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ALCOHOLIC PAIRED YOYO® BITTERS INDUCES LEYDIG CELL ATYPIA IN *RATTUS NOVERGICUS*

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ABSTRACT

YOYO ® bitters cleanser is Nigeria certified herbal products by National Agency for Food, Drugs and Control (NAFDAC) as the first non-alcoholic, coloring or artificial preservatives bitters produced in Nigeria and therefore alcoholic yoyo bitters is a modified form by end users called combined. This study was carried out to investigate the effect of alcoholic yoyo bitters on the histomorphology of the testis induced with binge alcohol drinking. Twenty four (24) adult male Wistar rats were randomly selected into three (3) groups n=8. Group A=control received 1.5ml of vita-milk and water per kg-^{1}b.wt, Group B received 1.5mLof 30% alcoholic vita- milk per kg-^{1}b.wt, and Group C received 1.5mL of 30% alcoholic vita- milk and 0.1mL of YOYO ® bitters cleanser per kg-^{1}b.wt by oral route. The substance was administered three times for four days daily and the animals were sacrificed on the 5th day by cervical dislocation and the testes excised following abdominal incision for histological observation using H/E stain. Blood samples was collected into EDTA and plain tubes for hematology and clinical chemistry respectively. Results showed degeneration of the germinal epithelium of the seminiferous tubule and widening of the interstitial space involving loss of Leydig cell compared with control. There was equally decreased in serum testosterone (ng/ml) level, Ca2+, Mean cell volume(MCV), and total Calcium was reduced while total protein and chloride ions was reduced in the alcoholic yoyo bitter group compared with the normal and was statistically significant at P<0.05. Alcoholic paired yoyo bitters has been confirmed as toxic to sperm cells. This interesting and comprehensive study showed that binge alcoholism alone and in combination with YOYO ® bitters alters testicular function and morphology and therefore alcoholics taking YOYO ® bitters for treatment of other ailments not investigated in the present work should avoid its combination with yoyo bitters to reduce male infertility.

KEYWORDS: YOYO, NAFDAC, histomorphology, abdominal, MCV.

INTRODUCTION

Alcohol is a powerful addictive and central nervous system depressant. The abuse or substance dependence has led to increased risk of major depression (Fergusson *et al.*, 2009). Binge drinking has been defined as episodic excessive drinking (Stolle *et al.*, 2009). It is a modern epithet for drinking alcoholic beverages with an intention of becoming intoxicated by heavy consumption of alcohol over a short period of time (Renaud *et al*., 2001). It is a style of drinking that is popular in several countries worldwide, and overlaps somewhat with social drinking since it is often done in groups. The degree of intoxication varies between and within various cultures that engage in this practice. A binge on alcohol can occur over hours, lasting up to several days, or in the event of extended abuse and even weeks. Due to the long-term effects of alcohol misuse and abuse, binge drinking is considered to be a major public health issue (Mathurin *et al.*, 2000) and is more common in men than it is in women. Approximately 50 percent of men and 39 percent of women of the student population in US are involved in binge drinking **(**Lopez-Caneda *et al., 2013).* Racial differences exist among binge drinking with Hispanics been the highest followed by Caucasians having the highest level of binge drinking. Caucasians have been found to be nearly twice likely to binge drinking than blacks (Davies *et al.*, 2013*).* Among the Australian youth population, 31% of males and 14% of women aged 15–17 years engage in risky alcohol consumption (Howat *et al.*, 2013).

Seminiferous tubules in alcoholics mostly contain degenerated spermatids with a consequent azoospermia has been reported in previous studies. These effects may be due to alteration of the endocrine system controlling the hypothalamic-pituitary-testicular (HPT) axis function and/or to a direct effect on testis and/or male accessory glands. Experimental evidence from previous studies suggests that alcohol is a Leydig cell toxin, (Fraser *et al.*,

2006) although dose dependent effects of alcohol on human spermatogenesis are not well known. Researchers has equally confirmed that alcohol significantly reduced sperm concentration and motility in experimental rats compared with control rats and that pre-treatment with ASA was not able to revert these effects (Dare *et al.*, 2002). Furthermore, Talebi *et al.*, (2011) evaluated the effect of ethanol consumption on sperm parameters and chromatin integrity of spermatozoa aspirated from the epididymalcauda of rats allowed to drink ad libitum ethanol compared to control rats and found testicular abnormalities. Also Yen *et al.*, (1991) proposed that alcohol may alter gonadotropin-releasing hormone receptor function at the pituitary levels or the interaction of these receptors with gonadotropin-releasing hormone, result in a diminished LH release and also interfere negatively with the LH biological activity contributing testicular dysfunction**.** Furthermore, the increased b-endorphin levels has observed in acute or chronic alcohol consumption which may contribute to testicular damage by suppresses testicular testosterone production and release (Gianoulakis *et al.*, 1990).

Testicular atrophy is caused by several factors, including alcohol's damaging effects on the testis; alcohol's effects on LH and FSH among other factors, stimulate testicular growth; and various confounding factors, such as malnutrition, concomitant treatment with various medications, and abuse of drugs other than alcohol by the subjects. Testicular atrophy results primarily from the loss of sperm cells and decreased diameter of the seminiferous tubules. Subsequent studies have confirmed alcohol's deleterious effects on the testosterone-producing Leydig cells, the Sertoli cells, and even on the off spring of alcohol-ingesting males independent of co-occurring liver disease or malnutrition. Alcohol's adverse effects on Leydig cell function and testosterone production has been demonstrated in young, healthy male volunteers with normal liver function who received alcohol over a 4-week period (Gordon *et al*., 1976).

MATERIALS AND METHODS

YOYO® bitters cleanser is a product of Abllat Company Nigeria Limited, Ikeja Lagos state. It was purchased from a major pharmacy shop in Bayelsa State, Nigeria with NAFDAC registration number: A7-1055L, manufactured date- June 2014; and batch number: RICOQO14. These details were checked to ensure the authenticity of the products purchased same for ethanol

Experimental animals care and design

Twenty four healthy Wistar rats of average weight (216.2±12.55-220.3±17.00g) were purchased from the Animal house of the Department of Pharmacology, Faculty of Basic Medical Sciences, Niger Delta University Wilberforce Island Bayelsa State. The animals were all allowed to acclimatize for two weeks and randomly divided into three (3) groups. The rats were housed in cages made of metal and metal nesting and were fed ad libitum with rat pellet and clean tap water with 12hours light and dark cycle. The cages were cleansed every morning and disinfected at intervals of three days. The rats were allowed to acclimatize for 14 days before drug administration. Calculated doses of YOYO ® bitters and alcohol in mL kg-^{1}b.wt as instructed by manufactures pamphlet and previous work on binge alcoholism and were administered as follows Group A=Control received 1.5ml of vita-milk and water per mL kg-^{1}b.wt., Group B = received 1.5ml of 30% alcoholic vita- milk per mL kg-^{1}b.wt.. Group C = received 1.5ml of 30% alcoholic vita- milk and 0.1ml of YOYO® bitters per mL kg-^{1}b.wt. Administration was by oral route, three times daily using oral gastric tube for 4days. The study was conducted in accordance with the institute of health guide for the care and use of laboratory animals (NIH, 1985). The rats were anaesthetized using light dose of diethyl -ether prior to sacrifice and blood samples collected by cardiac puncture using syringe and needle. Aliquot sample of blood samples were transferred immediately into ethylene diaminetetra acetic acid (EDTA) tube for complete blood count and some into plain containers for biochemical analysis in accordance with standard practice. The biochemical analysis was carried out using Selectra PRO-s, Chemistry system, Elitech Clinical System, AKA, Elitech GRP, USA. The testicular tissue samples were removed and fixed in 10 % formalin, processed and sectioned at 4µm using Rotary microtome (Leica RM 2125) The tissue sections were then stained with Erhlich's heamatoxylin and eosin method for microscopy described by Avwioro (2010). Microscopy was carried out using a binocular light microscope at x400 while **a** complete blood count analyzer was use for hematology studies.

Statistical analyses was performed using Graph Pad Prism software. All values were expressed as the Mean±SD, Mean±SEM, and $P<0.05$ was considered to be statistically significant.

RESULT

Table 1 shows the mean weight of adult wistar rat pre and post drug administration. There was a statistically significant reduction in weight among rats induced with binge alcoholism-group B (239.6±12.76) compared with control-group A(237.4±12.10) and no difference in group with YOYO$^{(R)}$ bitters.Table 2 shows the effect of alcohol alone and alcoholic paired YOYO® bitters on some serum electrolytes, total protien, urea and albumin. expressed. Result shows reduction in calcium ion(Ca2+) in group C administered with alcoholic paired YOYO® bitters (0.40±0.1(52.7)g compared with the groupA – control(0.87±0.1(24.5)g. Also the total calcium (Tca2+) was reduced in group C (0.80±00(49.8) and was statistically significant at $p<0.05$ compared with the control(1.69±0.2(19.6). Total protiens was increased in group C (81.00±0.4(1.0) while chloride ion(cl-)was stastically significantly increased in group C (114.7±6.5(11.4) compared with control(103±1.4(2.7). Table 3 shows the complete blood count of wistar rat

post alcohol, and alcoholic-YOYO® bitters administration compared with the control. There exist no statistically significant difference in packed cell volume (%PCV), Platelets count, Haemoglobin(g/dl), red blood cell count(RBC), mean cell haemoglobin concentration(MCHC), white blood cells count(WBC), lymphocytes, neutrophils and other granulocytes among the groups compared with control. The exist a statistically significant reduction in mean cell volume amongst the groups compared with control.

Photomicrographs of the testis

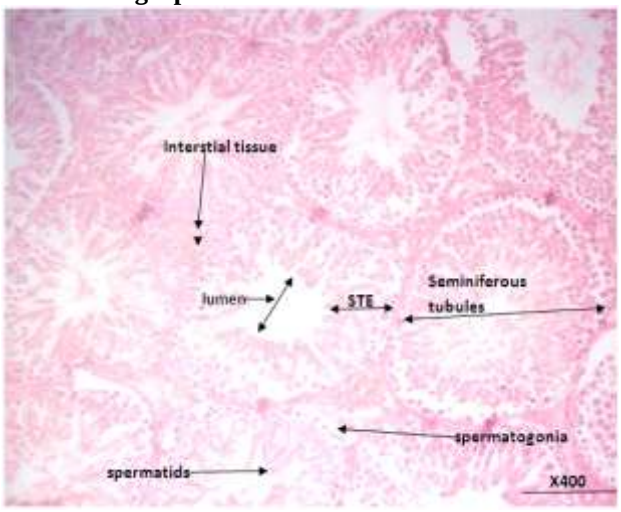

Figure 1: Group A shows normal histomorphology of the testis (following 1.5ml of vita-milk kg. body weight and water three times daily for four days) showing Seminiferous tubules containing different types of germ cells; spermatogonia Type A lying on the Basement Membrane beneath which is the myofibroblasts, Spermatogonia type B, spermatocytes, Spermatids, Spermatozoa and Somatic Sertoli Cells. The interstitial tissues found between seminiferous tubules contain interstitial cells and Leydig Cells.

Figure 2: Group B show a testis of binge alcohol drinking induced Wistar rat (1.5ml of 30% alcoholic vita-milk) kg-1-b.wt three times daily for 4days without feed. Histomorphology reveals exudation and necrosis of interstitial tissues and slight degeneration of germ cells lining seminiferous tubules, desquamation of germ cells and degeneration of Leydig cells.

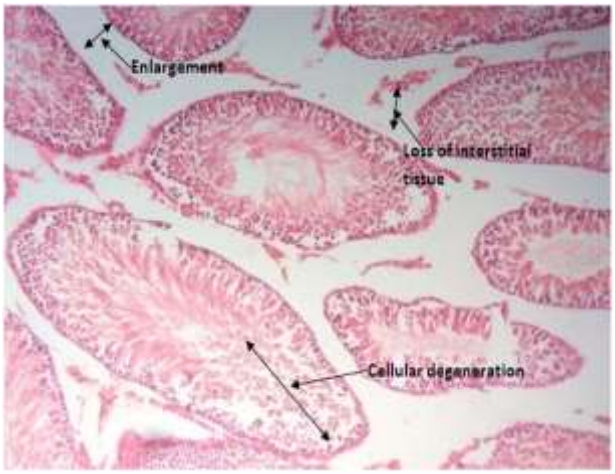

Figure 3: Group C show a testis of Wistar rat exposed to1.5ml of 30% alcoholic vita- milk and 0.1ml of YOYO® bitters per kg-1b.wt three times daily for 4days without feed. Histomorphology reveals loss of interstitial tissues and enlargement of seminiferous tubules amidst anisocytosis compared with normal.

Table 1: Mean weight of animals (g) before and after drug administration.

Groups	Initial Weight(g)	Final Weight(g)	X^2	P. value(0.05)
A	246.2±12.55	246.5±12.97	0.02	ns
B	267.4±12.10	239.6±12.76	17.5.	s
C	272.6±12.32	254.3±11.68	0.32	ns

Values were expressed as mean ± SEM.
Group a=control (1.5ml of vita-milk and water per kg-^{1}b.wt).
Group b = (1.5ml of 30% alcoholic vita- milk per kg-^{1}b.wt).
Group c = (1.5ml of 30% alcoholic vita- milk and 0.1ml of yoyo ® bitters cleanser per kg-^{1}b.wt).

Table 2: Effect of alcoholic-yoyo (R) bitters cleanser on electrolytes, urea and proteins of adult Wistar rat.

Parameters	GROUP A	GROUP B	GROUP C	P.0.05 X2
Ka+	12.18±1.1(18.19)	13.4±1.5(23.5)	14.01±1.1(16.17)	0.95 0.002
Na+	149.9±2.8(3.74)	144.1±2.3 (3.28)	150.80.4 (0.65)	0.82 0.87
Cl-	103.1±1.4 (2.7)	102.0±2.4 (2.7)	114.7±6.5 (11.4)	0.16 1.93
Ca2+	0.87±0.1 (24.5)	0.60±0.1 (26.1)	0.40±0.1 (52.7)	0.6 0.26
T Ca2+	1.69±0.2 (19.6)	1.18±0.2 (30.4)	0.80±0.1 (49.8)	0.02 5.3*
Urea	6.02±0.1 (4.3)	4.80±0.3 (14.52)	5.45±0.0 (2.37)	0.84 0.037
T. Protein	78.50±2.9 (7.3)	70.75±30.4 (1.3)	81.00±0.4 (1.0)	0.95 0.002
Albumin	43.5o±0.6 (2.9)	40.75±0.4 (2.3)	42.25±0.4 (2.2)	0.91 0.01

Values were expressed as Mean ± SD (95% Confident limit).
Group A=control (1.5ml of vita-milk and water per kg-^{1}b.wt).
Group B = (1.5mLof 30% alcoholic vita- milk per kg-^{1}b.wt).
Group C = (1.5mL of 30% alcoholic vita- milk and 0.1mL of YOYO ® bitters cleanser per kg-^{1}b.wt).

Table 3: Effect of alcoholic-YOYO ® bitters cleanser on hematology parameters of adult wistar.

Param.	Group A	Group B	Group C	P. value (<0.05)
HB	12 ±2.2 (8.9-16)	13±1.2 (11-15)	13±2.0 (11-15)	ns
PCV	41±8.2 (28-54)	43±3.4 (38-48)	42±8.2 (32-52)	ns
WBC	17±4.5 (9.5-24)	13±2.7 (84-17)	12±3.2 (8.3-16)	ns
PLTS	865±51(784-946)	684±264(267-1101)	804±216(480-1129)	ns
RBC	6.9±1.4(4.6-9.1)	7.4±0.74 (6.2-8.6)	7.1±1.2 (5.7-8.6)	ns
MCV	61±2.5 (57-65)	59±2.2 (55-62)	59±1.7 (57-61)	s
MCH	18±0.62 (17-19)	18±1.2 (16-20)	18±0.35 (29-32)	ns
MCHC	30±1.7 (27-33)	31±1.3 (28-33)	31±1.3 (29-32)	ns
LYMP	53±16 (27-78)	67±6.1 (57-77)	65±8.4 (53-76)	ns
NEU	16±15 (-7-39)	13±9.6 (-2.0-29)	7.2±2.2 (4.5-9.9)	ns
MXD	31±13 (11-52)	20±8.6 (5.8-33)	28±8.5 (17-38)	ns

Values were expressed as Mean ± SEM (%Coefficient of variation).
Group A=control (1.5ml of vita-milk and water per kg-^{1}b.wt).
Group B = (1.5mLof 30% alcoholic vita- milk per kg-^{1}b.wt).
Group C = (1.5mL of 30% alcoholic vita- milk and 0.1mL of YOYO ® bitters per kg-^{1}b.wt.

DISCUSSION AND CONCLUSION

YOYO® bitters cleanser is one among the class of bitters produced by Abllat Nigeria Company Limited, an indigenous health care product provider. It is a botanical medicine launched into the Nigerian market in the year 2003. Since its introduction into the Nigerian drug market as prescription and natural remedies, it has been commonly used for cure of indigestion and other stomach ailments and for treatment of various diseases. The product has received wide acceptance and usage by the general populace in Nigeria (Ganong, 2003).The drug is certified by National Agency for Food, Drugs and Control (NAFDAC) as the first real bitters without alcohol, coloring or artificial preservatives produced in Nigeria. Alcoholics in the study area prefer taking it in combination with alcohol claiming to enhance sexual performance and a lot more. Although it has been well established that spermatogenic cells undergo apoptosis when treated with ethanol, the mechanisms is not clear (Klonoff-Cohen 2003). In this present study, adult male Wistar rats were given ethanol orally at a dose of 1.5ml (30% v/v) per kg body weight three times per day for 4 days without feed rather 4.5ml of vita milk supplemented as energy boaster to induced binge alcohol drinking. Testicular and germ cell morphology was evaluated and results revealed exudation and necrosis of interstitial tissues and slight degeneration of germ cells lining the seminiferous tubules, desquamation of germ cells and degeneration of Leydig cells. This result is similar previous studies carried out on C57B1 mice to evaluate the effects of ethanol on the testicular function and its reversal following alcohol withdrawal, resulted in significantly decreased in testicular and seminal vesicles, increased frequencies of germ cell desquamation, inactive seminiferous tubules spermatozoa with abnormal morphology as compared with their respective pair-fed control values (Abel, 1987). In another study by Kuller et al., (1978) that evaluated testicular and liver pathology and related the findings with the estimated alcohol consumption among men who had died suddenly from a variety of causes and reported a moderate to severe decrease in spermatogenesis and fatty infiltration of the liver and further suggested that testicular spermatogenesis seem to be more sensitive to alcohol than liver tissue. A subsequent prospective autopsy study further explored the relationship between alcohol consumption, spermatogenesis and morphometric analysis of the human testis and reported a toxic effect on testis morphology (Pajarinen and Karhunen, 1994). YOYO® bitters cleanser is well formulated to reduce

free radical damage and removal of harmful toxins in the body, thereby supporting the immune system to fight disease. However excessive reactive oxygen species (ROS) production that exceeds critical levels can over whelm antioxidants defense strategies of spermatozoa and seminal plasma causing oxidative stress that damages the biological membranes in the testes (Salisu, 2012). Also spermatozoa are highly susceptible to damage by excessive concentrations of ROS due to the high content of polyunsaturated fatty acids within their plasma membrane (Duru *et al*., 2000, Najafi *et al*., 2010, Khaik *et al*., 2009, Sharma and Agarwal, 1996). Elevation of the ROS concentration lead to remarkable increasing in lipid peroxidation which destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility (Bustos-Obregon, 2005).

In the present study YOYO® bitters was taken as alcoholic paired YOYO ® bitters commonly refers to as combined and histomorphology revealed loss of interstitial tissues, enlargement and seminiferous tubules anisocytosis in Group C Wistar rat exposed to1.5ml of 30% alcoholic vita- milk and 0.1ml of YOYO® bitters cleanser per kg-^{1}b.wt three times daily for 4days without feed as compared with the control. The present work is in agreement with the works of Salisu, (2012) who reported histological changes in the testis of rats treated with Alomo bitter, an alcoholic based bitter. Hematological parameter result obtained showed that binge alcoholism and alcoholic paired YOYO® bitters groups has no effect on granulocytes, a granulocytes and packed cell volume except on red cell indices basically mean cell volume(MCV) of the rats as compared with normal. The presence of both testosterone and Follicle-Stimulating Hormone (FSH) is needed to support spermatogenesis (Michael and Wojciech, 2006).The testes contain germ cells that differentiate into mature spermatozoa, supporting cells called Sertoli cells, and testosterone-producing cells called Leydig (interstitial) cells. In the present study binge alcoholism alone and in combination with YOYO® bitters cleansers is seen to decrease circulating blood testosterone level implicating a negative effect on spermatogenesis as seen in the histology of the testis. Other biochemical variable affected were total calcium(TCa^{2+}) , calcium ion (Ca^{2+}), and total protein while electrolytes, urea and albumin not affected supported evidence of reduction in bone mass among alcoholics.

CONCLUSION

Testis is a pair of male gonads and is responsible for making testosterone, the primary male sex hormone, and for generating sperm cells needed for conception. This comprehensive study showed that binge alcoholism alone and in combination with YOYO ® bitters alters testicular function and morphology. And we therefore advice the public and alcoholics taking YOYO ® bitters for treatment of other ailments not investigated in the present work should avoid its combination to reduce male infertility and testicular atrophy.

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