

ORIGINAL ARTICLE

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Naringenin attenuates highly active antiretroviral therapy-induced sperm DNA fragmentations and testicular toxicity in Sprague-Dawley rats

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SUMMARY

Highly active antiretroviral therapy has evolved over the years, leading to a boost in the quality of life in people living with HIV and AIDS. However, growing evidence has shown that highly active antiretroviral therapy has deleterious effects on the testes and the overall reproductive capacity. Therefore, this study is to determine the adjuvant potential of Naringenin on highly active antiretroviral therapy-induced perturbations in fertility of male Sprague-Dawley rats. Thirty adult male Sprague-Dawley rats were divided into six groups viz – Control; H: 30 mg/kg of highly active antiretroviral therapy (EFV, 600 mg + FTC, 200 mg + TDF, 300 mg); N40: Naringenin, 40 mg/kg; N80: Naringenin, 80 mg/kg; HN40: highly active antiretroviral therapy + Naringenin, 40 mg/kg; HN80: highly active antiretroviral therapy + Naringenin, 80 mg/kg. The rats were euthanized after 4 weeks. Results showed that there was a significant decrease in sperm count ($p < 0.001$), spermatozoa with normal morphology ($p < 0.001$) and progressive sperm motility ($p < 0.05$) of H compared to the control and the HN groups. Likewise, fragmentations increased ($p < 0.05$) in tail lengths of sperm DNA in H compared to control. HN40 and HN80 decreased tail lengths compared to H ($p < 0.001$). There was also a decrease in %tail DNA and tail moment in HN40 ($p < 0.001$) compared to H. Luteinizing hormone significantly increased ($p < 0.05$) in HN40, HN80, and N40 ($p < 0.001$) but decreased in H ($p < 0.05$) compared to control. The diameter of the seminiferous tubules also decreased ($p < 0.05$) in H compared to control, N80, and HN40. Likewise, the area of the seminiferous tubules in group H decreased ($p < 0.05$) compared to N80 and HN80. The seminiferous tubules epithelium increased ($p < 0.05$) in N40 and HN40 compared to H. This study establishes that highly active antiretroviral therapy has deleterious effects on the testicular microanatomy, sperm parameters, and sperm DNA of Sprague-Dawley rats, which may impair fertility but Naringenin is a potential complimentary adjuvant.

INTRODUCTION

Highly active antiretroviral therapy (HAART) has evolved over the years since the discovery of antiretroviral drugs in 1985 (Mitsuya *et al.*, 1985), leading to a boost in the quality of life in people living with HIV and AIDS (PLWHA). The increased life span has however been paralleled with several comorbidities (Nagiah *et al.*, 2015) including nervous disorders (Jain *et al.*, 2016), diabetes mellitus (Karamchand *et al.*, 2016), malignancies (Ledru *et al.*, 2000), sexual dysfunctions (Lamba *et al.*, 2004) as well as impaired fertility (Azu *et al.*, 2014).

Growing evidence has shown that HAART has deleterious effects on the testes and the overall reproductive capacity of

men (Frapsauce *et al.*, 2015; Jerónimo *et al.*, 2017). It has been observed that antiretroviral therapy is associated with an increased amount of collagen deposits in the testes evidenced by interstitial fibrosis, cellular infiltration, and thickening of the basement membrane of the seminiferous tubules (Ogedengbe *et al.*, 2016). In a 2014 study on Sprague-Dawley (SD) rats, HAART was shown to have induced tubular atrophy and altered morphometric indices in the testis (Azu *et al.*, 2014).

Hormonal perturbation and androgen deficiencies have also been associated with HAART (Bhangoo & Desai, 2013; Rama *et al.*, 2015; Zavattaro *et al.*, 2017), resulting in male gonadal dysfunction (Lachâtre *et al.*, 2017). This is mediated by a

dysregulation of the hypothalamo-pituitary-testicular axis (Collazos *et al.*, 2002; Jegede *et al.*, 2017). In a cohort study of male homosexual, bisexual, and HIV⁺ men, the use of HAART was associated with raised serum estradiol levels (Lamba *et al.*, 2004; Scanavino, 2011).

Highly active antiretroviral therapy has good permeability in the gonad (Lowe *et al.*, 2004), hence they may impact negatively on spermatogenesis. It has been suggested by Barboza *et al.* (2004) that HAART induces topographic changes in sperm cell. In the study by White *et al.* (2001), HAART-induced mitochondrial damage in sperm cells thereby impairing sperm motility and their fertilizing potential (Adaramoye *et al.*, 2015). Although the reproductive toxicity of antiretrovirals is of great concern, the pathophysiological pathway leading to many of the observed changes is yet to be unraveled. This has also led to the reviewing of the treatment regimens over the years to newer and less toxic ones. Continuous evaluation of the safety profile of these drugs is therefore of great importance to achieve maximal adherence and benefit of the therapy. Alternatively, the use of adjuvant therapy that may ameliorate the toxic effects of these drugs without impairing the efficacy will be highly beneficial. Phytochemicals such as flavonoids, derived from fruits, have been found to be particularly useful in reproductive sciences (Patel *et al.*, 2014; Abarikwu *et al.*, 2016). The beneficial properties include counteracting certain oxidative processes in the body (Ali *et al.*, 2008), which may be induced by medications (Sak, 2012; Kumar & Tiku, 2016).

Naringenin, the predominant flavanone in grapefruit, has been investigated for its beneficial properties. It is considered to have a bioactive effect on human health as an antioxidant (Dikshit *et al.*, 2016), free radical scavenger (Braca *et al.*, 2002), anti-inflammatory (Bodet *et al.*, 2008), carbohydrate metabolism promoter (Kapoor *et al.*, 2013), immunity system modulator (Maatouk *et al.*, 2016), and repairs DNA (Gao *et al.*, 2006). Because of these promising properties and the fact that it has been shown to display antiretroviral effects especially as it demonstrated strong inhibition of virion assembly in the study by Khachatoorian *et al.* (2012), and strong antiviral activity in Huh7.5 cells after dengue virus exposure (Frabasile *et al.*, 2017), Naringenin has been chosen as the phytochemical of choice to be investigated for use as adjuvant therapy for use with HAART in this study.

MATERIALS AND METHODS

Materials

Thirty (30) adult male Sprague-Dawley (SD) rats of five each weighing 200–220 g were used for the study. They were housed at the animal house of the Biomedical Resource Unit, University of KwaZulu-Natal (UKZN), South Africa. This research was approved by the Animal Research Ethical Committee, UKZN, South Africa (protocol reference number AREC/046/016D). All procedures were performed in accordance with the Principle of Laboratory Animal Care of the National Medical Research Council and the Guide for the Care and Use of Laboratory Animals. The animals were housed in standard cages with dimensions 20 cm long, 20 cm wide, and 13 cm high. Three rats were kept in a cage, under controlled environmental conditions (25 °C and a 12-h light/dark cycle) and had free access to standard rat pellet food and tap water. They were allowed to acclimatize for two weeks prior to the commencement of the study.

Natural Naringenin was purchased from Sigma-Aldrich, while, Atripla containing efavirenz (EFV, 600 mg), emtricitabine (FTC, 200 mg), and Tenofovir (TDF, 300 mg) (Meintjes *et al.*, 2014) was procured from Pharmicare Ltd, Port Elizabeth, South Africa. The therapeutic dose of Atripla was adjusted for animal weight using the human therapeutic dose equivalent for a rat model.

Experimental design

Adult male SD rats were randomly divided into six groups viz – Group DW: Control (Distilled water), Group H: HAART (Atripla, 30 mg/kg), Group N40: Naringenin, 40 mg/kg (Hegazy *et al.*, 2016), Group N80: Naringenin, 80 mg/kg (Hegazy *et al.*, 2016), Group HN40: HAART + Naringenin, 40 mg/kg, and Group HN80: HAART + Naringenin, 80 mg/kg. The experiment was conducted between 8:00 am and 10:00 am for a period of 4 weeks and all administrations were carried out via the oral route.

Weight determination

Animals were weighed on the first day of the experiment, thereafter weekly and then on the last day of the study. Weights were taken in the morning between 8:00 and 10:00 am using an electronic balance (Zeiss, West Germany (Pty) Ltd; 0.000 g).

Animal sacrifice and collection of samples

The animals were euthanized on day 28 by excess Halothane. Blood was collected via transcardial puncture. Five milliliters (5 mL) of blood was obtained and the serum extracted by centrifugation at $g = 9.78$ m/sec.

The testes were excised and separated from the cauda epididymis. Each testis was weighed using an electronic balance (Mettler Toledo; Microstep (Pty) Ltd., Greifensee, Switzerland), and the average of the two testes for each animal was recorded. One testis from each animal was fixed in Bouin's fluid for histological analysis.

Sperm motility, sperm count, and sperm morphology

The cauda epididymides from each animal were minced in 5 mL of normal saline and used for determination of sperm count, sperm motility, and sperm morphology. Sperm motility was determined using the procedure by WHO (2010). A drop of prepared epididymal fluid was collected on a glass slide and covered with a coverslip (22 × 22 mm). Thereafter, it was immediately examined under the light microscope. The field was scanned systematically, and the motility of spermatozoa subjectively assessed and graded as progressive, non-progressive, and dead. At least 10 high power fields were observed at 400× magnification, and the relative percentage of spermatozoa in the different categories were estimated and recorded to the nearest 5% using the subjective method (WHO, 2010).

The epididymal sperm counts were obtained by the standard hemocytometer method. The epididymal fluid was thoroughly mixed with 10 mL of normal saline using a vortex, and approximately 10 µL of this diluted specimen was transferred to slides of the Bio-Rad counting chambers and counted with a Bio-Rad automated cell counter. Both sides of the counting chamber were used for each specimen and the average recorded to the nearest millions/milliliter (Ogedengbe *et al.*, 2016).

Sperm morphology was accessed by staining dry smeared diluted epididymal fluid on a glass slide with eosin-nigrosine staining and observed under a light microscope (Leica DM 500)

at 400× magnification. The number of normal spermatozoa, spermatozoa with abnormal heads, spermatozoa with abnormal tail, and spermatozoa with abnormal midpiece was recorded in percentage (Jegade *et al.*, 2017).

Comet assay

DNA damage was assessed using the Comet assay with minor modifications (Singh *et al.*, 1988). Two slides per sample were prepared. The first layer consisted of 2% low melting point agarose (LMPA, 37 °C). A second layer of 10 µL of rat spermatozoa from each sample with 1% LMPA (37 °C) and a volume of 1 µL of GelRed™ nucleic acid gel stain (Biotium, California, cat. no. 41003) were added. A third layer of 1% LMPA (37 °C) was then added. Once solidified, the slides were submerged in cold lysing solution [2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris (pH 10), 10% DMSO] and incubated (4 °C, 1 h). Following incubation, the slides were placed in electrophoresis solution [300 mM NaOH, 1 mM Na₂EDTA (pH 13)] for 20 min and thereafter electrophoresed (25V, 35 min, RT) using Bio-Rad compact power supply. The slides were then washed 3× (5 min each) with neutralization buffer [0.4 M Tris (pH 7.4)]. The slides were viewed with a fluorescent microscope (Olympus IX51 inverted microscope with 510–560 nm excitation and 590 nm emission filters). Images of 50 cells/comets were captured per sample and the comet tail lengths (µm), Tail Moment (a.u.), and tail DNA (%) were measured (Olive & Banáth, 2006; Gyori *et al.*, 2014) using Soft imaging system (Life Science – ©Olympus Soft Imaging Solutions v5) and CASPLAB – COMET ASSAY SOFTWARE (v1.2.3beta2), Poland.

Relative organ weight

The relative organ weights were calculated for the testes using the following formula:

$$(\text{organ weight})/(\text{Final body weight}) \times 100$$

Histomorphometrical studies

Testes tissues were fixed in Bouin's fluid. Samples were transferred to 70% ethanol (Latendresse *et al.*, 2002). They were then processed using a graded ethanol series and embedded in paraffin. The paraffin sections were cut into 5-µm-thick slices using a microtome (microm HM 315 microtome, Walldorf, Germany) and stained with hematoxylin and eosin (H&E). The sections were viewed and photographed using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Optical Co. Ltd., Tokyo, Japan).

For histomorphometric analysis, seven vertical sections from the polar and equatorial regions were sampled and as an unbiased numerical estimation of the diameter and cross-sectional area of the seminiferous tubule, the seminiferous epithelial thickness was determined using systematic random sampling fair distribution (Gundersen & Jensen, 1987). Eighteen seminiferous tubules with round profiles were randomly selected on each slide. The vertical and horizontal diameters of each seminiferous tubule were measured as d1 and d2, respectively, and the mean diameter (D) was recorded as an observation. This was performed to minimize longitudinal profiles that exhibit different degrees of damage or irregular shrinkage (Christensen & Peacock, 1980). The height and tubular diameter of the seminiferous tubule

epithelium was scanned using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany), and measurements were taken using the image analyzer and Leica microsystem software.

The cross-sectional areas of the seminiferous tubules were determined from the formula:

Area = $\pi D^2/4$ (Ogedengbe *et al.*, 2016) where π is equivalent to 3.142 and D is the mean diameter of the seminiferous tubules.

Determination of reproductive hormones

The serum levels of Luteinizing hormone (LH) and testosterone were determined using Elabscience enzyme immunoassay (ELISA) Rat-specific kits with catalog numbers: E-EL-R0026 and E-EL-R0389 for LH and free testosterone, respectively, in accordance with manufacturer's protocols.

Statistical analysis

Variables were tested for normality using Shapiro–Wilk test. All parametric data were analyzed with one-way ANOVA, followed by Fisher least significant difference (LSD) post hoc test while the non-parametric data were analyzed with Kruskal–Wallis 1-way ANOVA pairwise comparisons test. Significant values were adjusted by Bonferroni correction for multiple tests using IBM Statistical Package for Social Science (SPSS) version 24, New York, USA. $p < 0.05$ was considered statistically significant.

RESULTS

Weights

There was no significant difference in the body weight gain and relative testicular weight across the groups. However, group H showed decreased body weight and relative testicular weight compared to the other groups (Table 1).

Sperm count, motility, and morphology

There was a statistically significant decrease ($p < 0.001$) in the sperm count of H group when compared to control, HN40, and HN80 groups (Fig. 1a). There was also a significant decrease ($p < 0.001$) in the sperm count of N40 and N80 groups compared to control (Fig. 1a).

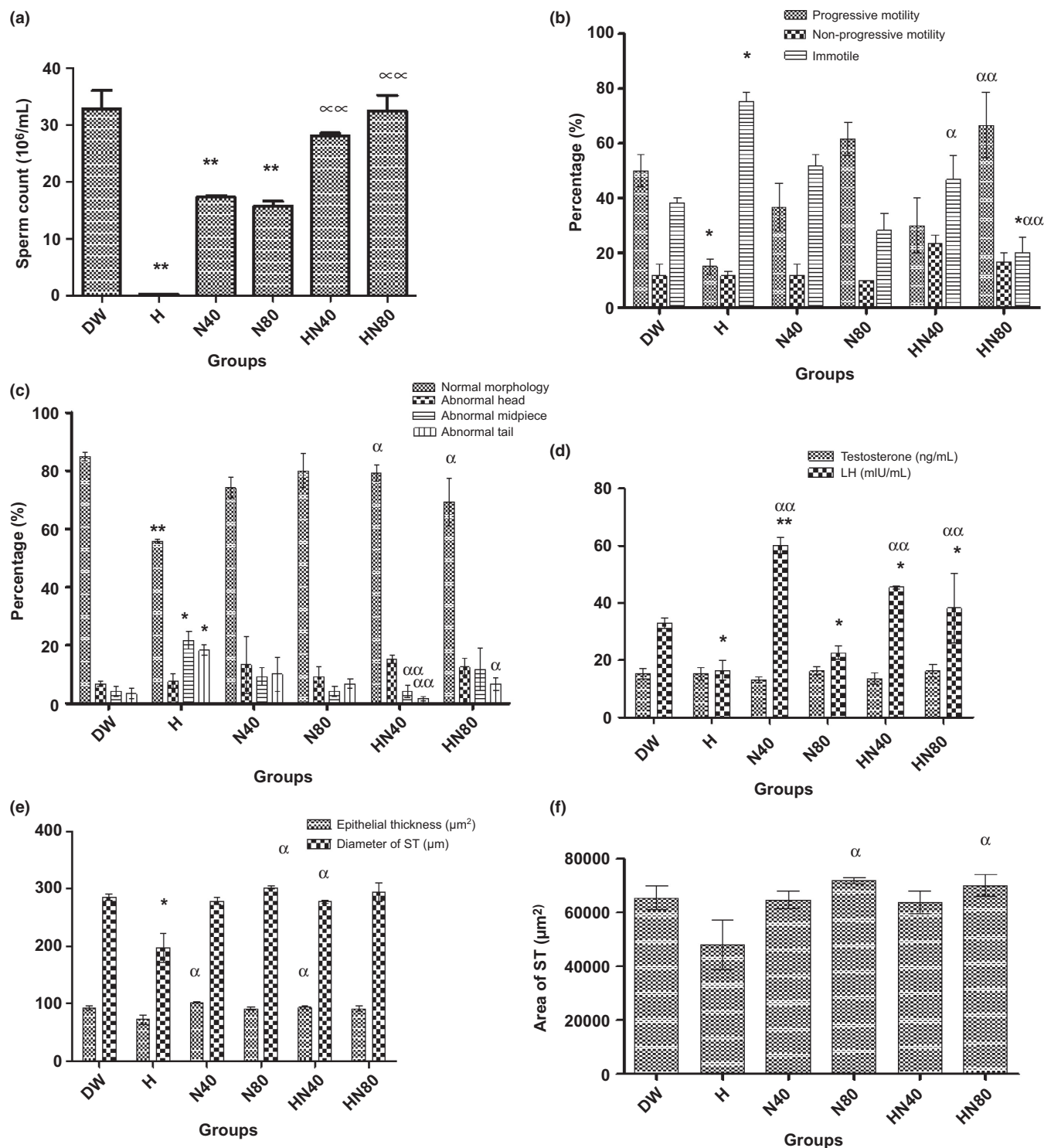
There was a statistically significant decrease ($p < 0.05$) in the progressive sperm motility of H group compared to control (Fig. 1b). There was also a significant increase ($p < 0.001$) in the progressive sperm motility of HN80 group compared to H group. In addition, the number of immotile sperm cells was increased significantly ($p < 0.05$) in H group compared to control but decreased significantly in the HN40 ($p < 0.05$) and HN80 ($p < 0.001$) compared to H group (Fig. 1b).

Table 1 Body weights, weight gain, and relative testicular weight

Group	Initial BW	Final BW	Weight gain	Relative TW
DW	182.00 ± 3.61	287.00 ± 7.57	105.00 ± 4.51	1.01 ± 0.04
H	167.66 ± 4.81	248.33 ± 12.68	80.67 ± 17.42	0.87 ± 0.29
N40	167.33 ± 1.76	267.33 ± 9.70	100.00 ± 11.35	1.09 ± 0.08
N80	208.67 ± 4.84	299.67 ± 6.69	91.00 ± 6.66	0.99 ± 0.02
HN40	167.00 ± 3.61	251.33 ± 6.49	84.33 ± 9.82	1.10 ± 0.04
HN80	177.44 ± 6.47	269.00 ± 6.64	83.33 ± 8.67	1.10 ± 0.01

Values are represented as mean ± SEM. DW (control), H (Atripla, 30 mg/kg), N40 (Naringenin 40 mg/kg), N80 (Naringenin 80 mg/kg), HN40 (Atripla + Naringenin 40 mg/kg), and HN80 (Atripla + Naringenin 80 mg/kg) after 4 weeks of treatment. BW, body weight; TW, testicular weight.

Figure 1 (a) Sperm count, (b) sperm motility, (c) Sperm Morphology, (d) Serum free testosterone and Luteinizing Hormones, (e) Epithelial thickness and diameter of seminiferous tubules (ST), (f) Areas of seminiferous tubules (ST) of SD rats in groups DW (control), H (Atripia, 30 mg/kg), N40 (Naringenin 40 mg/kg), N80 (Naringenin 80 mg/kg), HN40 (Atripia + Naringenin 40 mg/kg) and HN80 (Atripia + Naringenin 80 mg/kg) after 4 weeks of treatment. Bars indicate mean $\pm$ SD. *Represents significant difference ($p < 0.05$) between the group and control. **Represents significant difference ($p < 0.001$) between the group and control. α Represents significant difference ($p < 0.05$) between the group and H group. $\alpha\alpha$ Represents significant difference ($p < 0.001$) between the group and H groups.



The normal sperm morphology in H group was also significantly different ($p < 0.001$) compared to the control. In addition, the abnormalities in the tail and midpiece of rats in H group increased significantly compared to the control (Fig. 1c). Sperm

cells with normal morphology increased significantly ($p < 0.05$) in HN40 and HN80 compared to the control. In addition, there was a significant decrease ($p < 0.001$) in both abnormal mid-piece and abnormal tail in HN40 compared to H group while in

the HN80 there was a significant decrease ($p < 0.05$) in abnormal midpiece compared to H (Fig. 1c).

Sperm DNA fragmentation

There was a significant increase ($p < 0.05$) in the tail length of H compared to control while the N40 decreased significantly ($p < 0.001$) compared to control. The HN40 and HN80 also decreased significantly ($p < 0.001$) compared to H group (Table 2). The mean percent tail DNA decreased significantly ($p < 0.001$) in HN40 compared to both control and H groups while HN80 group increased significantly ($p < 0.05$) compared to control (Table 2). In addition, tail moment significantly increased in N80 group compared to the control ($p < 0.05$). However, tail moment in the HN40 group significantly decreased compared to the control ($p < 0.05$) and H group ($p < 0.001$) (Table 2).

Reproductive hormones

There were no statistically significant changes in the levels of free testosterone across the groups. However, LH significantly increased ($p < 0.05$) in HN40, HN80, and N40 ($p < 0.001$) but decreased ($p < 0.05$) in groups H and N80 compared to control, while groups N40, HN40, and HN80 increased ($p < 0.001$) compared to H (Fig. 1d).

Histology and morphometric parameters

The seminiferous tubules (ST) of the control animals displayed the normal progression of cells of the spermatogenic series with sperm cells in the lumen and Leydig cells in the interstitium (Fig. 2). Group H had distorted seminiferous tubules with dead immotile multinucleated cells in the lumen, whereas the N40, N80, HN40, and HN80 displayed the normal progression of cells of the spermatogenic series. Leydig cells in the interstitium and mature sperm cells in the lumen were similar to control (Fig. 2).

The diameter of the seminiferous tubules (ST) decreased ($p < 0.05$) in H compared to control, N80, and HN40. Likewise, the cross-sectional areas of the ST in group H decreased ($p < 0.05$) compared to N80 and HN80. The epithelial thickness of the ST in N40 and HN80 significantly increased ($p < 0.05$) compared to H (Fig. 1e).

DISCUSSION

The stage of once daily all-in-one single pill combination HAART has gained remarkable significance in the fight against the human immunodeficiency virus (HIV). This newer therapy

evolved in an attempt to improve compliance, reduce resistance, improve efficacy, and reduce side effects. Although these drugs are considered safer, they are not completely devoid of adverse effects (Chersich *et al.*, 2006; Venter *et al.*, 2017). Highly active antiretroviral therapy is a combination of three of the most efficacious and safest first-line antiretroviral drugs consisting of two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI)-efavirenz and emtricitabine and one non-nucleoside reverse transcriptase inhibitor (NNRTI)-Tenofovir (Esté & Cihlar, 2010).

In this study, HAART caused degeneration of seminiferous tubules and depletion of spermatogenic series in SD rats with a concomitant decrease in the diameter of the seminiferous tubule with the presence of multinucleated cells in the lumen. This affirms the reports of several authors concerning the toxicity of HAART (Azu *et al.*, 2014; Pathak *et al.*, 2015; Jegede *et al.*, 2017). This is suggestive of altered/arrested spermatogenesis, especially with the decreased seminiferous epithelium. Oyeyipo *et al.* (2015), in their study on obese Wistar rats, attributed the deleterious effects of HAART to increased oxidative stress. Although oxidative stress was not assessed in this study, literature is rife with reports on HAART's association with oxidative stress (Azu *et al.*, 2014; Sharma, 2014; Ogedengbe *et al.*, 2016; Weiß *et al.*, 2016).

The evidence of spermatogenic cells without spermatozoa in the lumen is indicative of unsuccessful spermiation (O'Donnell *et al.*, 2011). Moreover, failed spermiation is characterized by Sertoli cell phagocytosis leading to increased abnormally formed sperm cells and subsequently apoptosis (Russell & Clermont, 1977; O'Donnell *et al.*, 2011). Endocrine factors such as decreased levels of LH as observed in this study may also impair spermiation (Saito *et al.*, 2000). The increase in LH by Naringenin may have enhanced spermiation and the seamless growth of the spermatogenic cells. Phytochemicals are needful for the maintenance of cellular and tissue redox balance either directly or indirectly. Although at a higher dose of naringenin (80 mg/kg) we observed a converse effect on luteinizing hormone level compared to the lower dose (40 mg/kg), this may be attributed to possible biphasic phenomenon of some naturally occurring plant-based antioxidants in vivo. In addition to the modulatory and antioxidant effects, it has been reported that some bioflavonoids possess anti-androgenic properties in vivo depending on their bioavailability and kinetics related to metabolism and bio-transformation (Das *et al.*, 2004). Hence, they may display strong antioxidants or pro-oxidant properties depending on the physiological milieu and potential interactions thereof (Laughton *et al.*, 1989; Aruoma *et al.*, 1992; Lee & Lee, 2006; Martirosyan *et al.*, 2011). This may have been responsible for the diverse effects on the longer tail length observed in HN80 when compared to HN40. Furthermore, the antioxidant-bioflavonoid: Naringenin has been reported to neutralize oxidative stress in neurons (Al-Rejaie *et al.*, 2015) and hepatic cells (Chtourou *et al.*, 2015). Hence, Naringenin may have protected against HAART-induced testicular toxicity by possibly decreasing oxidative stress. Our findings plausibly reflect that the deleterious effects of HAART and the beneficial effects of Naringenin are not localized to the testis but extends to the hypothalamo-pituitary axis. This is in tandem with the report by Jegede *et al.* (2017) on HAART and the testis.

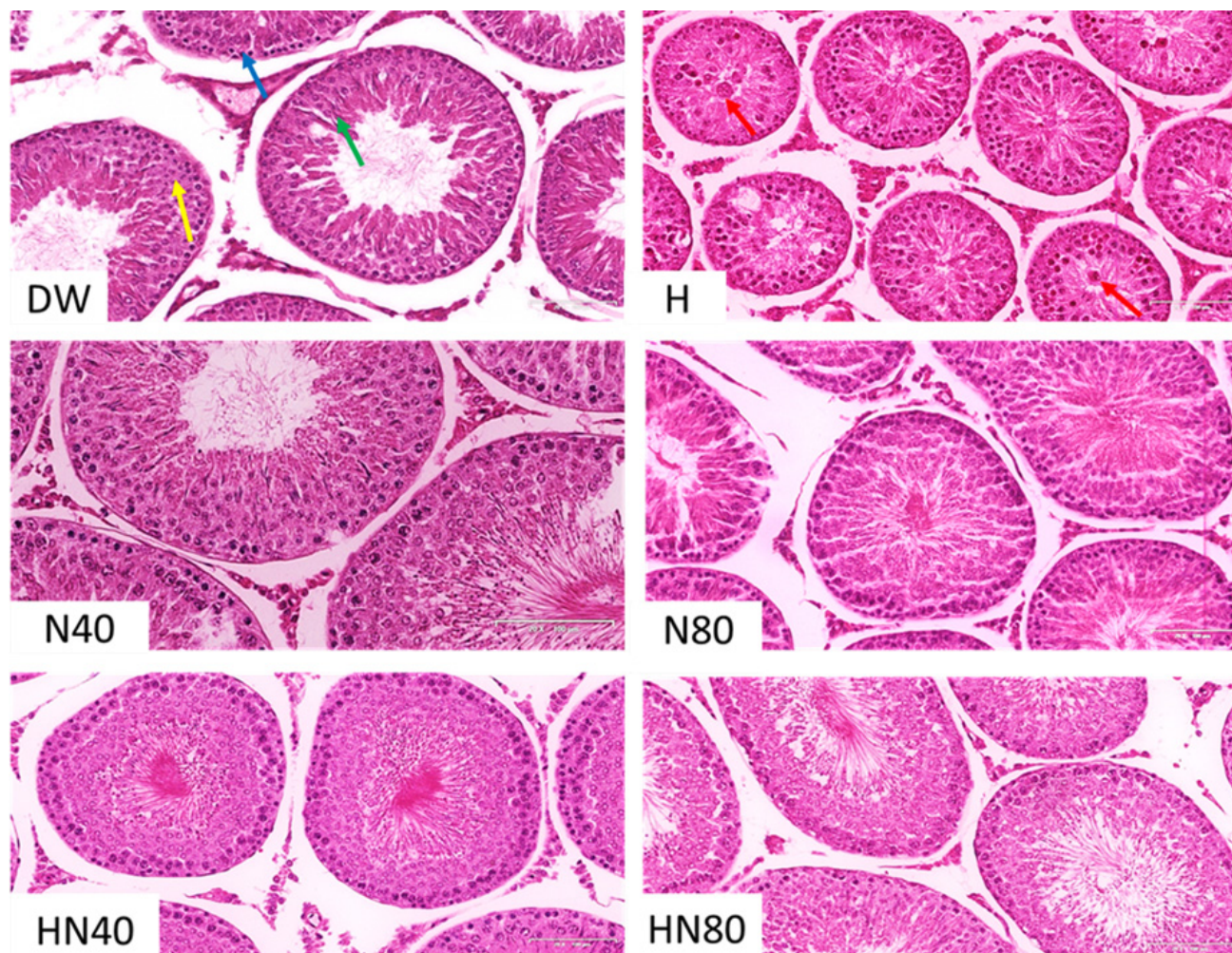
Highly active antiretroviral therapy also negatively affected the sperm quality in this study. The sperm count of the rats treated

Table 2 Tail length, tail moment, and %Tail DNA

Group	Tail length (μm)	Tail moment (a.u)	%Tail DNA
DW	4.58 ± 0.3	1.88 ± 0.71	24.3 ± 2.5
H	4.69 ± 0.4*	2.24 ± 1.09	24.6 ± 2.6
N40	4.15 ± 0.4**	1.85 ± 0.76	24.3 ± 2.4
N80	4.58 ± 0.3	2.36 ± 1.05*	24.9 ± 2.5
HN40	3.58 ± 0.4***	1.60 ± 0.77***	21.2 ± 3.6***
HN80	4.45 ± 0.3***	2.13 ± 1.06	25.0 ± 2.4*

Values are represented as mean ± SD. DW (control), H (Atripla, 30 mg/kg), N40 (Naringenin 40 mg/kg), N80 (Naringenin 80 mg/kg), HN40 (Atripla + Naringenin 40 mg/kg), and HN80 (Atripla + Naringenin 80 mg/kg) after 4 weeks of treatment. *Represents significant difference ($p < 0.05$) between the group and control. **Represents significant difference ($p < 0.001$) between the group and control. ***Represents significant difference ($p < 0.001$) between the group and H groups.

Figure 2 Representative photomicrographs of H&E stained testicular tissues. Distilled water (DW) Testis of control rats showing normal histological structure of active mature functioning seminiferous tubules (ST) associated with complete spermatogenic series. The peripheral layer of cells is composed of spermatogonia (blue arrow) and spermatocytes (yellow arrow) followed by a zone of spermatids (green arrow) and finally mature spermatids (green arrow) about to be released into the lumen. (H) Testis of HAART (Atripla, 30 mg/kg) treated rats, showing presence of immature spermatogenic series in tubular lumen (red arrow). There is loss of early and mature spermatids. The ST in the N40 (Naringenin 40 mg/kg), N80 (Naringenin 80 mg/kg), HN40 (Atripla + Naringenin 40 mg/kg), and HN80 (Atripla + Naringenin 80 mg/kg) after 4 weeks of treatment are similar to DW group.



with HAART decreased very significantly so much that values were less than 1% of the untreated/control animals, while the progressive motile sperm cells had comparatively decreased by 70% to the HAART-treated group. Likewise, the sperm morphology showed a marked increase in abnormalities especially tail abnormalities. Our findings may be an offshoot of the numerous aberrations observed in the testicular histology. In addition, the excessive accumulation of the polyunsaturated fatty acid (PUFA) on the plasma membrane of sperm cells makes it a target for oxidants (Oremosu & Akang, 2015; Aitken *et al.*, 2016). Increased lipid peroxidation impairs the flexibility of the sperm's flagellum thereby decreasing the sperm motility (Moazamian *et al.*, 2015). Moreover, oxidative stress is thought to play a role in many health conditions in both animal models and clinical trials by contributing to cellular dysfunction (Uygur *et al.*, 2013; Sies *et al.*, 2017). Reactive oxygen species (ROS) may have distorted the normal process of spermatogenesis resulting in the marked reduction in sperm count and increased morphological disorders. It has been reported that ROS may affect the fluidity of the plasma membrane of the sperm cell resulting in germ cell

apoptosis, thereby affecting the sperm count (Aitken & Fisher, 1994; Agarwal *et al.*, 2003). Although our study never assessed the effects of the individual component of HAART, Efavirenz has been reported to negatively affect sperm motility (Frapsauce *et al.*, 2015; Jerónimo *et al.*, 2017).

The use of phytochemicals have been proven to improve and protect against poor semen parameters (Abarikwu *et al.*, 2016; El-Desoky *et al.*, 2017), in this study, Naringenin proved potent enough to attenuate the HAART-induced toxicities on the spermatozoa. Although the marked decrease in sperm count in the Naringenin only groups depict that Naringenin may only serve as a prophylaxis rather than a potentiator in male fertility. This is in consonant with the study by Ranawat (Ranawat & Bakshi, 2017) who reported pro-oxidant effects of Naringenin. This paradoxical behavior of Naringenin is in tandem with reports on the importance of certain levels of ROS for normal physiological activities (Behrend *et al.*, 2003; Sies *et al.*, 2017). Another study by Dixit (Dixit & Cyr, 2003) highlighted the relevance of ROS in mitotic progression. Hence, an excessive increase in antioxidants neutralizing ROS beyond the requisite quantum for

spermatogenesis may hamper the entire process. This may also trigger a parallel production of mast cells and leukocytes in the semen ultimately initiating senescence of spermatozoa (Laroche, 2016; Tirado *et al.*, 2016). Furthermore, HAART has been shown to induce toxicity by targeting mitochondria leading to dysfunction and increased ROS production (Pérez-Matute *et al.*, 2013). The imbalance of increased ROS and insufficient antioxidant response results in oxidative stress (Pérez-Matute *et al.*, 2013). The Naringenin treated groups – although a decreased sperm count was noted compared to control – it was higher in comparison to the HAART-treated group (Fig. 1a). This was further seen in the combination treatment, where the sperm counts were decreased compared to the control but higher in comparison to HAART only and Naringenin only treatments. Under stress condition, Naringenin has the potential to chelate irons and scavenge ROS (Mostafa *et al.*, 2016). 5-hydroxy and 4-carbonyl groups in the C-ring of Naringenin play a role in ROS scavenging, Cu, and Fe ions interaction. Naringenin also restores mitochondrial membrane potential mitigating mitochondrial dysfunction and subsequent apoptotic cascade (Mostafa *et al.*, 2016). This could possibly explain the higher sperm count seen in HAART combined with Naringenin groups compared to HAART, and Naringenin treated groups as Naringenin mitigated the mitochondrial dysfunction and stress induced by HAART exposure.

The increased sperm DNA fragmentations is an indication of HAART perturbations at subcellular levels. Mitochondrial ROS has also been implicated in the induction of DNA damage. ROS penetrates the nucleopore of the nucleus causing DNA strand breaks (Villani *et al.*, 2010; Aitken & Nixon, 2013). Unfortunately, the spermatozoa lacks an inert mechanism to repair DNA strand breaks because it is deficient of the required cytoplasmic enzymes (Maneesh & Jayalekshmi, 2006). This induces germ cell apoptosis and subsequent degeneration posing a significant threat to the reproductive health of PLWHA as the safety of assisted reproduction is not guaranteed. Complimentary evidence shows that sperm DNA fragmentation is associated with increased spontaneous abortions (Cho *et al.*, 2003; Lewis & Aitken, 2005; Aitken *et al.*, 2009; Barratt *et al.*, 2010). Despite the use of intracytoplasmic sperm injection (ICSI), the safety remains evasive (Aitken & Curry, 2011; Boulet *et al.*, 2015). Especially, as it has been reported that ICSI men have poorer reproductive parameters (Belva *et al.*, 2016). It is suggested that the HAART-induced deleterious effects on the sperm DNA were minimized via the antioxidant effects of Naringenin. It will be highly speculative to postulate that Naringenin induced cytoplasmic enzymes that repaired sperm DNA strand breaks, however, it is possible that it upregulated the antioxidant pathway to neutralize the free radicals produced by HAART.

Antiretrovirals, specifically TFV and FTC, provide protection against the sexual transmission of HIV (Trezza & Kashuba, 2014). To achieve pregnancy and protection against transmission of HIV especially in a serodiscordant couple, the World Health Organisation (WHO) has laid down a consolidated guideline on sexual and reproductive health and rights of women living with HIV (WHO, 2017). This includes several options to support achieving pregnancy with minimal risk of HIV transmission in HIV serodiscordant relationships (Saleem *et al.*, 2017), such as antiretroviral therapy (ART) use by the person living with HIV to suppress viral load; use of oral pre-exposure prophylaxis (PrEP)

by the uninfected partner, which are also interventions that are important for health and well-being beyond attempting pregnancy and semen insemination or other assisted reproductive interventions. However, the result of this research and other previous ones (Ogedengbe *et al.*, 2016; Jegede *et al.*, 2017) has implications for the successful protection of male fertility. Thus, there may be need to include an adjuvant therapy with PrEP in the form of a phytochemical such as a flavonoid especially in those desirous of pregnancy to protect against the reproductive toxicity associated with the use of some ARTs. This can be either through the improvement in the oxidative status (Dikshit *et al.*, 2016; Kumar & Tikku, 2016) or through other pathways such as antihypercholesterolemic (Dikshit *et al.*, 2016), antiangiogenic, anti-ischemic, inhibition of platelet aggregation, and anti-inflammatory activities (Chu *et al.*, 2017).

CONCLUSION

This study reveals that the short-term use of HAART may predispose to degeneration of the seminiferous tubules, abnormality of sperm parameters, and increased sperm DNA fragmentations. This places PLWHA at high risk of infertility. However, the use of Naringenin, a bioflavonoid mitigates HAART-induced perturbation. More extensive research on the long-term use and a probe into the afore-discussed oxidant-antioxidant mechanism of action of HAART and Naringenin on the male fertility axis is encouraged to improve the activity of HAART on male reproductive health.

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CONFLICT OF INTEREST

None.

AUTHORS' CONTRIBUTIONS

M.Y.A. a Ph.D. student and E.N.A. a postdoctoral fellow designed the project, collected, analyzed/interpreted data, and drafted of the manuscript with close supervision by O.O.A. – the project leader. A.I.P. and E.C.S.N. took part in project execution and analysis/interpretation of data, drafting of the manuscript. A.I.J. did the sperm count, interpreted the results, and contributed to manuscript drafting. While, C.T. and A.A.C. conducted the comet assay, interpreted the data, and contributed to manuscript drafting.

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