

Lopinavir/ Ritonavir, an Antiretroviral Drug, Lowers Sperm Quality and Induces Testicular Oxidative Damage in Rats

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Background: Lopinavir/Ritonavir (Kaletra[®]) is a protease inhibitor used in the management of HIV infection. The increased incidence of toxicity of antiretroviral therapy (ART) has necessitated proper evaluation of their effects on reproductive health.

Purpose: Therefore, this study was designed to investigate the effects of Kaletra[®] on male reproductive system in Wistar rat.

Methods: Eighteen rats were assigned into three groups. The first group served as control while the second and third groups received Kaletra[®] at therapeutic dose (8.3 mg/kg) (Kaletra-T) and twice therapeutic dose (16.6 mg/kg) (Kaletra-2T). Kaletra[®] was given orally for 21 days.

Results: Administration of Kaletra[®] caused a significant ($p = 0.023$) decrease in body weight-gain of rats. Precisely, Kaletra-T and Kaletra-2T decreased body weight-gain by 43% and 48%, respectively. Kaletra-T and Kaletra-2T significantly ($p = 0.016-0.036$) decreased sperm motility and sperm count while Kaletra-2T increased total sperm abnormalities in the rats. Also, Kaletra[®] (at the two doses) caused a significant ($p = 0.02-0.04$) increase in the levels of testicular lipid peroxidation with a concomitant decrease in antioxidant indices. Specifically, Kaletra-T and Kaletra-2T decreased the activities of glutathione peroxidase by 38% and 57%, catalase by 40% and 48%, glutathione-s-transferase by 32% and 35% and superoxide dismutase by 47% and 52%, respectively while Kaletra-2T decreased reduced glutathione by 49%. Photomicrographs of testis from control and Kaletra-T groups showed normal seminiferous tubules with abundant spermatogenic cells while Kaletra-2T group had few and abnormal shape spermatogenic cells.

Conclusion: Kaletra[®] induces oxidative damage in testis of rats leading to changes in sperm characteristics and antioxidant status of the animals.

Key words: Lopinavir/Ritonavir, Kaletra[®], Sperm characteristics, Antioxidant enzymes, Testis

INTRODUCTION

Antiretroviral therapy (ART) is a treatment that suppresses replication of human immunodeficiency virus (HIV) among other retroviruses, and consequently stops progression of HIV infection to disease state referred to as acquired immune deficiency syndrome (AIDS). Different ART regimens have evolved over time from monotherapy to combination triple-drug therapy usually referred to as highly active antiretroviral therapy (HAART). Different classes of these antiretroviral drugs act on specific stages of the HIV replication cycle. With the advent of antiretrovirals and sustained major declines in opportunistic complications, HIV infection is increasingly a more chronic disease, and so more drugs are being used among many patients for longer periods. However, permanent and near-perfect adherence to antiretroviral therapy is needed to maximize its long-term benefits. On the other hand, the adverse effects of ART cause substantial morbidity and compromise adherence, which can lead to drug resistance [1]. The toxicity of antiretrovirals is an increasingly important issue in the management of patients with HIV [2], and study on the toxicity of

antiretrovirals is still an active area of research.

Lopinavir/ Ritonavir (Kaletra[®]) (Fig. 1), an antiretroviral drug, is a fixed dose combination drug for the treatment of HIV infection. It is a co-formulation of lopinavir and a sub-therapeutic dose of ritonavir, both of which belong to the class of antiretroviral drug known as protease inhibitors [3]. During replication, viral RNA is translated into a long polypeptide sequence comprising of several individual proteins. The viral protease cleaves the viral polypeptide into functional enzymes. However, protease inhibitors interfere with the function of the protease and consequently the production of new viruses. These protease inhibitors, especially lopinavir/ ritonavir, have been associated with several adverse effects such as diarrhea, nausea, hyperlipidemia, hyperglycemia, hyperbilirubinemia, insomnia, fatigue, e.t.c [4, 5]. However, due to the increased incidence of toxicity in ART, it is highly important to pay attention to their probable effects on reproductive health. Moreso, the perception of HIV has changed from an incurable deadly disease to a chronic manageable illness such that HIV-infected individuals would have to be on ART for longer years. In light of this development, the current study was designed to

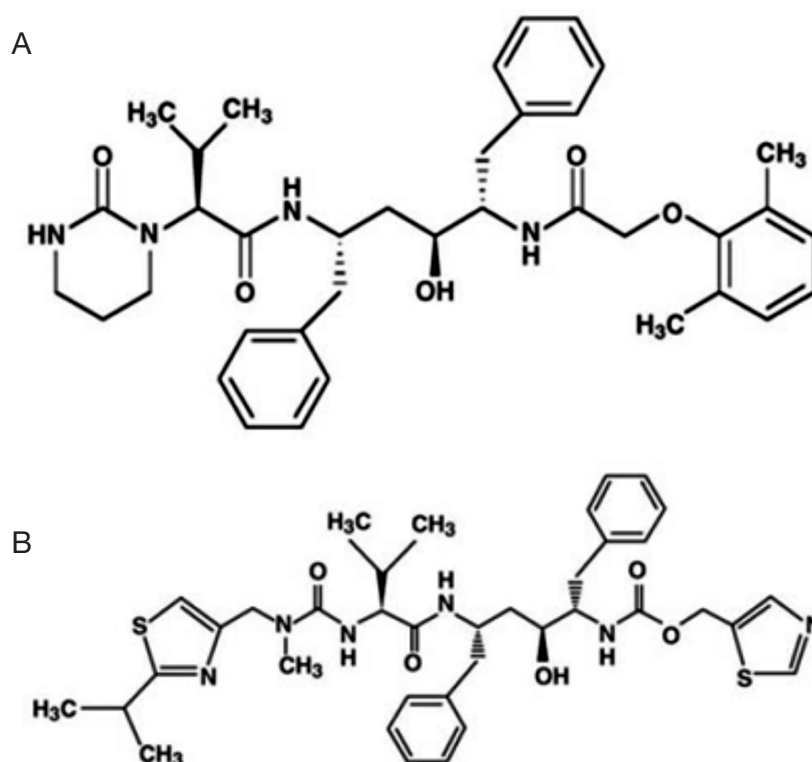


Fig. 1 Structure of kaletra[®] - 100 mg of **A** (Lopinavir, chemically designated as [1S-[1R*, (R*), 3R*, 4R*]-N-[4-[(2,6dimethylphenoxy)acetyl]amino]-3-hydroxy-5-phenyl-1-(phenylmethyl)pentyl]tetrahydro- α (1-methylethyl)-2-oxo-1(2H)-pyrimidineacetamide) and 25 mg of **B** (Ritonavir, chemically designated as 10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo-8, 11-bis(phenylmethyl)-2, 4, 7, 12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*, 8R*, 10R*, 11R*)]). (Chandwani and Shutter, 2008).

investigate the probable effects of lopinavir/ritonavir (Kaletra[®]) on sperm characteristics and testicular oxidant/antioxidant balance in male Wistar rats.

MATERIALS AND METHODS

Chemicals. Lopinavir/ Ritonavir (Kaletra[®]), manufactured by Abbott Laboratories (Abbott Park, IL, USA), was used for this study. The drug was obtained from PEPFAR (President's Emergency Plan for AIDS Relief) Clinic, University College Hospital, Ibadan, Nigeria. Glutathione, hydrogen peroxide, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and epinephrine were purchased from Sigma Chemical Co., Saint Louis, MO USA. Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from British Drug House (BDH) Chemical Ltd., Poole, UK. Other chemicals were of analytical grade and purest quality available.

Animals' protocol. Inbred male Wistar rats weighing between 180-185g were purchased from the central animal house located in the Department of Physiology, University of Ibadan, Nigeria. The animals were kept in well-ventilated cages at room temperature (28-30°C) and maintained on normal laboratory chow (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum*. Rats handling and treatments protocol conforms to the guidelines of Faculty of Basic Medical Sciences, University of Ibadan Animals' Ethical Committee as well as the National Institute of Health (NIH publication 85-23, 1985) for laboratory animals' care and use.

Experimental design. Eighteen male rats (Wistar strain) were randomly assigned into three groups of six

animals each. Animals were allowed to acclimatize for a period of one week before the experiment. The first group served as the control and was given corn oil (Vehicle for drug). The second group received Kaletra[®] at a dose of 8.3 mg/kg body weight (Kaletra-T) (Equivalent of human therapeutic dose) and the third group received Kaletra[®] at a dose of 16.6 mg/kg body weight (Kaletra-2T) (Equivalent of twice therapeutic dose for human). Kaletra[®] was given daily by oral gavage for 21 consecutive days.

Preparation of serum. Twenty-four hours after the last dose of the drug was administered, the animals were sacrificed after exposure to light ether anesthesia. Blood was collected from the animals into appropriately labeled plain tubes and allowed to stand for 1 hour before centrifuged at 3,000 g for 10 min in a bench centrifuge to obtain serum.

Preparation of tissue. The testes from the animals were quickly removed and washed in ice-cold 1.15% KCl solution to remove blood stain, dried and weighed. The tissue was homogenized in 4 volumes of 50 mM phosphate buffer, pH 7.4 and centrifuged at 10,000 g for 15 min to obtain post-mitochondrial fraction (PMF) of the testes, which was used for biochemical assay. The right testes from each group were fixed in Bouin's solution and used for histology.

Biochemical Assays. Protein levels in the serum and testis were assayed by the method of Lowry *et al.*, [6] using bovine serum albumin as standard. The activities of serum alanine and aspartate aminotransferases (ALT and AST) were assayed by the combined

Table 1 Effects of Lopinavir-Ritonavir (Kaletra^R) on body weight, weight of testis and relative weight of testis in male Wistar rats

Treatment	Weight (g)		Weight gain (g)	Weight of testis (g)	Relative weight of testis (% body weight)
	Initial	Final			
Control	184.00 ± 2.94	205.00 ± 1.40	21.00 ± 3.07	2.01 ± 0.14	0.98 ± 0.11
Kaletra -T	182.00 ± 1.30	194.00 ± 1.94	12.00 ± 2.94*	2.02 ± 0.26	1.04 ± 0.14
Kaletra-2T	180.00 ± 1.80	191.00 ± 2.14	11.00 ± 1.14*	1.98 ± 0.18	1.04 ± 0.10

Values are Mean ± SD of 6 animals per group

* Significantly different from control ($p < 0.05$)

Control = Animals treated with Corn Oil alone.

Kaletra-T = Animals treated with therapeutic dose of kaletra

Kaletra-2T = Animals treated with twice therapeutic dose of kaletra

methods of Mohun and Cook [7] and, Reitman and Frankel [8]. The estimation of serum alkaline phosphatase (ALP) activity was based on the method of King and Armstrong [9]. ALP activity was measured spectrophotometrically by monitoring the concentration of phenol formed when ALP reacts with disodium phenyl phosphate at 405 nm. Lipid peroxidation level was assayed by the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA), an end product of lipid peroxides as described by Buege and Aust [10]. PMF reduced glutathione (GSH) level was assayed by measuring the rate of formation of chromophoric product in a reaction between 5,5'-dinitrobis-2-nitrobenzoic acid (DTNB) and free sulphhydryl groups at 412 nm as described by Beutler *et al.* [11]. PMF superoxide dismutase (SOD) activity was measured by the method of McCord and Fridovich [12]. PMF catalase (CAT) activity was assayed spectrophotometrically by measuring the rate of decomposition of hydrogen peroxide at 570nm as described by Aebi [13], while glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities were determined by the methods of Habig *et al.* [14] and Rotruck *et al.* [15], respectively.

Determination of sperm characteristics. The caudal epididymis was minced in pre-warmed normal saline (37°C). One drop of sperm suspension was placed on a glass slide to analyze 200 motile sperm in 4 different fields. The motility of the epididymal sperm was evaluated microscopically within 2-4 minutes of their isolation from the epididymis, and data were expressed as percentage motility [16]. Epididymal sperm was obtained by mincing the epididymis in normal saline, and filtering through a nylon mesh (80- μ m pore size). The sperms were counted using a hemocytometer. The number of sperm in 5 squares (4 corners and the center) in the center grid of both sides were counted and averaged according to the method of Freund and Carol [17]. Sperm morphology was done using 2 drops of Wells and Awa's stain, air-dried, and examined under the microscope. The normal sperm cells were counted and the percentage calculated [18].

Histology of testis. Tissues fixed in Bouin's solution were dehydrated in graded concentrations of ethanol and then cleared in xylene before embedded in paraffin. Microsections (about 4 μ m) were prepared and stained with haematoxylin and eosin (H & E) dye and were examined under a light microscope by an histopathologist who was ignorant of the treatment groups.

Statistical analysis. All values were expressed as the

mean ± S.D. of six animals per group. Data were analysed using one-way ANOVA followed by the non-parametric Shirley-Williams post-hoc test. Values were considered statistically significant at $p < 0.05$.

RESULTS

Effects of Kaletra^R on body weight, relative weight and serum biochemical indices in rats

Administration of Kaletra^R at 8.3 and 16.6 mg/ kg/ day (Kaletra-T and Kaletra-2T) significantly ($p = 0.023$) decreased the body weight gain of the animals by 43% and 48%, respectively when compared to the control. However, there were no significant ($p = 0.37$) differences in the relative weight of testis of Kaletra^R-treated rats when compared to the control (Table 1). In addition, administration of Kaletra-2T increased the activities of serum aspartate and alanine aminotransferases (AST and ALT) by 1.2 and 1.6 folds, respectively relative to the control (Fig. 2). However, administration of Kaletra-T produced insignificant ($p = 0.41-0.58$) effects on the activities of ALT and AST. Similarly, there were no significant ($p = 0.39$) differences in the activities of serum alkaline phosphatase in Kaletra^R-treated rats relative to control (Fig. 7).

Effects of Kaletra^R on sperm and testicular antioxidant indices in the rats

Administration of Kaletra-T and Kaletra-2T caused a significant ($p = 0.016-0.036$) decrease in sperm motility and sperm count of the rats. Precisely, sperm motility and count were decreased by 1.2 and 1.3 folds in Kaletra-T, and by 1.5 and 1.4 folds in Kaletra-2T treated rats, respectively (Table 2). Furthermore, Kaletra-2T alone significantly ($p = 0.023$) increased total sperm abnormalities by 66% when compared to the control. There were no significant ($p = 0.31$) differences in the sperm live-dead ratio of Kaletra^R-treated rats relative to the control (Table 2). Administration of Kaletra^R caused a dose-dependent decrease in the activities of testicular glutathione peroxidase and catalase in the rats (Fig. 3). Specifically, the activities of glutathione peroxidase and catalase were decreased by 38% and 47% in Kaletra-T, and by 57% and 48% in Kaletra-2T treated rats, respectively. Kaletra-2T alone significantly ($p = 0.027$) decreased the level of testicular reduced glutathione by 50% when compared to the control (Fig. 4). In addition, Kaletra-T and Kaletra-2T decreased the activities of testicular glutathione-S-transferase by 29% and 44%, and testicular superoxide dismutase by 58% and 54%,

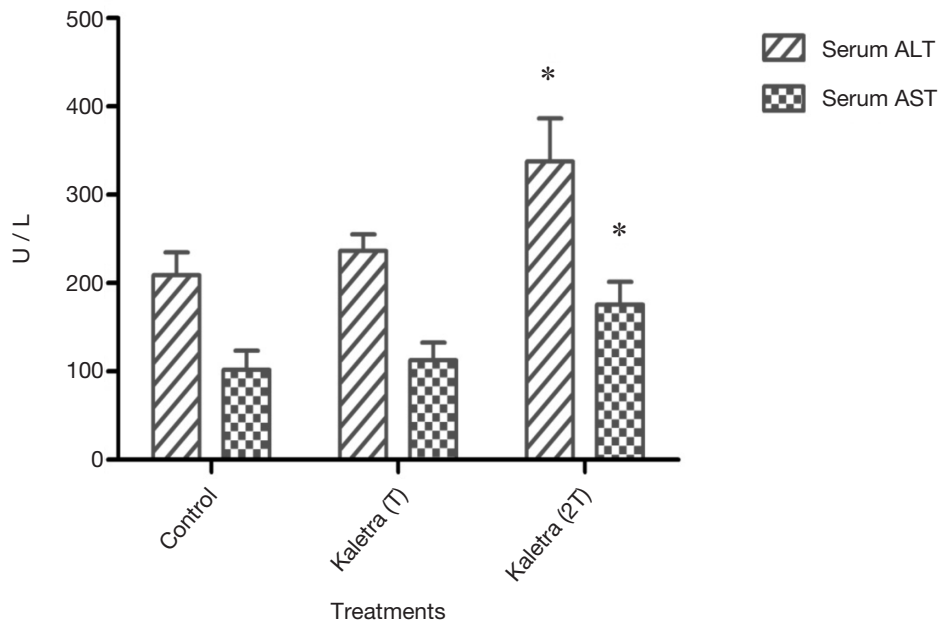


Fig. 2 Effects of Kaletra^R on the activities of serum alanine and aspartate aminotransferases (ALT and AST) in male Wistar rats.

*Significantly different from control ($p < 0.05$)

Table 2 Effects of Lopinavir-Ritonavir (Kaletra^R) on sperm characteristics in male Wistar rats

Parameters	Control	Kaletra-T	Kaletra-2T
Sperm motility (%)	90.0 ± 6.1	75.0 ± 5.5*	61.0 ± 3.1*
Sperm count (10 ⁶ /g testis)	129.2 ± 11.9	101.5 ± 12.8*	93.8 ± 4.2*
TSA (%)	10.5 ± 0.3	11.3 ± 0.6	17.4 ± 0.8*
Live-dead ratio (%)	96.8 ± 2.6	94.5 ± 1.9	92.7 ± 3.4
Sperm volume	5.2 ± 0.05	5.1 ± 0.03	5.3 ± 0.04

Values are Mean ± SD of 6 animals per group

* Significantly different from control ($p < 0.05$)

Control = Animals treated with Corn Oil alone.

Kaletra-T = Animals treated with therapeutic dose of kaletra

Kaletra-2T = Animals treated with twice therapeutic dose of kaletra

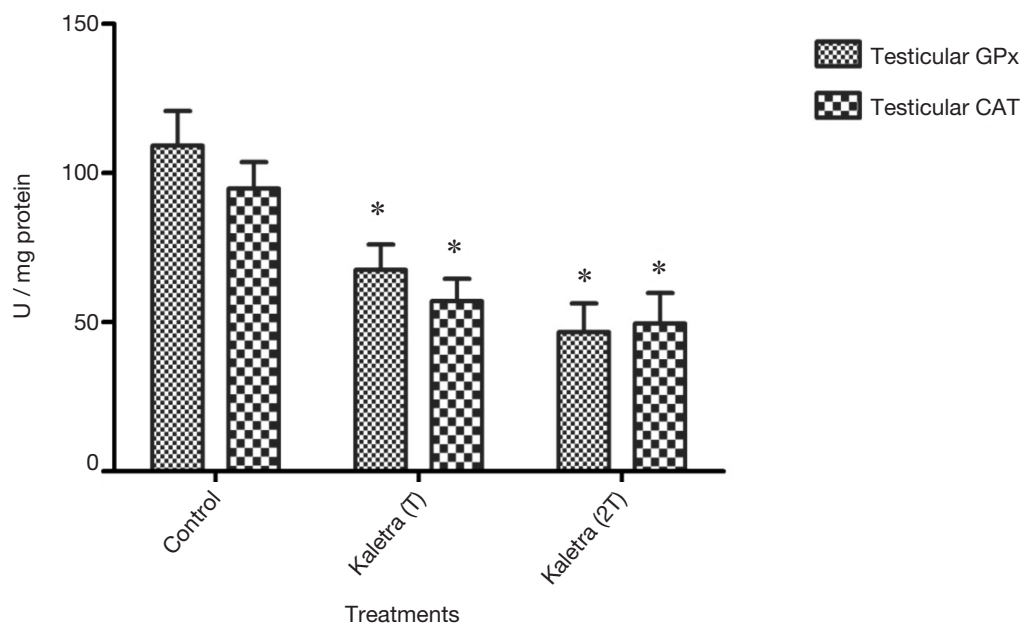


Fig. 3 Effects of Kaletra^R on the activities of testicular glutathione peroxidase (GPx) and Catalase (CAT) in male Wistar rats.

*Significantly different from control ($p < 0.05$)

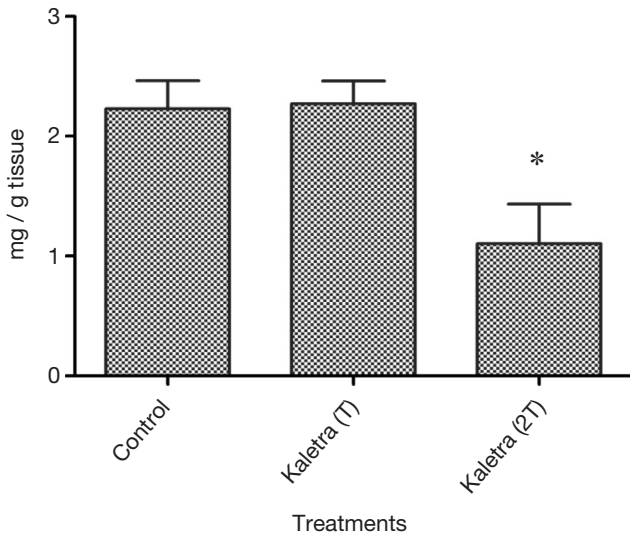


Fig. 4 Effects of Kaletra^R on testicular reduced glutathione (GSH) levels in male Wistar rats.
*Significantly different from control ($p < 0.05$)

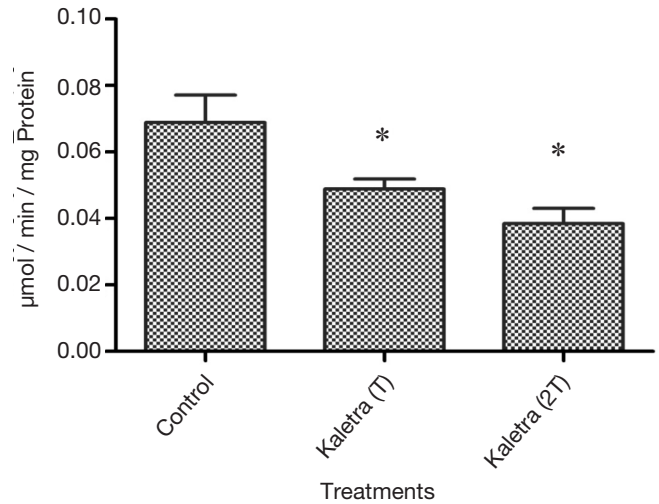


Fig. 5 Effects of Kaletra^R on the activities of glutathione-S-transferase in male Wistar rats.
*Significantly different from control ($p < 0.05$)

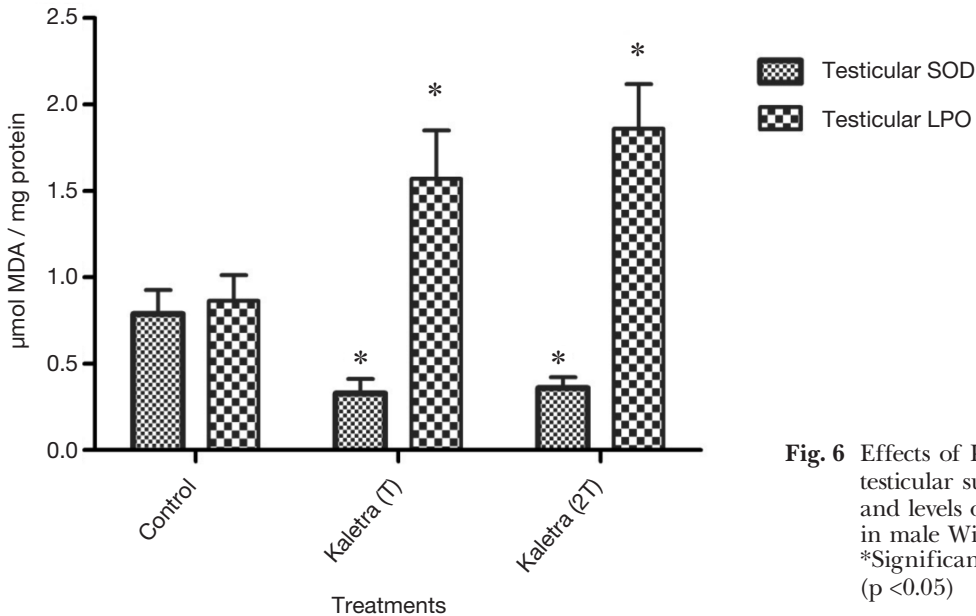


Fig. 6 Effects of Kaletra^R on the activities of testicular superoxide dismutase (SOD) and levels of lipid peroxidation (LPO) in male Wistar rats.
*Significantly different from control ($p < 0.05$)

respectively (Fig. 5 and 6). Significantly, administration of Kaletra-T and Kaletra-2T increased the levels of testicular lipid peroxidation by 81% and 115%, respectively when compared to the control (Fig. 6).

Effects of Kaletra^R on cyto-architecture of testis in the rats

Fig. 8 shows representative photomicrographs of testes from rats treated with Kaletra^R. In control and Kaletra-T treated rats, the tissue showed normal architectural layout with little or no visible lesions. Also, the seminiferous tubules contained spermatogenic cells in different stages of maturation. There is optimal maturation of spermatids as well. The sertoli and leydig's cells were abundant and normal. However in Kaletra-2T treated rats, though the seminiferous tubules appear normal, the spermatogenic cells were fewer in numbers when compared to the control.

DISCUSSION

The main outcomes of this study were that Kaletra^R promotes oxidative stress in testis of rats leading to elevation of lipid peroxidation with a concomitant decrease in the activities of enzymatic (SOD, CAT, GPx and GST) and non-enzymatic (GSH) antioxidant parameters. In addition, sperm motility and sperm count were decreased while total sperm abnormalities were increased in Kaletra^R-treated rats. It is safe to believe that Kaletra^R or its metabolites interact with major macromolecules within the testis of rats leading to the generation of reactive oxygen species (ROS) [19]. Overproduction of ROS may lead to oxidative damage to key biomolecules thereby causing chronic diseases [20]. Particularly, oxidative stress occurs in tissues when the concentration of ROS generated exceeds the antioxidant capacity [21] or when there is a decrease

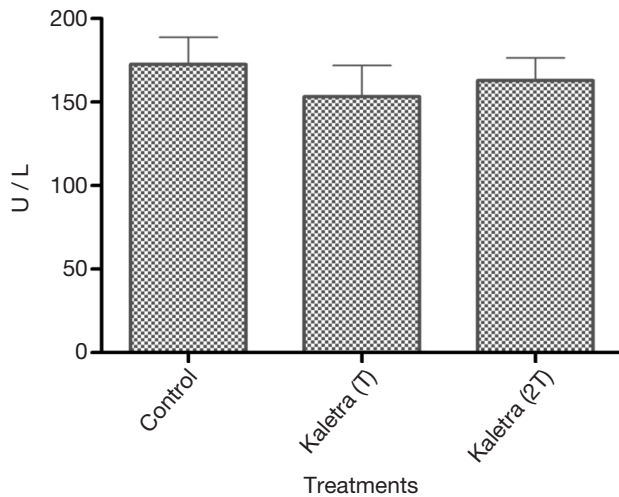


Fig. 7 Effects of Kaletra on the activities of serum alkaline phosphatase (ALP) in male Wistar rats.

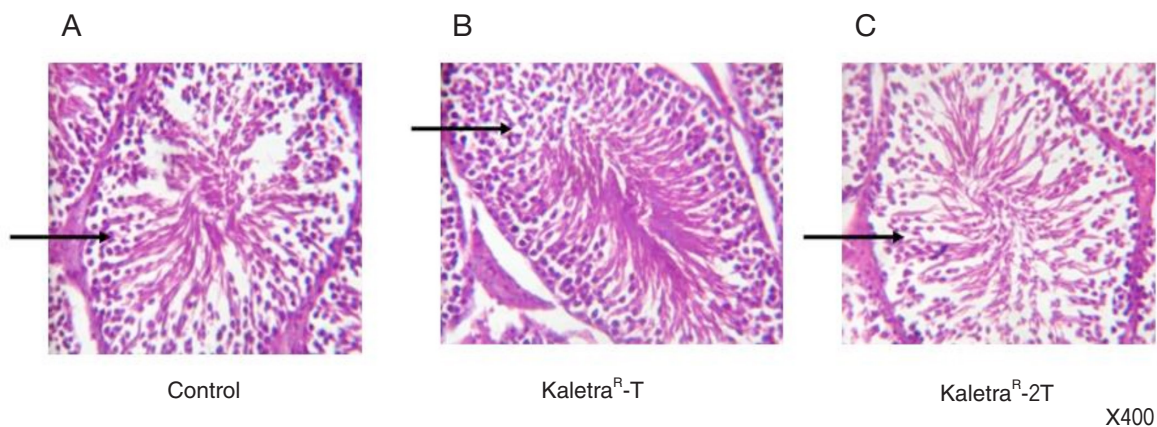


Fig. 8 Representative photomicrographs showing the effects of kaletra^R on testicular tissue of rats. Control and kaletra^R-T seminiferous tubules with spermatogenic cells in different stages of maturation. There is optimal maturation of sperm atids. Sentoli's cells (black arrow) and leydig's cells look normal. Kaletra^R-2T: seminiferous tubules look normal but scanty and abnormal shaped sentoli's cells (black arrow).

in the antioxidant capacity of the cell [22]. ROS attack cellular components involving polyunsaturated fatty acid residues of phospholipids, which are sensitive to oxidation [23]. The peroxy radicals formed by the attack can be rearranged through a cyclization reaction to endoperoxides which are precursors of malondialdehyde (MDA) [24]. Therefore, the increase in the levels of MDA observed in this study could be due to decrease in antioxidant capacity of the cells. Lipid peroxidation can lead to diverse cell damage and several studies have unequivocally demonstrated that oxidative damage to key macromolecules such as DNA, membrane functions and impairment of sperm characteristics such as reduction in motility and loss of capacity to undergo acrosomes reaction are common in the presence of ROS [25, 26]. In the present study, administration of Kaletra^R caused a significant decrease in epididymal sperm motility and count of the rats. This decrease may be linked to lipid peroxidation process in testis that destroyed the structure of the lipid matrix in the membrane of spermatozoa leading to rapid loss of intracellular ATP resulting to axonemal damage, decreased sperm viability and increased morphological defects, and may even inhibits spermatogenesis in ex-

treme cases [27, 28]. In addition, the lipo-peroxidative effects of Kaletra^R may adversely affect the structural integrity of acrosomal membrane, which ultimately may reduce motility and fertilization capacity of the spermatozoa.

In the present study, the antioxidant biomarkers-GSH, GST, GPx, CAT and SOD were significantly decreased in testis of Kaletra^R-treated rats. Both SOD and CAT are principal antioxidant enzymes that scavenge superoxide anion formed as the intermediate product of H₂O₂ breakdown and catalyzed reduction of H₂O₂, thus protecting tissue from highly reactive hydroxyl radicals. Thus, the inhibition of SOD and CAT may cause the accumulation of H₂O₂ or products of its decomposition [29] which may be deleterious to the cells. GSH is a naturally occurring antioxidant, which prevents free radical damage and helps detoxification by conjugating with chemicals and, also acts as an essential cofactor for antioxidant enzymes including GPx, GR and GST [30]. Depletion of intracellular GSH as observed in this study was a reflection of toxic effect of Kaletra^R which may expose the testes to damage. HIV-positive individuals have, in general, abnormally low antioxidant and glutathione levels [31,

32]. Therefore, combine effects of Kaletra^R therapy and HIV infection may further deplete the GSH or antioxidant status of the patients. In addition, the observed decrease in testicular antioxidant enzymes activities could result from their inactivation by ROS or direct inhibition by metabolites from Kaletra^R [33]. Importantly, Kaletra-2T interfered with the cyto-architecture of the testes leading to reduction in the number of spermatogenic cells in the seminiferous tubules of the rats. This histological observation was supported by our biochemical results.

In conclusion, our study has shown that Kaletra^R may produce adverse effect on male reproductive health, especially on sperm quality and structural integrity of testes. This finding is particularly important to HIV-infected individuals on ART who desire to have children irrespective of their positive serostatus. Therefore, we suggest an urgent need to develop policies to support availability and accessibility to relevant reproductive health services during ART.

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DECLARATION OF INTEREST

The authors have declared that there is no conflict of interest.

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