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The Role of Antiretroviral Therapy on Testicular Integrity in the Era of *Hypoxis Hemerocallidea*

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ABSTRACT

The consumption of *Hypoxis hemerocallidea* (*HH*) is of public health interest since preparations from them are highly promoted, especially in South Africa as agents that can also enhance immunity in HIV/AIDS patients. This study was designed to investigate the effect of this herb compared to highly active antiretroviral therapy (HAART) on the testis. Twenty four adult male Sprague-Dawley rats (188.98±4.5 g) were distributed into four groups (A - D) and treated with HAART and *HH* according to protocol. After eight weeks of treatment, blood samples were obtained for hormonal analyses. Testicles were fixed in buffered formaldehyde and paraffin processed. Histopathological, immunohistochemical, seminal fluid and morphometric analyses were carried out. There were no significant changes ($P<0.05$) both in the body and testicular weights between the groups compared with control. Serum testosterone was drastically reduced in all treated animals. Sperm concentration, motility, seminiferous tubule diameter, epithelium height were significantly ($P<0.001$) reduced in the *HH*-treated groups relative to control. Testicular morphology showed loss of interstitial space, seminiferous tubules distortion, cellular degeneration, in addition with altered pattern of collagen fibres and androgen receptors expressions. These effects were more pronounced in the HAART and 200 mg *HH*-treated groups. As the use of *HH* by people living with HIV/AIDS may be perceived cheap and convenient substance to boost the immune status, however, it uses require caution in view of its negative perturbations on the testicular integrity, especially in people of reproductive age.

Key words: *Hypoxis hemerocallidea*; HAART; Testis; Androgen receptor; HIV/AIDS

INTRODUCTION

Access to antiretroviral drugs for the management of HIV infection and increased awareness to control the rate of new infections have achieved tremendous measure of success, for low- and middle-income countries[1,2]. While highly active antiretroviral therapy (HAART) may lower the occurrence of infections in people living with HIV and AIDS (PLWHAs) and delay its progression to AIDS, there are still drawbacks to the use of conventional therapies; HAART comes with undesirable toxicities that have mitigated its use[3]. The most common toxicities of HAART include hepatotoxicity[4] linked to mitochondrial damage, especially in patients treated with zidovudine, stavudine or didanosine[5]. Others HAART-associated side effects include metabolic disorders especially with protease inhibitors[6], nephrotoxicity[7], poverty, drug resistance, shortage of health personnel[8,9].

As a result, the side effects of HAART on fertility and metabolic complications in a growing population has become a significant cause for concern as HIV is most prevalent among people of reproductive age, and reproductive desires have emerged as clinically important in PLWHAs[10]. More so, the deleterious effects of various antiretroviral regimes on the sperm cell morphology, motility and viability have been reported in various in vitro clinical trials[11,12] and animal studies[3,13,14] and human study[15]. These drawbacks in the use of HAART may lead to patients resorting to alternative and/or traditional medicines (TM) as a means of attaining the goal of immuno-restoration and mitigating HAART related toxicities[16].

The use of traditional herbal medicine (THM) for the management of HIV and treatment of related ailments is quite widespread among different communities globally, and particularly in Africa[16,17,18,19]. This includes *Hypoxis* species that are used against different ailments, with *Hypoxis hemerocallidea* (*HH*) *been recommended* among other herbal products for PLWHAs[20] in addition to antiretroviral drugs.

Commonly referred to as African potato, *HH* is one of southern Africa's most important medicinal plants used by PLWHAs to enhance their immunity. Major

constituents of the plant are hypoxoside, phytosterol glycosides and cytokinins (zeatin, zeatin riboside and zeatin glucoside). Anti-cancer, anti-HIV and anti-inflammatory activity is ascribed to hypoxoside which, once in the human gut, readily converts to rooperol. This is a biologically active compound that balances the immune system. Besides anti-inflammatory and anti-cancer activities, antidiabetic and anticonvulsant[21], infertility and anxiety[22], antimicrobial[23] and antioxidant[24] activities have been reported.

While the use of *HH* is widespread among PLWHAs purported to have beneficial effects, there is a shortage of scientific data examining the safety and efficacy of this plant upon concomitant use of HAART on the metabolic and reproductive status. Therefore, this study aimed at evaluating the toxicity and safety roles of *HH* adjuvant therapy with the aim of understanding its impact on testicular integrity, specifically on testicular morphology, morphometric and epididymal seminal fluid analyses) in animal models .

MATERIALS AND METHODS

Drugs and Chemical: Lamivudine (3TC), Zidovudine and Nevirapine (Aspen) were sourced from Pharmed Ltd., Durban, South Africa. Monoclonal Anti-antibody kits (N-20, Santa Cruz Biotechnology, CA, USA), Diaminobenzidine (DAB) which were of analytical grade quality were purchased from Capital Lab Supplies, Durban, South Africa.

Collection of Plant Material: Fresh corms of *Hypoxis* were purchased from a local 'Muthi' in Umbilo Road, Durban, KwaZulu-Natal. The corms were authenticated at the Department of Life Science, Westville Campus, University of KwaZulu-Natal, Durban, South Africa.

Extraction of *Hypoxis hemerocallidea:* *Hypoxis* fresh corms were extracted according to the procedure of Ojewole[25]. They were washed with water, cut into smaller pieces, air dried at room temperature (25 – 28 ^{0}C) and ground into powdered form in a blender. The milled corn was soaked in hot distilled water and extracted twice, on each occasion with 2.5 litres of hot distilled water (at 90 – 100 ^{0}C) for 12 hours. The combined extract soluble were concentrated to dryness under reduced pressure in a rotary evaporator at 70 ± 1^{0}C. The resulting crude aqueous extract was freeze dried, finally giving of a dark brown, and powdery aqueous extract residue. Without any further purification, aliquot portions of the aqueous extract residue were weighed and dissolved in distilled water (at room temperature) for use on each day of our experiments.

Animals: Twenty four adult male Sprague-Dawley rats aged 9-10 weeks old (188.98±4.5 g) were used for this study. The animals were bred and maintained at the Animal House of the Biomedical Resources Unit, University of KwaZulu-Natal. The animals received humane care 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 of the National Academy of Sciences (National Institute of Health Guide, 1985). The study protocol was approved by the University of KwaZulu Natal Animal Ethics Committee (Ethical clearance number: 056/15/Animals). All the rats were housed in well ventilated plastic cages having dimensions of (36 cm long × 24 cm wide and 15 cm high). They were maintained under standardized animal house conditions (temperature: 28 – 31 ^{0}C; light: approximately 12 h natural light per day) and were fed with standard rat pellets from (Meadow feeds, a Division of Astral Operations Limited, Durban, South Africa) and given tap water *ad libitum*.

Experimental Design: Animals (n = 24) were distributed into four groups: A - D with 6 animals per group follows:
Group A served as control;
Group B received HAART (Zidovudine, Lamivudine & Nevirapine) using the human therapeutic dose equivalents (600 mg, 300 mg and 400 mg/day respectively), as adjusted for animal weight to obtain corresponding therapeutic doses for the rat model therapeutic dose[26].
Group C received *HH* extract (100 mg/kg bwt);
Group D received *HH* extract (200 mg/kg bwt).
All administration was done daily by oro-gastric gavage, and the treatments lasted for 8 weeks.

Weight Changes: Body weight (BW) changes of the animals were also recorded weekly. BW of the rats was recorded on the first day before the commencement of the treatment (the initial body weight), thereafter weekly and on the last day shortly before animal sacrifice (final body weight). Testicular weights (TW) were measured by an electronic balance (Mettle Toledo; Microsep (Pty) Ltd, Greifensee, Switzerland) after laparotomy. The testes of each rat were measured individually and the average value obtained for each of the two measurements was taken as one observation. Values are expressed as g for all weight measurements.

Sample Collection and Analysis: Eight weeks post-treatment, the animals were sacrificed by exposure to Halothane® for 3 min via gas anesthetic chamber. Thereafter, blood samples were collected by cardiac puncture into pre-cooled heparinized tubes and centrifuged in a desktop centrifuge model 90-1 (Jiangsu Zhangji Instruments Co., China) for 15 minutes at 3000 revolutions per minute. The serum was decanted into Eppendorf tubes and stored at -80^{0}C for subsequent analysis. The testicles were excised, weighed and immediately fixed in 10% neutral buffered formalin. After proper fixation, tissues were dehydrated in graded series of alcohol, cleared in Xylene and embedded in paraffin wax using a cassette.

Epididymal Sperm Preparation and Seminal Fluid Analysis: The epididymis were macerated using an anatomical scissors and minced in 0.8 ml of 1% trisodium citrate solution for 7 - 8 minutes, then more solution was added (up to total amount 8 ml) and mixed for about 1 minute. The sperm suspension was diluted 1:1 in 10% buffered formalin. Spermatozoa were counted using the Biorad® automated Cell Counter 1450101TC 20TM with double-chambered counting slides loaded with 10 µl of caudal epididymal sperm solution. For the estimation of percentage motile sperm cells, we adopted the procedures described by Azu[27]. Briefly, motility and concentration estimation was carried out at room temperature between 24 and 28^{0}C. Sperm movement analysis was done by standard haemocytometer and light microscope (Olympus Co., Tokyo, Japan). Motility was expressed as the percentage of progressive motile spermatozoa, immotile spermatozoa and dead. The procedure was repeated once and the average reading was taken.

Histopathological Examination of the Testis: For routine histological study, testicular tissues were sectioned at 5 µm thickness using Leica RM 2255 microtome. The slides were deparaffinized in xylene and rehydrated in graded ethanol (100%, 90%, 80%, 70%, 50%) and rinsed in water. Slides were stained in Haematoxylin for 5 minutes and rinsed with water, and counterstained with eosin for general assessment of the testicular microanatomy. For histochemical studies, the testicular sections were stained with Periodic acid Schiff' (PAS) for glycogen, neutral polysaccharides and seminiferous tubule basement membrane and Masson trichrome (MT) for collagen fibres[28]. The stained slides were then cover slipped using DPX mounting glue directly over the tissue section ensuring no air bubbles were trapped. Thereafter, the slides were left overnight to dry for examination under the light microscope. The sections were examined using a binocular microscope and image acquisition was done using the Nikon Eclipse 80i, Tokyo, Japan. An independent Histopathologist blinded to the treatment groups reported on the qualitative assessments of the slides.

Immunohistochemistry: The Avidin Biotin Complex (ABC) method was used. Androgen receptor (AR) within the testicles tissue sections were localized using rabbit anti-AR. Antigenic sites were retrieved using citric acid solution and pressure cooker. Peroxidases, protein and biotin blocks were done using Hydrogen peroxide, avidine and biotin respectively. The BRCA 1 and 2 antibodies were diluted 1.100 and incubated on sections. These were followed by the biotylinated secondary antibody, streptavidine and DAB/substrate reaction according to Nasra[29].

In brief, formalin fixed and paraffin embedded tissues were sectioned on the rotary microtome and placed on the hot plate at 70 degrees for at least 1hour. Sections were brought down to water by passing on 2 changes of xylene, then 3 changes of descending grades of alcohol and finally to water. Antigen retrieval was performed on the sections by heating them on a citric acid solution of PH 6.0 using the microwave at power 100 for 15 minutes. The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5 minutes for the section to cool. Peroxidase blockings were done on the sections by covering section with 3% hydrogen peroxide (H_2O_2) for 15 minutes. Sections were washed with PBS and protein blocking were performed using avidin for 15 minutes. Sections were washed with PBS and endogenous biotins in tissue were blocked using biotin for 15 minutes. After washing with PBS sections were incubated with the respective diluted primary antibodies (BRCA1) antibody diluted 1:100 for 60 minutes. Excess antibodies were washed off with PBS and secondary antibodies (LINK) were applied on section for 15 minutes. Sections were washed and the (LABEL) which is the horsradish peroxidase (HRP) were applied to the sections for 15 minutes. A working DAB solution was made up by mixing 1 drop (20 microns) of the DAB chromogen to 1ml of the DAB substrate. The working solution was applied on sections after washing off the HRP with PBS for at least 5 minutes. The brown reactions begin to appear at the moment especially for a positive target. Excess DAB solution and precipitate were washed off with water. Sections were counterstained with Haematoxylin solution for at least 2 minutes and blued briefly. Sections were then dehydrated in alcohol, cleared in xylene and mounted in DPX.

Cells with specific brown colours in the cytoplasm, cell membrane or nucleus depending on the antigenic sites are considered to be positive. Haematoxylin stained cells without any form of brown colours were scored negative. Non-specific binding/brown artifacts on cells and connective tissue are disregarded. For testicular cells, Sertoli cells were characterized by their large irregularly shaped nuclei on the basal layer of the seminiferous epithelium; myoid cells by their elongated nuclei around the periphery of the tubule; and Leydig cells by their round nuclei in the interstitial tissue. Assessment for androgen receptors (Table 4) was performed according to the following semi-quantitative scale: (-) negative; (+) weakly positive; (++) positive; and (+++) strongly positive[30].

Morphometric analyses: To ensure an unbiased estimate of morphometric data, we adopted the following steps in obtaining our results. For each testis, seven vertical sections from the polar and the equatorial regions were sampled and an unbiased numerical estimation of the following morphometric parameters was determined using systematic random scheme[31]: seminiferous tubular diameter, seminiferous epithelial height/thickness and basement membrane thickness. The seven vertical sections were selected by a systematic sampling method that ensured fair distribution between the polar and equatorial regions of each testis. Diameters (D) of approximately 18 randomly selected seminiferous tubules with profiles that were round or nearly round was

measured for each slide and a mean D was determined by taking the average of two diameters, D1 and D2. D1 and D2 were taken only when D1/D2 $\geq$0.85 (1.0 = a perfect circle). This is to eliminate longitudinal profiles which might exhibit different degrees of damage along their length and/or show irregular shrinkage as previously reported[31,32]. The tubular diameter and height of the seminiferous tubule epithelium was scanned using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at 9100 magnification using image analyzer Leica (DMLB) and Leica QWIN software. The diameter of the seminiferous tubule was measured across the minor and major axes, and the mean diameter obtained as stated above.

Seminiferous Tubule Basement Membrane (STBM): Five micrometre-thick sections obtained from each animal were stained with Periodic acid Schiff (PAS). All sections were viewed under a microscope with an attached video camera and image analyzer system (MiniVid DCE-1, *LW Scientific Inc.*). The measurement was performed on thirty randomly selected STBM from 3 randomly taken sections and averaged[33].

Testes Size and Gonadosomatic Index (GSI): The length and width of the testes were measured with the aid of digital Vernier calipers according to the method of Parhirzkar[34]. The size of the testes was estimated using the formula for a prorate spheroid:

Size of testis = width^2 x length x 0.523 ---------- Equation 1

The gonadosomatic index (GSI) shows the percentage of body mass allocated to the testis was calculated based on body (BW) and TW as shown in equation 2 below.

GSI = (TW/BW) × 100) ---------- Equation 2

Testes Volume: The water displacement method commonly adopted for irregularly-shaped objects was used[35]. Briefly, a graduated cylinder was filled with a certain amount of water. The testis was then carefully placed into the graduated cylinder. The volume of the testis (V_t) was calculated by subtracting the initial (Vi) from the final (Vf) volume of water as in equation 3 below.

$V_t = V_f - V_i$ (cm^3) ---------- Equation 3

Leydig Cell Stereology: The volume of each Leydig cell was estimated from the volume of the nucleus. To obtain the nucleus volume, the diameters of 30 spherical nuclei showing evident nucleoli were measured for each animal[33]. Leydig cell nuclear volume was expressed in μm^3 and calculated using the formula below.

Leydig $= (4/3)\pi R^3$ ---------- Equation 4

Where R = nuclear diameter/2

Assessment of Serum Testosterone: Testosterone was determined in duplicate using the Testosterone Enzyme Immunoassay kit (Assay Design Inc., Ann Arbour, USA) according to the procedure described by Wang[36].

Statistical Analysis: All continuous variables were collated and analyzed and expressed as mean ± standard deviation (SD). Statistical comparison of the differences between the control and experimental groups was performed with GraphPad InStat Software (version 6.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. The $p < 0.05$ was considered statistically significant.

RESULTS

Weight Changes: There were no significant ($p < 0.05$) differences in the body and organ (testes) weights as well as organ body weight ratios of groups treated with HAART or *HH* in this study, but there were observational changes as shown in Table 1.

Table 1: Weight Changes in Experimental Groups

GROUP	A (Control)	B (HAART)	C (HH_{100})	D (HH_{200})
BWi (g)	173.8±9.2	185.6±9.8	184.3±7.5	174.0±5.5
BWf (g)	289.8±23.4	299.2±23.5	295.8±17.7	291.8±17.1
BW diff (g)	116.0	113.60	111.5	117.8
% BW diff	66.7	61.2	60.5	67.7
TW (g)	3.1±0.2	3.2±0.4	3.1±0.2	3.1±0.2
TBWR	1.1	1.1	1.1	1.1

Data are shown as the mean ± SD; $p<0.05$ vs Group A[α] (Tukey-Kramer multiple range post-hoc test). BWi= initial body weight, BWf=final body weight, TW=Testis weight, TBWR=Testis-body weight ratio.

Seminal Fluid Analysis: The sperm count and motility showed significant reduction ($p < 0.0001$ and $p < 0.05$ respectively) in the parameters of all treated groups (B, C and D) compared with control group A. There were also increase numbers of dead sperm cells in the treated animals compared with the control animals, Table 2.

Table 2: Seminal Fluid Analysis

GROUP		A (Control)	B (HAART)	C (HH_{100})	D (HH_{200})
SPERM COUNT ($x10^6$)		46.8±3.1	35.3±5.7	38.2±1.6	11.1±2.0$^{\alpha}$
MOTILITY (%)	MOTILE	64.3±7.4	46.8±4.8	40.0±6.4$^{\alpha}$	34.0±7.2$^{\alpha}$
	IMMOTILE	19.3±7.5	16.8±5.4	26.0±4.0	25.7±3.9
	DEAD	16.3±5.9	36.4±4.1	34.7±7.3	40.3±4.5

Data are shown as the mean ± SD; p<0.0001 vs Group A$^{\alpha}$ (Tukey-Kramer multiple range post-hoc test).

Morphometric Indices: The testicular length and width, gonadosomatic index (GSI) of treated groups were not significantly different from the control (Table 3). The morphometric measurement of testicular volume components was similar to the control ($p < 0.001$). While there was a significant ($p < 0.001$) reduction in the seminiferous tubule diameter, seminiferous tubule epithelium height of all the treated groups, there was observational increase (not significant) in the thickness of the basement membrane of the animals treated with HAART and 200 mg/kg of *HH* respectively when compared with the control group A (Fig. 1AP, Table 3). However, no changes in the average Leydig cell nuclear volume or in the number of these cells in the testis could be detected in the groups after the treatments ($p < 0.0001$).

Table 3: Testicular and Leydig Cell Morphometric Indices in Experimental Groups

GROUP	A (Control)	B (HAART)	C (HH_{100})	D (HH_{200})
TL (mm)	19.9±0.9	20.3±0.4	20.0±0.7	20.3±0.8
TWd (mm)	12.1±0.4	12.3±1.1	12.9±0.3	12.5±0.6
GSI (%)	1.1±0.1	1.1±0.1	1.1±0.1	1.1±0.1
STD (µm)	293.5±2.7	265.9±3.6$^{\alpha}$	263.9±4.6$^{\alpha}$	252.5±3.6$^{\alpha}$
SEH (µm)	93.3±5.6	82.5±1.4$^{\alpha}$	80.3±2.8$^{\alpha}$	79.1±4.2$^{\alpha}$
STBM (µm)	1.7±0.0	1.8±0.0	1.6±0.3	1.8±0.0
TS (mm)	1532±62.2	1625±64.7	1729±47.0	1668±62.2
TV (mm^3)	3.2±0.3	3.3±0.1	3.3±0.2	3.2±0.2
LCND (µm)	24.6±1.5	20.3±3.5	22.0±2.3	21.8±4.2
LCNV (μm^3)	1314±85.4	875±65.1	955±52.5	964±71.7

Data are shown as the mean ± SD; p<0.0001, p<0.0001 vs Group A$^{\alpha}$ (Tukey-Kramer multiple range post-hoc test) GRP=group, TL= testis length, TWd=testis width, GSI=gonadosomatic index, STD=seminiferous tubule diameter, SEH=seminiferous epithelium height, STBM=seminiferous tubule basement membrane, TS=testicular size, TV=testicular volume, LCND=Leydig cell nuclear diameters, LCNV=Leydig cell nuclear volume.

Serum Testosterone and Testicular Androgen Receptors (AR): There were significant reductions in testosterone levels of all the treated groups relative to the control and changes the AR distribution pattern as shown in table 4.

Table 4: Androgen Receptors (AR) Expression, Serum Testosterone and Lipoproteins

GROUP		A (Control)	B (HAART)	C (HH_{100})	D (HH_{200})
AR	Sertoli cells	++	-	+	+
	Leydig cells	+++	+	++	+
	Myoid cells	+++	+	++	+
	Germ cells	-	-	+*	-
Testosterone (nmol//L)		20.1±6.8	19.4±8.11	16.8±8.0	14.9±7.7

Data are shown as the mean ± SD; p<0.001 vs Group A$^{\alpha}$ (Tukey-Kramer multiple range post-hoc test). *III/IV stages of the seminiferous epithelium cycle AR = Androgen receptor.

Histopathological and Immunohistochemical Evaluation of the Testes: It was observed that the testes in the control group A was covered with an albugineous layer and the complete spermatogenic series present (Fig. 1AH) with basal lamina well preserved and interstitial spaces populated with Leydig cells. The histological structures in the HAART group was mildly distorted with loss of interstitial spaces (Fig. 1BH), the *hypoxis* treated groups (*HH* 100 mg and 200 mg) also showed loss of interstitial space, seminiferous tubule distortion and cellular degeneration in the 200 mg group (Fig. 1CH and DH).

The testis in the control group was well outline with complete spermatogenic series (Fig. 2AM) and no areas of fibrosis. The histological structures in the HAART group was distorted with extensive areas of fibrosis as shown in green (Fig. 2BM), the *hypoxis* treated groups (*HH* 100 mg and 200 mg) showed cellular distortions and mild to moderate areas of fibrosis shown in the green stain of MT, this defect was more pronounced in the 200 mg *HH*-treated group (Fig. 2CM and DM).

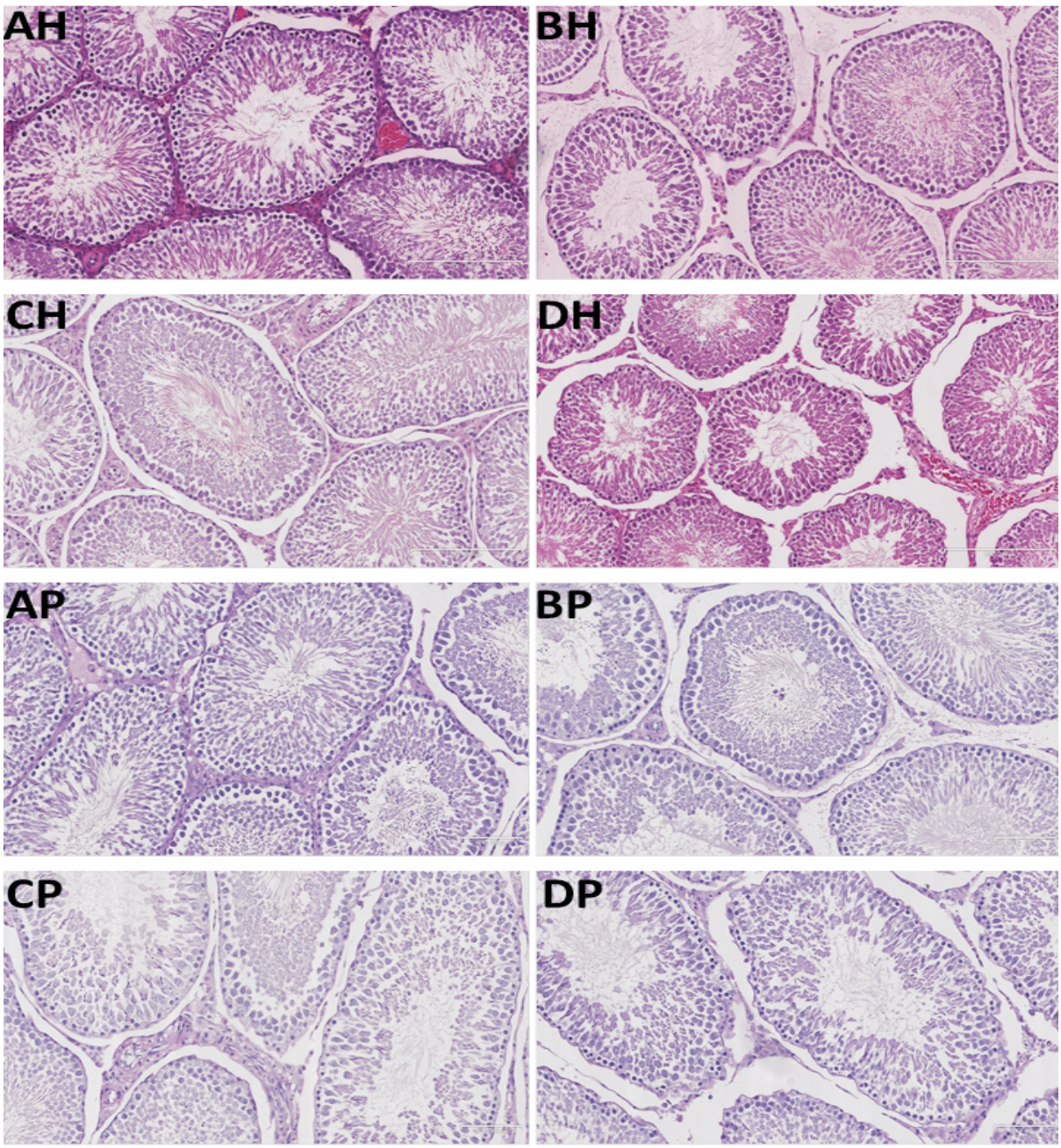

Figure 1: Photomicrographs showing representative seminiferous tubules. (AH): Control, (BH): rats treated with HAART, (CH): rats treated with 100 mg *HH,* (DH): rats treated with 200 mg *HH* (H&E Stain) and (AP): Control, (BP): rats treated with HAART, (CP): rats treated with 100 mg *HH* (DP): rats treated with 200 mg *HH* (PAS Stain).
Immunolocalization of AR in the wall of the seminiferous tubules of normoglycemic rat testis was found in the cytoplasm and nuclei of Sertoli cells. Expression of AR was also observed in the nuclei of peritubular myoid and

Leydig cells (Fig. 3AA, Table 4). As shown in Fig. 2BA, CA and DA, there was a change in the pattern of AR expression in the testes of HAART and *HH* treated animals (Table 4). The expression was almost negative in the testis of HAART treated animals (Fig. 2BA). Positive reaction was shown not only in Sertoli cells, but also in the cytoplasm of spermatogonia and spermatocytes in III/IV stages of the seminiferous epithelium cycle of the animals (Fig. 2CA, Table 4). Germ cells of other stages of the seminiferous epithelium cycle did not show immunoreactivity for AR. However, no AR-positive cells were detected when the primary antibody was omitted.

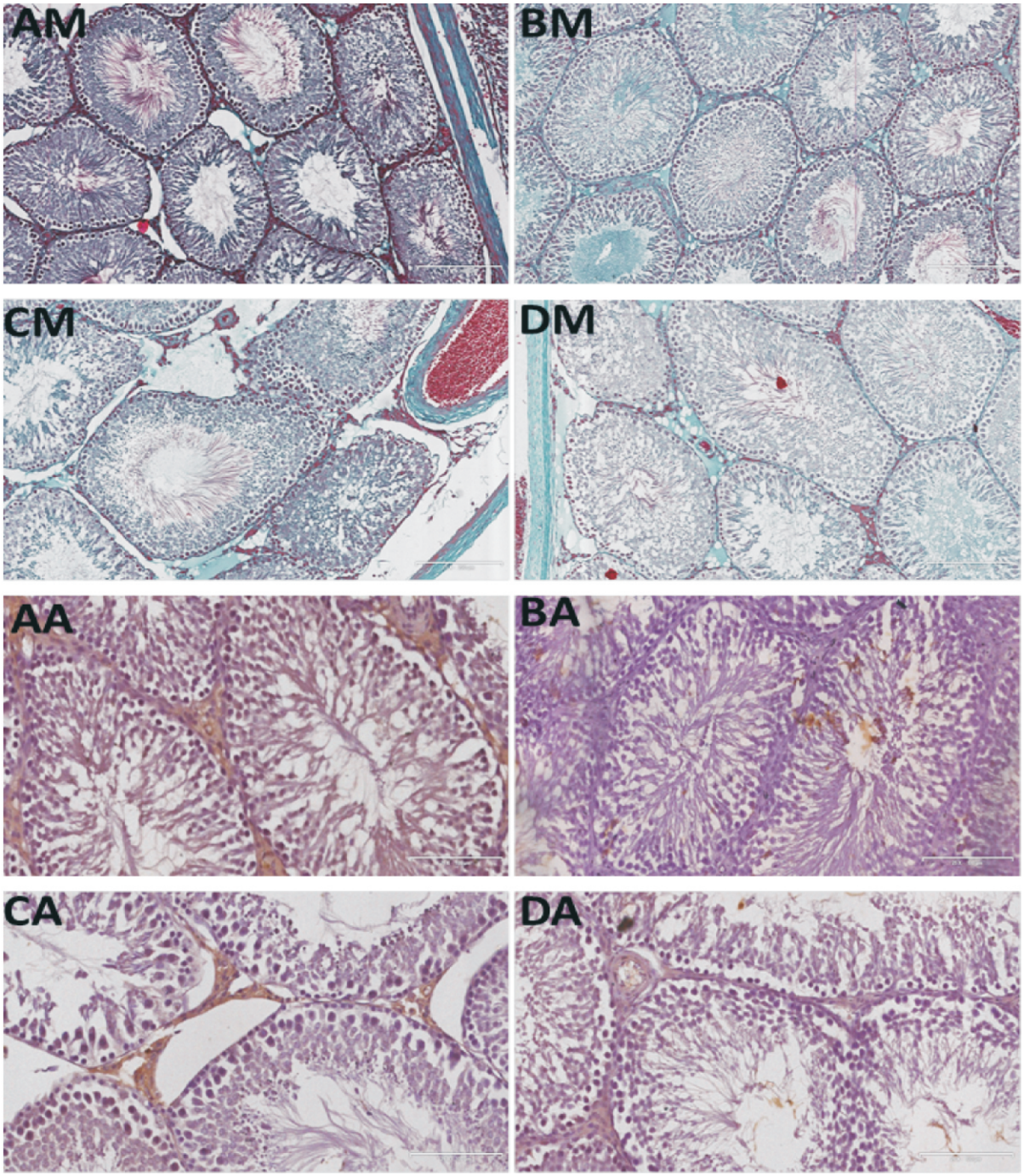

Figure 2: Photomicrographs showing representative histochemical expression of testicular collagen fibres and androgen receptor. (AM): Control, (BM): rats treated with HAART, (CM): rats treated with 100 mg *HH,* (DM): rats treated with 200 mg *HH* (Masson' Trichrome Staining). (AA): Control, (BA): rats treated with HAART, (CA): rats treated with 100 mg *HH,* (DA): rats treated with 200 mg *HH* (Androgen receptor).

DISCUSSION

The use of herbal medicine as a treatment modality has significantly increased over the last decade[37]. This is due to several factors, principal of which is that herbal medicine is perceived to be cheaper alternative with fewer undesired side effects[38,39]. Our current study revealed HAART and *HH* induced perturbations in the testicular morphology, morphometric indices, quality of epididymal seminal fluid and serum metabolic parameters evaluated.

Sperm concentration and motility were significantly reduced in the HAART and *HH* treated groups, when compared with the control, this supported the findings earlier reported[14]. While several factors were considered in the evaluation of male fertility[40], sperm count and motility are essential components, and therefore could be a good indicator for fertility assessment[41] .As spermatozoa motility is a key factor determining the fertility ability of any sperm cell and the likelihood of damage to flagella structures/or energy supply due to negative interactions with HAART and *HH* components is possible. Though the herb had been reported to possess anti-inflammatory and antidiabetic properties, however, its usage may be associated with adverse tissue effects. This corroborated with findings of studies that have shown that herbs like Viagra which are widely used lowers sperm count[42].

While albuginea layer, the complete spermatogenic series with basal lamina were well preserved and interstitial spaces populated with Leydig cells of control animals, the histological structures in the HAART group was mildly distorted with the *hypoxis* treated groups showing loss of interstitial space, seminiferous tubule distortion and cellular degeneration. The impact of HAART treatment on the morphology of the testis has generated diverse interest owing to different views surrounding viral "sanctuary sites" and the blood-testis barrier[43]. With evidence[44], HAART may be deposited in the semen with unruly disruption of the testicular structure.

Furthermore, morphometric analyses of seminiferous tubule diameter and epithelial height following HAART and adjuvant administration of *HH,* the duo did alter these parameters as against the control animals. These observations closely correlate with qualitative histological assessments and further support HAART-induces seminiferous tubular and epithelial atrophy with a nonspecific disruption and reduction of germ cells from the tubules. More so, epithelial atrophy is also known to accompany increased seminiferous lumen. Consequently, the eventual generalised hypoplasia in many tubules and dysfunctional microtubular supporting framework of spermatogenic cell series, and therefore, ultimately resulting in the sloughing of the germinal cells with compromise in spermatogenesis process[16] .

There was an increase in basement membrane thickness with extensive areas of fibrosis in the HAART treated and mild to moderate areas of fibrosis in the *HH* treated animals compared with the control group, these changes were dose dependent. This was closely correlated with MT and PAS staining findings which demonstrated the presence of fibrosis and thickening of basement membrane in the HAART and *HH* groups. These findings indicated tissue inflammatory changes and toxicity. The implication of these findings implies that unknown to PLWHA who are using *HH*, the herb has a deleterious effect on the testis in addition to negative consequential use of HAART. The production of reactive oxygen species and mitochondrial damage could account for this damage[45], leading to negative reproductive consequences because of altered spermatogenesis. Subsequently this can lead to poor reproductive outcome and infertility[45,46].

Androgen receptor (AR) belongs to a large family of ligand-activated nuclear receptors and is highly expressed in the testes, especially in Sertoli cells[47]. Sertoli cells are thought to be the major cellular target for the testosterone signaling that is required to support male germ cell development and their survival[48]. In the current study, there were changes in the expression pattern of AR localized to the cytoplasm and nuclei of Sertoli cells, peritubular myoid cells and Leydig cells of HAART and *HH* treated animals. The expression was almost negative in the HAART treated animals. Though, It has been reported that the absence of AR immunoexpression in germ cells and intense staining in Sertoli cells (SCAR), which has been universal result among all laboratories are thought to be the major cellular target for the testosterone signaling that is required to support male germ cell development and survival[47,49]. Several spermatogenic disorders are associated with impaired androgenic stimulation of Sertoli cells; thus, low levels of the AR are likely to be involved in spermatogenic disorders[50].

Testosterone also plays a significant role in the maturation of Sertoli cells[51]. This substance was significantly depleted in both HAART and *HH* treated animals in the current protocol, though the level was much lower in the *HH* groups, which might have been as result of insult on the Leydig cells. While the process of spermatogenesis is influenced by hormonal changes, sexual dysfunction and hypogonadism have long been recognised in HIV-infected men[52]. However, with the advent of HAART, studies have shown a positive correlation between testicular and hypothalamo-pituitary hormones (GnRH and LH), and patients with low levels of testosterone also had lower levels of gonadotrophins[53]. Both testosterone and FSH have additive effects on SCAR expression[50]. Rather than FSH stimulation, AR expression has been shown to be primarily dependent on the intra testicular testosterone level, and when the former is knocked out, its affects the ability of Sertoli cells to support maturing germ cells, resulting in spermatogenesis arrest before the first

meiotic division and during the transition from round to elongated spermatids, causing progressive loss of fertility[49].

CONCLUSION

In conclusion, the study further upholds the negative assault of HAART on reproductive activities of the testis. However, the perceived accessibility, cheap and convenient use of *HH* by PLWHA as immune booster require caution in view of the current findings that 100 and 200 mg/kg body weight for eight weeks in the Sprague Dawley rat which could not mitigate the resulting assault on the testis from the use HAART. Though, further studies are required to establish the mechanism of toxicity of this herb on the testis and its long term effect on the quality of life of PLWHA.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

O.O.I. is a postgraduate student who co-designed the project, executed the project, collected samples and analyzed the data and drafted the manuscript under the supervision of the project leader. OOA is the project leader who co-designed the project, supervised data collection, analyses/interpretation and oversaw manuscript writing. ECSN and PAI also took part in manuscript writing. A.I.J, U.O and O.O are postgraduate students under the project leader; they participated in the execution of the project and data collection.

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