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Investigations into the Risk of Reproductive Toxicity Following Exposure to Highly Active Anti-Retroviral Drugs in Rodents

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With the introduction of Highly Active Antiretroviral Therapy (HAART), there has been drastic decline in morbidity and mortality associated with HIV/AIDS. However, many patients experience adverse drug reactions perhaps due to the inherent toxic nature of HAART. The possible toxic effect of HAART (combination ARVs) on reproduction and sexual dysfunction in seropositive HIV patients remains a subject of intense research. This study was designed to investigate the toxic effects of HAART on the reproductive hormones and organs of male and female rats.
Sexually mature adult male and female rats were administered therapeutic doses of single and combination antiretroviral drugs for 48 days and thereafter sacrificed under anaesthesia. Morphological and histopathological examination of the testes and ovaries were carried out. Serum biochemical assay, semen quality analysis and hormonal assays were also conducted using standard methods.
Results show significant ($p < 0.05$) reductions in the weight of testes and epididymis across all groups versus control; sperm count and motility were also significantly reduced in the test groups while hormonal analysis in males revealed significant reductions in LH, FSH and Testosterone. In the females, there was a significant ($p < 0.05$) reduction in the number of ovarian follicles, prolactin, estrogen and progesterone.
We thus conclude that the administration of single and combined antiretroviral drugs have potential reproductive toxic effects.

Key words: HAART, Testosterone, Estrogen, LH, FSH

INTRODUCTION

The Human Immunodeficiency Virus (HIV) has been identified as the cause of Acquired Immunodeficiency Syndrome (AIDS) [1, 2]. HIV infection is managed using Antiretroviral drugs (ARVs); these have come to play an important role in arresting the progression of HIV infection to the dreaded stage of Acquired Immune Deficiency Syndrome (AIDS) in individuals and also help prevent the transmission of the disease among individuals in the community [3, 4].

With the introduction of Highly Active Antiretroviral Therapy (HAART), there has been drastic decline in morbidity and mortality associated with HIV/AIDS [5, 6]. However, besides these positive effects, many patients who are on therapy experience drug toxicities that include metabolic, hepatic, neurological and cardiovascular complications [7]; HAART may itself increase chemically reactive species in circulation [8], possibly by producing more oxidized metabolites deriving from the interaction between Reactive Oxygen Species (ROS) and infected-cell biomolecules [9-11]. This is supported by several biochemical mechanisms, such as mitochondrial interference following treatment like as observed with Nucleoside Reverse Transcriptase Inhibitors- NRTIs [11]. A putative mechanism of these toxicities has been shown to involve the inhibition of mitochondrial DNA polymerase-γ, resulting in mitochondrial DNA depletion and dysfunction [12]. As the mitochondrion is known as the 'power-house' of cells, the sperm and ovarian cells inclusive, these drugs may adversely affect cellular energy and hence compromise fertility.

Most ARVs show good penetration in the male genital tract (MGT) and therefore may affect spermatogenesis [13]; reports on single agents have however shown little or no reproductive risks in animal studies [7]. Combination therapies are known to induce biochemical or metabolic changes (with negative consequences on systems) that would not have arisen in the use of a single drug or the combination affecting pharmacokinetic parameters ultimately leading to negative effects or changes that are absent in the use of the drug singly [14].

The possible toxic effect of HAART (combination ARVs) on reproduction, particularly on the MGT is thus of interest because semen quality is a key factor for reproductive success [15, 16], [17] reported a decreased pregnancy rate among HIV-infected females on HAART as compared to un-infected women; the same observation was recorded when these women underwent *in vitro* fertilization (IVF) with their own oocytes. However, counter results have emerged wherein it was observed that there were no differences in

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semen parameters that may affect fertility following antiretroviral therapy [18]. The association of HAART and sexual dysfunction in seropositive HIV patients remains a subject of intense research amongst researchers; it has been postulated that the discussion revolving around HIV/HARRT and reproductive function may remain unresolved till such a time when exhaustive tools would have been employed to assess and correlate morphological changes with other associated parameters of fertility [19].

This study intends to make such correlation by investigating the toxic effects of HAART on the reproductive hormones and reproductive organs of male and female rats. Oxidative stress-mediated toxicity and HAART histologically-induced reproductive aberrations would also be investigated.

METHOD

This study was designed to investigate the toxic effects of combined HAART on the reproductive organs and hormones in male and female rats.

Drugs

Anti-retroviral drugs were obtained (with permission) from the Aids Prevention Initiative in Nigeria (APIN) HIV/AIDS Clinic of the University of Lagos Teaching Hospital (LUTH), Idi Araba.

- Efavirenz, EFV (600 mg/tablet)
- Zidovudine, AZT (300 mg/tablet)
- Tenofovir/Lamivudine, TDF/3TC (300 mg/300 mg/tablet)
- Atazanavir/Ritonavir, ATV/r (300 mg/50 mg/tablet)
- Zidovudine/Lamivudine/Nevirapine, AZT/3TC/NVP (300 mg/150 mg/200 mg/tablet)
- Tenofovir/Lamivudine/Efavirenz, TDF/3TC/EFV (300 mg/300 mg/600 mg/tablet)

Animals

Sexually mature adult Sprague- Dawley (Albino) male and female rats with average weight of 200 g were obtained from Laboratory Animal Centre of College of Medicine, University of Lagos, Nigeria. The animals were authenticated in Zoology department, Faculty of Science, University of Lagos, Nigeria. They were made to acclimatize for two weeks before the commencement of the experiment. The animals were fed on Pfizer Animal Feed cubes and water ad libitum. The investigation conforms to The Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) for studies involving experimental animals. Ethical clearance for use of animals for this research was obtained from the College of Medicine University of Lagos, Research Ethics Committee.

Treatment groups

Male and female rats were separately divided into 7 groups each with 8 animals per group. Based on current clinical (human) doses, animals were administered daily doses of treatment as follows:

Group 1 received EFV (9 g/kg); Group 2 received AZT (9 mg/kg); Group 3 received a combination of TDF (5 mg/kg) and 3TC (5 mg/kg); Group 4 received ATV/r (5/0.7 mg/kg); Group 5 received a combination of AZT (9 mg/kg), 3TC (5 mg/kg) and NVP (3 mg/kg); Group 6 received a combination of TDF (5 mg/kg), 3TC (5 mg/kg) and EFV (9 mg/kg); Group 7 (Control group) received distilled water (10 ml/kg).

Morphological and Histopathological Examination

The rats were subjected to light ether anesthesia and sacrificed on day 48 by cervical dislocation; the reproductive organs were harvested into normal saline contained sample bottles through abdominal incision for physical examination. Histopathological analyses of the organs were done, reported and micrographs taken at the Morbid Anatomy Department of the College of Medicine, University of Lagos. The following parameters were also measured using Mettler sensitive weighing balance:

- Total weight (includes weight of testis and epididymis) for male rats
- Weight of ovaries for female rats.

Examination of Semen

The testes from each rat were carefully exposed and removed along with its adjoining epididymis. One of the testes was separated from the epididymis and the caudal epipidymal tissue was removed and placed in a petri dish containing 1mL normal saline solution. An incision of about 1mm was made in the caudal epididymis to liberate its spermatozoa into the saline solution.

Progressive sperm motility, sperm count and sperm viability were then determined as previously described by [20]. Epididymal sperm motility was assessed by calculating motile spermatozoa per unit area and expressed as percent motility. Epididymal sperm count was done using the improved Neubauer hemocytometer and expressed as million/ml of suspension. The sperm viability was also determined using Eosin/Nigrosin stain. The motile (live) sperm cells were unstained while the non-motile (dead) sperms absorbed the stain. The stained and unstained sperm cells were counted and an average value for each was recorded from which percentage viability was calculated.

Sperm morphology was evaluated by staining the sperm smears on microscope slides with two drops of Walls and Ewa stain after they were air-dried. The slides were examined under the microscope.

Biochemical analysis

The method of [21] was followed in estimating the levels of glutathione (GSH). Glutathione-S-transferase (GST) activity was determined according to [22]. The level of superoxide dismutase (SOD) activity was determined by the method of [23]. Catalase (CAT) activity was determined according to the method of [24]. Glutathione Peroxidase (GPx) activity was determined according to the method of [25]. Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of [26].

Hormonal analysis

Serum concentrations of reproductive hormones were measured using micro plate enzyme-linked immunosorbent assay (ELISA) and expressed as Units/Litre. Direct immune-enzymatic determination of the Luteinizing (LH) and Follicle-Stimulating Hormones

Table 1 Weight of Testis and epididymis of antiretroviral exposed male rats

Group	Testis (g)	Epididymis (g)
1	1.37 ± 0.11	0.30 ± 0.06*
2	0.41 ± 0.09*	2.10 ± 0.29*
3	1.30 ± 0.39	1.00 ± 0.06*
4	0.33 ± 0.07*	1.30 ± 0.66*
5	1.20 ± 0.05	0.27 ± 0.06*
6	0.34 ± 0.08*	2.20 ± 0 .90*
7	1.70 ± 0 .01	3.40 ± 0.10

Table 1 shows the weight of testes and epididymis of male rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p<0.05$ as compared to the control (Group 7). Group 1: EFV (9 mg/kg); Group 2: AZT (9 mg/kg); Group 3: TDF (5 mg/kg) and 3TC (5 mg/kg); Group 4: ATV/r (5/0.7 mg/kg); Group 5: AZT (9 mg/kg), 3TC (5 mg/kg) and NVP (3 mg/kg); Group 6: TDF (5 mg/kg), 3TC (5 mg/kg) and EFV (9 mg/kg); Group 7 (Control group): distilled water (10 ml/kg).

Table 2 Weight of Ovaries of Antiretroviral Exposed Female Rats

Groups	Ovaries (g)
1	0.20 ± 0.06
2	0.17 ± 0.02*
3	0.18 ± 0.02*
4	0.20 ± 0.02
5	0.15 ± 0.02*
6	0.17 ± 0.02*
7	1.60 ± 0.03

Table 2 shows the weight of ovaries of female rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7). Groups 1–7: as in Table 1.

Table 3 Semen Analysis for Male Rats Exposed to ARVs

Group	Count (10^6/ml)	Motility (%)	Live/Death Ratio (%)	Morphology (%)
1	19.40 ± 0.81*	15.80 ± 0.71*	52.00 ± 0.71*	60.20 ± 1.43
2	47.80 ± 0.94*	56.20 ± 0.70*	71.00 ± 1.26*	76.20 ± 1.22
3	55.33 ± 0.11*	69.66 ± 2.47	73.33 ± 2.20*	79.00 ± 2.78
4	15.00 ± 0.20*	38.00 ± 0.35*	54.40 ± 0.54*	60.60 ± 0.65
5	70.00 ± 1.35*	60.20 ± 1.19*	85.20 ± 2.38	91.00 ± 1.73
6	80.40 ± 1.90*	54.80 ± 0.76*	85.20 ± 2.11	89.40 ± 0.54
7	134.40 ± 8.67	89.60 ± 1.67	90.80 ± 1.30	92.40 ± 1.81

Table 3 summarizes the results of semen examinations in male rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7); Groups 1–7: as in Table 1.

(FSH) in human serum were determined by the methods of [27] based on the manufacturer's manual. Testosterone activity was determined following the principle described by [28]. The method of [29] was followed in estimating the levels of progesterone and oestrogen levels while prolactin levels were determined according to the method of [30] based on the manufacturer's manual.

Statistical analysis

Results were expressed as mean±SEM. The data were subjected to one way analysis of variance (ANOVA) test and differences between samples were determined by Dunnett's Multiple Comparison Test, using Graph Pad Prism (statistical) software. Results were considered to be significant at $p < 0.05$.

RESULTS

Result on table 1 shows the mean weight of the testis which ranged from 0.33 ± 0.09 g to 1.7 ± 0.01 g. There was a significant reduction ($p < 0.05$) in measured testicular weight observed in groups 2, 4 and 6 compared to the control. The mean epididymis weight ranged from 0.27 ± 0.06g and 3.4 ± 0.10 g.There was an observed significant reduction ($p < 0.05$) in weight in all the treatment groups compared to control.

Results on table 2 shows mean weight of the ovaries of female rats exposed to ARVs. The weight ranged from 0.15 ± 0.02 g to 1.6 ± 0.03g. There was significant ($p < 0.05$) reduction in mean weights observed in groups 2, 3, 5, 6 compared to the control group.

Table 3 shows the semen analysis of the male rats from the treatment groups. The results showed that there was significant reduction ($p < 0.05$) in number of sperms in all test groups compared to control (group 7). There were also significant reductions in sperm motility across the groups, except group 3. The live/ death ratio in groups 1–4 were significantly lowered compared to the control. However, no significant changes in the morphology of the sperm cells were observed across all groups.

Table 4 results show the mean number of follicles in the ovaries of treated rats. There was a significant reduction ($p < 0.05$) in number of follicles in groups 1, 2 and 4, while a significant increase ($p < 0.05$) was observed in group 3.

Table 5 presents the result of the histopathological analysis of harvested testes from male rats exposed to ARVs. Analysis showed that the Tunica were normal except in groups 1 and 4 that had congested peritesticular vessels. Group 4 also presented with incomplete spermatogenic series and disorganised stratal and proliferated leydigs cells.

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Table 4 Number of Follicles in the Ovaries of Female Rats Exposed to ARVs

Group	Number of follicles in ovaries
1	2.00 ± 0.05*
2	0.0 ± 0.00*
3	11.00 ± 1.58*
4	2.00 ± 0.70*
5	8.00 ± 1.00
6	6.00 ± 0.70
7	7.00 ± 1.58

Table 4 shows the number of follicles in the ovaries of female rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7); Groups 1–7: as in Table 1.

Table 5 Summary of Histopathological Assessment of Rat Testes Exposed to ARV Treatment

Groups	Tunica	Seminiferous tubules	Spermatogenic series	Stroma
1	Normal, Congested peritesticular vessels	Transverse and longitudinal section- regular, uniformly spaced.	Organised, stratified. All cells of the spermatogenic series are present. Sertoli cells are normal.	Few small congested vessels. Leydig cells are normal.
2	Normal	Transverse and longitudinal section- regular, widely spaced.	Organised, stratified. Reduced cells of the spermatogenic series are present.	Few small congested vessels. Leydig cells are normal.
3	Normal	Transverse and longitudinal section- regular, widely seperated.	Organised, stratified. All cells of the spermatogenic series are present.	Few small congested vessels. Mildly inflamed. Leydig cells are normal.
4	Normal, Congested vessels	Transverse section- irregular, widely spaced.	Disorganised. 50% are empty or contains fragmented cells. Cells of the spermatogenic series are incomplete in majority of tubules.	Proliferated leydig cells.
5	Normal	Transverse and longitudinal section- regular, uniformly spaced.	Organised, stratified. All cells of the spermatogenic series are present. Sertoli cells are normal.	Few small congested vessels. Leydig cells are normal.
6	Normal	Transverse and longitudinal section- regular, uniformly spaced.	Organised, stratified. All cells of the spermatogenic series are present. Sertoli cells are normal.	Few small congested vessels. Leydig cells are normal.
7	Normal	Transverse and longitudinal section- regular, uniformly spaced.	Organised, stratified. All cells of the spermatogenic series are present. Sertoli cells are normal.	Few small congested vessels. Leydig cells are normal.

Table 5 shows the observations on histopathological assessment of testes harvested from male rats exposed to antiretroviral agents. Groups 1–7: as in Table 1.

topathological analysis of the epididymis harvested from control and test groups. Analysis showed that the epididymis were normal except for group 4, that had focal hyperplastic proliferation of the sterocilia.

Table 7 shows the results of the histopathological assessments of the ovaries harvested from control and test groups.

There were ($p < 0.05$) reductions in GSH levels in test groups 4 and 6, of SOD in group 2 and CAT in groups 1 and 4 compared with control. There was also an observed significant reduction ($p < 0.05$) in MDA in groups 1, 2, 3 and 5 compared to control (Table 8).

Table 9 results show a significant reduction ($p < 0.05$) in GSH in groups 4 and 5 compared to control.

Table 10 shows the effect of ARV exposure on hormonal parameters in treated male rats. There were significant ($p < 0.05$) reductions in the blood levels of the hormones measured (LH, FSH and Testosterone) across all the test groups when compared to the control.

Table 11 shows the effect of ARV exposure on hormonal parameters in treated female rats. There were significant ($p < 0.05$) reductions in the blood levels of the hormones across all the test groups when compared to the control except in group 1 where no statistically significant change in progesterone was observed.

Table 6 Summary of Histopathological Assessment of Rat Epididymis Exposed to ARV Treatment

Groups	Size	Sperm cells	Sterocilia and Basal cells	Intraepithelial lymphocytes and macrophages
1	Normal	Mature sperm cells fill tubules	Normal and organised	Several and scattered throughout the tissue
2	Normal	Mature sperm cells within tubules	Normal and organised	Few and scattered throughout the tissue
3	Mostly dilated	Mature sperm cells fill tubules	Normal and organised	Several and scattered hroughout the tissue
4	Normal	Mature sperm cells fill tubules	Focal hyperplastic proliferation of sterocilia	Few and scattered throughout the tissue
5	Mostly dilated	Mature sperm cells fill tubules	Normal and organised	Few and scattered throughout the tissue
6	Mostly dilated	Mature sperm cells fill tubules	Normal and organised	Few and scattered throughout the tissue
7	Mostly dilated	Mature sperm cells fill tubules	Normal and organised	Few and scattered throughout the tissue

Table 6 shows the observations on histopathological assessment of testes harvested from male rats exposed to antiretroviral agents. Groups 1–7: as in Table 1.

Table 7 Summary of Histopathological Assessment of Rat Ovaries Exposed to ARV Treatment

Groups	Ovarian stroma	Fallopian tube
1	Normal (fibrocellular)	Mucosal atrophy
2	Normal (firocellular	Mucosal atrophy
3	Normal (fibrocellular)	Normal
4	Increased stromal vascular channels	Normal
5	Normal (fibrocellular)	Mucosal atrophy
6	Increased stromal vascular channels	Normal
7	Normal (fibrocellular)	Normal

Table 7 shows the observations on histopathological assessment of ovaries and fallopian tubes harvested from female rats exposed to antiretroviral agents. Groups 1–7: as in Table 1.

Table 8 Oxidative Stress Analysis for Male Rats exposed to ARVs

Groups	GSH	SOD	CAT	MDA
1	26.53 ± 1.01	138.31 ± 6.92	602.43 ± 9.16*	0.63 ± 0.09*
2	34.45 ± 2.97	105.76 ± 12.66*	633.32 ± 22.10	0.72 ± 0.32*
3	25.87 ± 0.95	146.94 ± 13.35	635.36 ± 13.66	0.777 ± .09*
4	24.26 ± 2.2*	138.38 ± 7.72	591.30 ± 7.37*	0.48 ± .05
5	25.17 ± 1.46	133.37 ± 7.37	639.99 ± 31.56	1.19 ± 0.203*
6	20.54 ± 1.60*	127.92 ± 7.76	646.50 ± 24.02	0.588 ± 0.27
7	28.23 ± 0.31	143.44 ± 16.06	642.26 ± 32.53	0.25 ± 0.07

Table 8 shows the results of biochemical analysis of oxidative parameters in male rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7); **CAT** is serum catalase; **SOD** is superoxide dismutase; **GSH** is reduced glutathione; **MDA** is Malondialdehyde. Groups 1–7: as in Table 1.

Table 9 Oxidative Stress Analysis for female Rats exposed to ARVs

Groups	GSH	SOD	CAT	MDA
1	22.62 ± 1.67	127.02 ± 2.62	703.05 ± 8.49*	0.85 ± 0.08*
2	27.38 ± 1.05	82.69 ± 3.79	686.05 ± 9.08*	0.19 ± .03*
3	23.97 ± 1.43	88.63 ± 3.64*	685.28 ± 10.42*	1.00 ± 0.08*
4	19.15 ± 0.52*	143.77 ± 3.89*	628.24 ± 8.39	0.86 ± .01
5	19.72 ± .70*	131.08 ± 4.97*	659.89 ± 6.99	2.74 ± 0.08*
6	22.84 ± 0.55	73.13 ± 2.57*	546.99 ± 6.10*	0.32 ± 0.04
7	21.92 ± 1.09	112.13 ± 3.71	622.46 ± 9.81	0.484 ± 0.07

Table 9 shows the results of biochemical analysis of oxidative parameters in female rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7); **CAT** is serum catalase; **SOD** is superoxide dismutase; **GSH** is reduced glutathione; **MDA** is Malondialdehyde; Groups 1–7: as in Table 1.

Table 10 Effect of ARV Exposure on Hormonal Parameters in Male Rats

Group	LH	FSH	Testosterone
1	2.20 ± 0.31*	2.12 ± 0.44*	0.83 ± 0.22*
2	2.54 ± 0.19*	2.64 ± 0.01*	1.14 ± 0.44*
3	1.96 ± 0.99*	2.30 ± 0.95*	2.78 ± 1.55*
4	2.37 ± 0.50*	2.66 ± 0.98*	1.68 ± 0.34*
5	2.35 ± 0.74*	2.42 ± 1.15*	1.28 ± 0.33*
6	2.40 ± 0.91*	2.60 ± 0.58*	0.66 ± 0.26*
7	3.36 ± 0.77	4.12 ± 1.29	12.60 ± 1.98

Table 10 shows the results of biochemical analysis of hormonal parameters in male rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7); **LH** is Luteinizing Hormone, **FSH** is Follicle Stimulating Hormone. Groups 1–7: as in Table 1.

Table 11 Effect of ARV Exposure on Hormonal Parameters in Female Rats

Group	Prolactin	Progesterone	Estrogen
1	2.06 ± 0.17*	9.08 ± 1.48	2.42 ± 0.20*
2	0.88 ± 0.11*	2.72 ± 0.37*	2.68 ± 0.17*
3	1.24 ± 0.08*	1.64 ± 0.41*	2.66 ± 0.17*
4	1.66 ± 0.15*	3.98 ± 0.05*	3.48 ± 0.19*
5	1.70 ± 0.24*	3.82 ± 0.47*	4.16 ± 0.27*
6	2.04 ± 0.14*	4.44 ± 0.45*	3.24 ± 0.15*
7	3.58 ± 0.14	7.02 ± 0.39	7.50 ± 0.21

Table 11 shows the results of biochemical analysis of hormonal parameters in female rats exposed to antiretroviral agents. Results are presented as Mean ± SEM (n = 8). * represents result of statistical analysis where $p < 0.05$ as compared to the control (Group 7); Groups 1–7: as in Table 1.

DISCUSSION

Toxic injury affecting male and female reproductive competencies are largely due to influence on physiological mechanisms or processes; particularly in the male, those processes that regulate spermatozoa viability and motility [31–34]. Drugs such as psychotropics, chemotherapeutic agents, alcohol, steroids, 5-alpha-reductase inhibitors, α-blockers (silodosin, tamsulosin, alfuzosin, cardura), Selective Serotonin Reuptake Inhibitors (SSRIs), ketoconazole, spironolactone, cimetidine, nifedipine, sulfasalazine, and colchicine have been shown to possess negative effects on male fertility through induction of adverse biochemical and histological effects [35, 36].

In the current study, we aimed to evaluate the potential for adverse effects in the reproductive system by ARVs using rat models. Form the results, following administration of ARVs and subsequent data collation and analyses, we observed a significant reduction in the weight of testes (groups 2, 4, 6), epididymis (all test groups) (Table 1) and ovaries (all test groups except group 1) of rats exposed to ARVs compared to the control (Table 2). Perhaps more importantly, semen analysis revealed a significant reduction ($p < 0.05$) in sperm counts and sperm motility across all groups when compared to control; the live/death sperm ratio was also significantly reduced in groups 1–4 (Table 3). Thus, these results may suggest significant reduction in male fertility.

The report by [37] found significant relationships between certain measures of semen quality and fertility with men aged 20 to 35 who lived with a partner and had no children. Significant relationships with pregnancies were found for sperm concentration, number of sperm, and sperm morphology using traditional WHO (1992) evaluation.

From literature, two studies looked into effects on semen parameters before and after antiretroviral therapy: semen parameters were normal according to WHO criteria and remained stable after administration of zidovudine (AZT) monotherapy in 5 HIV-1-infected men [38]. In the other study, parameters improved in 20 men after 4 or 12 weeks of HAART [39]. The observed improvement in the latter study has been suggested to be because of improved general health resulting from HAART.

Conversely, mitochondria are abundant in spermatozoa and are necessary for progressive motility. Deletions in mitochondrial DNA of spermatozoa have been described as a result of antiretroviral therapy [40]. Thus, theoretically, penetration of nucleoside reverse transcriptase inhibitors or their precursors into spermatozoa could result in mitochondrial toxicity and thereby may lead to impaired progressive motility [41].

It should be taken into consideration that studies of drug effects on male fertility with the sperm function as target are difficult for reasons such as the inherent, large variability in semen parameters, even for a single individual, the large range of normal semen values the variability of technique and consistency of analyses of semen parameters among different laboratories, and the lack of specific guidelines for what constitutes clinically significant changes in a particular semen parameter or threshold values for impaired fertility [42, 43].

Our results also showed a significant reduction in number of ovarian follicles in treated female rats from groups 1, 2 and 4; however a significant increase was observed in group 3 (Table 4). It is generally accepted that reproductive ageing is directly related to the remains of the stock of primordial follicles, which is established during fetal life [44]. This pool progressively empties as a woman grows older and is (almost) completely exhausted when menopause is reached [45]. It was shown in an earlier study that the pattern of age-dependent loss of antral follicle numbers is strikingly similar to that of the primordial follicle pool [46]. It seems plausible that the number of antral follicles reflects what is left of the primordial follicle pool and, thus, the reproductive age of an individual woman. This number of antral follicles correlated much better than other presumed basal markers for reproductive age, including FSH, E_2 and ovarian volume [44]. Thus, the significant reduction in follicles in the current study may indicate an adverse effect to the female reproductive system.

Histopathologically, assessments of the male and female rats' reproductive organs following ARV exposure were largely normal. However, we observed epididymal focal hyperplastic proliferation of sterocilia in group 4 and mucosal atrophy in the fallopian tubes of female rats in groups 1, 2 and 5 (Figure 1).

Results of the oxidative stress analysis of the reproductive system of male rats exposed to ARVs did not show a clear association across groups neither across measured parameters. Significant reductions in GSH levels in test groups 4 and 6, of SOD in group 2 and CAT in groups 1 and 4 and of MDA in groups 1, 2, 3 and 5 were observed compared to control (Table 8). The same not so clear effect was observed with the female system (Table 9). Results show a significant reduction in GSH in groups 4 and 5; SOD levels were significantly increased in groups 4 and 5, while significant reduction was observed in groups 3 and 6 when compared with the control group.

Oxidative stress is an imbalance between production and elimination of chemically reactive species, such as reactive oxygen species (ROS) [47]. Cellular defenses to Reactive oxygen species include antioxidant scavengers, such as ascorbate, glutathione, and thioredoxin, and antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin reductase [48]. The male accessory sex gland secrete antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase as well as free radical scavengers such as Vitamins C and E, hypotaurine, taurine, uric acid and albumin [49] and these secretions are important to fertility in terms of sperm transport, motility acquisition and capacitation in diverse mammalian models and perturbations of which have resulted in diverse pathologies [50, 51]. Antioxidant enzyme levels are sensitive to oxidative stress and alterations have proved that cell damage and weakened antioxidant defense is common in combination antiretroviral therapy (cART).

Because ART may increase chemically reactive species in circulation, many patients who are on ART experience drug toxicities that include metabolic, hepatic, neurological and cardiovascular complications [52]. This is supported by biochemical mechanisms, such as mitochondrial interference subsequent to treatment with NRTIs [11], and activation of the P450 hepatic system by PIs [9] and mitochondrial DNA depletion and dysfunction due to the inhibition of mitochondrial DNA polymerase-γ [12].

Reduction in activity of CAT has been reported [53] and may reflect an inability of tissues to eliminate the hydrogen peroxide produced following treatment with NNRTI-nevirapine. It may also be due to overutilization of the enzyme caused by excess reactive oxygen species.

Polyunsaturated fatty acids, which are major components of cell membranes, can also undergo free radical attack, producing lipid peroxidation products like malondialdehyde (MDA) and 4-hydroxynonenal. Under normal circumstances, the body is protected from such damage by a careful balance between pro-oxidants and antioxidants [54].

The observed decrease in SOD activity suggests inactivation (or overutilization) of the enzyme probably caused by increased superoxide radical production, or an inhibition by the hydrogen peroxide as a result of corresponding decrease in the activity of CAT in nevirapine metabolism [53]. Testicular lipid peroxidation process induced by nevirapine may destroy the structure of lipid matrix in membranes of spermatozoa and may lead to rapid loss of intracellular ATP resulting to axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and even

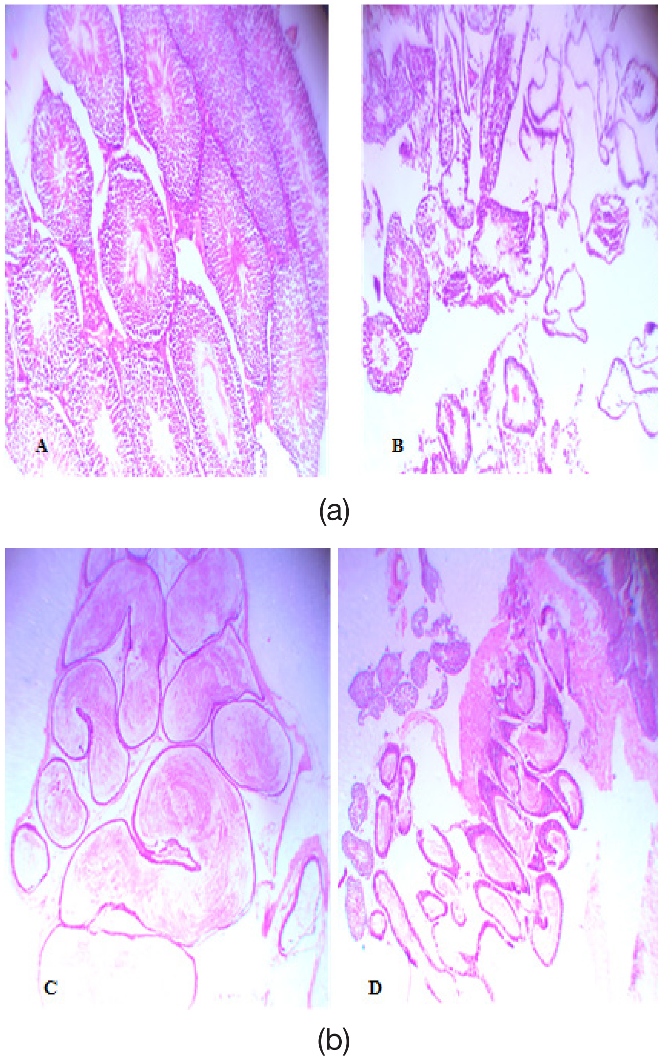

Figure 1 (a) Microscopic (photomicrograph) illustration of harvested testes from male rats exposed to ARVs. A shows the testes from control rats (group 7) with organized stratified withal cells of the spermatogenic series present, magnification x40. B shows the testes from rats treated with ATV/r (group 4) with disorganised, empty or fragmented cells. Cells of the spermatogenic series are incomplete in majority of tubules, magnification x40. (b) Microscopic (photomicrograph) illustration of epididymis from male rats exposed to ARVs. A shows the epididymis from control rats (group 7) normal and organised with mature sperm cells filling the tubules, magnification x40. B shows the epididymis from rats treated with ATV/r (group 4) with focal hyperplastic proliferation of sterocilia, magnification x40.

inhibits spermatogenesis in extreme cases [55].

Certain reports however advocate caution in linking ART with increased oxidative stress. In our earlier report [8], we concluded that while it may be advisable to incorporate exogenous antioxidants in the regimen of HIV patients on ART, the general understanding of drug-induced free radical generation may not be absolutely applicable to ART.

The effects of ARV exposure on the hormonal parameters in the male and female reproductive system seem clearer though. Our results show a significant reduction in levels of LH, FSH and Testosterone in the male and in Prolactin, Estrogen and Progesterone in the female across all the test groups when compared to the control.

LH is a glycoprotein that serves to regulate the function of the gonads. In males, LH stimulates the production and secretion of testosterone from the testes via leydig cells. FSH stimulates the maturation of germ cells within the testes and ovaries. In the female, it also stimulates follicular development and estrogen synthesis. Prolactin exerts a stimulatory effect on milk synthesis within the mammary glands. It has also been shown to have some degree of gonadal function in some domestic species and rodents. Estrogen stimulates follicular growth and maturation, induce the female to begin displaying estrous behavior to facilitate mating, prepare the external genitalia for copulation and create favorable conditions for the development of fertilized egg cells. Estrogen also contributes to the growth and development of mammary tissue and prepares the uterus for parturition. Progesterone prepares the uterus for reception of fertilized oocytes. It also prepares the mammary tissues for milk production.

From the foregoing, a significant reduction in the levels of these hormones, both in the male and female rats under study following administration of ARVs, may indicate a significant reduction in reproductive competency and point to a possible and potentially damaging adverse effect of ARVs/ART.

In a review of antiretroviral agents, most ARVs had no toxic/adverse effects on reproduction or fertility in animal studies [7]. In contrary to the aforementioned, didanosine at approximately 12 times the estimated human exposure was slightly toxic to female rats and their pups during mid and late lactation. Also, TDF caused an alteration of the estrous cycle in female rats while evidence of impaired fertility was seen in female rats at NVP doses resulting in systemic exposure comparable to human therapeutic exposure. More so, our current findings also revealed the reproductive toxicity of ARVs in rats.

Combination therapies are known to induce biochemical or metabolic changes (with negative con-

sequences on systems) that would not have arisen in the use of a single drug or the combination affecting pharmacokinetic parameters ultimately leading to negative effects or changes that are absent in the use of the drug singly [14]. Thus, it is plausible that effects not seen on single ARV exposure may occur during HAART.

CONCLUSION

From this study, administration of single and combined ARVs have potential reproductive toxic effects as shown by significant reduction in weight of testis, epididymis and ovaries and also evident by significantly reduced sperm count, sperm motility, and reduced ovarian follicules in animal models. This is further corroborated by a hormonal analysis that showed significant reduction in the levels of reproductive hormones.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

REFERENCES

1) Barre-Sinoussi F, Chermann J, Rey F, Nugeyre M, Chamaret S, Gruest J, *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 1983, 220(4599): 868–71.
2) Gallo R, Salahuddin S, Popovic M, Shearer G, Kaplan M, Haynes B, *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science. 1984, 224(4648): 500–3.
3) Hoffmann C, Mulcahy F, editors. ART, Perspective. Paris, Cagliari, and Wuppertal: Flying Publisher; 2006.
4) Federal Ministry of Health N. National Guidelines for HIV/AIDS Treatment. Treatment and care in Adolescents and Adults. 2007.
5) Pace GW, Leaf CD. The role of oxidative stress in HIV disease. Free Radical Biology and Medicine. 1995, 19(4): 523–8.
6) Tang AM, Smit E, Semba RD, Shah N, Lyles CM, Li D, *et al.* Improved Antioxidant Status Among HIV-Infected Injecting Drug Users on Potent Antiretroviral Therapy. Journal of Acquired Immune Deficiency Syndromes. 2000, 23(4): 321–6.
7) The Antiretroviral Pregnancy Registry. The Antiretroviral Pregnancy Registry: Interim report. 2009.
8) Awodele O, Olayemi SO, Nwite JA, Adeyemo TA. Investigation of the levels of oxidative stress parameters in HIV and HIV-TB co-infected patients. J Infect Dev Ctries. 2012, 6(01).
9) Martin JA. Hepatic γ-Cystathionase Deficiency in Patients With AIDS. JAMA. 2001, 285(11): 1444.
10) Hagmann M. Study confirms effectiveness of antiretroviral drugs for HIV patients. Bulletin of the World Health Organization. 2003, 81(12): 918–9.
11) Hulgan T, Morrow J, D'Aquila RT, Raffanti S, Morgan M, Rebeiro P, *et al.* Oxidant Stress Is Increased during Treatment of Human Immunodeficiency Virus Infection. Clinical Infectious Diseases. 2003, 37(12): 1711–7.
12) Brinkman K, Smeitink JA, Romijn JA, Reiss P. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. The Lancet. 1999, 354(9184): 1112–5.
13) Lowe SH, Sankatsing SUC, Repping S, Veen Fv, Reiss P, Lange JMA, *et al.* Is the male genital tract really a sanctuary site for HIV? Arguments that it is not. AIDS. 2004, 18(10): 1353–62.
14) Awodele O, Popoola D, Odunsi P, Akinde R, Akintonwa A. Assessing the risk of birth defects associated with exposure to highly active anti-retroviral therapy during organogenesis in rats. The Tokai journal of experimental and clinical medicine. 2013, 38(2): 82–92.
15) Hunault C, Habbema J, Eijkemans M, Collins J, Evers J, Te Velde E. Two new prediction rules for spontaneous pregnancy leading to live birth among subfertile couples, based on the synthesis of three previous models. Human reproduction. 2004, 19(9): 2019–26.
16) Sanchez-Palacios A, Shaman F, Garca JA, MA S-P. Prevalencia de serrsihilizaciones a coslneticos en prot'esionales de la estetica. Allergologia et Immunopathologia (Madr). 1995, 23(4): 148–52.
17) Coll O, Suy A, Figueras F, Vernaeve V, Martínez E, Mataró D, *et al.* Decreased pregnancy rate after in-vitro fertilization in HIV-infected women receiving HAART. AIDS. 2006, 20(1): 121–3.
18) Krieger JN, Coombs RW, Collier AC, Koehler JK, Ross SO, Chaloupka K, *et al.* Fertility Parameters in Men Infected with Human Immunodeficiency Virus. Journal of Infectious Diseases. 1991, 164(3): 464–9.
19) Azu OO. Highly Active Antiretroviral Therapy (HAART) and Testicular Morphology: Current Status and a Case for a Stereologic Approach. Journal of Andrology. 2012, 33(6): 1130–42.
20) Raji Y, Oloyo AK, Morakinyo AO. Effect of methanol extract of Ricinus communis seed on reproduction of male rats. Asian journal of Andrology. 2006, 8(1): 115–21.
21) Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. The Journal of laboratory and clinical medicine. 1963, 61: 882–8.
22) Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. Journal of biological Chemistry. 1974, 249(22): 7130–9.
23) Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. Journal of biological Chemistry. 1972, 247(10): 3170–5.
24) Sinha AK. Colorimetric assay of catalase. Analytical Biochemistry. 1972, 47(2): 389–94.
25) Ellerby LM, Bredesen DE. Measurement of Cellular Oxidation, Reactive Oxygen Species, and Antioxidant Enzymes during Apoptosis. Methods in Enzymology: Elsevier BV; 2000. p. 413–21.
26) Varshney R, Kale RK. Effects of Calmodulin Antagonists on Radiation-induced Lipid Peroxidation in Microsomes. International Journal of Radiation Biology. 1990, 58(5): 733–43.
27) Odell WD, Parlow AF, Cargille CM, Ross GT. Radioimmunoassay for human follicle — stimulating hormone: physiological studies. Journal of Clinical Investigation. 1968, 47(12): 2551–62.
28) Joshi UM, Shah HP, Sudhama SP. A sensitive and specific enzymeimmunoassay for serum testosterone. Steroids. 1979, 34(1): 35–46.
29) Devilla GO, Roberts K, Wiest WG, Mikhail G, Flickinger G. A Specific Radioimmunoassay of Plasma Progesterone 1. The Journal of Clinical Endocrinology & Metabolism. 1972, 35(3): 458–60.
30) Shome B, Parlow AF. Human Follicle Stimulating Hormone (Hfsh): First Proposal For The Amino Acid Sequence Of The Α-Subunit (Hfshα) And First Demonstration Of Its Identity With The Α-Subunit Of Human Luteinizing Hormone (hlhα). The Journal of Clinical Endocrinology & Metabolism. 1974, 39(1): 199–202.
31) Folgerø T, Bertheussen K, Lindal S, Torbergsen T, Øian P. Andrology: Mitochondrial disease and reduced sperm motility. Human reproduction. 1993, 8(11): 1863–8.
32) Ruiz-Pesini E, Diez C, Lapeña AC, Pérez-Martos A, Montoya J, Alvarez E, *et al.* Correlation of sperm motility with mitochondrial enzymatic activities. Clinical Chemistry. 1998, 44(8): 1616–20.
33) Donnelly ET. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. Human reproduction. 2000, 15(7): 1552–61.
34) May-Panloup P. Increased sperm mitochondrial DNA content in male infertility. Human reproduction. 2003, 18(3): 550–6.
35) Nudell DM, Monoski MM, Lipshultz LI. Common medications and drugs: how they affect male fertility. Urologic Clinics of North America. 2002, 29(4): 965–73.
36) Sigman M. Medications that impair male fertility. 2007.
37) Bonde JPE, Ernst E, Jensen TK, Hjollund NHI, Kolstad H, Scheike T, *et al.* Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. The Lancet. 1998, 352(9135): 1172–7.
38) Crittenden JA, Handelsman DJ, Stewart GJ. Semen analysis in

human immunodeficiency virus infection. Fertility and sterility. 1992, 57(6): 1294–9.

39) Robbins Wendie A, Witt Kristine L, Haseman Joseph K, Dunson David B, Troiani L, Cohen Myron S, *et al*. Antiretroviral Therapy Effects on Genetic and Morphologic End Points in Lymphocytes and Sperm of Men with Human Immunodeficiency Virus Infection. The Journal of Infectious Diseases. 2001, 184(2): 127–35.
40) White DJ, Mital D, Taylor S, St John JC. Sperm mitochondrial DNA deletions as a consequence of long term highly active antiretroviral therapy. AIDS. 2001, 15(8): 1061–2.
41) van Leeuwen E, ter Heine R, van der Veen F, Repping S, Beijnen JH, Prins JM. Penetration of Atazanavir in Seminal Plasma of Men Infected with Human Immunodeficiency Virus Type 1. Antimicrobial Agents and Chemotherapy. 2006, 51(1): 335–7.
42) Clark RV, Sherins RJ. Treatment of Men with Idiopathic Oligozoospermic Infertility Using the Aromatase Inhibitor, Testolactone Results of a Double-Blinded, Randomized, Placebo-Controlled Trial with Crossover. Journal of Andrology. 1989, 10(3): 240–7.
43) Clark RV, ed. Male infertility. 3rd ed. Philadelphia: Lippincott; 2001.
44) Scheffer GJ. The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. Human reproduction. 2003, 18(4): 700–6.
45) Faddy M, Gosden R, Gougeon A, Richardson SJ, Nelson J. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Human reproduction. 1992, 7(10): 1342–6.
46) Scheffer GJ, Broekmans FJM, Dorland M, Habbema JDF, Looman CWN, te Velde ER. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. Fertility and sterility. 1999, 72(5): 845–51.
47) Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The Lancet. 1994, 344(8924): 721–4.
48) Xia S. Sensitization of Glioma Cells to Fas-Dependent Apoptosis by Chemotherapy-Induced Oxidative Stress. Cancer Research. 2005, 65(12): 5248–55.
49) Wai-Sum O, Chen H, Chow P. Male genital tract antioxidant enzymes — their ability to preserve sperm DNA integrity. Molecular and Cellular Endocrinology. 2006, 250(1): 80–3.
50) Carpino A, Sisci D, Aquila S, Salerno M, Siciliano L, Sessa M, *et al*. Adnexal Gland Secretion Markers in Unexplained Asthenozoospermia. Archives of Andrology. 1994, 32(1): 37–43.
51) Topper EK, Killian GJ, Way A, Engel B, Woelders H. Influence of capacitation and fluids from the male and female genital tract on the zona binding ability of bull spermatozoa. Reproduction. 1999, 115(1): 175–83.
52) Morse CG, Kovacs JA. Metabolic and Skeletal Complications of HIV Infection. JAMA. 2006, 296(7): 844.
53) Adaramoye O, Adesanoye O, Adewumi O, Akanni O. Studies on the toxicological effect of nevirapine, an antiretroviral drug, on the liver, kidney and testis of male Wistar rats. Human & Experimental Toxicology. 2011, 31(7): 676–85.
54) Ngondi JL, Oben J, Forkah DM, Etame LH, Mbanya D. The effect of different combination therapies on oxidative stress markers in HIV infected patients in Cameroon. AIDS Research and Therapy. 2006, 3(1): 1.
55) Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. Molecular and Cellular Endocrinology. 2004, 216(1–2): 31–9.