (PBR) (Papadopoulos V, 2004). Further research will be required to supplant a clear mechanism of transport in cholesterol, but may appear that the task of steroidogenic acute regulatory protein is to ferry cholesterol to peripheral type benzodiazepine receptor, which goes ahead to directly form a pore for its internal conveyance. Located in the inner or matrix of the mitochondrial membrane is cytochrome P450 cholesterol side chain cleavage enzyme ($P460_{Sec}$). The cleavage reaction of the side chain uses three oxygen molecules, three NADPH molecules and an electron transfer system of the mitochondrion leading to pregnenolone synthesis (Porter T.D). Located in the smooth endoplasmic reticulum (SER) membranes in the cytoplasm are the enzymes necessary for succeeding steps of steroidogenesis. Pregnenolone, in its bulk, formed in the mitochondrion, spreads into the smooth endoplasmic reticulum and functions as a substrate for the synthesis of progesterone. However, based on recent evidence, it may be converted within the mitochondria by a mitochondrial form of the enzyme, 3β-hydroxysteroid dehydrogenase enzyme to progesterone (Cherradi *et al.,* 1994). The testicular 3β-HSD is coded by type I and II 3β-HSD gene (HSD3b2), even though multiple isoforms of 3β-HSD have been detected (Lachance *et al.,* 1991). Progesterone is coveted by P450cl7 once formed by two steps; 17 α- hydroxylation and C17-C20 cleavage, into androstenedione (Nakajin *et al.,* 1981; Nakajin, Hall Pf, 1989). The gene for cytochrome P45017α-hydroxylase/C17-20-lyase and cytochrome $P450_{17}$-α hydroxylase/ c17-20-lyase, in the human, is situated on the cytochrome 10 (Matteson, 1986). Conversion of androstenedione into testosterone which is the final step of androgen synthesis has 17β-hydroxysteroid dehydrogenase (17β-HSD) as its catalyst. 17β-HSD exhibits multiple isoforms and type III 17β-HSD (17β-HSD3) is the testicular isoform, coded by 17β-HSD3 gene, (hsd 17b3) (Geissler *et al.,* 1994). Testosterone is either further metabolized into separate steroid, some of which are more active biologically like dihydrotestosterone in reactions mediated by types 1 and 25α-reductase or associates with androgen binding protein and diffuses into the seminiferous tubules lumen to stabilize spermatogenesis.

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## MEASUREMENT OF BIOCHEMICAL PARAMETERS AND ANTIOXIDANTS

According to Tothova *et al.,* 2013, the resolution of markers of oxidative stress and antioxidant status will be such that samples about 0.2g testes would be homogenized with 2ml buffered saline of cold-ice phosphate of $P^H$ 7.2 using Tissue lysine (ii) homogenizer. The sample homogenates would be centrifuged under a mass of 4000g at $4^0$c for 10 minutes and further analyses were made from the supernatant. Concentration of proteins in sample could be estimated with the use of commercial bicinchroninic acid kit. With due instructions serine serum albumin standard could be used for construction of the calibration curve (Tothova 2013). According to Munch *et al,* 1997, advanced glycation end product levels in the testes could be assessed fluorometrically at $\lambda_{ex}$ =370nm and $\lambda_{em}$ = 440nm after phosphate buffered saline have seen added to samples.

In evaluating fructosamine concentration, San-Gil et al., 1985, showed that testicular homogenates and standards were transferred into microplate and could be incubated at $37^0$C with nitro blue tratrazolium for fifteen minutes and specific absorption at 5300nm could be measured. Calibration curve could be constructed with 1-deoxy- morpholino-D fructose. Advance oxidation protein products, (AOPPs) could be spectrophotometrically measured at 340nm, as was described previously (Tothova *et al.,* 2012; Witko-Sarsat *et al.,* 1996). Advanced oxidation protein products concentration could be determined on the basis of chloramine T using potassium Iodide (K.I). Quantification of lipid peroxidation in samples was determined by analysis of thiobabituric acid reactive substance (TBARSs) according to the protocol established.

## MATERIALS AND METHODS

This is an experimental study to determine the effects of soft drinks consumption on the reproductive hormones of males using wistar rats model. 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).

## STUDY DESIGN

**Table 1. GROUPINGS OF THE ANIMALS AND DOSAGE ADMINISTERED**

| Groups | Route of Administration- orally | Daily Dosage Administered (ml) |
|---|---|---|
| 1 | Distilled Water | 1.0 |
| 2 | Coca-Cola | 1.2 |
| 3 | Coca-Cola | 2.4 |
| 4 | Bullet Drink | 0.6 |
| 5 | Sugar solution | 1.2 |

**The Administration were carried out daily for a period of 40 days. The 35 wistar rats were randomly divided into five (5) groups of seven (7) rats each.**

## 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 surgical blades.

## SERUM HORMONAL ASSAY

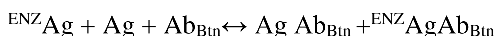
While being pinned in a supine position on the dissecting board prior to dissection, 5ml of blood sample was collected from each animal through cardiac punctures and was temporarily stored in a plain centrifuge tube and labelled. After some time, the plain centrifuge tube containing the blood samples were centrifuged at 3000rev/min for 15 minutes using a centrifuging machine with model No.800D Ocean made$^+$, England; then the sera were separated from the cells and stored in a sample bottles labelled and frozen at $4^o$c before being used for hormonal assays. The stored sera were analyzed for testosterone, follicle stimulating hormone (FSH) and luteinizing hormones (LH) at the Divic specialist medical laboratory (Diagnostic Centre and blood transfusion services) by Rumuosi, Timber Market East West Road, Port Harcourt Nigeria following the outline of Uotila *et al.,* (1981) with the hormonal kit supplied by Monobind Inc. Lake Forest, CA 92630 USA with product code: 3725-300 for testosterone test system and Biocheck Inc. 323 Vintage Park Dr. Foster city, CA 94404; for FSH and LH.

## PRINCIPLES OF THE ASSAYS

The principle, for testosterone is based on the competitive enzyme immunoassay type 7. The essential reagents include antibody-antigen conjugate and native antigen. As biotinylated antibody is mixed enzyme antigen conjugate and testicular supernatant containing the native antigen, a

competitive reaction results between the antigen of the native type and the conjugate of enzyme – antigen of binding sites of antibodies having a limited number.

The interaction is illustrated below:



$^{ENZ}Ag + Ag + Ab_{Btn} \leftrightarrow Ag\ Ab_{Btn} + {}^{ENZ}AgAb_{Btn}$

$Ab_{Btn}$= Biotinylated antibody (Constant)

Ag = native antigen (variable quantity)

$^{ENZ}Ag$ = conjugate of enzyme – antigen (constant).

Ag $Ab_{Btn}$ antigen – antibody complex

A simultaneous reaction between the biotin attached to the antibody and streptavidin immobilized on the micro-well occurs.

$AgAb_{Btn}$ + $ENZAgAb_{Btn}$+ streptavidin = immobilized complex

$Streptavidin_{Cw}$ = streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid area.

The enzyme activity in the fraction that is bound to antibody is inversely proportional to the concentration of native antigen. Through the utilization of several references of serum with unknown antigen concentrations, a dose-response curve can be produced from which the concentration of unknown antigen can be ascertained.

Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) assays share the same principle which is based on a solid enzyme linked immunosorbent assay (ELISA). This system makes use of the mouse monoclonal anti-$\alpha$-hormone antibody for solid phase (microwells) immobilization and another mouse monoclonal anti-β-hormone antibody in the conjugate solution of antibody-enzyme. The test is kept for a simultaneous reaction with the antibodies resulting in the sandwiching of hormone molecules between the enzyme- linked and solid-phase antibodies. After forty – five (45) minutes incubation at room temperature, the wells are washed with water to remove inbound – labeled antibodies. A TMB reagent solution is added and incubated under room temperature for a period of twenty (20) minutes, resulting in the blue colour development. This colour development is stopped by adding stop solution and the colour turned to yellow and at 450nm, it was spectrophotometrically measured. The hormone concentration is directly proportional to the intensity of sample used for test.

## MATERIALS FOR THE ASSAYS

The materials used are:

A. 1ml of seven testosterone calibrates containing serum references for testosterone at concentrations of (a)0.1 (b) 0.5 (c) 1.0 (d) 2.5, (e) 5.0 (f) and (g) 12.0 all in ng/ml.

B. Mouse monoclonal anti-$\alpha$-FSH antibody coated micropiece with 96 wells.

C. Mouse monoclonal anti- $\alpha$-LH antibody coated microplate with 96 well.

D. Follicle stimulating Hormone and luteinizing Hormone reference standards.

E. Enzyme and conjugate reagent

F. One 7.0ml steroid conjugate buffer.

G. 6ml of one testosterone biotin reagent

H. Streptavidin coated plate contains 96 wells coated with 1.0µg/ml streptavidin.

I. 20ml of one wash solution concentrate.

J. One substrate A – 7ml and contains tetramethylbenzidin (TMB) in buffer.

K. One stop solution of 8ml and contains a strong acid (1NHCL). All these materials came with the testosterone kit and are stored at a temperature of $2.8^0$C.

Other materials which are provided in the laboratory are

i. Pipette capable of delivering 10µl, 50µl and 100µl with a precision of at least 1.5%.

ii. Dispenses for repetitive delivery of 0.100ml and 0.350ml with precision of 1.5%.

iii. Adjustable volume (200-1000µl) dispenses for conjugate

iv. Microplate washer

v. Microplate reader with 450mm and 650mm wavelength absorbance capability.

vi. Absorbent paper

vii. Microplate cover

viii. Vacuum aspirator

ix. Times

## PREPARATION OF THE REAGENTS USED IN THE ASSAY

**Working Enzyme Reagent:** This reagent is stable for one year and it is being prepared by measuring 0.7ml of testosterone reagent enzyme. This preparation was added to the bottle containing steroid conjugate buffer and stored at 2-$8^0$C.

**Wash Buffer:** This is prepared by diluting the contents of the wash solution with 1000ml of distilled water. It is stored at 2-$30^0$C and stable for 60 days.

**Working Substrate Solution:** This is prepared by pouring the content of the bottle labeled solution A into the bottle labeled solution B. It is stored at 2-$8^0$C and stable for one year.

## PROCEDURE USED FOR TESTOSTERONE ASSAY

Firstly, the required number of microplates to be used was selected. This included 25 test and 7 references. Then 10µl of references and specimen were pipetted into appropriate wells. 5µl of working testosterone enzyme reagents was added into all selected wells and swirl gently for thirty (30) seconds to mix. Thereafter, 50µl of testosterone biotin reagent was added to all wells, swirl again for thirty (30) seconds, and covered with microplate cover and incubate for 60 minutes at room temperature. After 60 minutes, the content of

microplate was discarded by decantation and blotted with dry absorbent paper to dry them; then 350µl of wash buffer was added tapped and blotted for a total of three times, before 100µl of working substrate solution was added to each well and incubated for 15 minutes at room temperature. Stop solution of about 50µl was added to each well. Mixed gently for twenty (20) seconds after 15 minutes' incubation and read within 30 minutes of adding the stop solution with a microplate reader at a wavelength of 450nm using a reference wavelength of 630nm.

**PROCEDURE FOR LUTEINIZING HORMONE (LH) ASSAY**

The kit used for this assay was Biocheck, INC with the catalog number: BC-1031. The materials in the kit are; Mouse monoclonal anti-$\alpha$-LH antibody coated microliterplate with 96 wells 13ml of enzyme conjugate reagents, LH reference standards containing 0.5, 15, 50, 100, and 200ml µ/ml and TMB reagent (11ml).

The required number of microplates was secured and 50µl of standard and specimen were appropriate wells followed by addition of 100µl of enzyme conjugate reagents. The mixture was whirled gently for thirty seconds, at room temperature for 45 minutes to mix before incubating. After incubation the incubation mixture was discarded, raised with distilled water for 5 times before striking gently and sharply onto absorbent paper to remove all water droplets. 100µl of TMB reagent was added to each well gently mixed for ten (10) seconds and incubated again for 0 minute. Thereafter, 100µl of stop solution was added to each well, mix gently before reading the microplate reader with optical density at 450nm within 15 minutes.

**PROCEDURE FOR FOLLICLE STIMULATING HORMONE (FSH) ASSAY**

The kits used for this assay were Biocheck INC. with catalog number: BC 1029. The materials in the kit are; mouse monoclonal anti-$\alpha$-FSH antibody coated microliter plate with 96 wells, 13ml enzyme conjugate reagents, FSH reference standards containing 0,5,15,50,100 and 200Iµ human FSH lyophilized, TMB reagent (11ml) and 11ml of stop solution.

The required number of microplates was secured and 50µl of standard and specimen were appropriate wells followed by the addition of 100µl of enzyme conjugate reagent. The mixture was swirled gently for thirty (30) seconds to mix before incubation at room temperature for forty-five (45) minutes. After incubation, the incubation mixture was discarded rinsed with distilled water for five (5) times before striking gently and sharply onto absorbent paper to remove all water droplets. 100µl of TMB reagent was added to each well, gently mix for ten (10) seconds and incubate again for 70 minutes at room temperature in the dark. Thereafter, 100µl of stop solution was added with the microplate reader with optical density at 450nm within 15 minutes

**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 $37^0$C overnight.

**ANALYSIS OF OXIDATIVE PARAMETERS**

**SUPEROXIDE DISMUTASE (SOD) ANALYSIS**

The superoxide dismutase (SOD) of the sample activity was analyzed using modified method from Marklund and Marklund 1974. Contained in the reaction is 2500µl 50mM Tris – HCL buffer ($P^H$ 8.2), 100µl 0.04mM pyrogallol and a specific amount of distilled water appropriate for mixing summing up to a volume of 3ml. Except pyrogallol, all reagents were added into the blank after which proper mixing was done. The blank absorbance and samples were recorded continually for 2 minutes at 20 seconds interval at 420m. The amount of enzyme that lowers the auto – oxidation rate of pyrogallol by 50% at a standard assay condition was the definition of one unit of SOD.

SOD activity (U/ml) = $\frac{V_p - V_s}{(V_p \times 0.5)} \times \frac{V_t \times n}{V_e}$

Where

Vp = Pyrogallol auto-oxidation rate (control)

$V_s$ = samples with enzymes auto-oxidation rate

$V_t$ = Total volume of reaction

$V_e$ = Volume of enzymes used in performing the assay

n = Dilution fold of SOD sample

0.5 = Factor for 50% inhibition.

**ASSESSMENTS OF LIPID PEROXIDATION**

The peroxidation level of lipid was indicated by the content of testicular Malondialdehyde (MDA). MDA in the tissue was determined with the use of thiobabituric acid reactive substance assay, following the description of Buege & Aust (1978) although modified slightly. Briefly, 0.5g of a tissue specimen was homogenized in 0.15mol/l Kcl at a ratio of 1:9ml using a glass homogenizer.

A thorough mixing of one homogenate volume with two thiobabituric acid reagent volumes was performed. The solution was then heated for 15 minutes in a boiling of water bath. The removal of the precipitate by centrifugation was ensured after cooling for 10 minutes at 1000g. The clear supernatant absorbance was determined at 535nm and MDA concentration was calculated using 1.56, 105 $mol^{-1}cm^{-1}$ molar absorbance coefficient. The expression of MDA results was carried out of wet tissue.

**MEASUREMENT OF BODY WEIGHTS**

The weight of the experimental animals was measured weekly with an electric scale weight Balance-Golden Meter USA calibrated in grammes for four weeks.

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## RESULTS

**Table 2. NUTRITIONAL INFORMATION PER 25CL OR 250ML OF REGULAR COCACOLA BEVERAGE DRINK** (www.thecocacolacompany.com)

| INGREDIENTS | AMOUNT REQUIRED | % GDA |
|---|---|---|
| Energy | 105kcal | 5 |
| Sugars | 27g | 29 |
| Fat | 0g | 0 |
| Saturates | 0g | 0 |
| Sodium | 18g | <1 |

According to a publication made by www.thecocacolacompany.com, published in 2015, the general content of the regular Coca-Cola drink are Carbonated water, sugar, colour, caramel and acidulant which include Phosphoric acid, flavor and Caffeine. Also, and specifically for every 25cl or 250ml content, the specific amount of the major ingredients was given and the percentage of Guideline Daily Amounts (% GDA) based on a 2000kcal diet were also given above.

**Table 3. EFFECT OF SOFT DRINKS ON TESTICULAR TISSUES OXIDATIVE PARAMETERS**

| Group | Superoxide Dismutase (SOD) (µg/mg) | | Malondialdehyde (MDA) (µg/mg) | |
|---|---|---|---|---|
| | Mean±sem | Relative Difference (%) | Mean±sem | Relative Difference (%) |
| 1 (Control) | 12.40±0.25 | 0.00 | 2.60±0.25 | 0.00 |
| 2 (Coca cola® ×1) | 4.25±0.75* | -65.73±0.50* | 7.50±0.65* | 188.46±3.12* |
| 3 (Coca cola® ×2) | 5.00±1.23* | -60.48±1.24* | 6.60±0.51* | 153.85±1.90* |
| 4 (Bullet drink) | 3.00±1.00* | -75.81±1.72* | 13.60±1.63* | 423.08±3.31* |
| 5 (Sugar solution) | 5.80±1.50* | -53.23±0.87* | 5.00±0.45* | 92.31±0.95* |

Values are presented in mean ± sem. N= 5. P ≤ 0.05 * means values are statistically significant when compared with the control.

**Table 4. EFFECT OF SOFT DRINKS ON REPRODUCTIVE HORMONES**

| Groups | Serum Follicle Stimulating Hormone (µg/mol) | Serum Luteinizing Hormone (mg/mol) | Serum Testosterone (µg/mol) |
|---|---|---|---|
| 1 (Control) | 0.60±0.40 | 0.40±0.40 | 2.80±0.49 |
| 2 (Coca cola® ×1) | 2.60±0.11* | 2.40±0.03* | 5.20±0.49* |
| 3 (Coca cola® ×2) | 2.60±0.15* | 2.00±1.05* | 5.40±1.66* |
| 4 (Bullet drink) | 1.00±0.08* | 0.80±0.09* | 5.40±0.87* |
| 5 (Sugar solution) | 1.40±0.00* | 1.20±0.09* | 1.60±1.12 |

Values are presented in mean ± sem. N= 5. P ≤ 0.05 * means values are statistically significant when compared with the control.

**Plate.1: A Photomicrograph showing the Histological Variations of the various Testicular Tissues in the Bullet Drink Group of Rats**

(i) Spermatogonia cells (i.e. Spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids) are properly lined and articulated within the seminiferous tubules.

(ii) Interstitial cells and interstitial space not well defined. The wide space and the absence of Leydig cells seem to be an indication of pathologic condition of such slide.

(iii) Seminiferous tubules are loosely attached to each other due to the lack of no normally defined boundary.

(iv) Spermatids and mature spermatozoa are lined with their flagella pointing to the luminal border of the seminiferous tubule

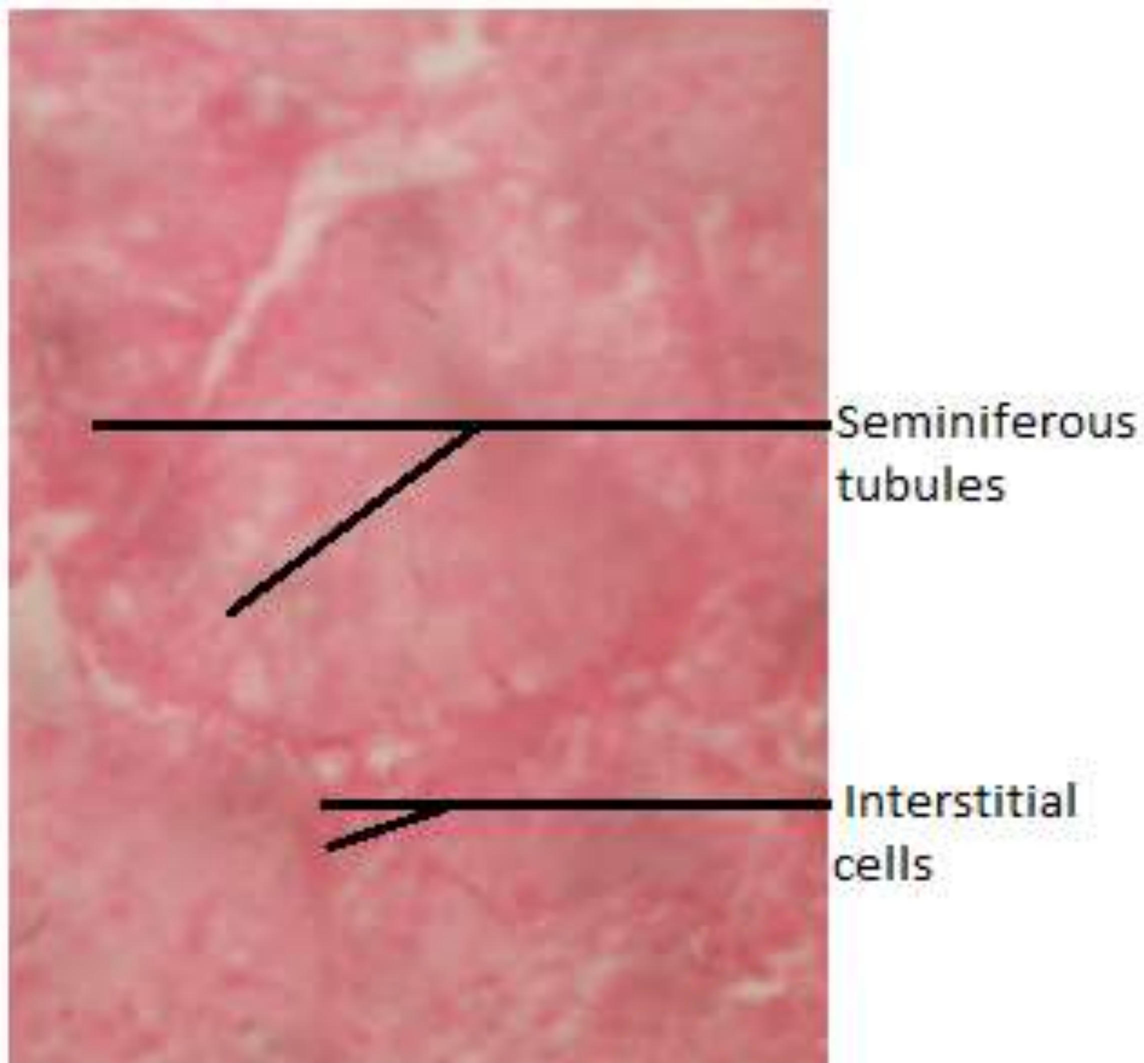


**Plate 2: A Photomicrograph showing the Histological Variations of the various Testicular Tissues in the Sugar Solution Group of Rats**

(i) Spermatogonia are lined at the luminal border of the basal lamina
(ii) Other Spermatogonia cells are also lined and located in their appropriate positions. They are also found to be maintaining their normal histologic conditions.
(iii) The interstitial cells of Leydig are observed in their normal positions in the interstitial spaces
(iv) The tubular cells are held in contacts with each other via tight junctions
(v) Intercellular spaces between tubules are largely visible in some areas in the slide but absent in some other regions.

**DISCUSSION**

It was observed from the results of the present study that there is a unanimous significant increase in the serum level of follicle stimulating hormone, luteinizing hormone and testosterone after forty (40) days of soft drinks administration. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are known as gonadotropins having possessed stimulatory affinity with the Gonads, the male testes. They are produced by the cells of the anterior pituitary gland categorized as gonadotrophs. Luteinizing hormones (LH) binds to receptors on Leydig cells in the testes to stimulate the synthesis and secretion of testosterone. Luteinizing hormone increases cAMP after binding to cells which increases the side chain cleavage of cholesterol and protein secretion together with other likely steps to increase the steroidogenesis and testosterone production of all other androgens. Hence increased in FSH and LH levels is is a signal of testicular damaged which could result in infertility (https://medlineplus.gov).

Testosterone acts on the peritubular and sertoli cells of the seminiferous tubules and directly stimulates spermatogenesis (Singh *et al.,* 1995; Bhasin *et al.,* 1988; O'Donnell *et al.,* 1994). In this light, both FSH and LH inhibit the gonads either from sperm production or synthesizing sufficient quality of testosterone (Nieesclag, 1997). FSH stimulates the various phases of development from Spermatogonia to spermatocytes

and also maintains the spermatogenic processes (Connel and Eik-Nes, 1968; Johnson and Ewing, 1971; Holt *et al,* 1973; Darrington and Armstrong, 1975) while both FSH and LH are necessary for meiotic development of the spermatids (Lostroch, 1963). The androgens (Testosterone) are also necessary for meiosis induction, spermatids development and formation in response to FSH (Chemes *et al.,* 1979; Haneji *et al.,* 1984; Russel *et al.,* 1987; Hall, 1994).

The significant increases in serum FSH of all test groups suggest that all soft drinks have stimulatory effects on the hypothalamic-pituitary axis. The gonadotrophic effects by the various test soft drinks give the indication that the drinks may enhance the normal functioning of the Sertoli cells which will increase the sperm cell maturation. (Cheng, Mruk, 2002). Also, the significant increase in serum LH concentration in all test soft drinks administered rats conferred the increase in testosterone concentration, which is the case in this study. This may result from an indication that the various soft drinks possess unique contents that exert stimulatory effects on the hypothalamic-pituitary axis of the soft drinks administered rats. However, previous studies have revealed the fact that, at peak level, increased concentration of serum testosterone exerts a negative feedback that directly inhibit the hypothalamus and anterior pituitary gland to decrease testosterone production (Rhoden & Morgentaler, 2004).The elevation in the concentration of gonadotrophic hormones in all test groups suggests a direct stimulation of the anterior pituitary homogenesis and indeed an indication of elevating testicular protein, cholesterol and glycogen.

Testosterone is the main hormone of the gonads in males produced by the interstitial cells of Leydig in the testis. Also, in addition to luteinizing hormone and follicle stimulating hormone, it is the major hormonal marker of androgenicity (Walton *et al*, 1995). The development and maintenance of male reproductive organs are also enhanced by testosterone (Mooradan *et al.,* 1987). Testosterone also acts in association with follicle stimulating hormone to stimulate the seminiferous tubules which in turn initiate and maintain spermatogenesis (Woode *et al*, 2012).

In this study, with the exception of sugar solution administered rats, the significant increases in the concentration of testosterone is a direct indication of the increased Luteinizing hormone concentration, as observed and hence, a stimulation of the hypothalamic-pituitary-testicular axis (Guyton and Hall, 2011).

Based on the concurrent increases in FSH, LH, and Testosterone, it is expected that spermatogenesis will be increased but this is not the case in the present study since sperm motility, count and viability were all reduced in all test substances with significant reductions observed in sperm count concentration of the double dose Coca-Cola and bullet drinks. The possible reason for this anomaly is that there might have been a lack or reduction in androgen binding protein (ABP), a substance secreted by the sertoli cells that function to maintain high stable supply of androgen in the seminiferous tubular fluid. Thus the depleted ABP possibly have caused a deprivation of the tubular fluid of this already secreted testosterone thereby impairing spermatogenesis (Ganong, 2013).

## CONCLUSION

It is a common observation in our society today that people consume different types of drinks especially beverages without considering their contents, daily requirements and their nutritional and the detrimental consequences associated with over consumption on the various systems and organs of the body particularly reproduction. SOD were significantly reduced and MDA were significantly increased in virtually all test groups indicating marked Oxidative stress/damage to the testes and pronounced lipid peroxidative activities of the test substances. FSH, LH and Testosterone were significantly increased in all test groups indicating the stimulatory effects of all test substances on the hypothalamic-pituitary-testicular axis.

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