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Evaluation of Ogogoro-Induced Testicular Toxicity in Adult Male Wistar rats.

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ABSTRACT

Alcohol has been implicated in the aetiology of male infertility. Ogogoro, a locally brewed alcohol, is processed by distilling fermented sap of Raphia palms (*Raphia hookeri*) or coconut palm (*cocus nucifera*). The aim of this study was to evaluate Ogogoro induced testicular toxicity in adult male Wistar rats. Twenty (20) adult male Wistar rats were assigned into four groups (n=5) each. Group I (control) was administered with normal saline 1ml/kg, groups II-IV: Ogogoro 3.5ml/kg, 7ml/kg, and 14ml/kg respectively orally for eight weeks. Following animal sacrifice, blood samples, seminal fluid and testes were used for assay of reproductive hormones, seminal analysis, and testicular histology and oxidative stress biomarkers respectively. Serum testosterone and luteinizing hormone (LH) in groups III and IV were significantly ($p<0.05$) lower compared to the control. A significant decrease ($p< 0.05$) in sperm motility and increased number of dead spermatozoa in group IV compared to control. Testicular weight and gonadosomatic index were significantly reduced in group IV rats. Testicular malondialdehyde (MDA) level in groups III and IV was significantly (<0.05) higher compared to the control. Testicular superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) activities in groups III and IV were significantly lower compared to the control. Group IV testes showed irregular seminiferous tubules with epithelial sloughing, cellular degeneration and fibrohyalinisation of testicular parenchyma. Ogogoro causes testicular toxicity via oxidative stress induction, direct testicular atrophy and hormonal dysregulation which could adversely impact male reproduction.

Keywords: Ogogoro, Testicular toxicity, Reproduction, Oxidative stress, lipid peroxidation

INTRODUCTION

Alcohol consumption is common to many societies all over the world, with 60% of the population over 15 years identified to have taken alcoholic drinks at least once, and about 15 million having developed alcohol abuse disorder in the United States [1,2]. Alcohol is made up of hydroxyl compounds of branched or straight chain hydrocarbons, with ethanol being both water and lipids soluble, it is highly permeable to all tissues of the body, thereby affecting vital organs including the reproductive system [3,4].

Excessive consumption of alcoholic beverages are associated with numerous disorders such as withdrawal syndromes characterized by sudden change in mood, irritability, agitation and anxiety, sleep disturbances, anhedonia and increased pain sensitivity. Also, social ill behavior and clinical conditions such as congenital abnormalities, cancer, cardiovascular disorders, liver diseases, mental illness and consequent increase in morbidity and mortality have been associated with alcohol abuse [5,6]. Chronic alcohol consumption affects the male reproductive system - the hypothalamus, the pituitary gland and the testes. The testes produce sperm and vital hormones that regulate male sexual characteristics and behaviour, notably among these hormones is testosterone [7,8]. Studies have demonstrated that alcohol abuse can cause impaired follicle stimulating hormone, luteinizing hormone and testosterone production and testicular atrophy, decrease in sperm concentration in cauda epididymis; decrease in motility, viability and density of sperm cells, and increased abnormal sperms in males. The consequent results of these changes are impotence, infertility, and impaired male secondary sexual characteristics [4,9,10,11]. It has been reported that about 15% of couples of reproductive age are infertile, of these approximately 50% have male related causes, for which 42% of them consume alcohol [12,13,14]. There is an increased metabolic demand in chronic alcoholics, which requires high oxygen consumption in the body; the free radicals produced in these processes are the main source of lipid peroxidation that results in cellular and tissue injury [15,16]. Alcohol induced toxicity occurs via different mechanisms which include: inflammation (release of excessive pro-inflammatory cytokines), apoptosis (increase expression of pro-apoptotic Bax and lower expression of anti-apoptotic Bcl-2), genotoxicity (high rate of DNA damage) and oxidative stress (high level of oxidative stress and reduced antioxidant capacity) [2, 16,17].

Alcoholic beverages have varying ethanolic contents which range from 20-78 % [18,19]. Ogogoro is prepared by distilling fermented sap of Raphia palms (*Raphia hookeri*) coconut palm (*cocus nucifera*) and oil palm (*Elaeis guinesis*) [18]. The physico-chemical property of ogogoro includes specific gravity (0.9897), PH (6.3), titratable acidity (0.8), total solid (1.2), high percentage of ethanol content (37.6-78%) and impurities such as lead [20]. Excessive consumption of the local gin (Ogogoro) increases the concentration of contaminants (impurities-present in the local gin) in the body provoking increased toxic and carcinogenic effects in addition to increased socio-political ills and vices [6,18]. Though local gin (Ogogoro) is widely consumed in various part of Nigeria, however, there is paucity of literature on its effects on male reproduction. The aim of this study was to examine the testicular toxicity associated with chronic administration of local gin (Ogogoro) in adult male Wistar rats.

MATERIALS AND METHODS

Equipment, Drugs and Reagent: The local gin: Ogogoro (with 32% ethanol content) made in Sapelle Delta state, Nigeria was purchased from the major distributor at Sabon Gari Zaria, Kaduna State, Nigeria; normal saline solution (0.9 %w/vol.), ketamine and diazepam (Fidson

pharmaceutical limited, India), vital feeds (Grand cereals limited, Jos, Plateau State); ELISA test kits (Wkea Med Supplies Corps, China), weighing balance, bench centrifuge and other routine laboratory materials used were of analytical grade.

Experimental Design and Animal Grouping: Twenty (20) adult male Wistar rats weighing about 150-200g were obtained from the National Veterinary Research Institute, Vom, Plateau State, Nigeria. Rats were acclimatized to the study environment for a week and were fed with compressed grower mash and given access to water ad libitum before the administration of Ogogoro commenced.

The study was conducted in the Animal House of the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, Ahmadu Bello University, Zaria, Nigeria. It was conducted following the guideline for use of animals in scientific research obtained from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC). The weights of the animals were estimated at procurement, during acclimatization, at commencement of the experiment and twice weekly throughout the duration of the experiment, using an electronic analytical precision balance [21].

The rats were randomly divided into four groups of five (5) animals each (n=5). A cannula was used to administer the local gin orally for eight (8) weeks.

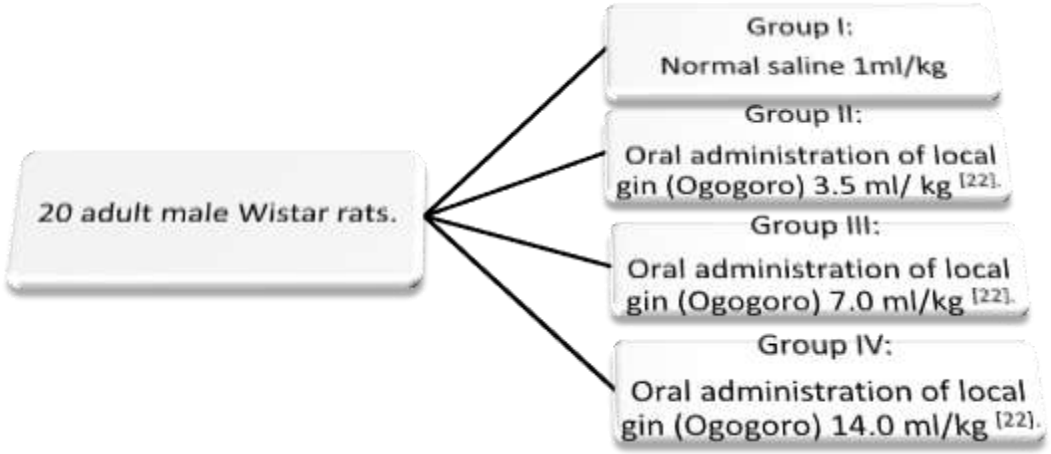

Figure 1: Experimental groupings

Animal Sacrifice and Sample Collection: At the end of the 8 weeks of administration, rats were weighed and anaesthetized using IM ketamine (10mg/kg) and diazepam (2mg/kg) [23]. Following anesthetic induction, the abdominal cavity was opened through a midline incision exposing the heart; 5mls of blood sample was collected through cardiac puncture, stored in plain sample bottles, centrifuged at 3000g and the serum used for hormonal assay.

The epididymis was carefully dissected from the testes, weighed after which seminal fluid was extracted for seminal analysis. Testicular tissues were harvested and weighed, one was homogenized in 7.4 pH phosphate buffer solution for biochemical assays, and the other fixed in 10% formol-saline for histological analysis [10].

Seminal Analysis - Estimation of sperm count: The sperm count was determined using the Neubauer improved

hemocytometer. Epididymal fluid ratio of 1:20 was prepared by adding 0.1 ml of fluid to 1.9 ml of water. The dilution was mixed thoroughly; the mixture was loaded into the counting chamber and mounted on the microscope and viewed at x 400 magnification. Spermatozoa within five of the red blood cell squares including those which lie across the outermost lines at the top and right sides were counted, while those at the bottom and left sides were left out. The number of spermatozoa counted was expressed in millions/ml [14,24].

Estimation of sperm motility: The cauda epididymis of the rats were incised and a drop of epididymal fluid placed on a glass slide, covered by a 22 x22 cover slip and examined under the light microscope at x 400 magnification while evaluating different microscopic field systematically and each spermatozoon encountered was assessed. In this study, motility was classified as motile, sluggish or non-motile (dead). The relative percentage of sperm motility was estimated and reported to the nearest 5% using the subjective determination of motility [14,24].

Estimation of sperm morphology: The sperm cells were evaluated with the aid of light microscope at x 400 magnification. Caudal epididymal sperm were taken and diluted 1:20 with 10% neutral buffered formalin. Abnormal sperm cells were classified using presence of one or more abnormal features, such as tail defects (short, irregularly coiled or multiple tails); neck and middle piece defects (absent, distended, irregular, bent middle piece, abnormally thin middle piece); and head defects (round head, small or large size, double or detached head). Findings were expressed as a percentage of morphologically normal sperm [14, 25].

Gonadosomatic Index (GSI): Calculated using the formula: Weight of gonads X 100 /Body weight [26].

Hormonal Assay: Serum follicle stimulating hormone, luteinizing hormone and testosterone assay were carried out using appropriate rats ELISA kit (Wkea Med Supplies Corp, China). The procedures for the assay as contained in the manufacturer's manual were strictly followed.

Determination of the Testicular homogenate biomarkers of Oxidative Stress: Assay of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activity and lipid peroxidation (MDA) levels were determined using appropriate ELISA kits according to the manufacturer's manual (Wkea Med Supplies Corp, China).

Histological Analysis: The histological profile of the testes was determined in Histopathology Department, Ahmadu Bello University Taching Hospital, Zaria, Kaduna, Nigeria. The method of Hematoxylin and Eosin staining technique was used. Briefly, the tissue section was hydrated in descending grades of alcohol from 100%, 95%, 90% and 70% for three minutes in each step. Thereafter, tissues were washed in running tap water and stained with haematoxylin for twenty five (25) minutes, then washed with water and differentiated in acid alcohol. The tissues were counter stained with eosin blue in Scott water. The tissues were then hydrated in ascending grades of alcohol and cleared in xylene for three (3) changes in five (5) minutes each. The tissues were then mounted with cover slips using a mounting media; then viewed under a light microscope and the photomicrographs were taken [27].

Statistical Analysis: Data obtained were expressed as mean ± standard error of mean (SEM). The results were analyzed by one-way analysis of variance (ANOVA) using SPSS version 23 and appropriate post-hoc test used for multiple comparisons. $p < 0.05$ was taken as accepted level of significance.

RESULTS

Table 1: Sperm counts, motility and viability in adult male Wistar rats treated with Ogogoro.

Treatment Groups	Counts ($\times 10^6$/mL)	Motile (%)	Sluggish (%)	Dead (%)
Group I (Normal saline 2ml/kg)	64.00 ± 1.87	80.00 ± 2.7	13.60 ± 2.29	6.40 ± 1.12
Group II (Ogogoro 3.5ml/kg)	62.00± 1.76	75.00 ± 4.18	18.60 ± 3.60	6.40 ± 1.57
Group III (Ogogoro 7.0 ml/kg)	57.60 ± 1.44	36.80 ±4.92**	40.00 ±5.48	18.60±1.86**
Group IV (Ogogoro 14.0 ml/kg)	58.00± 1.89	38.00± 6.44**	45.00± 3.73**	22.00 ± 3.39**

**= within columns are statistically significant compared to control ($P < 0.01$).

Table 2: Sperm morphology (normal, abnormal head and abnormal tail) in adult male Wistar rats treated with Ogog.

Treatment Groups	Normal (%)	Abnormal head (%)	Abnormal tail (%)
Group I (Normal saline 2ml/kg)	76.00 ± 1.87	16.00 ± 1.87	7.00 ± 1.22
Group II (Ogogoro 3.5ml/kg)	75.00 ± 3.54	16.00 ± 1.87	8.00 ± 1.22
Group III (Ogogoro 7.0 ml/kg)	71.00 ± 1.87	19.00 ± 1.97	9.00 ± 1.87
Group IV (Ogogoro 14.0 ml/kg)	70.00 ± 1.22	23.00 ± 3.39	10.00 ± 1.58

Table 3: Body weight, Testicular weight and Gonadosomatic index (GSI) in adult male Wistar rats treated with Ogogoro.

Treatment groups	IBW(g)	FBW(g)	TW (g)	GSI
Group I(Normal saline1.0 ml/kg)	121.40 ± 2.44	137.60 ± 1.57	1.41 ± 0.17	0.83±0.01
Group II (Ogogoro 3.5ml/kg)	144.00 ± 2.21	129.80 ± 2.71	1.17 ± 0.07	0.81± 0.01
Group III(Ogogoro 7.0 ml/kg)	164.60 ± 2.14	144.40± 3.02	1.12 ± 0.06	0.78 ±0.02
Group IV(Ogogoro 14.0 ml/kg)	192.00 ±4.70	151.80 ± 3.02	0.79 ± 0.13*	0.56±0.02*

*= statistically significant ($P < 0.05$) compared to control within columns.
IBW=Initial body weight, FBW=Final body weight, TW=Testicular weight, GSI=Gonadosomatic index

Table 4: Serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone concentrations in adult male Wistar rats treated with Ogogoro

Treatment Groups	FSH (ng/ml)	LH (ng/ml)	Testosterone (ng/ml)
Control (1ml/kg normal saline)	8.22 ± 1.21	11.58 ± 1.69	7.14 ± 1.01
Ogogoro (3.5 ml/kg)	7.24 ± 0.66	9.64 ± 0.83	5.04 ± 0.23
Ogogoro (7 ml/kg)	6.22 ± 0.24	7.92 ± 0.36*	3.70 ± 0.26*
Ogogoro (14 ml/kg)	5.36 ± 0.50	7.38 ± 0.39*	3.70 ± 0.26*

*=statistically significant when compared to control

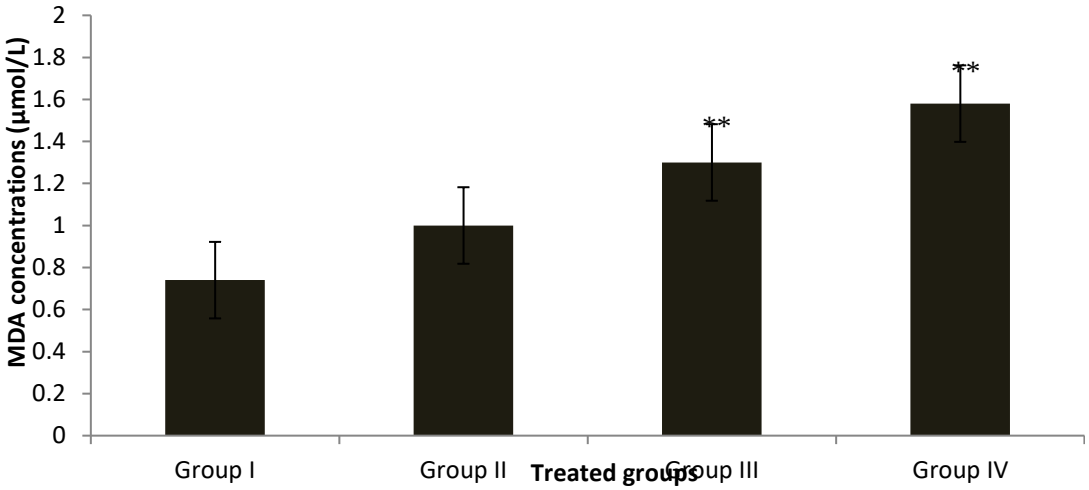

Figure 2: Testicular homogenate MDA level in rats administered with Ogogoro

**= statistically significant (P< 0.01) compared to control.

Table 5: Testicular homogenate level of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in adult male Wistar rats fed with Ogogoro.

Treatment Groups	SOD (IU/L)	GPx (IU/L)	CAT (IU/L)
Group I (Control)	1.96 ± 0.08	41.40 ± 0.75	45.00 ± 0.84
Group II (Ogogoro 3.5 ml/kg)	1.78 ± 0.04	37.60 ± 0.98	42.20 ± 0.66
Group III (Ogogoro 7.0 ml/kg)	1.60 ± 0.45*	35.80 ± 1.39*	38.40 ± 0.68*
Group IV (Ogogoro 14.0 ml/kg)	1.36 ± 0.05*	30.60 ± 0.40*	33.80 ± 0.58*

* = statistically significant ($P < 0.05$) compared to control.

HISTOLOGY OF THE TESTES

The testicular histological profile of Ogogoro treated rats as shown in plates I-IV

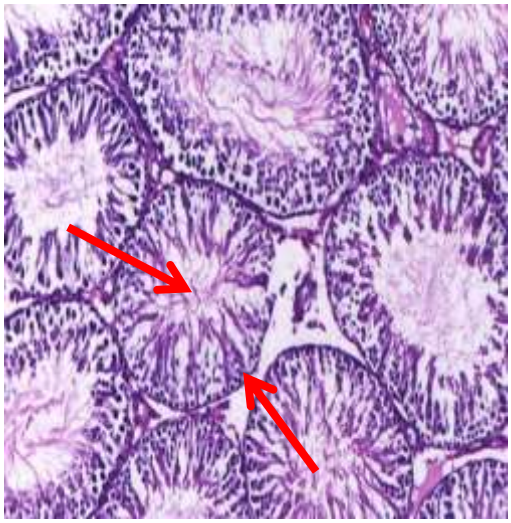

Plate I: Photomicrograph of group I (Control) rats, showing normal seminiferous tubules containing spermatogenic cells (black arrows) and interstitial cells of Leydig (blue block arrow). H&E stain, x 200.

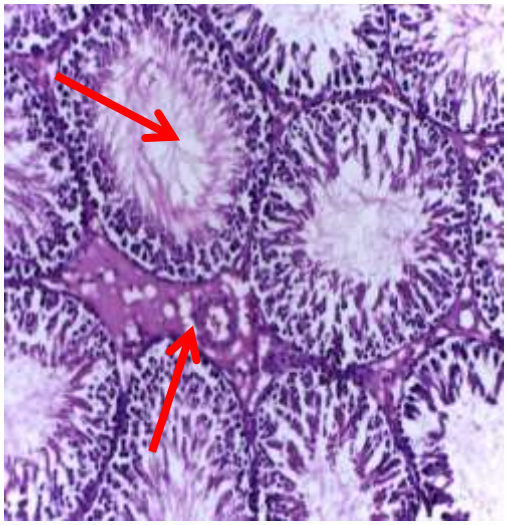

Plate II: Photomicrograph of group II (Ogogoro 3.5 ml/kg) rats, showing normal tubules (black arrows) and interstitium (blue block arrow). H and E stain, (x 200).

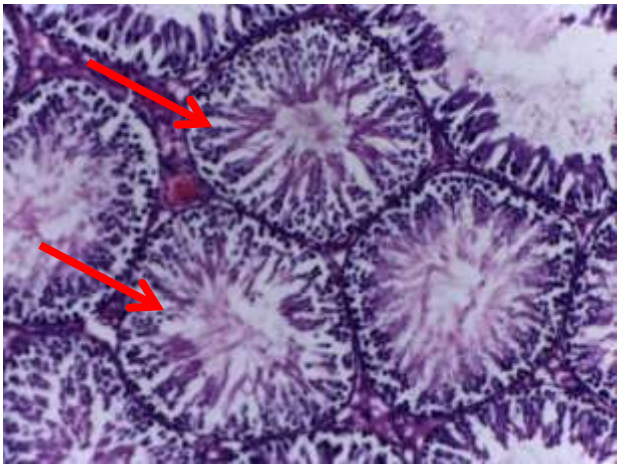

Plate III: Photomicrograph of group III (Ogogoro 7.0ml/kg) rats showing normal seminiferous tubules (black arrows) and interstitium (block blue arrow). H&E stain, x200.

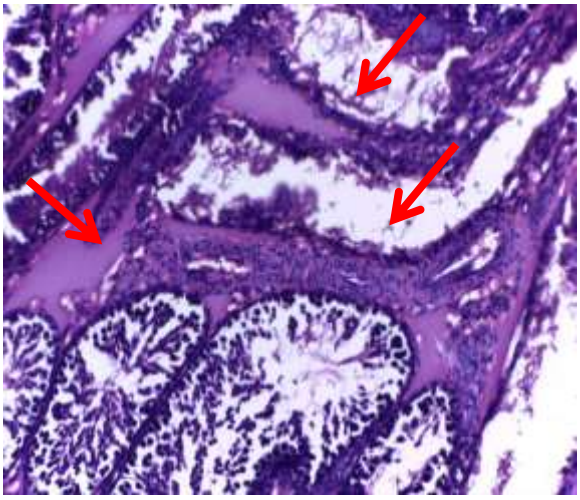

Plate IV: Photomicrograph of group IV (14.0 ml/kg) rats, showing areas of fibrohyalinization of testicular parenchyma (black arrows) and abnormal tubules devoid of spermatogenic cells (blue block arrows). H&E stain, x200.

DISCUSSION

Sperm vitality measurement is one of the basic elements of semen analysis [14]. Motility is indispensable for the spermatozoa, as it travels the female reproductive tract to reach the site of fertilization [13]. This study revealed a significant dose-dependent reduction (doses 7ml/kg and 14ml/kg) in the percentage of motile, sluggish, and dead sperms when compared to control. Chronic oral administration of alcohol has been found to significantly decrease spermatozoa motility and sperm concentration in adult rats [10,28,29,30] and in humans [31,32]. In the pathogenesis, alcohol causes testicular injury through direct effect on the spermatozoa, formation of sperm DNA adducts and increased lipid peroxidation of the spermatozoa lipid rich plasma membrane resulting in reduced motility and sperm concentration as seen in our study [2,33,34].

This study showed a significant reduction in mean testicular weight and gonadosomatic index in group IV experimental rats compared to control rats. This observation is similar to that made for adult rats exposed to alcohol, indicating the tendency of Ogogoro at high doses to cause testicular atrophy and infertility suggesting testicular toxicity [11,28,35].

From the present study, there was a significant decrease in serum luteinizing hormone and testosterone levels. Several studies have demonstrated that alcohol use can cause impaired testosterone production and shrinkage of the testes [9,28,36]. The hormonal alteration could possibly be as a result of an impairment of spermatogenesis in experimental rats, evidenced by the spermatogenic cell loss or damage to Sertoli cells, resulting in alteration of the feedback regulation of luteinizing hormone secretion from the pituitary gland. The reduction in serum testosterone could have been due to oxidative damage to leydig cells thus resulting in decreased testosterone levels overtime as shown in our result. This result is in conformity with those of previous studies [10,28], that reported low levels of testosterone and luteinizing hormone following 8 weeks of oral alcohol administration. However, it is contrary to the findings of Priya et al., [11] who reported increased levels of FSH and LH in rats exposed to alcohol indicating an intact hypothalamo- pituitary–testicular axis. A recent review showed that alcohol directly inhibit the hypothalamic production of GnRH with onward reduction in the levels of LH and testosterone, possibly through distortion in the nitric oxide (NO) mechanism, reduction in NAD+/NADH levels or inhibition of enzymes involved in testosterone synthesis [2].

A significant increase in testicular homogenate MDA level in the groups III and IV compared to the control indicating increased lipid peroxidation. The increase in the level of MDA observed in the ogogoro treated groups III and IV may be linked to the generation of free radicals, lipid peroxidation of membrane. This result is in concert to the result of Renate *et al*., [37] who suggested that free radical generation and lipid peroxidation might be an important mechanism in the toxicity of ethanol within the testes. More so, the major pathway for alcohol metabolism involves the enzyme alcohol dehydrogenase; which metabolizes alcohol into toxic acetaldehyde, whose interaction with cell proteins and lipids can result in free radical generation and cellular damage which is common in testicular tissue rich in polyunsaturated lipid plasma membrane [15,16,38]. This study revealed a significant decrease in the levels of testicular super oxide dismutase (SOD), Glutathione peroxidase (GPX) and Catalase (CAT) activities in the group treated with 7.0ml/kg and 14.0ml/kg ogogoro compared to the control. The decrease in the level of endogenous antioxidant enzymes obtained from this study could be due to their utilization to restore balance between

oxidants and anti-oxidants. Alcohol metabolism result in increasing production of reactive oxygen species (ROS) as evident by increased MDA; this causes an intracellular redox imbalance and reducing the concentration of antioxidants [16,39]. This is in accordance with Oremosu and Akang, [10] and Taati *et al*., [40] who reported a decrease in the SOD concentration and glutathione peroxidase respectively in alcohol treated rats.

Histological slides of groups I-III (plates I-III) showed testes with normal seminiferous tubule, parenchyma and interstitial cells of leydigs. However, group IV (plate IV) showed irregular seminiferous tubules with epithelial sloughing, cellular degeneration and fibrohyalinization of testicular parenchyma and abnormal tubules devoid of spermatogenic cells. Germ cells showed disorganized maturation, and nonattachment to the epithelium. Interstitium showed edema and vacuolization and presence of multinucleated giant cells and alteration in the interstitial cells of Leydig evidenced by fibrosis, which further confirmed that Ogogoro has effect on the Leydig cells and seminiferous epithelium. Leydig cells in the interstitium secrete "testosterone", required for spermatogenesis. The observed alteration in the interstitium is an indication that the cells may be affected thus ultimately affecting spermatogenesis. This is in agreement with the previous work on the effect of alcohol on Leydig cells of the testes in adult rats, which revealed that alcohol caused deleterious histological changes in the seminiferous tubules resulting in suppression of spermatogenesis [11,33,34,35,41,42]. Free radicals (ROS) attack germ cells in the seminiferous tubules causing extensive necrosis and disruption of spermatogenesis resulting infertility [43,44]. Thus, the histological changes seen in this study could also be due to the oxidative stress produced by the ogogoro administration evidenced by significant lipid peroxidation and decreased endogenous antioxidants.

CONCLUSION

In conclusion, oral administration of Ogogoro at doses 7.0ml/kg and 14.0ml/kg have been found to cause a significant change in sperm viability and motility, decreased testicular weight and gonadosomatic index (GSI), with increased number of abnormal spermatozoa; significant decrease in testosterone and luteinizing hormone. Also, this study observed an increase in lipid peroxidation and decreased endogenous antioxidants (SOD, GPx and CAT) activity in the testicular tissue of the treated rats; and fibro-hyalinization of testicular parenchyma, thus confirming the tendency of Ogogoro to cause testicular toxicity and impede fertility in male Wistar rats.

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CONFLICT OF INTEREST

All the authors of this manuscript declare no conflict of interest.

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